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**Analysis of the Autoantibody Response directed
against Tissue Transglutaminase in Coeliac Disease**

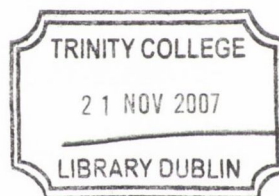
A thesis submitted for the degree Doctor of Philosophy

by

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**Trinity College
University of Dublin**

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Declaration

I declare that this thesis is my own work and has not been submitted for any degree at this or any other university and except where otherwise stated; it is entirely my own work.

I agree that the library of the University of Dublin, Trinity College may lend or copy this thesis on request.


Greg Byrne

This thesis is dedicated to Mum 'n' Dad

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Communications from this thesis

Journal Article in press

- **Byrne, G.**, Ryan, F., Jackson, J., Feighery, C., Kelly, J. "Mutagenesis of the catalytic triad of Tissue Transglutaminase Abrogates Coeliac Disease Serum IgA Autoantibody Binding" Accepted for publication in Gut August 2006.

Oral presentations

- **Byrne, G.**, Ryan, F., Feighery, C., Kelly, J. "Analysis of Recombinant Tissue Transglutaminase as a Target for Autoantibodies in Coeliac Disease"
 - 11th International Symposium on Coeliac Disease, April 2004, Belfast, Northern Ireland

Poster presentations

- **Byrne, G.**, Ryan, F., Feighery, C., Kelly, J. "Analysis of Recombinant Tissue Transglutaminase as a Target for Autoantibodies in Coeliac Disease"
 - 11th International Symposium on Coeliac Disease, April 2004, Belfast, Northern Ireland
- **Byrne, G.**, Ryan, F., Feighery, C., Kelly, J. "Analysis of Autoantibody Binding to Recombinant Tissue Transglutaminase in Coeliac Disease Using Site-Directed Mutagenesis"
 - 12th International Congress of Immunology and 4th Annual Conference of FOCIS, July 2004, Montreal Canada.
- **Byrne, G.**, Ryan, F., Feighery, C., Kelly, J. "The core region of tissue transglutaminase is specifically targeted by mucosal autoantibodies in coeliac disease"
 - 8th International Conference on Protein Cross-linking and Transglutaminases (PCL8), September 2005, Lübeck, Germany
 - Irish Society for Immunology Conference, October 2005, Dublin, Ireland.

Abbreviations

Abbreviation	Meaning
%CV	Percentage co-efficient of variation
3D PSSM	Three-dimensional position-sensitive scoring matrix
AAA	Anti-actin antibodies
AGA	Anti-gliadin antibodies
AMV	Avian myeloblastoma virus
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Antigen-presenting cell
APS	Ammonium persulphate
AU	Arbitrary units
bp	Base pairs
BSA	Bovine serum albumin
CD	Coeliac Disease
cDNA	Complimentary DNA
CGG	Carbobenzoxy-L-glutaminyglycine
CGG-DNS	1- <i>N</i> -(carbobenzoxy- <i>L</i> -glutaminyglycyl)-5- <i>N</i> -(5' <i>N</i> ' <i>N</i> '-dimethylaminonaphthalenesulfonyl) diamidopentane
DAB	3,3'-Diaminobezidine
DEPC	Diethylpyrocarbonate
dH ₂ O	Distilled water
DMC	<i>N</i> ' <i>N</i> -dimethylcasein
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-triphosphate
DTT	1,4-Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ESPGAN	European Society of Pediatric Gastroenterology and Nutrition
GALT	Gut associated lymphoid tissue
gptTG	Guinea pig tissue transglutaminase
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEL	Intraepithelial lymphocyte
IPTG	Isopropyl b-D thiogalactoside
kb	kilobases
kDa	Kilodalton
L	Litre
LAP	Latency associated protein
LB	Luria-Bertani
LTBP	Latent TGF binding protein
mA	Milli-amp
MADCAM 1	Mucosal addressin cell-adhesion molecule 1
mg	Milligram
ml	Millilitre
nm	Nanometre
OD	Optical density
PCR	Polymerase chain reaction
PDB	Protein database
PP	Peyer's patch
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute

RT	Room temperature
RT-PCR	Reverse transcriptase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SNRPN	Small nuclear riboprotein polypeptide N
TAE	Tris-acetate EDTA
<i>Taq</i>	<i>Thermus aquaticus</i> DNA polymerase
TBE	Tris-borate EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
T _m	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tissue necrosis factor
tTG	Tissue transglutaminase
ug	Microgram
WASP	Wiskott-Aldrich syndrome protein

Summary

Coeliac disease is an inflammatory disease of the proximal small intestine caused by wheat gluten ingestion in genetically susceptible individuals. The mucosal pathology in coeliac disease is characterised by villous atrophy, crypt hyperplasia and increased levels of mononuclear cells in the epithelium and lamina propria. Although coeliac disease is one of the best characterised complex diseases, the mechanisms leading to the associated mucosal pathology are not well understood. A related gluten-sensitive disorder called dermatitis herpetiformis is characterised by a bullous skin rash caused by IgA deposits at the dermal-epidermal junction and a milder intestinal lesion.

The diagnosis of coeliac disease involves detection of IgA class serum antibodies directed against the ubiquitous enzyme tissue transglutaminase. Recent advances in the field have suggested that the enzyme has a role in the deamidation of specific glutamine residues in gliadin resulting in more efficient antigen presentation. The mechanism underlying the autoantibody response against tissue transglutaminase in coeliac disease is not known but it is thought to require the formation of covalent tissue transglutaminase-gliadin complexes.

The aim of this study was to determine whether the active site of tissue transglutaminase is a major epitope for coeliac serum autoantibodies and whether, as a consequence, the activity of the enzyme is inhibited by these autoantibodies. This was investigated by (1) recombinant protein production and site-directed mutagenesis, (2) ELISA assays with the recombinant antigens and (3) tissue transglutaminase cross-linking inhibition assays.

In the first part of the study, recombinant human tissue transglutaminase and a novel site-directed mutagenesis variant with the three amino acids of the catalytic triad (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) replaced by alanine residues were produced in *E.coli*. The recombinant proteins were expressed with a

glutathione-S-transferase affinity tag and were routinely examined by SDS-PAGE and found to be highly pure.

In the second part of the study, full-length wild type and mutant tissue transglutaminase were tested for their antigenicity in ELISA assays with sera from coeliac disease and dermatitis herpetiformis patients. While coeliac disease and dermatitis herpetiformis serum IgA bound wild type recombinant transglutaminase at a range of values that correlated well with their reactivity in the Celikey[®] commercial anti-tTG ELISA system, all sera showed a profoundly reduced binding to the mutant tissue transglutaminase. There was no significant difference in coeliac disease serum IgG binding to wild type or mutant tissue transglutaminase, although IgG reactivity to the wild type tissue transglutaminase was at a lower level than IgA responses. We concluded from these studies that the region of tissue transglutaminase responsible for its transamidation and deamidation reactions is targeted by IgA autoantibodies in coeliac disease and dermatitis herpetiformis and that IgG anti-tissue transglutaminase autoantibodies are less specifically targeted and may react with other regions of the enzyme.

In the third part of the study, a highly sensitive, microtitre plate, fluorescence-based assay was developed to measure the cross-linking activity of wild type tissue transglutaminase in the presence or absence of inhibitors or enhancers. Tissue transglutaminase was shown to have an absolute requirement for calcium and was inhibited by zinc, in accordance with the literature. Purified coeliac serum IgA was shown to be a stronger inhibitor of tissue transglutaminase activity, at certain concentrations. Also, the depletion of anti-tissue transglutaminase was shown to, at least partially, remove the inhibitory capacity of coeliac serum IgA. We can conclude from these studies that the IgA response against tissue transglutaminase in coeliac disease is targeted at the region of the enzyme responsible for its cross-linking activity. These antibodies are clearly responsible for some level of enzyme inhibition and the possible implications of such an inhibition are discussed.

Chapter 1

General Introduction

1.1 Coeliac Disease

1.1.1 The Gut-Associated Lymphoid Tissue

The intestinal epithelium is a single layer of cells that must function in the digestive absorption of nutrients and, at the same time, control the access of potential antigens and pathogens. The intercellular tight junctions between cells in the gut contribute to these functions by restricting the passage of even very small (2kDa) molecules (Madara 1998). The gut-associated lymphoid tissue (GALT), which functions to protect against luminal antigens, encompasses the Peyer's patches, mesenteric lymph nodes, and the lymphocytes of the lamina propria and epithelium, which are dispersed throughout the intestinal tract (Kohne, *et al.* 1996).

Enterocytes are the primary cell type in the epithelial monolayer, which is interspersed in some regions by a specialised epithelial cell type known as the M cell. M cells lack the normal glycocalyx that is present on the apical surface of villous enterocytes and instead has microfolds (hence the "M" cell nomenclature). Soluble protein antigens do cross the enterocyte barrier but the M cells, which overlay the Peyer's patches, are thought to be specialised cells for antigen sampling (Nagler-Anderson 2001).

Peyer's patches are aggregations of lymphoid follicles found primarily in the distal ileum of the small intestine. Like other secondary lymph nodes, they consist of varying numbers of follicles with central domes of B cells surrounded by T cells and macrophages (Janeway and Travers 1997). Naïve T and B cells encounter antigen in the Peyer's patch and become activated. They then leave via the mesenteric lymph nodes, lymphatics and the thoracic duct and re-circulate back to mucosal surfaces where they act as effectors of specific immunity (Mowat 2003).

The homing of lymphocytes to the gut mucosa occurs because lymphocytes that are primed by antigen in the GALT lose expression of L-selectin and selectively upregulate expression of $\alpha 4\beta 7$ integrin. This directs the emigration of lymphocytes from the bloodstream by interacting with the ligand for $\alpha 4\beta 7$ integrin, mucosal addressin cell-adhesion molecule 1 (MADCAM 1), which is expressed at high levels by the vasculature of mucosal surfaces (Butcher, *et al.* 1999). Also, expression of the chemokine receptor CCR9 is induced by gut derived T cells, allowing them to respond to the chemokine CCL25 which is expressed selectively by small intestinal epithelial cells (Campbell and Butcher 2002).

The main source of potential antigenic stimulation in the gut comes from the abundance of food proteins that are ingested and processed. A large body of evidence suggests that the default response to the ingestion of orally administered antigen is the induction of tolerance (Mowat and Weiner 1999). The mechanisms of oral tolerance, as it is most commonly known, are unclear but are thought to involve clonal deletion of reactive cells (Chen, *et al.* 1995), induction of anergy to the antigen (Melamed and Friedman 1993), the action of regulatory T cells (Faria and Weiner 2005) and the action of specific regulatory cytokines (Marth, *et al.* 1996, Rizzo, *et al.* 1999). The potential sites for antigen uptake, antigen presentation and the subsequent immune response are illustrated in Figure 1.1.

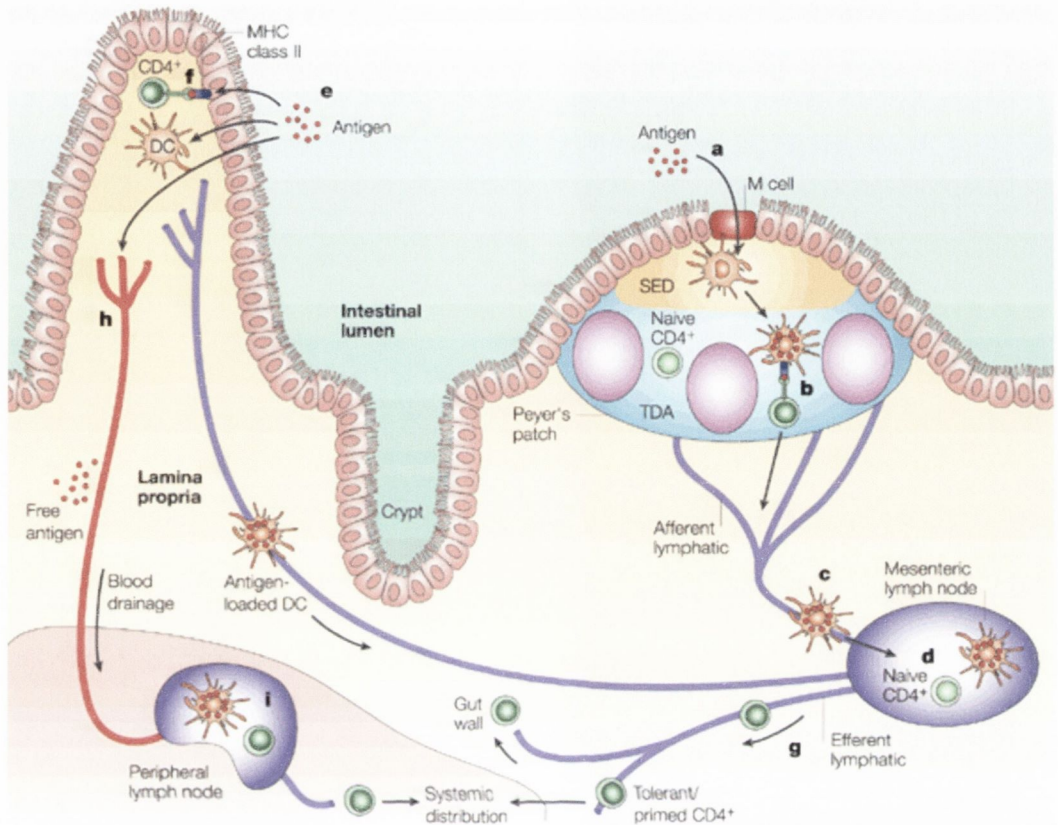


Figure 1.1: The fate of ingested antigen. Antigen may enter the GALT via the M cell route (a) and be processed by local antigen presenting cells such as dendritic cells (b). Antigen loaded dendritic cells leave the Peyer's patch via the draining lymph (c) and migrate to the mesenteric lymph node (d). Antigen may also enter through the intestinal epithelium (e) with the enterocyte acting as antigen presenting cell (f). In either case, T cells leave the mesenteric lymph nodes via the efferent lymph (g). Antigen may gain direct access to the bloodstream (h) and interact with T cells in the peripheral lymphoid tissues (Image taken from Mowat *et al.* 2003).

1.1.2 History of Coeliac Disease

In the second century AD, Aretaeus of Cappadocia described what is believed to be the first reference to coeliac disease. The observations made by Aretaeus were translated from the Greek into English for the Sydenham Society of England by Francis Adams in 1856 (Thomas 1945). Adams was the first to refer to individuals suffering from the disorder as “coeliacs”. The Greek word “koiliakos” used by Aretaeus had originally meant “suffering in the bowels” when used to describe people. Passing through Latin, ‘k’ became ‘c’ and ‘oi’ became ‘oe’. Dropping the Greek adjectival ending ‘os’ gave us the word coeliac. In 1888 Dr. Samuel Gee published a set of clinical details of adults and children suffering from coeliac disease (Gee 1888). Gee was the first to recognise that treatment of the disorder must be mediated by diet stating, “to regulate the food is the main part of treatment. The allowance of farinaceous foods must be small, but if the patient can be cured at all, it must be by means of diet”. In 1908, Herter described that fats were much better tolerated in these patients (Herter 1908). In 1918, George Frederick Still further characterised the causative agent of coeliac disease stating, “Unfortunately, one form of starch seems particularly liable to aggravate the symptoms is bread. I know of no adequate substitute” (Still 1918).

The most significant dissection of the disorder came about when Dicke demonstrated that the anorexia, steatorrhoea and increased amount of faeces observed in coeliac patients was in fact caused by wheat and that the removal of the cereal from the patient’s diet caused the symptoms to disappear (Dicke 1950). Dicke also demonstrated that wheat flour and not starch was responsible for the increased faecal fat seen in coeliac disease patients (Dicke, *et al.* 1953). While other starch-containing foods were shown to be harmless, Dicke showed that rye, flour and oats had unfavourable effects on patients (Dicke, *et al.* 1953). In 1952 Anderson *et al.* demonstrated that the reintroduction of wheat into the diet of a coeliac disease patient resulted in loss of appetite, diarrhoea, loss in weight and change in temperament, thus inventing the “gluten challenge” that became a tool for coeliac disease diagnosis.

1.1.3 Clinical Symptoms

Coeliac disease is caused by an inappropriate immune response to cereal proteins and generally presents in young children with chronic diarrhoea, failure to thrive, abdominal distension, poor appetite and unhappy behaviour (Kelly, *et al.* 1990). In the early 1980s it was noted that the clinical features of coeliac disease had changed (Swinson and Levi 1980, Logan, *et al.* 1983). Adults often present with milder symptoms such as indigestion or iron deficiency (Maki and Collin 1997).

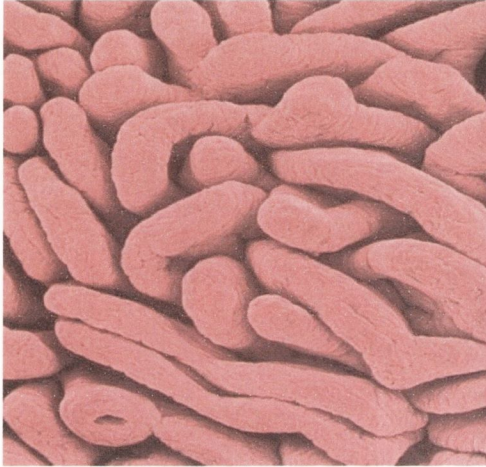
Atypical symptoms in coeliac disease are often a result of malabsorption in the gut. The mucosal damage associated with coeliac disease results in impaired absorption of many nutrients. Infertility or miscarriages have been described in women and reversible infertility in men (Sher, *et al.* 1994). Low bone mineral density is a common feature in untreated coeliac disease patients; this problem is resolved upon adopting a gluten-free diet (Molteni, *et al.* 1990). Mouth ulcers, arthritis or other joint symptoms as well as elevated liver enzyme concentrations sometimes lead to coeliac disease diagnosis (Collin, *et al.* 1991, Cernibori and Gobbi 1995, Hadjivassiliou, *et al.* 1996, Gobbi 2005). Patients with coeliac disease may suffer from neurological symptoms such as peripheral neuropathy, ataxia, brain atrophy, and epilepsy. Gluten sensitivity is also a presenting symptom associated with the related IgA-mediated skin disease, dermatitis herpetiformis (Nicolas, *et al.* 2003). Dermatitis herpetiformis patients usually present with an erythematous rash on the elbows, knees and buttocks (Karpati 2004). Taken together, these complex presentations of coeliac disease mean diagnosis can be difficult and misdiagnosis is common (Hin, *et al.* 1999).

1.1.4 The Coeliac Mucosal Lesion

Biopsy of the proximal part of the small intestine remains the gold standard for coeliac disease diagnosis (Green, *et al.* 2005). The lesion is characterised by villous atrophy, crypt hyperplasia and increased levels of lymphocyte density in the epithelium and small intestine (Figure 1.2). A study investigating the usefulness of biopsies from the terminal ileum showed that although villous atrophy was only demonstrated in 1 out of 30 coeliac disease patients, intraepithelial lymphocyte (IEL) levels were significantly higher in coeliac disease patients compared to controls (Dickey and Hughes 2004). These results suggest that although the effects are most pronounced in the proximal ileum, coeliac disease affects the entire small bowel.

The histological changes in the coeliac lesion characterised by Marsh (Marsh 1992b, Marsh 1995) and subsequently modified (Rostami, *et al.* 1999, Amsterdam 2001) provided a major advance for the histological diagnosis of coeliac disease (Figure 1.3). The earliest manifestation of disease, Marsh I, is characterised by normal villous architecture with increased IEL infiltrate (>30 lymphocytes per 100 enterocytes). It should be noted that an intraepithelial lymphocytosis is not specific for coeliac disease and occurs in tropical sprue, giardiasis, acute infective enteropathies, Crohn's disease during non-steroidal anti-inflammatory drug usage and in various autoimmune diseases (Marsh 1992a, Shah, *et al.* 2000, Kakar, *et al.* 2003, Lebwohl, *et al.* 2003). The lesion is classified as Marsh II when the lymphocytic infiltrate is accompanied by crypt hypertrophy. The majority of untreated coeliac disease patients (50-60%) fall into the category of Marsh III, which is characterised by a moderate to severe reduction in villous height.

a.



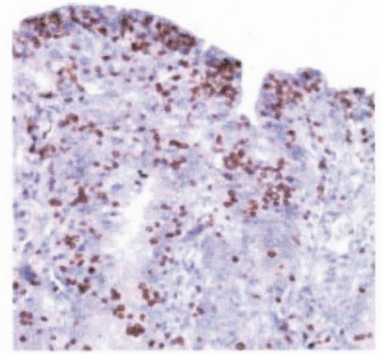
b.



Figure 1.2: Scanning electron micrographs of (a) normal duodenal (x150) and (b) coeliac mucosa (x285). The coeliac mucosa appears flat and atrophied due to the loss of villi, which normally project from the gut wall. Large holes correspond to hyperplastic crypts. Yellow particles are migrating leucocytes. (Images are taken from the Science Photo Library at www.sciencephoto.com).



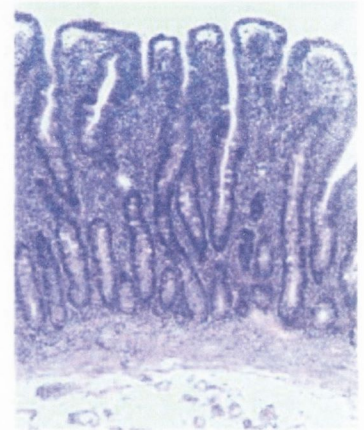
Marsh I: lymphocytic enteritis



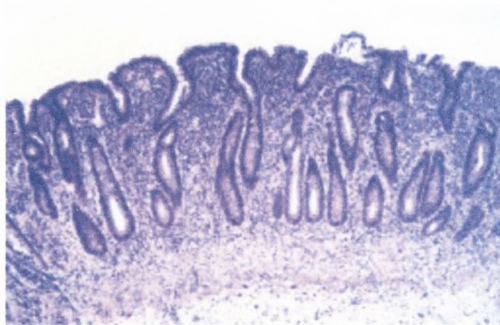
Intraepithelial lymphocytosis
HE CD3 IEL



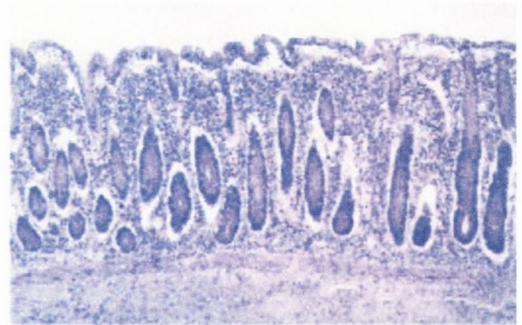
Marsh II: lymphocytic enteritis with crypt hyperplasia



Marsh III A: partial villous atrophy



Marsh III B: subtotal villous atrophy



Marsh III C: total villous atrophy

Figure 1.3 The Marsh classification of intestinal damage in coeliac disease. (Image taken from Green *et al.* 2005).

1.1.5 Diagnosis

Biopsy

The diagnosis of coeliac disease is made on the basis of an abnormal duodenal biopsy. Patients may come for biopsy as a result of clinical suspicion, positive serological tests or because of biopsies for unrelated reasons (Green and Jabri 2003). In 1970 the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) established criteria for the diagnosis of coeliac disease (Meeuwisse 1970). According to these guidelines, three biopsies were required: an initial biopsy showing a flat mucosa, a second demonstrating improvement after adherence to a gluten-free diet and a third showing deterioration after a gluten challenge. These criteria were recently revised to recognise the increased use of sensitive and specific serological tests (Walker-Smith, *et al.* 1990). By the current criteria, if atypical symptoms are present and the patient shows positive serology, a single biopsy is sufficient for diagnosis. A demonstration of mucosal improvement on a gluten-free diet may be required for some asymptomatic patients (Farrell and Kelly 2001).

Serology

Anti-gliadin antibodies

The anti-gliadin antibody (AGA) test was one of the first tests developed for the serological diagnosis of coeliac disease and was introduced in the late 1970s (Haeney and Asquith 1978). Patients with active disease often show AGA of both IgA and IgG classes, which disappear upon treatment. AGA can be measured by enzyme linked immunosorbent assay (ELISA). The AGA test has only moderate sensitivity (IgA: 75-90%, IgG: 69-85%) and specificity (IgA: 82-95%, IgG: 73-90%) (Farrell and Kelly 2001). AGA can also be detected in other gastrointestinal conditions such as inflammatory bowel disease or milk protein intolerance as well as occurring in normal individuals (Uibo, *et al.* 1993). AGA tests only retain their diagnostic usefulness in cases of IgA deficiency (IgG-AGA only) or patients younger than 2 years old.

IgA class Endomysial antibodies

The production of IgA class antibodies directed against endomysium, a connective tissue protein found between myofibrils in the gastrointestinal tract of primates, was found to be associated with coeliac disease (Chorzelski, *et al.* 1983). By using monkey oesophagus as antigenic substrate, EMAs can be visualised in an indirect immunofluorescence assay and produce a brilliant green fishnet pattern. The replacement of primate oesophagus with human umbilical cord has facilitated a wider uptake of the test (Volta, *et al.* 1995). The test has high sensitivity and specificity (89% and 98% respectively) (Collin, *et al.* 2005). It is of particular use in diagnosing latent coeliacs since EMA positive individuals with normal mucosa often later develop a flat mucosa (James and Scott 2000). However, the technique requires a suitable substrate and is labour intensive and subjective.

Anti-tissue transglutaminase antibodies

In 1997 Dieterich *et al.* identified the target of the EMA as an enzyme called tissue transglutaminase (tTG) (Dieterich, *et al.* 1997). On this basis, a solid-phase ELISA was developed for the quantitative detection of IgA class anti-tTG antibodies in serum (Dieterich, *et al.* 1998). Initial tests used tTG extracted from guinea pig liver (gptTG) as substrate in ELISAs. Since gptTG shares only 80% homology with human tTG (Dahele, *et al.* 2001) and preparations often contain antibodies, liver proteins and other contaminants, these ELISAs had high rates of false positives (Leon, *et al.* 2001). The test sensitivity was improved by replacement of gptTG with recombinant human tTG (Sblattero, *et al.* 2000) and a simple dot blot assay has been developed for use by the general physician in the clinic (Baldas, *et al.* 2000). The sensitivity (96%) and specificity (99%) of the IgA anti-tTG ELISA test has been reported to be comparable to (Burgin-Wolff, *et al.* 2002) and sometimes even more sensitive than the EMA test (Sblattero, *et al.* 2000, Kumar, *et al.* 2001). While the IgA anti-tTG is a good predictor of coeliac disease, the IgG anti-tTG test has been reported to have lower sensitivity and specificity (Dieterich, *et al.* 1998, Sulkanen, *et al.* 1998). In one such study, the IgG anti-tTG sensitivity was only 13% (Feighery, *et al.* 2003).

Anti-actin antibodies

In recent years it has been suggested that the presence of anti-actin antibodies (AAAs) correlates with severe mucosal damage in coeliac disease (Clemente, *et al.* 2000). AAAs have been detected using both immunofluorescence (Clemente, *et al.* 2003) and ELISA techniques (Granito, *et al.* 2004). Actin is the major component of cytoskeletal microfilaments and is regarded as the target of smooth muscle antibodies detected by indirect immunofluorescence on kidney sections and is closely associated with type I autoimmune hepatitis (Bottazzo, *et al.* 1976). Unlike the anti-tTG ELISA, the AAA ELISA test may represent a serological marker that correlates well with mucosal damage

1.1.6 Epidemiology

In the 1950s the incidence of coeliac disease was reported to be as low as 1/8000 in England and Wales and 1/4000 in Scotland (Davidson and Fountain 1950). Diagnosis was based upon the presence of classical symptoms such as steatorrhea, weight loss and failure to thrive. Diagnosis was subsequently improved by the development of peroral biopsy tests and malabsorption tests. Consequently, reported incidence of coeliac disease increased to 1/450-500 in Ireland (Mylotte, *et al.* 1973) and Scotland (Logan, *et al.* 1986).

As a consequence of serological testing and atypical symptom recognition, the reported incidence of coeliac disease has increased dramatically over recent years. Today coeliac disease is considered one of the most frequent genetically based diseases, occurring in 1/100-1/200 individuals (Catassi, *et al.* 1996, Csizmadia, *et al.* 1999, Hill, *et al.* 2000). Cases have been reported in the Middle East (Rostami, *et al.* 2004), South America (Trevisiol, *et al.* 2004), North Africa (Bouguerra, *et al.* 2005) and Asia (Malekzadeh, *et al.* 2005) but there is generally a greater incidence in Caucasians.

Although historically coeliac disease was often considered to be a paediatric disorder, at present more than 60% of newly diagnosed patients are adults with 15-20% being over 60 years of age (Mulder and Cellier 2005). The advent of more sophisticated screening tests such as the anti-tTG ELISA assay has allowed the identification of individuals with “latent” or “silent” coeliac disease. Owing to the diverse presentation of the disease and varying levels of severity, the “coeliac iceberg” is an analogy often applied to coeliac disease epidemiology (Figure 1.4). The majority of people with coeliac disease are asymptomatic or have mild symptoms and do not approach health care professionals for a diagnosis. If individuals have positive coeliac disease serology and flat biopsy but no symptoms, they are described as having “silent” coeliac disease. Individuals with abnormal serological results but no mucosal lesion have “latent” coeliac disease (Fraser and Ciclitira 2001).

In adults the peak incidence of coeliac disease diagnosis is in the fifth decade (Feighery 1999). A gradual rise in age of diagnosis is apparent. This could be due to the delayed introduction of gluten into the diet in childhood and increased diagnosis of older children and adults with atypical symptoms (Murray 1999, Kennedy and Feighery 2000). Females are more commonly affected than males with a female to male ratio of 3:1 having been reported (Feighery 1999).

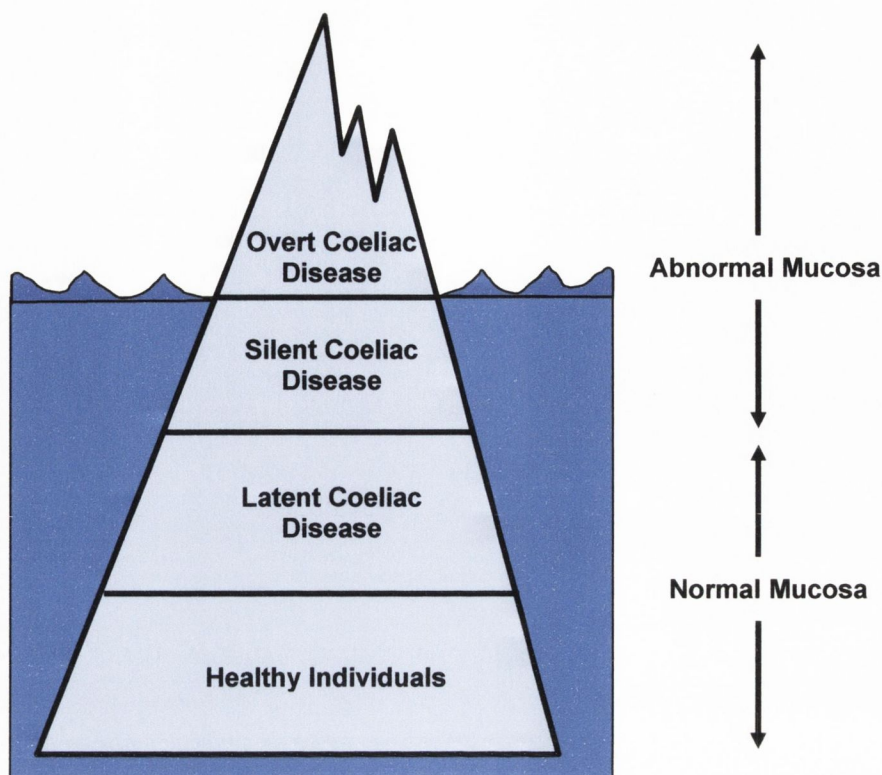


Figure 1.4: The coeliac iceberg. (Image adapted from (Fraser and Ciclitira 2001).

1.1.7 Genetics

In studies investigating the coincidence of coeliac disease in monozygotic and dizygotic twins, disease concordance rates of 75% and 11% respectively were determined (Greco, *et al.* 2002). Since monozygotic twins share 100% genetic variability and dizygotic twins share 50%, this provides good evidence for a strong genetic component in coeliac disease susceptibility.

The Human Leukocyte Antigen (HLA) Complex

The expression of the HLA-DQ2 variant is required for the presentation of disease causing dietary antigens to T cells. The HLA complex occupies a 4Mb region of chromosome 6p21 and contains some 200 genes over half of which have been associated with immune function. Association of the HLA complex with coeliac disease was first reported in 1972 by serological methods (Falchuk, *et al.* 1972). Strong linkage disequilibrium

occurs around the HLA region (Trowsdale 2005) and the first reports of association were with B8 and DR3 alleles. These variants occur on the highly conserved A1-B8-DR3 extended HLA haplotype. Interestingly, diseases such as type I diabetes and autoimmune thyroid disease are associated with this haplotype and coeliac disease patients are at substantially higher risk for these disorders. More recent studies have determined that the strongest association with coeliac disease is with HLA-DQ2. Particular susceptibility is associated with the combination of alleles encoding the alpha and beta chain variants DQA1*05 and DQB1*02 of the DQ2 heterodimer (Sollid, *et al.* 1989).

The function of the DQ2 molecules is in the presentation of exogenous peptide antigens (gliadin peptides in the case of coeliac disease) for scrutiny by helper T cells (Eisenlohr and Rothstein 2005) and is therefore expressed on antigen presenting cells (APCs) such as monocytes, macrophages and dendritic cells. The evidence for the involvement of DQ2 provided by initial genetic association studies was strengthened by subsequent functional studies. An immuno-dominant gliadin peptide was shown to be recognised by DQ2-restricted T cells in the intestine (Arentz-Hansen, *et al.* 2000) and in peripheral blood after antigen challenge (Anderson, *et al.* 2000). The crystal structure of dominant epitope-DQ2 binding was also resolved (Kim, *et al.* 2004).

The HLA-DQ heterodimer can be encoded in *cis* (on the same haplotype) or more rarely in *trans* where the monomer subunits are encoded on separate haplotypes (van Heel, *et al.* 2005) (Figure 1.5). Although the *cis* and *trans* variants are known to vary by one amino acid in each subunit, the ability of the variants to present antigen is thought to be identical.

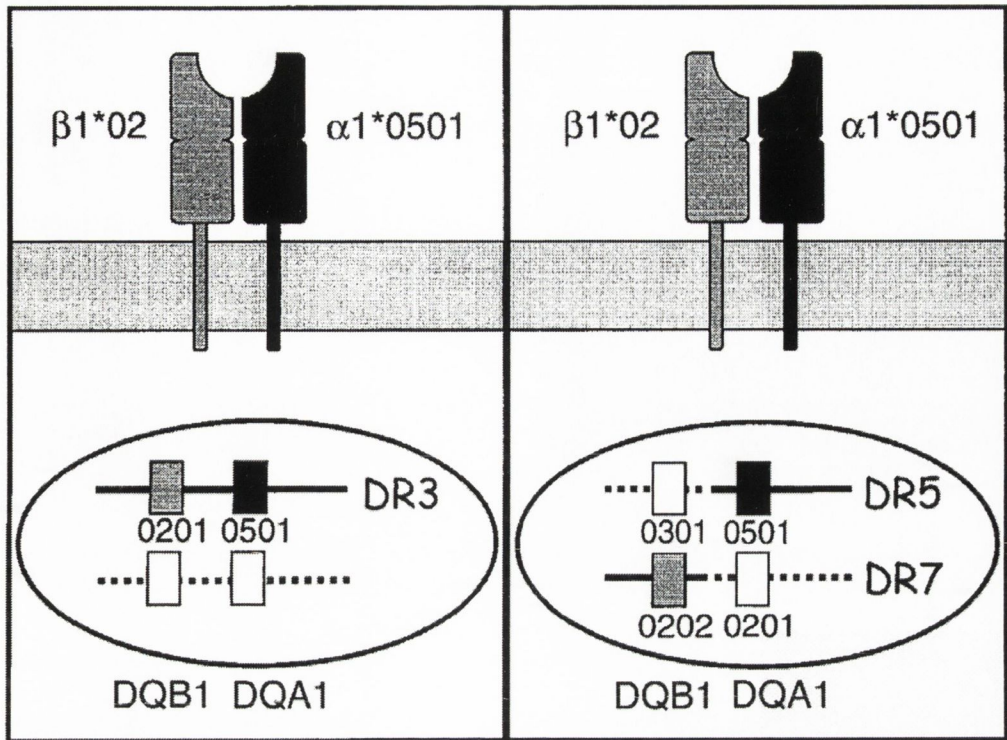


Figure 1.5: The HLA-DQ2 molecule can be encoded in *cis* or *trans*. Individuals who are DR3 or DR5/DR7 heterozygous express the same HLA-DQ2 molecule, HLA-DQ($\alpha 1^*0501$, $\beta 1^*02$). The DQA1*0501 and DQB1*02 genes are located in *cis* (on the same chromosome) in DR3 individuals, whereas they are located in *trans* (on opposite chromosomes) in DR5/DR7 heterozygous individuals. (Image taken from Sollid 2000).

Around 90% of coeliac disease patients express the DQ2 molecule but in more rare cases the disease is associated with other related HLA molecules. Expression of the HLA-DQ8 molecule (serologically DR4-DQ8 and at the genetic level DQA1*03-DQB1*0302) without DQ2 was found in 6% of coeliac disease patients in a recent large scale study (Karell, *et al.* 2003). Interestingly, this study also identified 6% of patients without the classical DQ2 molecule that expressed only either the alpha or beta chain of the heterodimer. Only 0.4% of cases did not express DQ2 in part or DQ8.

Since the HLA-DQ2 allele is expressed in 30% of the Caucasian population (Sollid, *et al.* 1989) and only a very small proportion of these individuals develop coeliac disease, there must be other genetic or

environmental factors that contribute to susceptibility. Several studies have attempted to map susceptibility loci within the HLA region. McManus and co-workers (1996) have described associations between coeliac disease and microsatellite polymorphisms close to the tumour necrosis factor genes (McManus, *et al.* 1996). Two separate studies have found significant associations between coeliac disease and polymorphisms in the MICA gene (Fernandez, *et al.* 2002, Bolognesi, *et al.* 2003). Unfortunately, the strong linkage disequilibrium in the HLA region is difficult to control for and may be responsible for at least some of these findings.

Genome-Wide Linkage Studies

Eleven genome-wide searches for linkage regions have been performed in coeliac disease, as well as numerous region specific linkage studies (van Heel, *et al.* 2005). Most studies have identified strong linkage to the HLA region, thus validating previous HLA associations. Two loci of major interest on chromosome 5 and 19 have emerged as a result of genome-wide linkage studies.

CD28-CTLA4-ICOS Variants

Although the region coding for the CTLA4 gene has shown linkage to coeliac disease in several genome-wide scans (van Heel, *et al.* 2005), initial studies on CTLA4 were based upon known function (Djilali-Saiah, *et al.* 1998). The T cell regulatory genes CD28, CTLA4 and ICOS are found within a 300kb block of chromosome 2q33 and much is known about their function. Engagement of CD28 by the B7 ligands on APCs provides a potent co-stimulatory signal to T cells. Engagement of ICOS by B7-H2 also augments activation and cytokine secretion by T cells. CTLA4 is upregulated on T cells upon activation and has a higher affinity for the B7 complex than CD28, providing a negative signal to regulate T cell activation (Greenwald, *et al.* 2005). Thus, all three molecules have important functions in T cell regulation. High levels of CTLA4 expression on CD4⁺CD25⁺ regulatory T cells also suggests an important role for the molecule in T cell regulation (Tang, *et al.* 2004).

Independent analysis of the 2q33 region have been carried out by twelve groups (van Heel, *et al.* 2005). Most studies have analysed single nucleotide polymorphisms (SNPs) within the CD28/CTLA4/ICOS region. Djilali-Saiah *et al.* (1998) discovered an SNP at the +49 position in the first exon of CTLA4 associated with coeliac disease (Djilali-Saiah, *et al.* 1998). This association has been confirmed in only 3 out of 13 follow-up studies genotyping this variant. The +49 mutation results in a threonine to alanine change in the leader peptide that is associated with decreased glycosylation of the signal peptide, altered processing in the endoplasmic reticulum and lower cell surface levels on transfected cells (Anjos, *et al.* 2002).

Tissue transglutaminase

Since tissue transglutaminase plays an important role in the pathogenesis of coeliac disease (see Section 1.2.10) the possible contribution of germline mutations in the tissue transglutaminase gene (TGM2) to the pathogenesis of coeliac disease have been investigated in 2 studies (Aldersley, *et al.* 2000, Popat, *et al.* 2001). In a study carried out in England the TGM2 sequence of eight controls and four coeliac disease patients were compared. Although the DNA sequencing revealed five differences from the previously published sequence (Genbank M55153), there was no difference between the control and coeliac disease TGM2 sequence (Aldersley, *et al.* 2000). In a larger Swedish study analysing the TGM2 sequence of 52 children with coeliac disease and their parents, three novel polymorphisms were discovered but there was no evidence that they contributed to pathogenesis (Popat, *et al.* 2001).

Gene expression profiling

The application of microarray technology has become a powerful tool in the search for candidate disease-causing genes (Gu, *et al.* 2002). Gene expression profiling has been carried out in two coeliac disease studies (Diosdado, *et al.* 2004, Juuti-Uusitalo, *et al.* 2004). The Finnish study (Juuti-Uusitalo, *et al.* 2004) used a 5,000 gene array to compare duodenal biopsies from untreated coeliac disease patients, coeliac disease patients on a gluten-free diet and normal individuals (n=4 each).

Sixty genes showed changes of >1.25-fold in expression levels in all sample pairs. No genes from previously described candidate regions (2q, 5q or 15q) showed consistent expression changes although only 12% of genes from these regions were included in the array. In the study carried out in the Netherlands the biopsies of 15 coeliac disease patients with villous atrophy were compared to 7 controls using arrays containing 20,000 human genes (Diosdado, *et al.* 2004). Genes and pathways were identified that may have roles in the pathogenesis of coeliac disease enteropathy.

1.1.8 Cereals

Cereals are members of the monocotyledonous grass family, Gramineae, which contains four subfamilies. The subfamily Pooidea includes the Aveneae or oats tribe and the Triticeae to which wheat, rye and barley belong (Shewry 1992). There is much debate as to the safety of oats in the coeliac diet (Kilmartin, *et al.* 2003, Lundin, *et al.* 2003, Storsrud, *et al.* 2003) and the reduced harmfulness of oats is reflective of the relative evolutionary distance from the more damaging Tritaceae.

Cereal Proteins

Cereal grains contain about 7-16% extremely complex protein. For example, isoelectric focusing and SDS-PAGE of wheat kernel proteins yields over a thousand individual bands (Tkachuk and Mellish 1987). Three types of cereal protein can be recognised: structural, metabolic and storage proteins. The latter makes up 70-80% of total grain protein and is the only fraction that acts as causative agent of coeliac disease.

Cereal proteins have been traditionally separated into four groups according to their solubility (Osborne fractions): albumins (water soluble), globulins (soluble in dilute salt concentrations), prolamins (soluble in aqueous alcohols) and glutelins (insoluble in the applied solvents). Prolamins constitute the storage proteins of cereals (Weiser 1992). The prolamin fractions of the different cereals have been named individually

as gliadin (wheat), secalin (rye), hordein (barley) and avenin (oats). The glutelin fraction of wheat has been named glutenin.

The amino acid content of prolamins show a close relationship to the phylogeny of the cereals and to cereal toxicity (Weiser, *et al.* 1980). The prolamins of the Tritaceae are characterised by high levels of proline (17-23 mol-%) and glutamine (35-37 mol-%), hence the title "prolamins". In contrast the prolamins of rice, millet and maize are lower in glutamine and proline, but rich in alanine (9-14 mol-%) and leucine (12-19 mol-%). Oats lie somewhere in between with glutamine content similar to the Triticeae but proline and Leucine values similar to the other species.

Gliadin and glutenin form gluten, which is formed when a ball of wheat flour dough is exhaustively washed in tap water. Gluten is involved in the trapping of CO₂ in bread dough and ultimately its elasticity and strength. No nutritional value has been attributed to gluten (Ciccocioppo, Di Sabatino and Corazza 2005).

Gliadin

Gliadin and the related prolamins are responsible for the mucosal pathology associated with coeliac disease but only gliadin has been studied in great detail. Gliadin is an extremely heterogeneous mixture of proteins with at least 40 components that have been traditionally assigned to four major groups (α -, β -, γ -, or ω -gliadins) based upon their electrophoretic mobility at acidic pH (Jones, *et al.* 1959). More recently gliadins have been separated into three groups (α -, γ -, or ω -gliadins) according to their N-terminal amino acid sequences (Wieser 1987).

1.1.9 Dermatitis Herpetiformis

Dermatitis herpetiformis is a skin manifestation of the intestinal response to ingested gluten and is characterised by the deposition of IgA granules at the dermal-epidermal junction (Fry 1995). The extremely itchy and bullous rash associated with the condition is usually found at the extensor surfaces of the limbs, trunk and scalp (Murray 1999). The incidence of dermatitis herpetiformis was described as 11.2 per 100,000 population in a North American study but reports of incidence vary (Zone 2005). Dermatitis herpetiformis affects men slightly more frequently than women (Nicolas, *et al.* 2003). Although dermatitis herpetiformis is initiated in the gut, the small bowel morphology in dermatitis herpetiformis patients is characterised by a more patchy distribution of a much milder pathology when compared to coeliac disease. In 2002, Sárdy and co-workers demonstrated that the predominant autoantigen of dermatitis herpetiformis is epidermal transglutaminase (Sardy, *et al.* 2002). The targeting of epidermal transglutaminase by dermatitis herpetiformis IgA helps explain the skin manifestations seen in this subclass of gluten-sensitive disease. Dermatitis herpetiformis patients also produce autoantibodies directed against tTG (Karpati 2004). Epidermal transglutaminase and tTG share an overall homology of 38% at the amino acid level, but with up to 64% homology in certain regions (Kim, *et al.* 1993).

1.2 Tissue Transglutaminase

The transglutaminases are a widely distributed group of enzymes primarily responsible for the post-translational modification of proteins by the introduction of cross-links. The cross-linked products, often of high molecular mass, are highly resistant to mechanical challenge and proteolytic degradation and their accumulation is found in a number of tissues where such properties are important including skin, hair, blood clotting and wound healing (Griffin, *et al.* 2002). Transglutaminase mediated cross-links are carried out by the exchange of primary amines for ammonia at the γ -carboxamide group of glutamine or lysine residues (Greenberg, *et al.* 1991).

The term “transglutaminase” was first used by Heinrich Waelsch and co-workers in 1959 to describe the calcium dependent transamidating activity present in the liver and some other organs of the guinea pig (Mycek, *et al.* 1959). This transglutaminase, now designated tissue transglutaminase (tTG) or TG2, is considered a very enigmatic enzyme with putative roles in wound healing (Verderio, *et al.* 2004), extracellular matrix (ECM) interactions (Gaudry, Verderio, Aeschlimann, *et al.* 1999, Gaudry, Verderio, Jones, *et al.* 1999, Balklava, *et al.* 2002), cell signalling (Hwang, *et al.* 1995, Mhaouty-Kodja 2004), apoptosis (Fesus, *et al.* 1987, Melino and Piacentini 1998), CD8⁺ T cell extravasation (Mohan, *et al.* 2003) and guanosine triphosphate (GTP) hydrolysis (Iismaa, *et al.* 1997). tTG is also recognised as the predominant autoantigen in coeliac disease and the detection of antibodies directed against tTG is a very sensitive and specific test for coeliac disease (Reif and Lerner 2004).

1.2.1 Enzymology

Transglutaminases are recognised by the Enzyme Commission on Nomenclature as an R-glutaminy-peptide: amine γ -glutaminy transferase (EC 2.3.2.13) (Greenberg, *et al.* 1991). The transglutaminase-catalysed acyl-transfer reaction results in the formation of new γ -amide bonds between γ -carboxamide groups of peptide-bound glutamine residues and

various amines (Figure 1.6). A glutamine residue serves as an acyl-donor and the most common acyl acceptors are ϵ -amino groups of peptide-bound lysines or primary amino groups of some naturally occurring polyamines, like putrescine or spermidine (Aeschlimann and Paulsson 1994). While transglutaminases have a broad specificity for primary amine acceptors, relatively few proteins contain glutamine residues that form acyl-enzyme intermediates (Greenberg, *et al.* 1991). The number of proteins that can serve as glutamine substrates is restricted as both primary structure (including charge) and conformation determine whether a glutamine residue is available for reaction with transglutaminase (Folk and Finlayson 1977, Gorman and Folk 1981, Gorman and Folk 1984, Aeschlimann, *et al.* 1992).

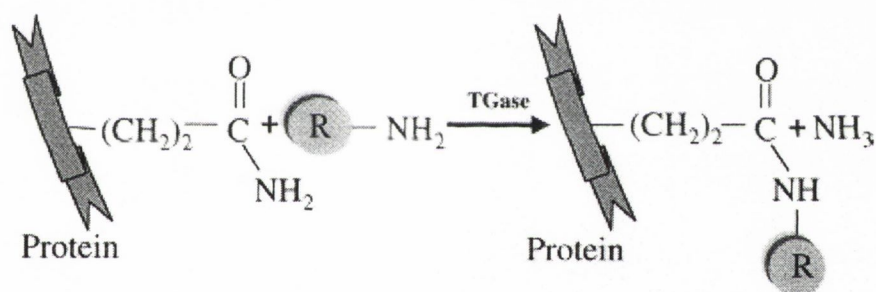


Figure 1.6: The transamidation reaction catalysed by transglutaminases. Transglutaminases catalyse an -acyl transfer reaction between the γ -carboxamide group of a protein-bound glutamine residue and the primary amine group of either a protein-bound lysine residue or other polyamine molecules (designated $R-NH_2$). The reaction results in a posttranslational modification through either the formation of an irreversible protein cross-link or the incorporation of a polyamine into an -acyl donor protein. (Image adapted from Chen and Mehta 1999).

The cross-linking reaction is a multi step process. Initially, the active site cysteine reacts with the γ -carboxamide group of a glutamine residue to form the acyl-enzyme intermediate with the simultaneous release of ammonia. In the second step, the complex reacts with the primary amine to form an iso-peptide bond and liberate the reactivated enzyme. Enzyme kinetics proceed with a Michaelis-Menton type of saturation kinetics (Aeschlimann and Paulsson 1994).

1.2.2 The Transglutaminase Family

In mammals, eight distinct transglutaminase isoenzymes have been identified by genetic analysis (Grenard, *et al.* 2001). Six of these have been isolated and characterised to varying extents and include (1) the zymogen Factor XIII which is activated by thrombin and has a critical role in the generation of a viable haemostatic plug (Lorand 2001); (2) keratinocyte transglutaminase (transglutaminase 1) which is involved in the terminal differentiation of keratinocytes (Rice, *et al.* 1992); (3) tTG (transglutaminase 2) a ubiquitous, multifunctional enzyme and the predominant autoantigen in coeliac disease; (4) the epidermal/hair transglutaminase (transglutaminase 3) which, like transglutaminase 1, is involved in the terminal differentiation of keratinocytes (Hitomi, *et al.* 2003); (5) the prostatic secretory transglutaminase (transglutaminase 4) which is essential for fertility in rats (Dubbink, *et al.* 1998) and (6) transglutaminase 5 which cross-links loricrin, involucrin and small proline-rich proteins and can trigger cell death (Candi, *et al.* 2001, Cadot, *et al.* 2004). Details of all known transglutaminases are summarised in Table 1.1.

Table 1.1 Characterised members of the transglutaminase family

Transglutaminase	Synonyms	Residues (Molecular Mass)	Gene	Gene locus	Function
Factor XIII	Fibrin stabilising factor	732 (83 kDa)	F13A1	6p24-25	Blood clotting Wound healing
Type 1	Keratinocyte transglutaminase	814 (90 kDa)	TGM1	14q11.2	Cell envelope formation in keratinocyte differentiation
Type 2	Tissue transglutaminase	687 (77 kDa)	TGM2	20q11-12	Cell death, differentiation, adhesion, wound healing etc.
Type 3	Epidermal transglutaminase	692 (77 kDa)	TGM3	20q11-12	Cell envelope formation in keratinocyte differentiation
Type 4	Prostate transglutaminase	683 (77 kDa)	TGM4	3q21-22	Reproductive functions in rats
Type 5	Transglutaminase X	719 (81 kDa)	TGM5	15q15.2	Epidermal differentiation
Type 6	Transglutaminase Y		TGM6	20q11 15	Not characterised
Type 7	Transglutaminase Z	710 (80 kDa)	TGM7	15q15.2	Not characterised

Transglutaminase enzymes are ancient with equivalent isoenzymes detectable in bacteria. The SWISS-PROT protein knowledge database (PDB) contains transglutaminase sequences from *Streptovorticillium* sp, the filarial worm *Dirofilaria immitis* and the nematode *Caenorhabditis elegans*. All mammalian transglutaminases have some structural homology and are the product of different genes arising from duplication, rearrangement and chromosomal shifts (Grenard *et al.* 2001) and are all members of the papain-like superfamily of cysteine proteases. All member of this papain-like superfamily possess a catalytic triad of Cys-His-Asp or Cys-His-Asn.

1.2.3 Structure of Tissue Transglutaminase

A four-domain organisation is highly conserved among members of the transglutaminase family (Griffin, *et al.* 2002). The 3D structure and some of the functional domains of tTG are shown in Figure 1.7 a & b. The four domains of the enzyme consist of an amino terminus β -sandwich domain (amino acids 1-139), a prevalently α -helical catalytic core (amino acids 147-460) and two carboxy terminal β -barrel domains (amino acids 472-583 and 584-687) (Fesus and Piacentini 2002). The conservation of amino acid sequence across species is quite high when compared to the human sequence at 88% from bovine, 84% from mouse, 80% from guinea pig and 65% from chicken (Aeschlimann and Paulsson 1994). The active site of human tissue transglutaminase has been identified as cysteine 277 with the other elements of the papain-like catalytic triad found at histidine 335 and aspartic acid 358 (Murthy, *et al.* 2002). tTG has also been shown to have GTPase activity (Nakaoka, *et al.* 1994). GTP binding and hydrolysis is thought to involve S171, K173, Y174, R476, R478, V479, M483, R580 and Y583 (Fesus and Piacentini 2002, Griffin, *et al.* 2002). tTG is not glycosylated (Folk and Finlayson 1977) and contains no disulphide bonds (Folk 1968, Boothe and Folk 1969) although it contains 17 cysteine residues and 5 or 6 potential sites for N-linked glycosylation.

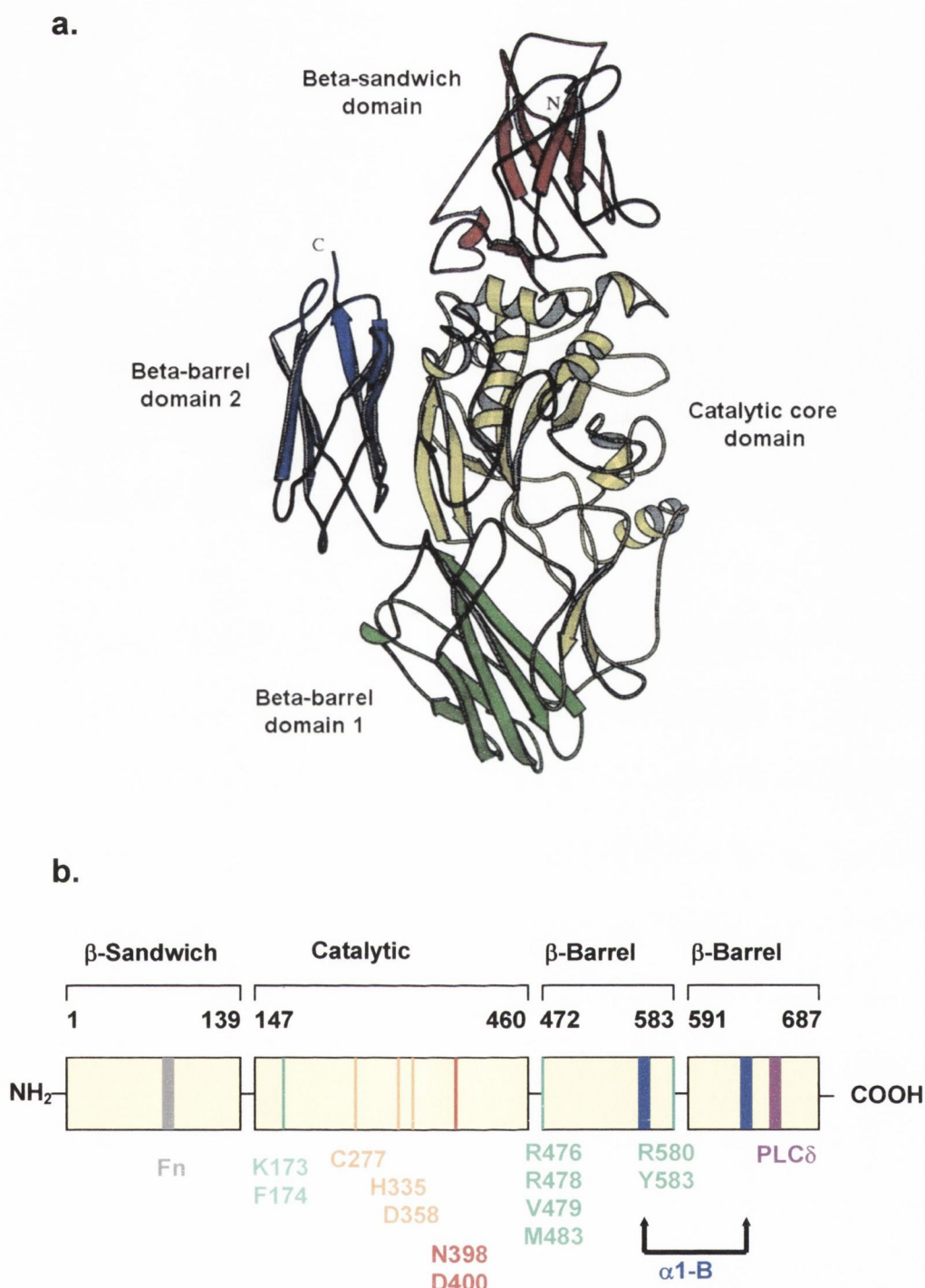


Figure 1.7: Structure and functional domains of tissue transglutaminase. (a) The 3D structure of tTG. (Image adapted from (Casadio, *et al.* 1999b)) (b) Domain organisation of tTG. The guanine nucleotide binding sites are shown in green. Fibronectin binding, catalytic residues (C277, H335, D358), putative Ca²⁺ binding sites (N398, D400), α1B-adrenergic receptor and PLCδ1 binding domains are respectively shown in grey, orange, red, blue and purple. (Image adapted from Mhaouty-Kodja, 2004).

Although tTG is associated with extracellular function, it does not contain a hydrophobic leader sequence necessary for translocation of proteins to the endoplasmic reticulum (Gaudry, Verderio, Aeschlimann, *et al.* 1999). There is evidence for the externalisation of tTG in various tissues (Barsigian, *et al.* 1991, Aeschlimann, *et al.* 1993, Kojima, *et al.* 1993) and is likely to take place via an alternative pathway of protein secretion.

The amino terminus region of tTG has been shown to interact with fibronectin (Gaudry, Verderio, Aeschlimann, *et al.* 1999). The interaction was believed to involve amino acids 1-4 (Upchurch, *et al.* 1987) but more recent work using deletion mutant analysis suggests the fibronectin domain is located between amino acids 88 and 106 (Hang, *et al.* 2005). Amino acids 665-672 have been shown to interact with phospholipase C and play a role in signal transduction events (Hwang, *et al.* 1995).

1.2.4 Regulation of Tissue Transglutaminase Activity

The transglutaminase and GTPase activities of tTG are regulated by the binding of Ca^{2+} and GTP. In the absence of calcium ions, the enzyme assumes a latent conformation and the activity of Cys²⁷⁷ is decreased either by hydrogen-bonding with the phenolic hydroxyl group of Tyr⁵¹⁶ or by formation of a disulphide bond with the neighbouring Cys³³⁶ (Noguchi, *et al.* 2001). In this “closed” conformation the enzyme exhibits GTPase activity. The nucleotide is thought to bind at Lys¹⁷³ and is hydrolysed in a process thought to involve Ser¹⁷¹. tTG mutants lacking Lys¹⁷³ and Ser¹⁷¹ lack GTPase activity but retain transamidation activity (Iismaa, *et al.* 2000).

Studies involving small-angle scattering (Mariani, *et al.* 2000), protein dynamics (Casadio, *et al.* 1999a), site-directed mutagenesis (Murthy, *et al.* 2002) and crystallography (Liu, *et al.* 2002) show that the binding of calcium by tTG results in a conformational change, revealing the catalytic triad (Figure 1.8). Upon calcium binding to α -helix H₄, the structure unfolds, perturbing the structure of the neighbouring loop 455-478, which connects domains 2 and 3, and also the spatial location of domains 3 and

4, which move from each other and domain 2. Trp²⁴¹ is also crucial to this process and is thought to stabilise the transition-state intermediate (Murthy, *et al.* 2002).

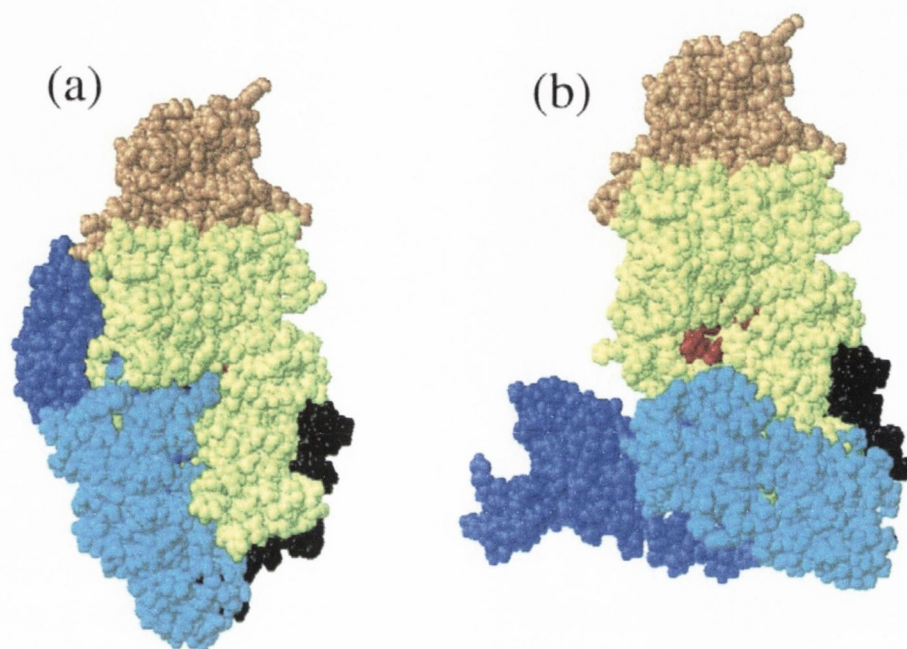


Figure 1.8: Three-dimensional representation of possible structures of tTG with GTP and with Ca²⁺, reconstructed from SANS profiles by a Monte Carlo method and starting from the computer-designed model and molecular dynamics results. (a) Conformation of GTP-bound tTG. (b) Conformation of Ca²⁺-bound tTG. Domains are colour coded: β -sandwich (*orange*); core (*yellow*); β -barrel 1 (*cyan*) and β -barrel 2 (*blue*). Catalytic triad residues are coloured red (Image taken from Mariani *et al.* 2000).

Given the concentration of calcium ions in the cytosol, it is generally accepted that the transamidating activity of tTG is absent intracellularly (Lai, *et al.* 1997). However, in circumstances associated with calcium influx, such as apoptosis, the cross-linking activity of tTG is thought to be activated (Oliverio, *et al.* 1999). In fact, it has been well established that tTG is one of the few genes induced during apoptosis (Piacentini, *et al.* 1991, Knight, *et al.* 1993, Amendola, *et al.* 1996, Nagy, *et al.* 1997).

1.2.5 Regulation of Tissue Transglutaminase Expression

The expression of tTG has been shown to be upregulated several-fold by retinoic acid *in vitro* and *in vivo* (Chiocca, *et al.* 1988, Nakanishi, *et al.* 1991, Zhang, *et al.* 1995). This increase in tTG expression is evident at mRNA and protein level in several cell types such as peritoneal macrophages (Moore, *et al.* 1984), aortic endothelial cells (Nara, *et al.* 1989) and hepatocytes (Piacentini, *et al.* 1992). The mediators of retinoic acid-induced tTG expression have been shown to be nuclear receptors for *trans*-retinoic acid, RAR- β and RAR- γ , as well as for *cis*-retinoic acid, RXR's. Characterisation of the promoter region of guinea pig liver tissue transglutaminase showed the presence of consensus motifs of the estrogen receptor-type binding which may function as retinoic acid responsive elements (Suto, Ikura, Shinagawa, *et al.* 1993).

Several cytokines have been implicated in tTG expression induction. The guinea pig liver transglutaminase promoter contains presumptive response elements for IL-6 (Suto, Ikura, Shinagawa, *et al.* 1993) and the induction of tTG expression by IL-6 has been demonstrated in hepatocytes (Suto, Ikura and Sasaki 1993). Given the involvement of tTG in coeliac disease and the increased levels of tumour necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β) and interferon- γ associated with the condition, Kim and co-workers (2002) investigated the ability of these cytokines to induce tTG expression in rat small intestinal cells. Their results showed that while TGF- β decreased tTG mRNA and cross-linking activity, interferon- γ treatment resulted in a 2-fold increase after 24 hours and a 5-fold increase after 5 days (Kim, *et al.* 2002). Conversely, Ritter and Davies (1998) identified a TGF- β response element in the tTG promoter region (Ritter and Davies 1998). TGF- β has also been shown to induce IL-6 expression by intestinal epithelial cells (McGee, *et al.* 1992), which could, in turn, induce tTG expression.

1.2.6 Tissue Transglutaminase and the Extracellular Matrix

tTG serves as an integrin-binding adhesion co-receptor for fibronectin. It has been demonstrated that tTG strongly promotes fibronectin assembly mediated by $\alpha 5\beta 1$ integrin (Fesus and Piacentini 2002). It has been estimated that 5-40% of $\beta 1$ integrins could be complexed with tTG and that all tTG on the cell surface is complexed with integrins in a ratio of 1:1. This interaction does not involve the cross-linking activity of tTG and facilitates adhesion, spreading and motility of cells (Akimov and Belkin 2001b, Balklava, *et al.* 2002).

tTG has been assigned a role in wound-healing and angiogenesis. Haroon and co-workers (1999) showed that tTG is expressed, active and involved in the process of dermal wound healing in rats (Haroon, *et al.* 1999). tTG has also been shown to be involved in the assembly, remodelling and stabilisation of the ECM in various tissues. This is carried out by cross-linking fibronectin, fibrinogen/fibrin, von Willebrand factor, vitronectin, lipoprotein 'a', dermatane sulphate proteoglycans, collagen V, osteonectin, laminin, nidogen and osteopontin (Aeschlimann and Thomazy 2000).

tTG has also been implicated in the activation of several soluble factors. TGF- β is produced in a latent form, bound to latency-associated protein (LAP). This small latent complex can form a large latent complex by binding to latent TGF- β binding proteins (LTBPs). This large latent complex is incorporated into the ECM by tTG (Taipale, *et al.* 1996, Nunes, *et al.* 1997). Since TGF- β is constitutively expressed by many cell types in culture, this suggests that regulation of activity is dependent upon subsequent activation (Munger, *et al.* 1997). It is possible that the sequestering of the large latent complex to the ECM is an important step in the process of activating latent TGF- β (Kojima, *et al.* 1993). TGF- β promotes transcriptional regulation of many ECM genes (Fesus and Piacentini 2002) possibly including tTG itself (Ritter and Davies 1998).

1.2.7 Tissue Transglutaminase and Apoptosis

tTG has been shown to be induced in cells undergoing apoptosis *in vivo* and $\epsilon(\gamma\text{-glutaminy})$ lysine cross-links have been detected in apoptotic bodies (Fesus, *et al.* 1987). In one study, the overexpression of tTG has been associated with an increased rate of apoptosis (Piacentini, *et al.* 2002). It is thought that tTG sensitises cells to apoptosis by interacting with mitochondria and shifting them to a higher polarised state and altered redox status. In other studies it has been shown that inhibition of tTG has been associated with an increased survival of cells (Oliverio, *et al.* 1999). It is thought that the cross-linking activity of tTG is activated by the influx of calcium ions that is associated with the initiation of apoptosis. This cross-linking activity leads to the polymerisation of cytoskeletal proteins such as actin (Nemes, *et al.* 1997) and produces a detergent insoluble scaffold (Fesus, *et al.* 1989) responsible for stabilising the apoptotic body before its phagocytosis. In this fashion tTG may play a role in the prevention of autoimmunity that is potentially caused by the leakage of components from apoptotic bodies (Szondy, *et al.* 2003).

It should be noted that other studies have observed that apoptosis proceeds in the absence of tTG induction (Monczak, *et al.* 1997) and, indeed, its overexpression might even promote cell survival by inhibition of programmed cell death (Benedetti, *et al.* 1996, Grabarek, *et al.* 2002).

1.2.8 Tissue Transglutaminase and Signal Transduction

Gh protein is a protein complex made up of α (74-85kDa) and β (50kDa) subunits that has been associated with signalling downstream of the α 1-adrenergic receptors (Mhaouty-Kodja 2004). The α 1-adrenergic receptors play an important role in glycogenesis and sympathetic nervous system responses, particularly those involved in arteriolar smooth muscle and cardiac contractions (Im, *et al.* 1997). In 1994, Nakoaka and co-workers identified the Gh α subunit as tTG (Nakoaka, *et al.* 1994) and more recently, the Gh β subunit was identified as calreticulin (Feng, *et al.* 1999). Interaction of the Gh complex with α 1-adrenergic receptors switches

transglutaminase activity off and dissociates GTP-bound tTG from calreticulin. tTG then activates phospholipase C δ 1. This interaction involves the region spanning Leu⁶⁶¹-Lys⁶⁷² of tTG and the region spanning Thr⁷²¹ and Leu⁷³⁶ of the C2 domain of phospholipase C δ 1 (Feng, *et al.* 1996, Kang, *et al.* 2002). Activation of phospholipase C δ 1 results in phosphoinositide hydrolysis and an increase in intracellular calcium. This process is illustrated in Figure 1.9.

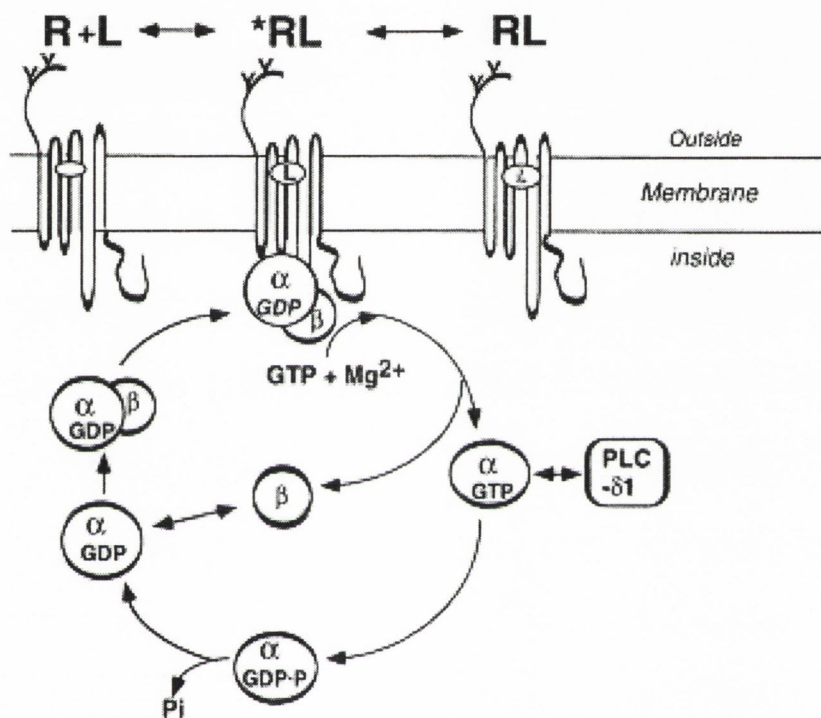


Figure 1.9: The GTPase cycle of the Gh singling complex and the α_{1B} -adrenoceptor-mediated transmembrane signalling. R is the receptor and R* is agonist-bound receptor. RL is agonist-bound receptor, but not associated with G_h . G_h consists of tTG ($G\alpha_h$) and calreticulin ($G\beta_h$). The β -subunit regulates the α -subunit function by locking the α -subunit in a GDP-bound form. The α -subunit directly interacts with the receptor and PLC in the activation process. (Image taken from Im *et al.* 1997).

1.2.9 Tissue Transglutaminase in Disease

Neurodegenerative disease

Several studies investigating Alzheimer's disease have reported increased levels of tTG in the brains of affected individuals (Selkoe, *et al.* 1982, Ikura, *et al.* 1993, Appelt, *et al.* 1996, Zhang, *et al.* 1998, Kim, *et al.* 1999). This is in agreement with the fact that there are more $\epsilon(\gamma\text{-glutamyl})$ lysine linkages in insoluble proteins in Alzheimer's disease brain tissue than in normal tissue (Karpuj and Steinman 2004). Whether this transglutaminase activity is a result or a cause of disease activity is, as of yet, unknown.

A more specific role for tTG has been described in Huntington's disease. Genes with expanded CAG triplet repeats within their coding regions cause at least seven adult-onset neurodegenerative diseases including Huntington's disease (Cooper, *et al.* 1997). The number of CAG repeats is generally ≥ 40 in affected individuals and ≤ 36 in unaffected individuals. It has been shown that these poly-Q proteins are excellent substrates for tTG and are cross-linked to lysine-containing proteins contributing to the brain plaques associated with Huntington's disease (Kahlem, *et al.* 1996, Cooper, *et al.* 1997).

Coeliac Disease

tTG is the predominant autoantigen in the gluten-sensitive enteropathy, coeliac disease (CD) (Dieterich, *et al.* 1997). Antibodies directed against tTG provide a very sensitive and specific test for coeliac disease (Reif and Lerner 2004) and in recent years a role has been assigned to tTG in the molecular pathogenesis of coeliac disease (van de Wal, Kooy, van Veelen, Pena, Mearin, Papadopoulos, *et al.* 1998).

1.2.10 The Role of Tissue Transglutaminase in Coeliac Disease

Gluten and DQ2

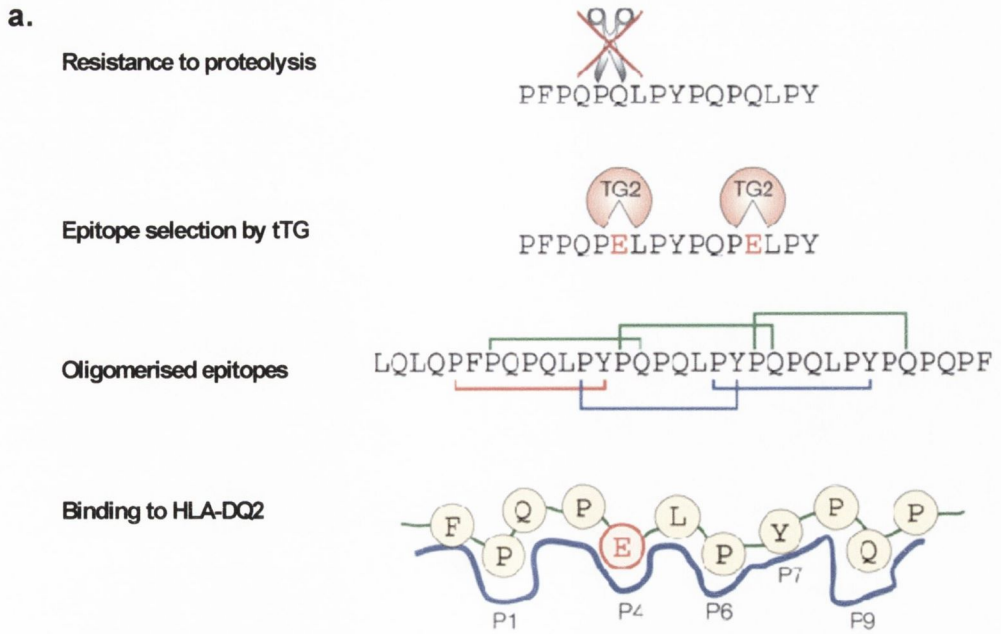
The near exclusive association of coeliac disease with DQ2 and DQ8 suggests an important role for these MHC class II molecules in the disease pathogenesis. In fact, it has been shown in many studies that gliadin peptides that are shown to be immunogenic in coeliac disease have a pivotal interaction with the DQ2 molecule (Dewar, *et al.* 2004). The peptide-binding groove of DQ2 has been extensively studied. Analysis of peptide fragments eluted from DQ2 have shown a predilection for negatively charged residues at positions 4, 6 and 7 of the nonapeptide binding-groove (van de Wal, *et al.* 1996). The fact that gluten does not contain significant numbers of negatively charged residues initially confounded the proponents of a role for DQ2 in coeliac disease pathogenesis.

Several studies have identified peptide fragments from gliadin or glutenin that stimulate T cell clones derived from coeliac disease patients in a DQ2 or DQ8-restricted manner (Johansen, *et al.* 1996, van de Wal, Kooy, van Veelen, Pena, Mearin, Molberg, *et al.* 1998, van de Wal, *et al.* 1999). The optimal requirement for MHC-binding and T cell stimulation is a 10-15 residue peptide, many of which are generated during peptic-tryptic digest of gluten (Dewar, *et al.* 2004). An example of one such sequence is derived from α -gliadin and contains two overlapping immunodominant epitopes, to which the vast majority of adult coeliac T cell lines respond (Arentz-Hansen, *et al.* 2000, Arentz-Hansen, *et al.* 2002). These peptides have also been shown to be resistant to normal luminal digestion (Hausch, *et al.* 2002, Shan, *et al.* 2002). However, a study carried out by Vader *et al.* (2002) showed that approximately half of T cell clones from children with coeliac disease do not respond to these immunodominant epitopes, suggesting a possible difference between childhood and adult coeliac disease. It seems that coeliac disease small intestinal T cells respond to a variety of epitopes but it has been suggested that after response to a wide array of epitopes in childhood, the response focuses on immunodominant sequences in later life (van de Wal, *et al.* 1999).

Tissue transglutaminase activity increases gliadin-DQ2 binding

In coeliac disease, tTG has been shown to have an important role in modifying gliadin epitopes prior to recognition by T cells (Arentz-Hansen, *et al.* 2000, Arentz-Hansen, *et al.* 2002). It has been shown that in the absence of a suitable lysine donor and in the presence of dH₂O, tTG deamidates its target glutamine, creating a glutamic acid residue (Molberg, *et al.* 2000). The deamidation of specific glutamine residues in gliadin peptides by tTG results in much stronger binding to DQ2, and hence, stronger T cell activation. The discovery of a protease-resistant 33-mer in gliadin generated much interest. This peptide is resistant to gastric proteolysis, an excellent substrate for tTG and contains 3 overlapping immunodominant epitopes (Shan, *et al.* 2002). The proposed mechanism for immune stimulation by protease-resistant gliadin fragments is shown in Figure 1.10a.

In order to determine the specificity of tTG, Vader and colleagues (2002) investigated the factors that determined whether synthetic peptides were deamidated by tTG or not (Vader, *et al.* 2002). Their work showed that the deamidation of glutamine residues depended upon the relative spacing of glutamine and proline residues in peptides. The specificity turned out to be quite straightforward in the case of gluten: in the sequence QP and QXXP the Q is not deamidated by tTG while in the sequences QXP, QXXF(Y, W, M, L, I or V) and QXPF(Y, W, M, L, I or V), the Q is a target for tTG deamidation. This data was used to design an algorithm to predict whether a glutamine residue would be targeted by tTG or not. Strikingly, these algorithms detected many peptides from other cereals that are known to cause illness in coeliac disease patients, particularly in barley and rye (Vader, *et al.* 2002). Interestingly, this algorithm did not detect similar sequences in oats, a cereal that generates much disagreement regarding its toxicity to coeliac disease patients (Kilmartin, *et al.* 2003, Lundin, *et al.* 2003, Storsrud, *et al.* 2003).



b.

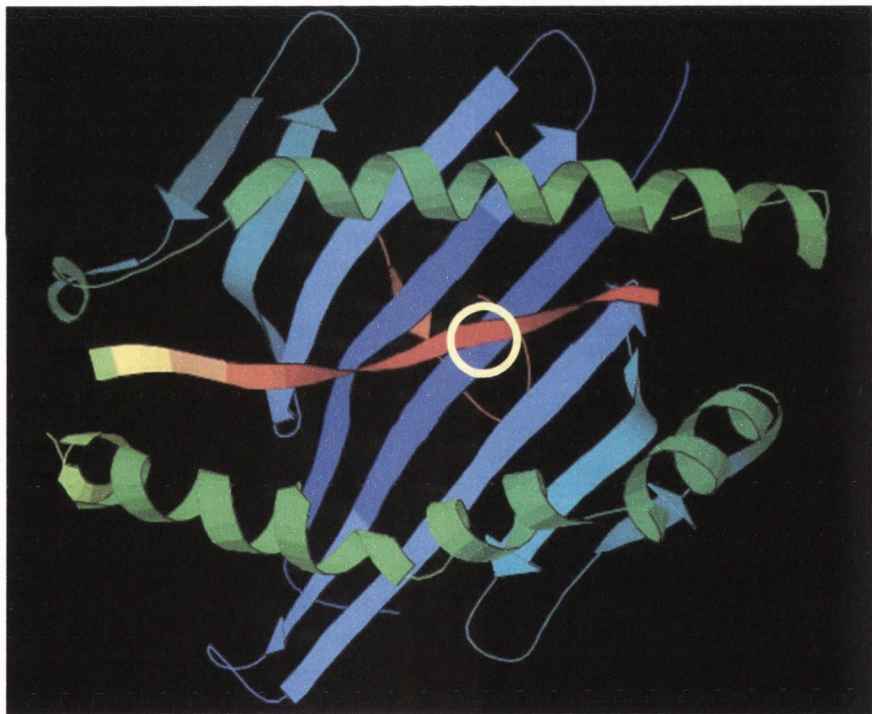


Figure 1.10: The binding of deamidated gliadin peptides to HLA-DQ2. (a) A schematic representation of the interaction between gliadin peptides, tTG and DQ2 (Taken from Sollid *et al.* 2002). (b) A 3D diagram of a deamidated gliadin peptide (red) in the binding groove of HLA-DQ2. A gold circle denotes the position of a deamidated glutamine residue. Image generated using KiNG software and PDB file 1S9V (Kim, *et al.* 2004).

Further studies of the conditions of gliadin deamidation have shown that lower pH values strongly favour deamidation, rather than transamidation reactions (Fleckenstein, *et al.* 2002). These results have led to speculation that the deamidation of gluten peptides takes place in the slightly acidic environment of the early endosomal vesicle.

The strength of T cell responses to gluten peptides is influenced by the level of DQ2 expression. It has been shown that gluten presentation by DQ2 homozygous antigen presenting cells results in at least five-fold higher T cell responses compared to heterozygous antigen presenting cells (Vader, *et al.* 2003). In fact, HLA-homozygous individuals have been shown to be at increased risk of coeliac disease development (Mearin, *et al.* 1983).

The origin of the immune response against Tissue Transglutaminase

The mechanisms underlying the antibody response generated against tTG in coeliac disease are unknown but given that anti-tTG titres fall, and can become undetectable during a gluten-free diet, B cell activity must depend upon persistent antigen presentation. Sollid (1997) put forth the hypothesis that gliadin-tTG complexes act as a hapten-type complex (Sollid, *et al.* 1997). In this model, tTG-gliadin complexes are endocytosed by IgM on the surface of tTG-specific B cells and gliadin epitopes are presented to gliadin-specific CD4⁺ T cells which then provide help for the production of anti-tTG antibodies (Figure 1.11). This hypothesis does not require the presence of tTG-specific T cells. This theory is supported by the demonstration that covalently bound gliadin-tTG complexes can be generated *in vitro*. Gliadin is bound to tTG at the catalytic triad or to particular lysine residues of the enzyme (Fleckenstein, *et al.* 2004).

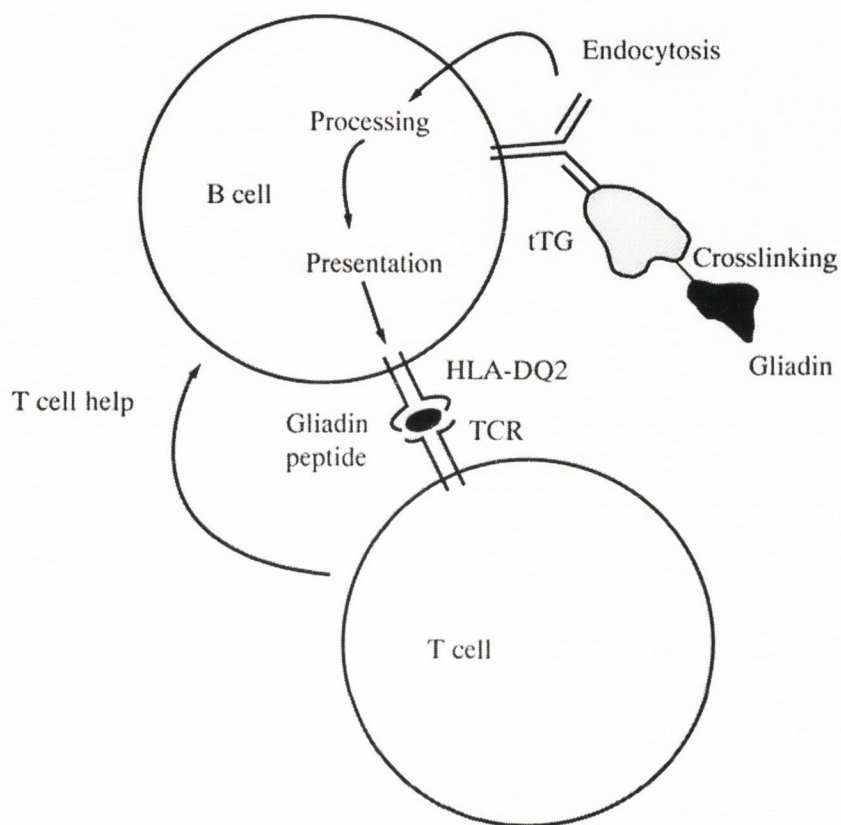


Figure 1.11: The hapten carrier complex theory for anti-tTG production. By this mechanism anti-tTG could be produced in the absence of tTG-specific T cells (Taken from Sollid *et al.* 1997).

It has also been suggested that sera from coeliac disease patients contains antibodies recognising not only gliadin or tTG, but also neoepitopes on tTG-gliadin complexes. It is conceivable that the antibody response against tTG arises from intermolecular epitope spreading from gliadin to tTG. Intermolecular epitope spreading has been demonstrated in animal models of autoimmune diseases such as autoimmune diabetes and experimental autoimmune encephalomyelitis (Vanderlugt and Miller 1996). In systemic lupus erythematosus (SLE), autoantibodies often arise in grouped, or linked sets, that target macromolecular complexes such as chromatin, snRNPs or Ro/La (SS-A/SS-B). This observation was first made in 1973 when it was demonstrated that the nRNP and Sm antigens are physically associated and that autoantibodies to these antigens often occur together in patient sera (Mattioli and Reichlin 1973). Hence, as a consequence of the close co-localisation of gliadin and tTG through complex formation, the T cell response might ultimately involve epitopes on tTG itself. This model would require the action of tTG-specific T cells for antibody production, and, while the presence of tTG-specific cells in the periphery has not been conclusively shown, mechanisms that allow autoreactive T cells to escape negative selection in the thymus have been described (Liu, *et al.* 1995, Yan and Mamula 2002).

1.2.11 Project Aims

The overall aims of this project were

- To determine whether the catalytic triad of tTG (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) is targeted by coeliac disease autoantibodies by production of recombinant tTG and a site-directed mutagenetic variant lacking elements of the catalytic triad and testing these antigens in ELISA assays with sera from coeliac disease patients.
- To determine whether serum antibodies from dermatitis herpetiformis patients recognise wild type and mutant tTGs in a pattern that is comparable to coeliac disease autoantibodies.
- To determine whether autoantibodies inhibit the cross-linking activity of tTG using a sensitive fluorescence-based transglutaminase cross-linking assay.

Chapter 2

Recombinant tissue transglutaminase production and site-directed mutagenesis

2.1 Introduction

2.1.1 Reverse Transcriptase polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR) is a powerful technique that allows *in vitro* amplification of DNA sequences using short oligonucleotide primers that flank the region of choice (Markham 1993). The development of the PCR method was a major breakthrough in molecular biology because it allows the rapid, sensitive and inexpensive amplification of DNA sequences. The method was developed by Mullis *et al.* in 1984 and was made possible by the thermal stability of the DNA polymerase enzyme from *Thermus aquaticus* (*Taq*) (Saiki, *et al.* 1985). During the PCR reaction, oligonucleotide primers that have been designed to flank the sequence of choice bind single stranded DNA. *Taq* polymerase carries out the synthesis of a complimentary strand of DNA from a 5' to 3' direction by extension from the primer binding sites. Because the *Taq* polymerase is stable at 94°C, it remains active through successive thermal cycles of denaturing double stranded DNA (94°C), primer annealing (~55°C) and DNA extension (72°C) (Innis, *et al.* 1989). In some cases, PCR can be improved by preventing pre-PCR mis-priming and primer-dimerisation prior to PCR initiation (Chou, *et al.* 1992). In this study, Platinum[®] *Taq* (Invitrogen) was the DNA polymerase of choice. Platinum[®] *Taq* is kept inactive prior to thermal cycling by an antibody inhibitor (Sharkey, *et al.* 1994).

Since DNA polymerases are unable to extend RNA, in order to amplify gene transcripts RNA must first be reverse transcribed into cDNA using a reverse transcriptase enzyme. This complimentary DNA (cDNA) then acts as template for a PCR reaction. Primers designed for RT-PCR are usually designed to span intron-exon boundaries in order to avoid amplification of any chromosomal DNA that may contaminate RNA purifications. Reverse transcription is carried out using an enzyme such as avian myeloblastoma virus (AMV) reverse transcriptase. An oligo dT primer can be used to target the polyadenosine tail that is present on

every RNA transcript in order to prevent 5' exonuclease degradation (Krug and Berger 1987).

RT-PCR reactions can be carried out in one or two steps. One step reactions are performed by combining RNA, reverse transcriptase enzyme, *Taq* enzyme, and PCR primers in one tube. After an initial reverse transcription incubation the PCR cycle begins and cDNA is amplified (Murakawa, *et al.* 1988, Wang, *et al.* 1992, Aatsinki, *et al.* 1994). However, most RT-PCR protocols recommend a two-step approach. In a two-step reaction, RNA is first reverse transcribed and the resulting cDNA can be stored and used as a template in subsequent PCR reactions. This method allows for easier optimisation of the PCR and avoids the inhibitory effect reverse transcriptase can have on *Taq* polymerase (Santini, *et al.* 1996). The overall scheme for a two-step RT-PCR is shown in Figure 2.1.

Efficiency of oligonucleotide primers is dependent on several factors including how well they match the complimentary sequence in the target DNA, length, annealing temperature (T_m), G/C content and tendency to form secondary structure. PCR primers are typically 16-30 base pairs in length and should have a G/C content of between 40 and 60%. All primer design considerations can be taken into account by using primer design software such as Primer Premier™, which calculates T_m using the nearest neighbour thermodynamic theory (Freier, *et al.* 1986).

In conditions with insufficient magnesium chloride *Taq* polymerase will not work, and, conversely, excess of magnesium reduces enzyme fidelity (Eckert and Kunkel 1990) and increases the likelihood of non-specific amplification of contaminating PCR products (Ellsworth, *et al.* 1993). The concentrations of DNA template, primers and dNTPs also affect the efficiency of a PCR reaction. Careful primer design is required to ensure that oligonucleotide primers have little secondary structure and a T_m that will accommodate PCR.

2.1.2 DNA sequencing

In order to confirm the identity of the RT-PCR product and ensure that the PCR reaction has introduced no errors into the sequence, DNA sequencing was carried out. To facilitate DNA sequencing it is common practice to ligate the RT-PCR product to a vector containing M13 forward and reverse priming sites downstream and upstream of the insert respectively. The M13 priming sites originate from bacteriophage M13 DNA and correspond to universal DNA sequencing primers (Randhawa and Easton 1999).

In this study, the vector TOPO-XL™ (Invitrogen) was chosen for DNA sequencing purposes. This vector is designed for easy DNA ligation with the only insert pre-requisite being the presence of a deoxyadenosine (A) overhang at the 3' end of PCR product. *Taq* polymerase has a non-template dependent terminal transferase activity that results in the necessary A overhang (Campbell 1996). TOPO™ vectors are kept in a linear format by topoisomerase I from *Vaccinia* virus. This enzyme binds double stranded DNA at specific sites and cleaves the phosphodiester backbone on one strand after the sequence 5'-CCCTT. The addition of a double-stranded DNA sequence with a 3' A overhang results in displacement of the topoisomerase I enzyme and ligation of the DNA sequence to the vector in the correct orientation (Figure 2.2). As well as M13 priming sites, this vector also contains *EcoRI* restriction sites and antibiotic resistance loci (Figure 2.3).

Since insertion of DNA into the cloning site of XL-TOPO™ results in interruption of the *LacZ* gene, blue/white screening can be used to screen bacterial colonies after transformation. The *LacZ* gene product, β -galactosidase, is capable of turning its substrate, X-gal, into an insoluble blue dye. If β -galactosidase activity is present in a bacterial colony, the *LacZ* gene has not been interrupted and hence, DNA has not been inserted into the vector (Sambrook, *et al.* 1989).

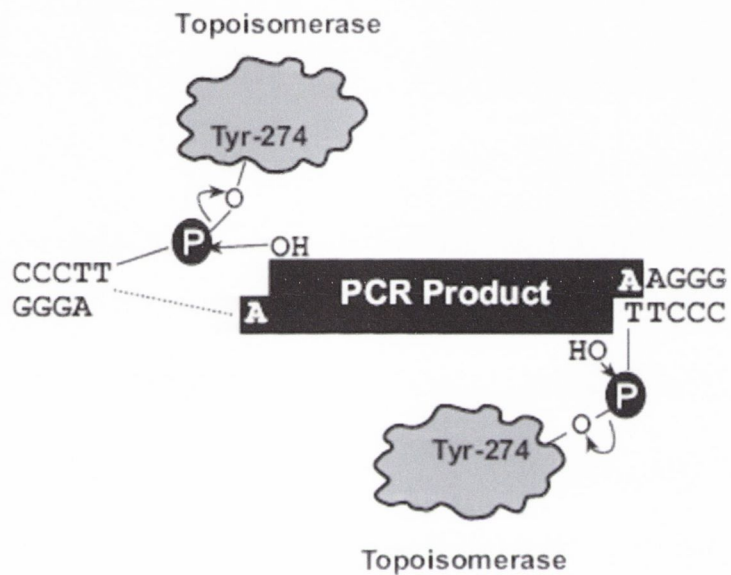


Figure 2.2: Mechanism of ligation of PCR product to TOPO-XL™ vector. Mechanism involves the displacement of topoisomerase I and the ligation of PCR product via a 3' deoxyadenosine overhang. (Image taken from www.invitrogen.com)

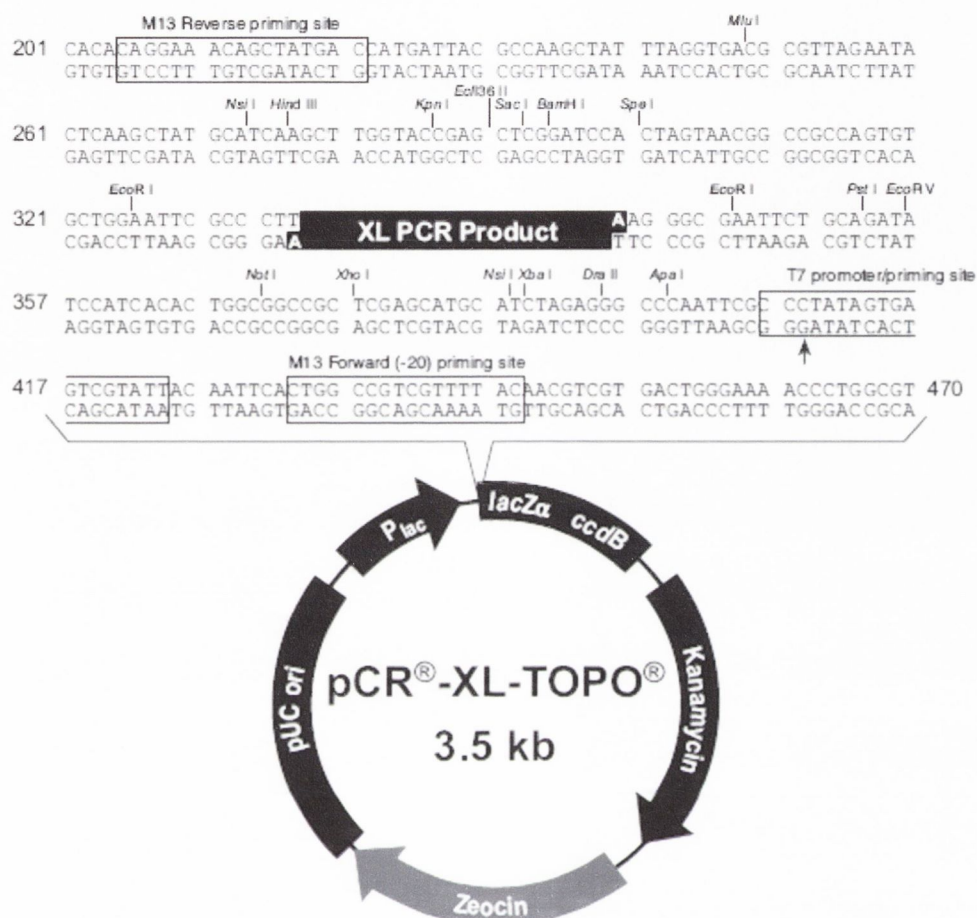


Figure 2.3: TOPO-XL™ Map. This map represents the layout of the TOPO-XL™ vector. The multiple cloning site detail illustrated the location of the PCR product ligation site, antibiotic resistance genes, M13 priming sites and restriction sites for EcoRI. Insertion of DNA sequence involves the displacement of topoisomerase I and the ligation of PCR product via a 3' deoxyadenosine overhang. (Image taken from www.invitrogen.com)

2.1.3 Site-directed mutagenesis

In a recent study Sblattero *et al.* (2002) produced a series of tTG fragments and applied them to ELISA assays with coeliac disease sera in an attempt to map the regions of tTG that are important for coeliac disease anti-tTG binding (Sblattero, *et al.* 2002). Their study found that the most antigenic tTG fragments were those that contained amino acids 140-376 which includes the catalytic triad and an intact amino or carboxy terminus. These results provided evidence for an important conformation-dependent epitope within the core region of tTG. The core region contains the catalytic triad of tTG (Cys²⁷⁷, His³³⁵, Asp³⁵⁸), the three amino acids responsible for the deamidation and transamidation reactions that the enzyme catalyses (Liu, *et al.* 2002).

Several studies report the antibody-binding enhancing effects of calcium in ELISA coating buffers (Feighery, *et al.* 2003, Roth, *et al.* 2003). The presence of calcium ions in solution results in a conformational change in tTG, revealing the catalytic triad (Mariani, *et al.* 2000). Sulkanen and colleagues (1998) reported that the pre-treatment of tTG with calcium ("calcium activation") dramatically improved the differentiation between coeliac and non-coeliac disease sera in a guinea pig liver tTG based ELISA, and also increased the binding affinity of coeliac disease serum antibodies for tTG (Sulkanen, *et al.* 1998). The catalytic triad also represents a site for gliadin-tTG interaction (Fleckenstein, *et al.* 2004) and hence, a possible site for intermolecular epitope spreading.

For the above reasons, this study was designed to investigate the importance of the catalytic triad of tTG in the tTG-autoantibody interactions in coeliac disease. Since previous studies investigating tTG-autoantibody interactions by producing truncated tTG proteins have produced contradictory results (Seissler, *et al.* 2001, Sblattero, *et al.* 2002, Tiberti, *et al.* 2003, Nakachi, *et al.* 2004), it is possible that these short tTG proteins did not assume a similar conformation to the tTG found *in vivo*. For this reason, site-directed mutagenesis represents a more precise method to investigate tTG-autoantibody interactions while

preserving overall protein conformation. In this study, full-length tTG and a novel site-directed mutagenic tTG lacking the Cys-His-Asp catalytic triad were produced and applied to ELISA assays with cohorts of coeliac disease and dermatitis herpetiformis sera in order to determine the importance of the catalytic core in tTG-autoantibody interactions.

DNA mutagenesis is defined as any process through which an altered DNA sequence is produced. It is a technology that plays a role in determining the functions of different protein domains, the identification of transcriptional regulatory sequences and the importance of specific amino acids in protein-protein interactions (Cseke, *et al.* 2003). Mutagenesis methods have been developed based on PCR methodology (Ling and Robinson 1997, Ishii, *et al.* 1998) and oligonucleotide site-directed mutagenesis was used in this study to alter the catalytic triad of tTG.

Oligonucleotide site-directed mutagenesis is a widely used method to specifically alter the sequence of cloned DNA. The basic principle is that using mismatched oligonucleotide primers, a mutated DNA sequence is produced by PCR (Sambrook, *et al.* 1989). The Quickchange[®] system (Stratagene) is a highly efficient and widely used site-directed mutagenesis kit that uses the restriction enzyme DpnI (target sequence '5-Gm⁶ATC-3') to digest parental bacterial DNA, leaving behind only mutated sequences (Escovar-Kousen, *et al.* 2004, Zheng, *et al.* 2004).

Alanine screening is a common method in site-directed mutagenesis (Hobba, *et al.* 1998, Jonson and Petersen 2001). Alanine is an aliphatic amino acid with a CH₃ side-chain and is the second simplest amino acid. The replacement of the catalytic triad with alanine residues should remove the catalytic and steric features of the region. The codons coding for Cys²⁷⁷, His³³⁵ and Asp³⁵⁸ were mutated to sequences coding for alanine residues.

2.1.4 Recombinant DNA technology and protein production

Once amplified, PCR products can be ligated to vectors for recombinant protein production. Choice of vector and host cell depends on several factors. In this study, *E.coli* was chosen as the organism for protein production as these bacteria are easily manipulated and transformed. The cells also multiply very rapidly and can produce large amounts of protein on inexpensive substrates (Baneyx 1999). A potential disadvantage of using *E.coli* to produce a human protein is that, being a prokaryotic cell, *E.coli* carry out no post-translational modifications such as glycosylation or formation of disulphide bonds. Since tTG has no disulphide bridges or glycosylation (Gaudry, Verderio, Aeschlimann, *et al.* 1999), *E.coli* should be capable of producing antigenic and functional tTG. BL21 is a strain of *E.coli* that is deficient in lon and omp T proteases (Grodberg and Dunn 1988) and is commonly used for recombinant protein production.

The vector pGEX-4T-1 was used in this study to produce recombinant tTG (Figure 2.4). pGEX vectors produce recombinant proteins fused to Glutathione-S-transferase (GST), a 26kDa protein from *Schistosoma japonicum*. The GST protein has a high affinity for glutathione and can be used to purify the fusion protein from cell lysates. An advantage of the GST-fusion system is its reported solubility-increasing properties (Nygren, *et al.* 1994, Smyth, *et al.* 2003). The solubility-increasing ability of the GST tag is of particular interest in this study since tTG has been reported to form insoluble inclusion bodies in *E.coli* (Shi, *et al.* 2002). An obvious disadvantage of the GST-fusion protein is that the protein of interest is fused to an unrelated 26kDa protein that may interfere with downstream experiments. Since this particular study involved using ELISA assays, it was necessary to bear in mind that antibody responses to GST can occur in the serum of patients suffering from glaucoma (Yang, *et al.* 2001). The GST tag can be removed by thrombin cleavage, but further manipulation of recombinant proteins often leads to reduced yield.

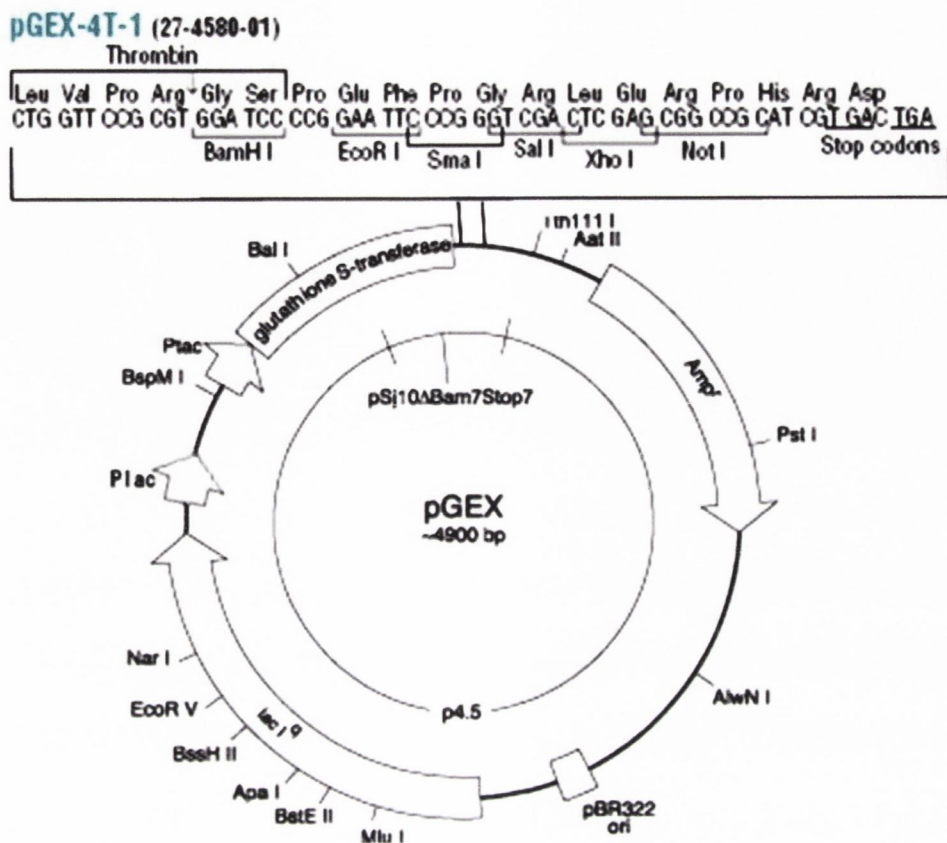


Figure 2.4: pGEX-4T-1: Map of the pGEX-4T-1 vector showing detail of the multiple cloning site, Glutathione-S-transferase domain and antibiotic resistance domain. (Image is taken from The Recombinant Protein Handbook, Amersham Biosciences)

GST-fusion protein expression is under the control of the *tac* promoter (Sawers and Jarsch 1996), which is induced by the lactose analog isopropyl β -D thiogalactoside (IPTG). All pGEX vectors are also engineered with an internal *lacIq* gene. The *lacIq* gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the insert (Sambrook, *et al.* 1989).

Production of large amounts of protein in *E.coli* can result in the formation of insoluble inclusion bodies. To prevent this occurring it is often necessary to minimize the amount of overall protein being expressed in order to maximise the amount of correctly folded, soluble protein expressed. In order to achieve this, parameters such as IPTG concentration, induction temperature, induction time and agitation must be optimized (Makrides 1996).

2.1.5 Aim of this chapter

The aim of the work described in this chapter was to produce a recombinant wild type tTG and a novel site directed mutagenic variant with the elements of the catalytic triad (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) replaced by alanine residues.

2.2 Materials and Methods

2.2.1 RNA Extraction

A major consideration in isolating undegraded RNA is contamination of samples with ribonucleases (RNases). For this reason, all solutions for RNA extraction were prepared with diethyl pyrocarbonate (DEPC) treated water (Appendix II). Due to the abundance of RNases found in dust particles and on skin, latex gloves were worn and surfaces were treated with RnaseZap (Sigma).

RNA extraction method was adapted from the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). RNA was extracted from 3.5ml aliquots of whole fresh blood with EDTA as anticoagulant. Blood was mixed with red blood lysis reagent (appendix II) and incubated at room temperature for 10 minutes. Lymphocytes were then pelleted by centrifugation at 2,000 rpm for 10 minutes at RT. The supernatant was removed by decanting, leaving ~50µl solution above the pellet. The pellet was resuspended by vortexing. 500µl denaturing solution (Appendix II) was added, followed by 50µl 2M sodium acetate pH 4.0. 500µl water-saturated phenol was then added and the solution was vortexed vigorously. 150µl of a chloroform/isoamyl alcohol (24:1) solution was then added and the resulting solution was incubated on ice for 15 minutes. The solution was then transferred to a 1.5ml tube and centrifuged at 13,000rpm for 5 minutes at 4°C. The upper (aqueous) phase was transferred to a fresh tube, taking care not to disturb the protein layer at the interface. An equal volume of isopropanol was added, the solution was vortexed and incubated at -20°C for 30-60 minutes. RNA was subsequently pelleted by centrifugation at 13,000rpm for 15 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 300µl denaturing solution (Appendix II). This was followed by addition of 300µl isopropanol and incubation at -20°C for 30-60 minutes. The RNA was centrifuged once again as above and the resulting pellet was washed once with 75% ethanol and briefly air-dried. The RNA pellet was resuspended in 50µl of RNase-free water and stored

at -70°C . RNA quantity and quality was assessed by spectrophotometric analysis of absorbance at 260/280nm.

2.2.2 Reverse transcription

RNA samples were incubated at 85°C for 2 minutes to denature any secondary structure, and were subsequently incubated on ice for 1 minute. 10 μl RNA was added to a 50 μl reverse transcription reaction containing 10 μl of a 5x reverse transcription buffer (Promega); 2U RNasin[®] RNase inhibitor (Promega); 5 μl 10mM dNTPs (Invitrogen); 3 μl oligo-dT primers (Promega) and 6 μl Avian Myeloblastosis Virus Reverse Transcriptase, (Promega). The reaction was carried out at 42°C for one hour and the resulting cDNA was stored at -70°C .

2.2.3 PCR Primer Design

Primer sequences were designed using Primer Premier[™] software. This software considers criteria such as primer length, GC content, annealing temperature and secondary structure considerations. tTG primers were designed to incorporate 5' restriction enzyme recognition sequences in the PCR product in order to facilitate subsequent ligation to the pGEX vector (Table 2.1). All tTG PCRs were run in tandem with multiplex control PCRs for two constitutively expressed transcripts. Small nuclear riboprotein polypeptide N (SNRPN) is a protein involved in Prader Willi syndrome (McEntagart, *et al.* 2000) while Wiskott-Aldrich syndrome protein (WASP) facilitates the reorganisation of the actin cytoskeleton in normal individuals (Orange, *et al.* 2004). All normal peripheral blood mRNA samples should contain transcripts for both of these and should therefore generate PCR products of SNRPN and WASP of 143 and 324bp respectively in an RT-PCR reaction.

Table 2.1 PCR Primers

Primer	Sequence
tTG Fwd	5' GATC GAATTC ATGGCCGAGGAGCTGGTCTTAG 3'
tTG Rev	5' GATC CTCGAG TTAGGCGGGGCCAATGATGAC 3'
SNRPN Fwd	5' CCCTAGGTCTTCAGAAGCAT 3'
SNRPN Rev	5' TGAAGATTCGGCCATCTTGC 3'
WASP Fwd	5' GCCACTGCAGTTGTTTCAG 3'
WASP Rev	5' GGCGTCTGTCTCCACTTTG 3'

Bold font represents restriction enzyme recognition sites. The filled arrow represents the site cleaved by EcoRI; the empty arrow represents the site cleaved by XhoI. Primers were ordered from Sigma Genosys.

2.2.4 Agarose Gel Electrophoresis

PCR products were visualised by gel electrophoresis. 1% gels were prepared by adding 1g agarose (Pronadisa) to 100mls tris-borate-EDTA (TBE) buffer (Invitrogen). The solution was heated in a microwave until the agarose had melted. Once the solution had cooled to ~60°C, 5µl of a 10mg/ml solution of ethidium bromide (Sigma) was added in a fume hood, the gel was poured into a template and a comb was inserted. After ~45 minutes the comb was removed. 10µl PCR product was mixed with 2µl 6x loading buffer (30% glycerol, 0.25% bromophenol blue) and pipetted into a well of the gel. A 100bp ladder (Invitrogen) was included in the gel to estimate product size. The gel was then electrophoresed at 100V for at least 45 minutes. Gels were visualised on a UV light box.

2.2.5 Optimisation of MgCl₂ concentration

PCR reactions contained 2.5µl of a 10x reaction buffer (Invitrogen), 0.5µl 10mM dNTP mix (Invitrogen), 1µl primer mix (100ng/µl), 0.2µl Platinum® *Taq* (5U/µl, Invitrogen), 2µl cDNA as template and dH₂O to 25µl. Annealing temperature was set at 55°C. MgCl₂ concentration was tested at 1.5mM, 2mM and 2.5mM. PCR products were visualised on 1% agarose gels.

2.2.6 Optimisation of cDNA concentration

Once MgCl₂ concentration was optimised, the amount of cDNA template added to the PCR reaction was varied in order to determine the optimal concentration. cDNA was added at volumes of 1, 2 and 5µl. PCR products were visualised on 1% agarose gels.

2.2.7 Final PCR protocol

After optimisation, a final PCR protocol was devised. The PCRs contained 10µl 5X PCR buffer (Invitrogen), 0.2mM dNTPs (Invitrogen), 2mM MgCl₂, 2U Platinum® *Taq* polymerase (Invitrogen), 10µl cDNA, 2µl primers (tTG Fwd + tTG Rev; 100ng/µl) and dH₂O to 50µl. Cycling conditions are shown in Table 2.2. To enhance quantity of tTG DNA, the PCR product was diluted 400 fold and 2µl of this was used as template in a second round of PCR.

Table 2.2 PCR cycling conditions

Initial denature	94°C – 2 minutes	
Denature	94°C – 30 seconds	} X 40
Annealing	55°C – 30 seconds	
Elongation	72°C – 30 seconds	
Final elongation	72°C – 7 minutes	

PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research).

2.2.8 Gel Purification of PCR products

Before ligation to vector, in order to remove dNTPs, salts or any other inhibitors of the ligation reaction, PCR products were purified by gel excision. PCR product was purified using the S.N.A.P™ UV-free gel purification kit (Invitrogen). Purification was carried out as per the manufacturer's protocol. Briefly, the PCR product was electrophoresed in a crystal violet (Invitrogen) stained, 0.8% low melt point agarose (Sigma) gel. The agarose gel was prepared with tris-acetate-EDTA (TAE) buffer (Appendix II) since the use of TBE buffer can result in inhibition of ligation reactions. The PCR product was visualised on a standard light box, excised with a fresh scalpel and incubated with ~2.5 volumes of 6.6M sodium iodide at 42°C until the agarose had melted. The resulting solution was loaded onto a S.N.A.P™ spin column (Invitrogen), washed and eluted in DNase free water.

In some cases, gel purification was carried out by phenol chloroform extraction. Briefly, the PCR product was electrophoresed in an ethidium bromide (Sigma) stained 0.8% low melt point agarose (Sigma). PCR product was visualised on a UV light box, excised using a fresh scalpel and incubated with 3 volumes TE buffer at 65°C until the agarose had melted. The resulting solution was incubated with an equal volume of water-saturated phenol (Ambion), vortexed and centrifuged at 14,000rpm for 5 minutes. The aqueous phase was removed to a fresh tube, mixed with an equal volume of chloroform (Sigma) and centrifuged at 14,000rpm for 5 minutes. The aqueous phase was removed and mixed with 1/10 volume of 5M sodium chloride (Sigma) and 2.2 volumes ethanol (Sigma). This solution was vortexed and stored at -70°C for 10 minutes before being centrifuged at 14,000rpm for 10 minutes. The resulting pellet was briefly air-dried and resuspended in 20µl TE buffer.

2.2.9 Preparation of chemically competent BL21 *Escherichia coli*

Preparation of competent *E.coli* was based on the method of Cohen *et al.* (1972). A sterile conical flask containing 50mls LB broth was inoculated with BL21 *Escherichia coli* (*E.coli*) and incubated in a 37°C orbital incubator with gentle agitation. After ~3 hours or when the culture was quite turbid ($OD_{600} = 0.5$) the culture was centrifuged at 3,000rpm for 10 minutes. The supernatant was discarded and the pellet was gently resuspended in 10mls ice-cold 100mM $MgCl_2$ and incubated on ice for 15 minutes. The bacteria were pelleted as before. The pellet was then gently resuspended in 100mM $CaCl_2$ and incubated on ice for at least 1 hour (Cohen, *et al.* 1972). The cells remained viable and competent at 4°C for several days.

2.2.10 Cloning to TOPO vector

PCR product was ligated to the TOPO-XL™ vector for DNA sequencing purposes. Ligation reaction was as per the manufacturer's protocol. Briefly, a 1 in 5 dilution of the gel-purified tTG PCR product was incubated with 1μl of the TOPO-XL™ vector. This mixture was incubated at RT for 5 minutes before the addition of 1μl 6x TOPO™ stop solution. This mixture was briefly stored on ice and then added to 100μl chemically competent BL21 *E.coli*. This mixture was incubated on ice for 15-30 minutes and subsequently heat-shocked at 42°C for 2 minutes. 500μl LB broth (Appendix II) was then added and the cells were incubated for 50 minutes in a 37°C orbital incubator with gentle agitation. 125μl of the transformation mixture was spread on an LB agar plate containing 50μg/ml kanamycin. For blue/white screening purposes 100μl of X-gal (20μg/ml) and 100μl IPTG (0.5mM) were spread on agar plates and allowed to dry at 37°C prior to plating of bacteria. Colonies resulting from this transformation were screened for successful transformation by plasmid purification and restriction digest. Successful transformants were selected and stored at -70°C on bacterial cryobeads (Innovatek Medical).

2.2.11 Plasmid isolation

Bacterial colonies were selected from transformation agar plates on the basis of blue/white colour. Autoclaved toothpicks were used to lift colonies from the agar plates and the toothpicks were placed in tubes with 3mls LB broth containing the appropriate selective antibiotic. After overnight culture at 37°C with gentle agitation, 1.5ml of each bacterial culture was pelleted at 14,000rpm for 3 minutes. The supernatant was discarded and the pellet was dissolved in 100µl TE buffer (10mM Tris, 1mM EDTA, pH 7.5). 200µl 0.2M NaOH, 1% SDS was added and the solution was incubated at RT for 5 minutes. 150µl 5M potassium acetate was added and the solution was incubated on ice for 5 minutes before centrifugation at 14,000rpm for 5 minutes. 400µl of the resulting supernatant was placed in a fresh tube and 800µl of 100% ethanol was added. This solution was vortexed and incubated on ice for 5 minutes. The pellet was centrifuged as before and the supernatant discarded. 1ml 70% ethanol (-20°C) was added and the solution was pelleted once again. The supernatant was discarded and after air-drying the pellet for 5 minutes, the pellet was resuspended in 25µl TE buffer containing 22µg/ml RnaseA (Qiagen).

2.2.12 Restriction digest analysis of purified plasmid

10µl plasmid DNA was incubated with 1.5µl 10X EcoRI buffer, 1µl EcoRI (20,000U/ml), 1µl XhoI (20,000U/ml) (New England Biolabs) and 2.5µl dH₂O for 1 hour at 37°C. After digest, the products were visualised on 1% agarose gels. If ligation was successful, plasmid (TOPO™-XL = 3.5kb or pGEX = 4.9kb) and tTG PCR product (2,067bp) were both visible on gel.

2.2.13 DNA sequencing

For DNA sequencing purposes, larger quantities of plasmid DNA were required. For this reason, plasmid purification was carried out using the Concert midiprep plasmid purification system (Invitrogen). DNA sequencing was carried out using the LiCor® GeneReader™ 4200 system.

The Thermo Sequenase[®] DYEnamic ET terminator cycle sequencing kit was used to carry out sequencing reactions. Sequencing reactions were carried out as per the Licor[®] Thermo Sequenase[®] protocol except for halving the Thermo Sequenase[®] reagent mix volume and increasing gel loading to 1.5µl. Briefly, four 0.5µl microcentrifuge tubes were labelled “A”, “C”, “G”, and “T”. 1µl Thermo Sequenase A reagent was added to tube “A”, etc. A pre-mix was prepared by mixing 2µl IRD800 labelled primer (1 pmol/µl), the appropriate volumes of DNA template, and dH₂O to 18µl. Four µl of pre-mix was added to each of the four reaction tubes and placed in a PTC-100 thermal cycler (MJ Research). After an initial denaturing step of 92°C for 2 minutes, the reactions were subjected to 30 cycles of 92°C for 20 seconds, annealing for 20 seconds (temperature depended upon individual primer) and extension at 70°C for 45 seconds.

Gel cassette and sequencing gel electrophoresis were carried out as per the manufacturer’s recommendations. Electrophoresis conditions are described in appendix I. Sequence information was processed and aligned to published tTG sequences using e-Seq[™] and AlignIR[™] softwares respectively. Sequencing primers were designed using Primer Premier[™] software (Table 2.3).

Table 2.3 DNA sequencing primers

Name	Sequence	Base Pair Target
M13F	5' –CAC GAC GTT GTA AAA CGA C- 3'	+96
tTGseq1	5' –CAG GGC TTT ATC TAC CAG GGC- 3'	490
tTGseq2	5' –CAG TTC GTG CCA TCA AGG AGG- 3'	1124
tTGseq3	5' –GTG GAG CCA GTT ATC AAC AG- 3'	1702
tTGRev1	5' –ATT GAC CTC CGC AAA GAC AAA G- 3'	1194
tTGRev2	5' –TAC TCG ATG AGA AGG TTG CTG TTC- 3'	944
M13R	5' –CAC ACA GGA AAC AGC TAT GA- 3'	-132

Primers were ordered from MWG Biotech and were attached to a 5' IRD800 fluorescent tag. All sequencing primers were purified by HPLC.

2.2.14 Cloning to pGEX-4T-1 vector

In order to isolate the wild type tTG sequence from the TOPO-XL™ vector, a restriction digest was performed using enzymes EcoRI and XhoI (as per Section 2.2.12). Digest products were separated on a 0.8% gel and the tTG products were excised from the gel and the DNA purified as described in Section 2.2.8. In order to linearise pGEX-4T-1 (Amersham Biosciences) and provide sticky ends for tTG ligation, pGEX-4T-1 also underwent restriction digest with enzymes EcoRI and XhoI (as per Section 2.2.12). Concentrations of vector (pGEX-4t-1) and insert (tTG) were estimated on agarose gels in order to create optimal conditions for ligation reaction (vector:insert ratio of 2:1). Insert DNA was concentrated by ethanol precipitation and the appropriate amount was incubated with 1µl pGEX-4T-1, 2µl 3M sodium acetate and 50µl ethanol. This solution was centrifuged at 14,000rpm for 25 minutes and the pellet was washed once with ice-cold, 70% ethanol. For the ligation reaction, this pellet was resuspended in 17µl Milli-Q water, 2µl 10X DNA ligase buffer and 1µl T4 DNA ligase (New England Biolabs). This solution was incubated overnight at 4°C. 10µl of the ligation reaction was then added to 100µl chemically competent *E.coli* and transformed as previously described. Colonies resulting from this transformation were screened for successful transformation by plasmid purification and restriction digest. Successful transformants were selected and stored at -70°C on bacterial cryobeads (Innovatek Medical).

2.2.15 Site-directed mutagenesis

Site directed mutagenesis was carried out using the Quickchange® system (Stratagene). Mutation reactions were carried out as per the manufacturer's recommendations except that the XL1-Blue super-competent *E.coli* provided with the kit were no longer competent upon receipt and were made competent manually by MgCl₂/CaCl₂ treatment as per section 2.2.9. Mutagenic primers (Table 2.4) were designed using Stratagene's online labtools website (<http://labtools.stratagene.com/QC>). Briefly, 150-300ng template DNA was used as template in a mutagenesis

PCR reaction with 5µl 10x buffer (Stratagene), 125ng of forward and reverse mutagenesis primers, 1µl dNTP mix (Stratagene) and 2.5U *PfuTurbo* DNA polymerase. Thermal cycling conditions were 16 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 1 minute and elongation at 68°C for 7 minutes. Bacterial chromosomal DNA was digested by incubation with 10U DpnI at 37°C for one hour. 1µl of this DNA solution was transformed into 100µl competent XL1-Blue cells and incubated overnight at 4°C. Successful transformants were screened for mutation by plasmid isolation and DNA sequencing as per Section 2.2.13.

Once mutagenesis rounds were complete, the plasmid was extracted from XL1-Blue *E.coli* and transformed into BL21 *E.coli* for protein production and purification purposes.

Table 2.4 Primers for site-directed mutagenesis

C277A-F	5'- GTCAAGTATGGCCAG <u>GCT</u> TGGGTCTTCGCCG- 3'
C277A-R	5'- CGGCGAAGACCCA <u>AGC</u> CTGGCCATACTTGAC- 3'
H335A-F	5'- CGAGATGATCTGGAACCTC <u>GCT</u> TGCTGGGTGGAGTCGTG- 3'
H358A-R	5'- CACGACTCCACCCAGCA <u>AGC</u> GAAGTTCCAGATCATCTCG- 3'
D358A-F	5'- GCAGGCCCTG <u>GCCCC</u> AAACGCCCC- 3'
D358A-R	5'- GGGGCGTTGG <u>GGCC</u> AGGGCCTGC- 3'

Primers were ordered from Sigma-Genosys. Primer sets C277A, H335A and D358A were used to perform mutagenesis of the catalytic triad of tTG.

The effect of the three mutations on secondary structure of tTG were predicted using 3D-PSSM (three-dimensional position-sensitive scoring matrix), a fast, web-based method for protein fold recognition using 1D and 3D sequence profiles coupled with secondary structure and salvation potential information. 3D-PSSM has been used to predict the structure of many recombinant proteins (Hsu, *et al.* 2002, Pons, *et al.* 2004, Roy, *et al.* 2005). The entire length of the tTG amino acid sequence and the amino acid sequence of the Cys-His-Asp mutant tTG were uploaded to the 3D-PSSM server (<http://www.sbg.bio.ic.ac.uk/~3dpssm>). The program is used to generate a three state predication (helix/strand/coil) for the protein of interest.

2.2.16 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures and coats proteins, conferring a negative charge. With all proteins in solution having a negative charge, they separate based upon molecular weight in an electric field (Cseke, *et al.* 2003). An SDS polyacrylamide gel is formed when the acrylamide monomer is mixed with initiator (ammonium persulphate) and accelerator (TEMED; N,N,N',N'-tetramethylethylenediamine) forming a linear polymer. The cross-linker (N,N'-methylene bis-acrylamide) joins the linear polymers together, side-by-side to form a three dimensional mesh (Gersten 1996).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely carried out to visualise recombinant tTG expression and purification. All solutions for SDS-PAGE are available in appendix II. SDS-PAGE method was taken from Molecular Cloning: A Laboratory Manual (Sambrook, *et al.* 1989). 12% gels were prepared in two steps; first a resolving gel was prepared and poured into a glass template (Atto) and overlaid with 0.1% SDS. After 20 minutes, the 0.1% SDS was washed away with dH₂O and a resolving gel solution was overlaid and a comb inserted. After 15 minutes the comb was removed and the gel rig was assembled and filled with cold running buffer. Sample preparation was carried out by adding 6x loading buffer to samples and heating them at 100°C for 5 minutes. Samples were loaded into wells and the gel was run at ~40mA for ~1 hour until the dye front had reached the bottom of the gel. A molecular weight standard (Protein Marker, Broad Range, 6-175 kDa (New England Biolabs); or Kaleidoscope marker, 10-250 kDa (Bio-Rad)) was included in each run. Protein was visualised by staining overnight in Coomassie blue solution and subsequent removal of excess Coomassie blue with destain solution.

2.2.17 Recombinant protein expression optimisation

Short-term cultures with induction of tTG expression were used in order to confirm GST-tTG expression in *E.coli*. Cultures of *E.coli* containing wild type tTG-containing pGEX vector were grown to mid-log phase ($OD_{600} = 0.5$). Protein expression was induced by the addition of 1mM IPTG and protein expression was monitored by SDS-PAGE of total *E.coli* proteins at various time points.

Given that recombinant tTG has been reported to generate insoluble inclusion bodies in *E.coli* (Shi, *et al.* 2002), various strategies were attempted in order to maximise yield from bacterial cultures. Conditions such as lysis buffer, induction temperature, induction time and IPTG concentration had to be optimised. Some of the methods and relative success are outlined in Table 2.5. The overall observations from these early experiments were that (1) lysate viscosity made purification difficult; (2) milder induction conditions resulted in better yields and (3) CellLytic™ reagent (Sigma) was a good reagent for *E.coli* cell lysis. In order to reduce viscosity, all subsequent tTG purification attempts included DNase (Promega) treatment of cell lysates. Once bacterial cell lysis conditions were settled upon, subsequent experiments were carried out in order to optimise IPTG induction concentration. tTG expression was examined at 0.5 μ M, 1 μ M, 5 μ M, 10 μ M and 20 μ M IPTG.

Table 2.5 Optimisation of recombinant tTG purification

Lysis conditions	Induction conditions	Yield/l <i>E.coli</i>	Purity
20mM Tris Acetate, 2mg/ml lysozyme	1mM IPTG, 3hrs, 37°C	None	-
20mM Tris-HCL 1% Triton X-100, 1mg/ml Lysozyme	1mM IPTG, 2hrs, 37°C	80µg	Moderate Lysozyme contamination
CellLytic™ Reagent, 0.2mg/ml Lysozyme	1mM IPTG, 2hrs, 37°C	49.07µg	Poor
CellLytic™ reagent	1mM IPTG, 2hrs, 37°C	24.27µg	Moderate
CellLytic™ reagent 0.1mg/ml Lysozyme	50µM IPTG, 2 hrs 30°C	38µg	Good
CellLytic™ reagent 0.1mg/ml Lysozyme	25µM IPTG, 2 hrs 30°C	18.56µg	Good
CellLytic™ reagent 0.5mg/ml Lysozyme	50µM IPTG, 2 hrs 30°C	65µg	Good

Recombinant tTG purity was assessed by SDS-PAGE

2.2.18 Final protein expression and purification protocol

Tissue transglutaminase GST fusion protein was routinely performed using 1 litre of bacterial culture. 1 litre of nutrient broth (Oxoid) containing 100µg/ml ampicillin (Sigma) was inoculated with 20mls of an overnight culture of BL21 *E.Coli* containing the wild type or mutant pGEX-tTG plasmid. After 3 hours of growth at 37°C with moderate shaking, the culture was induced to produce the fusion protein by addition of 1µM IPTG with incubation at room temperature overnight with 200rpm agitation. The following day the culture was centrifuged at 3,500xg for 15 minutes and resuspended in 40mls 20mM Tris, 150mM NaCl, 1mM EDTA, 1mM DTT, 15% glycerol pH 8.0 (Lai, *et al.* 1994). Pellets were stored at -80°C as yields were noted to improve following storage.

Pellets were de-frosted and resuspended in 80mls CellLytic™ B (Sigma) containing 0.1mg/ml lysozyme and ~1,000 units DNaseI (Invitrogen). Cell lysate was mixed at room temperature for 20 minutes and homogenised by passing it four times through a 21G x 1.5" needle (Becton Dickinson). The lysate was then centrifuged at 25,000xg for 15 minutes with no brake. 800µl glutathione sepharose was added and incubated with the lysate for 30-60 minutes with mixing at 4°C. Sepharose was pelleted and washed twice in 120mls PBS, once in 60mls PBS and finally in 2mls CellLytic™. Fusion protein was eluted from the beads with three 5-minute washes in 100mM glutathione, 20mM Tris, pH 8.0 giving a total elution volume of 2mls. 10µl of each batch was run on a 12% SDS-PAGE gel to assess purity. Recombinant tTGs were stored in 50% glycerol to prevent freezing at -20°C.

2.2.19 Recombinant tissue transglutaminase quantification

Since protein yields were consistently low and below the linear range for standard Bradford protein quantification, a highly sensitive fluorescent protein quantification reagent, NanoOrange® (Molecular Probes), was used. Protein quantification was carried out as per the manufacturer's protocol. Briefly, a standard curve was generated by dilution of bovine

serum albumin (BSA) in NanoOrange[®] working solution while analytes were diluted 1:100 with NanoOrange[®] working solution. All samples were heated to 96°C for 10 minutes and cooled at room temperature for 20 minutes. Fluorescence was measured at $\lambda_{\text{ex}}480$ $\lambda_{\text{em}}590$ using the Versafluor[™] cuvette fluorimeter system (Bio-Rad) or at $\lambda_{\text{ex}}485$ $\lambda_{\text{em}}595$ in a 96 well format using a Spectrafluor Plus instrument (Tecan).

2.2.20 DNA extraction from whole blood

DNA extraction was carried out based on alcohol precipitation methods (Sambrook, *et al.* 1989). Selective lysis of erythrocytes was followed by lysis of DNA-containing leukocytes. Proteins were removed by ammonium acetate precipitation and nucleic acids were subsequently purified by isopropanol precipitation and ethanol washing.

Chromosomal DNA was extracted from whole blood with EDTA as anti-coagulant. 3mls whole blood was added to 9mls red cell lysis buffer (Appendix II) and incubated at room temperature for 15 minutes with occasional inversion. White cells were pelleted by centrifugation at 3,000 rpm for 5 minutes. The supernatant was decanted off, leaving ~50 μ l of residual liquid above the pellet. The pellet was resuspended by vigorous vortexing and 3mls white cell lysis buffer was added (Appendix II). The solution was mixed well before addition of 1ml protein precipitation solution (Appendix II). The solution was then transferred to 1.5ml microcentrifuge tubes and centrifuged at 14,000 rpm for one minute to pellet proteins. The resulting supernatant was transferred to a fresh tube containing 3mls isopropanol. This solution was inverted gently until DNA precipitated. DNA was pelleted by centrifugation at 14,000 rpm for 5 minutes and the supernatant was discarded. The pellet was washed with ice-cold 70% ethanol and pelleted once again by centrifugation at 3,000 rpm for 5 minutes. The supernatant was then discarded and the DNA was air-dried for 10-15 minutes before being resuspended in 100 μ l TE buffer. The resulting solution was incubated at 65°C for one hour to ensure DNA had fully dissolved.

2.3 Results

2.3.1 RNA Extraction

RNA was successfully extracted from whole peripheral blood (Figure 2.5). 18s and 28s bands were visualised on 1% agarose gels and RNA was subsequently used for reverse transcription and PCR. RNA quantity and purity was assessed by spectrophotometric analysis. All samples used for RT and PCR had an OD 260/280 ratio of >1.6. RNA yields were typically 0.2-0.5µg/µl and were calculated by using the following formula.

$$\text{RNA } (\mu\text{g}/\mu\text{l}) = \frac{\text{OD}_{260} \times 40 \times \text{dilution factor}}{1000}$$

2.3.2 Reverse transcription

Reverse transcription was deemed to be successful because resulting cDNA could be used as template in control multiplex PCR reactions for WASP and SNRPN products (Figure 2.6). PCR reactions with no template DNA produced no bands (lane 2) while the PCR multiplex PCR control reaction (lane 3) produced a SNRPN PCR product (143bp) and WASP PCR product (324bp).

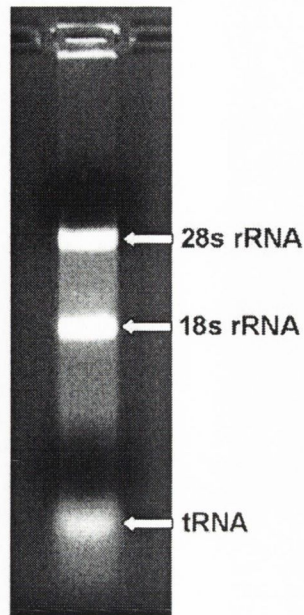


Figure 2.5: A representative example of RNA extraction. RNA was visualised on 1% agarose gels stained with ethidium bromide. 28s and 18s ribosomal RNA bands were visible as well as transfer RNA band

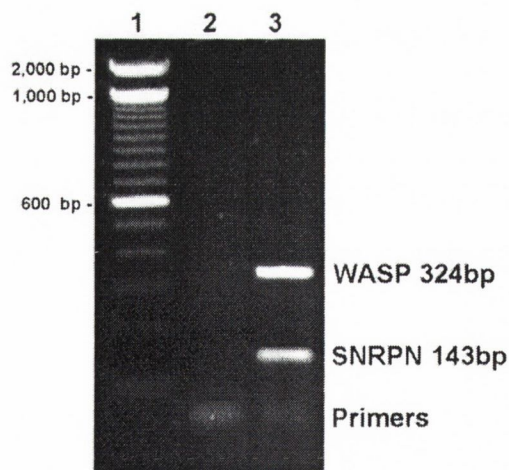


Figure 2.6: PCR of control products SNRPN and WASP. cDNA from reverse transcription was good a template for PCR. Lane 1: 100bp ladder. Lane 2: Blank (no cDNA). Lane 3: SNRPN/WASP PCR.

2.3.3 Optimisation of MgCl₂ concentration

PCRs for tTG were carried out at various MgCl₂ concentrations in order to determine the optimal molarity for tTG amplification (Figure 2.7a). 1.5mM MgCl₂ was sufficient to produce a tTG band at the appropriate molecular weight but also gave rise to a non-specific product at ~250bp (lane 4). 2mM MgCl₂ produced a similar tTG band with no significant contaminating bands (lane 5). 2.5mM MgCl₂ had the effect of inhibiting tTG PCR product formation and introducing a non-specific band at ~800bp (lane 6). The MgCl₂ concentration for subsequent PCRs was set 2mM.

2.3.4 Optimisation of cDNA concentration

PCRs for tTG were carried out at various cDNA concentrations in order to determine the optimal volume of cDNA template for tTG amplification. PCR reactions were carried out with 1μl, 2μl and 5μl cDNA template (Figure 2.7b). 1μl cDNA was found to be insufficient template to produce any detectable tTG PCR product (lane 4). 2μl cDNA template produced a clearly visible tTG band at the appropriate molecular weight but also yielded two non-specific products at ~550 and ~750bp (lane 5). The absence of these bands in any other PCR reaction suggests that they are the result of aerosol contamination of that particular reaction. 5μl cDNA template produced the strongest tTG band (lane 6) and therefore, the cDNA concentration was set at 5μl in subsequent PCR reactions.

2.3.5 Re-PCR of tTG PCR product

Since the levels of tTG PCR product generated were generally low, a dilution of tTG PCR product was used as template for a second round of PCR. This second PCR successfully amplified large amounts of the 2,067bp tTG product (Figure 2.7 c).

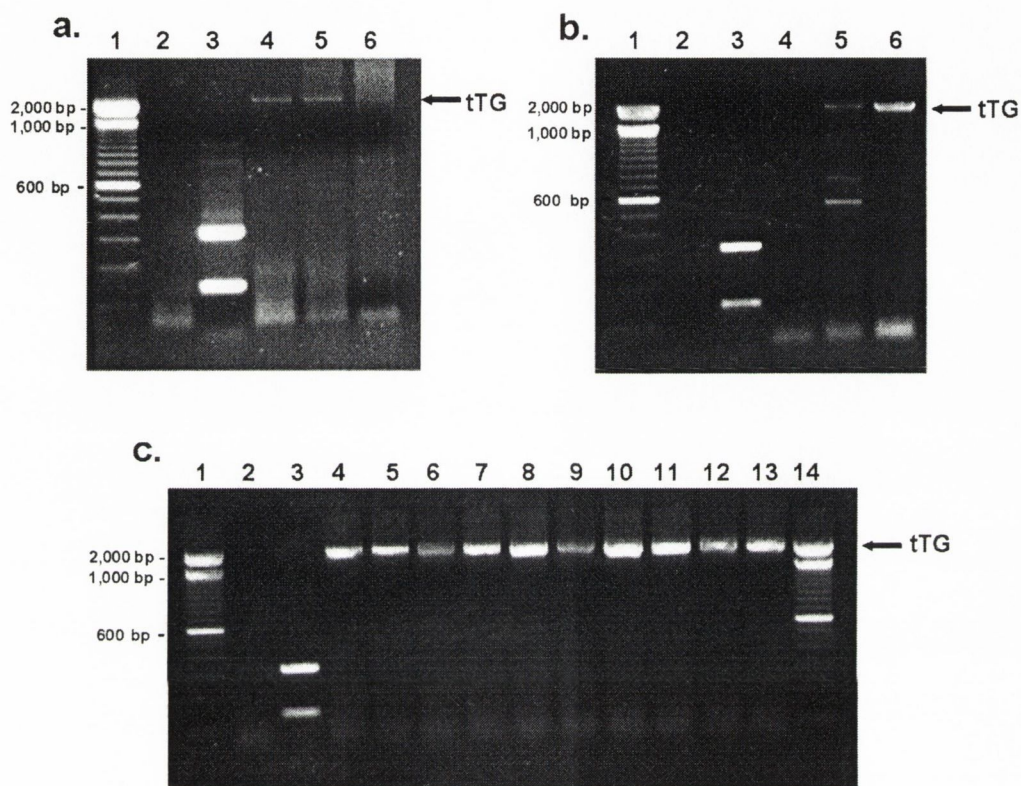


Figure 2.7: PCR optimisation. a: MgCl₂ optimisation. 1: 100bp ladder, 2: Blank, 3: SNRPN/WASP, 4: 1.5mM MgCl₂, 5: 2mM MgCl₂, 6: 2.5mM MgCl₂. **b. cDNA optimisation.** 1: 100bp ladder, 2: Blank, 3: SNRPN/WASP, 4: 1μl cDNA, 5: 2μl cDNA, 6: 5μl cDNA. **C. tTG second round of PCR.** 1&14: 100bp ladder, 2: Blank, 3: SNRPN/WASP, 4-13 tTG second round of PCR.

2.3.6 Restriction digest analysis of ligated vectors

Restriction digest of recombinant vectors with enzymes EcoRI and XhoI successfully identified tTG PCR product inserts (Figure 2.8). Wild type tTG and mutant tTG were 2,067bp and were easily distinguished from vectors (TOPO-XL: 3.5kb; pGEX-4t-1: 4.9kb).

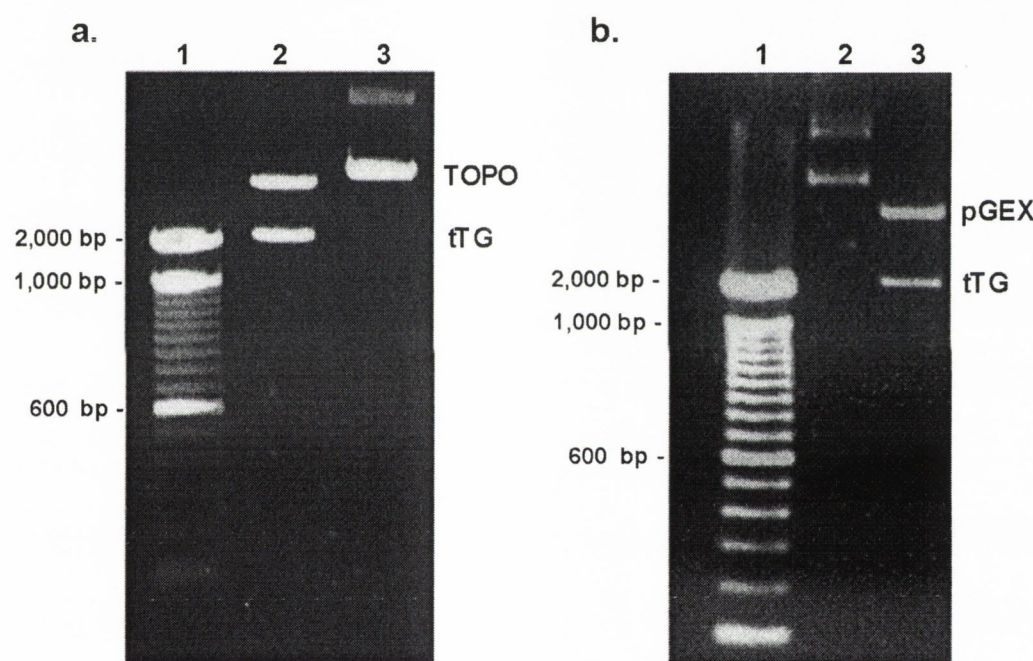


Figure 2.8: Restriction digest analysis of ligated vectors. (a) 1: 100 bp ladder; 2: Restriction digest of TOPO-tTG with EcoRI and XhoI; 3: Uncut TOPO-tTG. (b) 1: 100 bp ladder; 2: Uncut pGEX-tTG; 3: Restriction digest of pGEX-tTG with EcoRI and XhoI.

2.3.7 DNA sequencing

DNA sequencing was carried out using the LiCor GeneReadIR™ 4200 system. Four sense and three anti-sense primers were used to sequence the entire length of the wild type and mutant tTG sequences (Table 2.3). The wild type tTG sequence was found to be completely homologous to a previously characterised endothelial tTG sequence (Gentile, *et al.* 1991) except for an A→G shift at position 1592 which resulted in an asparagine to serine shift at amino acid 531. In order to determine whether this was a genuine single nucleotide polymorphism or an error introduced by PCR, the original subject's chromosomal tTG gene was sequenced. This mutation was absent in the blood donor's chromosomal DNA and was therefore deemed to be an error introduced during PCR. Site-directed mutagenesis was used to successfully correct this error (see Section 2.3.8) DNA sequencing was also performed to screen for successful mutants resulting from site-directed mutagenesis experiments

2.3.8 Site-directed mutagenesis

The 3D-PSSM system was used to investigate any possible effect the chosen mutations may have on the structure of tTG. The output generated by the program suggested that the secondary structure of tTG should not be significantly altered by mutation of the Cys-His-Asp catalytic triad to alanine residues (Appendix III). Mutation of Cys²⁷⁷ to alanine results in no change in secondary structure but even in the wild type protein this is a region with poor predictability since reliability scores are low. Mutation of His³³⁵ to alanine results in no change in secondary structure or reliability of score. Mutation of Asp³⁵⁸ to alanine results in no apparent change in secondary structure although the reliability score at this position is zero.

The Strategene Quickchange® site-directed mutagenesis system successfully repaired the error at codon 531 and mutated codons 277, 335 and 358 to alanine coding sequences. DNA sequencing was used to screen for successful mutants from site-directed mutagenesis experiments (Figure 2.9). In order to correct the A1592G error introduced during PCR, site-directed mutagenesis primers were designed to return the DNA sequence to its normal genotype (Table 2.6). The average mutagenesis efficiency was 58.32% (n=5) although 100% efficiency was observed in 2 experiments.

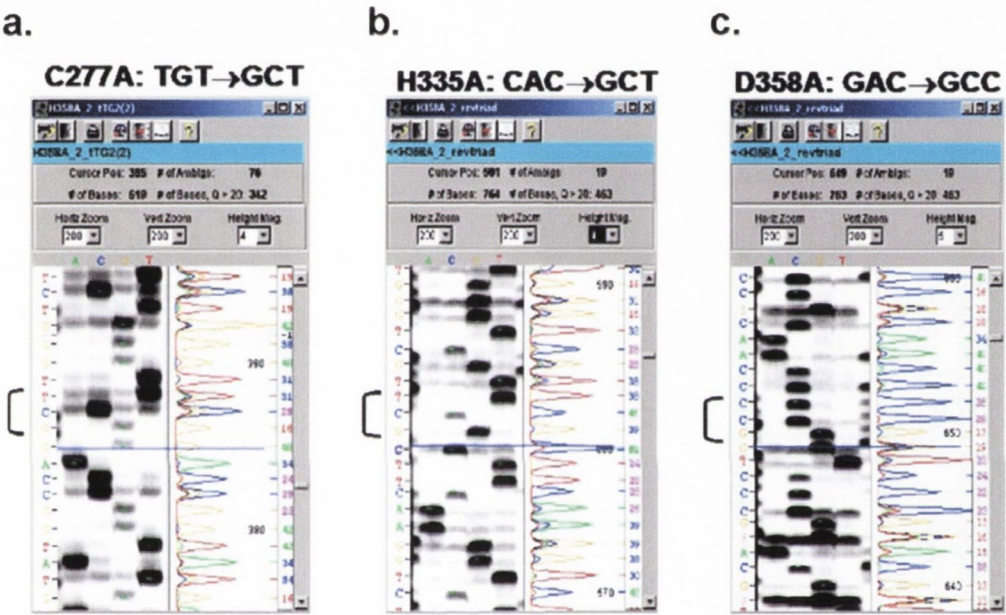


Figure 2.9: DNA sequencing of mutant codons. DNA sequencing was performed to screen plasmid isolated from bacterial colonies resulting from Quickchange® site-directed mutagenesis experiments. **a.** Cys²⁷⁷→Ala. **b.** His³³⁵→Ala. **c.** Asp³⁵⁸→Ala. Images are output from e-Seq™ software (LiCor). Brackets denote mutated codons.

Table 2.6: Site-directed mutagenesis primers designed to correct A1592G PCR error

S531N-F	5'- CCAAGTACCTGCTC <u>AAC</u> CTCAACCTGGAGCC- 3'
S531N-R	5'- GGCTCCAGGTTGAGG <u>TTG</u> AGCAGGTACTTGG- 3'

Primer set S531N were ordered from Sigma-Genosys to correct an error introduced during PCR (Ser to Asp shift at codon 531)

2.3.9 Optimisation of protein expression and purification

The results of protein expression experiments are shown in Figure 2.10. Total *E.coli* proteins were separated on an SDS-PAGE gel in order to visualise any bands influenced by IPTG induction. Before addition of 1mM IPTG (lane 2), there is no protein band visible at the molecular weight of the tTG-GST fusion protein (103 kDa). At subsequent sampling times, however, a distinct increase in protein at 103 kDa is clearly visible (lanes 3-7). Expression of a protein with the correct molecular weight was clearly being induced by derepression of the Ptac promoter.

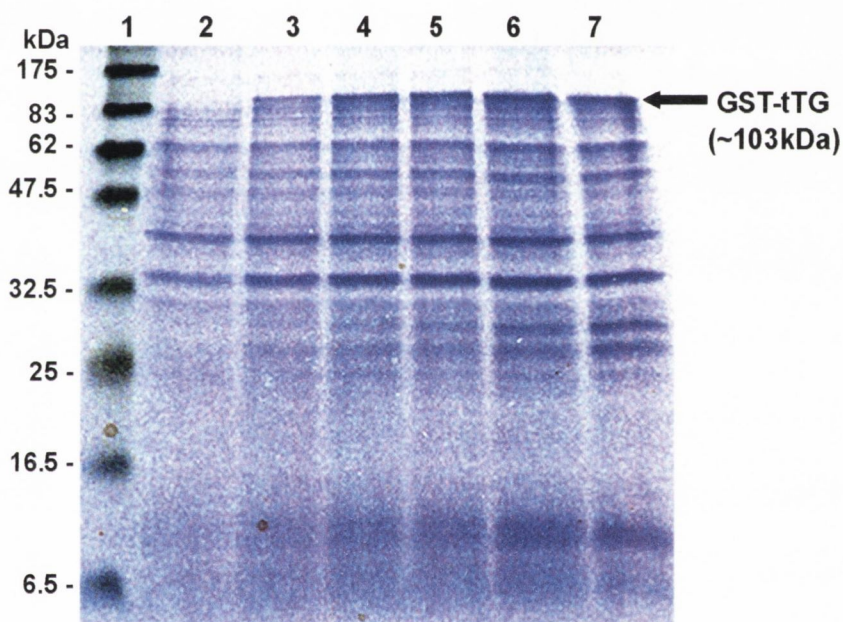


Figure 2.10: tTG expression in *E.coli*. tTG expression was induced by 1mM IPTG and tTG expression was monitored by visualising total *E.coli* protein on SDS-PAGE. 1: broad range protein marker, 2: 0 hours, 3: 0.5 hours, 4: 1 hour, 5: 1.5 hours, 6: 2 hours, 7: 3 hours.

Initial protein purification attempts showed that, in keeping with previous attempts to purify recombinant tTG from *E.coli* (Shi, *et al.* 2002), >90% of tTG was present in inclusion bodies. After unsuccessfully attempting various in-house methods for cell lysis and protein purification, a commercial detergent reagent, CellLytic™ B (Sigma), was used to lyse *E.coli*. This method proved more efficient than cell lysis using lysozyme alone but lysate viscosity was still very high. In order to reduce this viscosity, ~1,000 units Dnase I (Promega) were added. In order to maximise soluble tTG yield from *E.coli*, various methods were used in order to reduce the amount of tTG being produced by cells. These methods included vigorous agitation, reduced induction temperature and low IPTG concentrations. An SDS-PAGE gel from an experiment optimising IPTG concentration is shown in Figure 2.11. The strongest yield of soluble tTG fusion protein is clearly from cultures induces with 1μM IPTG (lane 3). A dramatic reduction in tTG fusion protein yield is noted when 5μM, 10μM or 20μM IPTG is used to induce protein expression (lanes 4-6). This provides evidence for the initiation of inclusion body formation above a threshold level of induction.

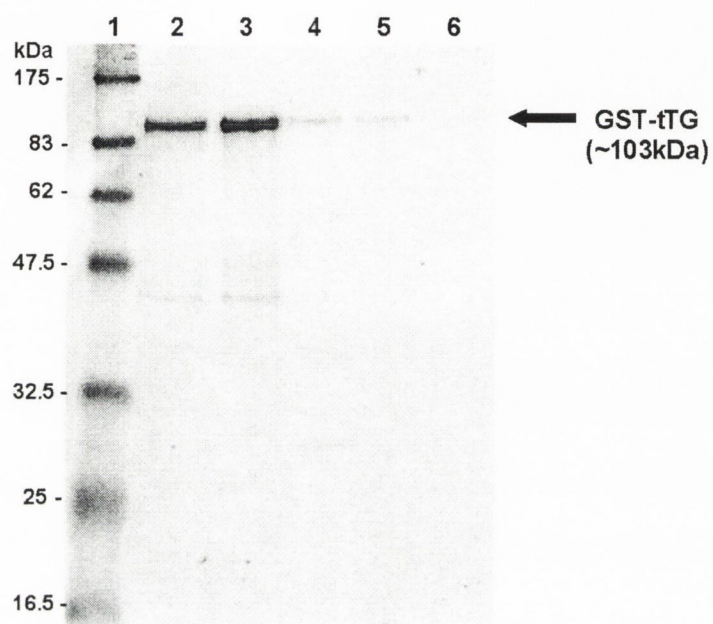


Figure 2.11: IPTG concentration optimisation. Various micromolar concentrations of IPTG were used to optimise tTG expression level. The fusion protein of tTG (77kDa) and GST (26kDa) results in a band of ~103 kDa. 1: broad range protein marker, 2: 0.5 μ M IPTG, 3: 1 μ M IPTG, 4: 5 μ M IPTG, 5: 10 μ M IPTG, 6: 20 μ M IPTG.

The optimisation conditions arrived at were 1 μ M IPTG induction overnight at room temperature with 200rpm agitation. An SDS-PAGE gel of a complete tTG purification experiment is shown in Figure 2.12. The tTG fusion protein is clearly absent prior to induction (lane 2) and present post-induction (lane 3). When a small sample of the insoluble pellet post-lysis was separated by SDS-PAGE (lane 4), the vast majority of cellular protein was clearly made up of tTG fusion protein in inclusion bodies. Total cell lysate (lane 5) was incubated with glutathione sepharose slurry to sequester GST-fusion protein and the unwanted proteins were washed away (lanes 6-9). The eluted tTG fusion protein is clearly visible and does not elute with any major contaminating bands (lane 10). Protocol for purification of wild type and mutant tTGs was identical. SDS-PAGE of purified wild type and mutant tTGs is shown in Figure 2.13.

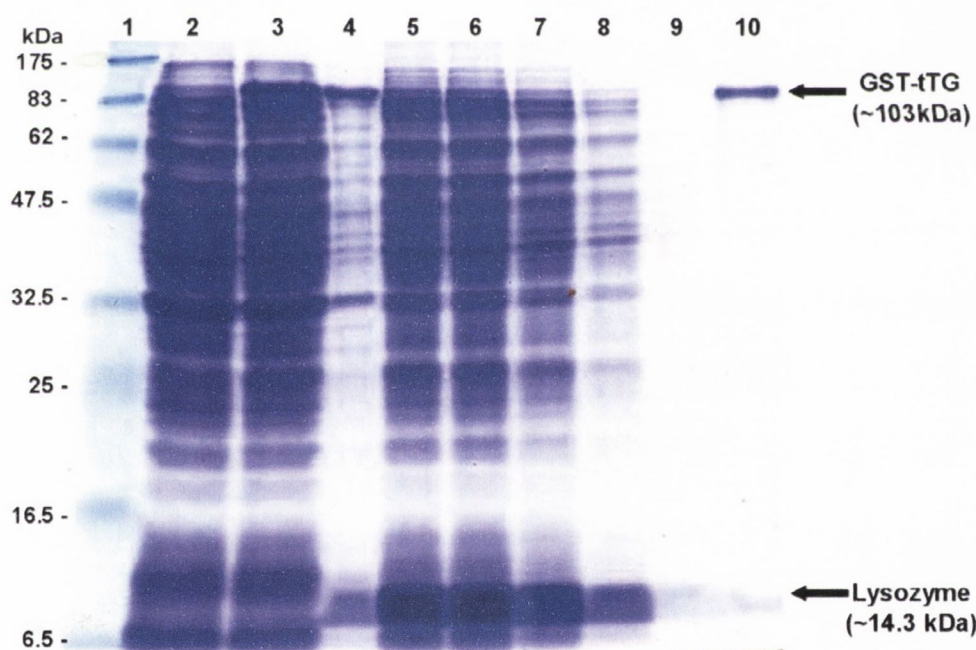


Figure 2.12: tTG expression and purification. 1: broad range protein marker. 2: *E.coli* protein pre-induction. 3: *E.coli* protein post-induction. 4: Insoluble protein fraction, post-lysis. 5: Soluble protein fraction, post-lysis. 6-9: Discarded washes. 10: Pure GST-tTG fusion protein.

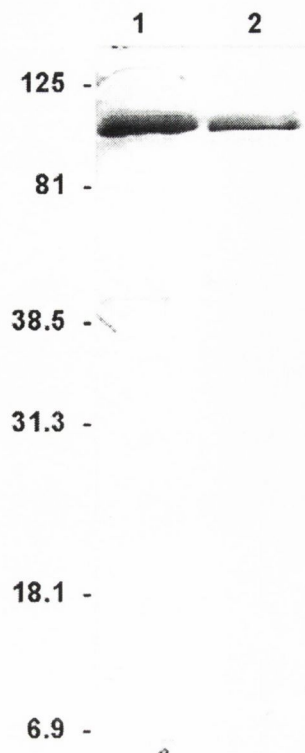


Figure 2.13: SDS-PAGE of wild type and mutant tTG. 1: Recombinant wild type tTG. 2: Recombinant mutant tTG.

2.3.10 Recombinant protein quantification

The NanoOrange[®] reagent was used to generate a standard curve using a BSA standard. This curve was used to measure the concentration of purified recombinant tTG. Yields of wild type tTG were, on average, 2-fold more concentrated than mutant tTG yields. Wild type tTG yields ranged from 0.519-2.159mg/L *E.coli* while mutant tTG yields ranged from 254-688μg/L *E.coli* (Table 2.6). An example of a NanoOrange[®] standard curve is shown in Figure 2.14.

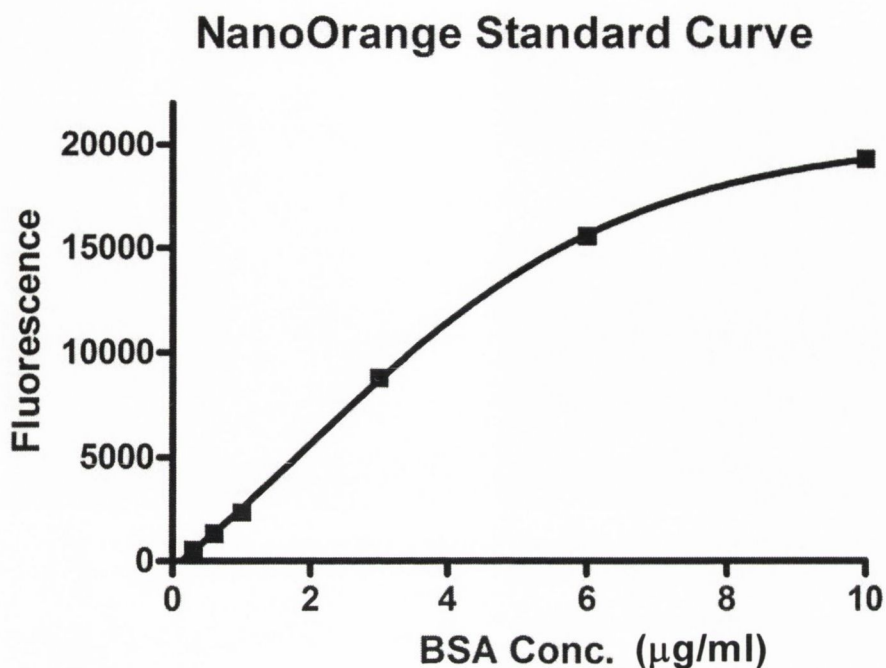


Figure 2.14: NanoOrange standard curve. Generated using BSA standard (Molecular Probes) and GraphPad Prism software.

Table 2.6: NanoOrange quantification results

Date	Wild type tTG yield	Date	Mutant tTG Yield
12/08/05	557.03µg	24/8/05	676.81µg
18/08/05	470.35µg	04/11/04	256.93µg
06/09/05	2159.079µg	10/11/04	688.78µg
10/09/05	686.27µg	17/11/04	607.84µg
13/09/05	1875.15µg	27/06/05	254.97µg
23/06/05	823.12µg		
28/06/05	519.22µg		
Mean	1012.89µg		502.61µg
SD	700.69µg		199.27µg
Median	686.27µg		569.09µg

SD: standard deviation

2.4 Discussion

2.4.1 RT-PCR and DNA sequencing

RT-PCR was successful in amplifying the 2kb transcript of tissue transglutaminase from peripheral blood RNA. In order to produce sufficient amounts of tTG PCR product, this PCR product was diluted 400-fold and used as template in a second PCR reaction. The resulting PCR product produced a strong band on agarose gels (Figure 2.7 c) but had been subjected to 80 cycles of PCR amplification, increasing the risk of error.

The error rate of *Taq* polymerase has been estimated between 2×10^{-5} (Lundberg, *et al.* 1991) and 2.1×10^{-4} (Keohavong and Thilly 1989) errors per base pair. Indeed, upon DNA sequencing, an error was discovered in codon 531 (AAC→AGC) resulting in an asparagine to serine shift. Sequencing of the original blood donor's chromosomal DNA confirmed that this was not a natural single nucleotide polymorphism and had been introduced during PCR. This error could probably have been avoided by using a *Taq* polymerase with proofreading ability such as Platinum[®] *Taq* high fidelity (Invitrogen). As a result of this error, a fourth and fifth round of mutagenesis were required to correct this error in the pGEX-4T-1 plasmids containing wild type and mutant tTG inserts.

2.4.2 Site-directed mutagenesis

Site-directed mutagenesis was successfully carried out for the three codons of the catalytic triad, generating a novel tTG construct. Mutation efficiency was calculated as the percentage of mutation-containing colonies screened for each mutation reaction. Stratagene claim that the Quickchange reaction kit has a mutation efficiency of greater than 80%. However, the average mutagenesis efficiency in these experiments was 58.32% (n=5) although 100% efficiency was observed in 2 experiments. Mutagenesis was also used to successfully repair an error introduced during PCR at codon 531.

2.4.3 Recombinant tTG expression and purification

The protein production strategy in this study was based upon previous studies generating recombinant human tTG proteins. Since previous reports generating recombinant tTG in *E.coli* have generally used either the His-tag (Liu, *et al.* 2002, Piper, *et al.* 2002, Sblattero, *et al.* 2002, Shi, *et al.* 2002) or the GST-tag (Lai, *et al.* 1998, Iismaa, *et al.* 2000, Murthy, *et al.* 2002) for affinity purification, the initial decision on which affinity method to use was made early in the experimental design. Since the poor solubility of His-tagged tTG has been reported elsewhere (Shi, *et al.* 2002), it was decided to produce the recombinant tTGs as GST-tagged proteins, given that previous reports have suggested that GST enhances solubility of difficult proteins (Nygren, *et al.* 1994, Smyth, *et al.* 2003).

The tendency for tTG to aggregate in inclusion bodies made protein purification troublesome. Purification of inclusion bodies and refolding of aggregated protein was not an option because although a protocol has been reported for the refolding of transglutaminase from inclusion bodies using polyethylene glycol (Ambrus and Fesus 2001), not all studies have been successful in producing active, antigenic tTG from inclusion bodies (Shi, *et al.* 2002). The results of optimisation experiments demonstrated that extremely gentle induction conditions were required to generate higher levels of soluble tTG. In fact, there is a general consensus in the literature that recombinant tTG produced in *E.coli* must be induced very gently in order to maximise protein yield. Several studies have employed micromolar levels of IPTG and low temperatures for recombinant tTG induction (Iismaa, *et al.* 2000, Liu, *et al.* 2002, Murthy, *et al.* 2002, Piper, *et al.* 2002, Sblattero, *et al.* 2002).

After exhaustive optimisation of conditions such as lysis buffer, lysis method, induction time, temperature and IPTG concentration, highly pure wild type and mutant tTGs were produced (as evidenced by SDS-PAGE and Western blotting).

Although pGEX-4T-1 produces GST fusion proteins with a thrombin cleavage site between the protein of interest and the GST tag, it was decided to leave the GST tag attached to tTG. There were two reasons for this decision. First, yields of tTG were extremely low. Any subsequent manipulation would have resulted in further depletion of tTG. Second, as previously reported (Nygren, *et al.* 1994, Smyth, *et al.* 2003), the GST tag enhances solubility of recombinant proteins and removal of the affinity tag could have resulted in reduced solubility of tTG. The GST tag should not have affected subsequent ELISA assays since both wild type and mutant tTGs have equimolar amounts of GST attached.

Quantification of recombinant tTG proteins using NanoOrange™ reagent revealed that yields of wild type tTG were, on average, ~2-fold greater than yields of mutant tTG. This result was possible evidence for increased insolubility of mutant tTG caused by mutagenesis of the catalytic triad. The lower yields of mutant tTG could suggest more problematic folding of the mutant protein. Since cysteine 277 represents the starting point for a coiled structure within the core region of tTG, *in silico* mutation using the 3D-PSSM system was used to confirm that secondary structure in the region did not seem to be affected by the mutagenesis. Also, experiments described in Chapter 3 will demonstrate that the mutant tTG and wild type tTG are recognised equally well by polyclonal antibodies raised against human tTG and are, therefore, likely to be folded in a similar fashion. It is also noteworthy that while average yields differed greatly, the median yields were quite similar at 686.27µg and 569.09µg per litre *E.coli* for wild type and mutant tTGs respectively.

2.4.4 Conclusions

To summarise, the 2kb tTG transcript was successfully amplified by RT-PCR and cloned into TOPO and pGEX-4T-1 vectors for DNA sequencing and protein production respectively. Site-directed mutagenesis was used to mutate the codons of the catalytic triad (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) to alanine residues. Mutagenesis was also used to correct a SNP error introduced during PCR at position 1592.

Protein production and purification proved problematic due to the insolubility of recombinant tTGs. After exhaustive optimisation of induction and lysis conditions, both wild type and mutant tTG were produced as GST fusion proteins in BL21 *E.coli* and purified from bacterial cell lysates. SDS-PAGE analysis showed that the recombinant tTGs were highly pure and corresponded to their predicted molecular weight. NanoOrange™ protein quantification analysis showed that while wild type tTG was purified in quantities that averaged 2-fold greater than mutant tTG, yields varied greatly. Yields of wild type and mutant tTGs averaged ~1mg and ~0.5mg per litre of *E.coli* respectively which compared well with yields of ~0.6mg His-tagged tTG per litre *E.coli* in a report by Shi (Shi, *et al.* 2002).

Chapter 3

The importance of the catalytic triad of tissue transglutaminase in autoantibody-tissue transglutaminase interactions

3.1 Introduction

3.1.1 Epitope mapping

Epitope mapping refers to methods used to characterise binding sites on protein antigens. Antigen sites conform to one of two possible architectural types. Epitopes may constitute part of a linear amino acid sequence on a polypeptide chain (linear epitope) or may be formed when more than one stretch of primary sequences which are distant from each other, are brought together in the folded molecule's secondary or tertiary structure. It has been well established that B-cell epitopes are conformation-dependent and require correctly folded protein antigen (Westwood and Hay 2000).

With knowledge of the amino acid sequence of a protein antigen, the precise residues involved in protein-antibody interactions can be determined by site-directed mutagenesis. To determine the residues involved in antibody-antigen complex formation, alanine-screening mutagenesis may be employed (McCarthy-Troke, *et al.* 1997). Alanine is an amino acid with a very simple structure, i.e. it has only has a single methyl group as side-chain. Therefore, alanine substitution has the advantage of effectively removing from the antigen-antibody interface the energetic contributions of all side chain atoms beyond the C β position, including intramolecular forces such as side-chain hydrogen bonds (Westwood and Hay 2000).

Given the conformation dependence of B-cell epitopes, a site-directed mutagenesis approach to epitope mapping is a superior tool to investigate tTG-autoantibody interaction when compared to previous tTG fragment analysis studies (Seissler, *et al.* 2001, Sblattero, *et al.* 2002, Nakachi, *et al.* 2004). For this reason, the antigenicity of wild type and mutant tTGs (generated in Chapter 2) were compared in ELISA assays with coeliac disease and dermatitis herpetiformis patient sera. While the traditional approach of site-directed mutagenesis is to alter one amino acid at a time, this study altered three amino acids (Cys²⁷⁷, His³³⁵ &

Asp³⁵⁸) that come together in the tertiary structure of tTG. Given that the tTG enzyme is 687 amino acids long, this “shotgun” approach was more likely to disrupt the steric properties of these three key amino acids and yield meaningful results.

3.1.2 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA assay is a solid phase assay that can be used to detect antigen or antibody in solution. The basic principle is the use of an antibody or antigen conjugated with an enzyme, which, upon reacting with its substrate, forms a soluble coloured reaction product. The coloured product is monitored visually or spectrophotometrically and used to determine the proportionality between the amount of colour and the amount of analyte present (Diamandis and Christopoulos 1996). Since a single unit of enzyme can amplify a product manifold, many ELISAs have been optimised to detect analytes at the picomole or attomole level (Lapresle and Lafaye 1985, Inouye, *et al.* 2002).

In this study, a non-competitive indirect solid phase ELISA assay was established in order to compare antigenicity of wild type and mutant tTGs generated in Chapter 2. This type of assay is frequently used in the clinical laboratory and involves coating the antigen of interest onto a solid phase. When patient sample is added, antibodies specific for the antigen of interest bind and these can be subsequently detected by using an enzyme labelled secondary antibody (Kricka 1999). Isotype-specific, enzyme labelled anti-immunoglobulin antibodies can be used as secondary antibodies in order to determine the specific immunoglobulin class present (Tijssen 1985). A schematic diagram of a non-competitive indirect solid phase tTG ELISA assay is shown in Figure 3.1.

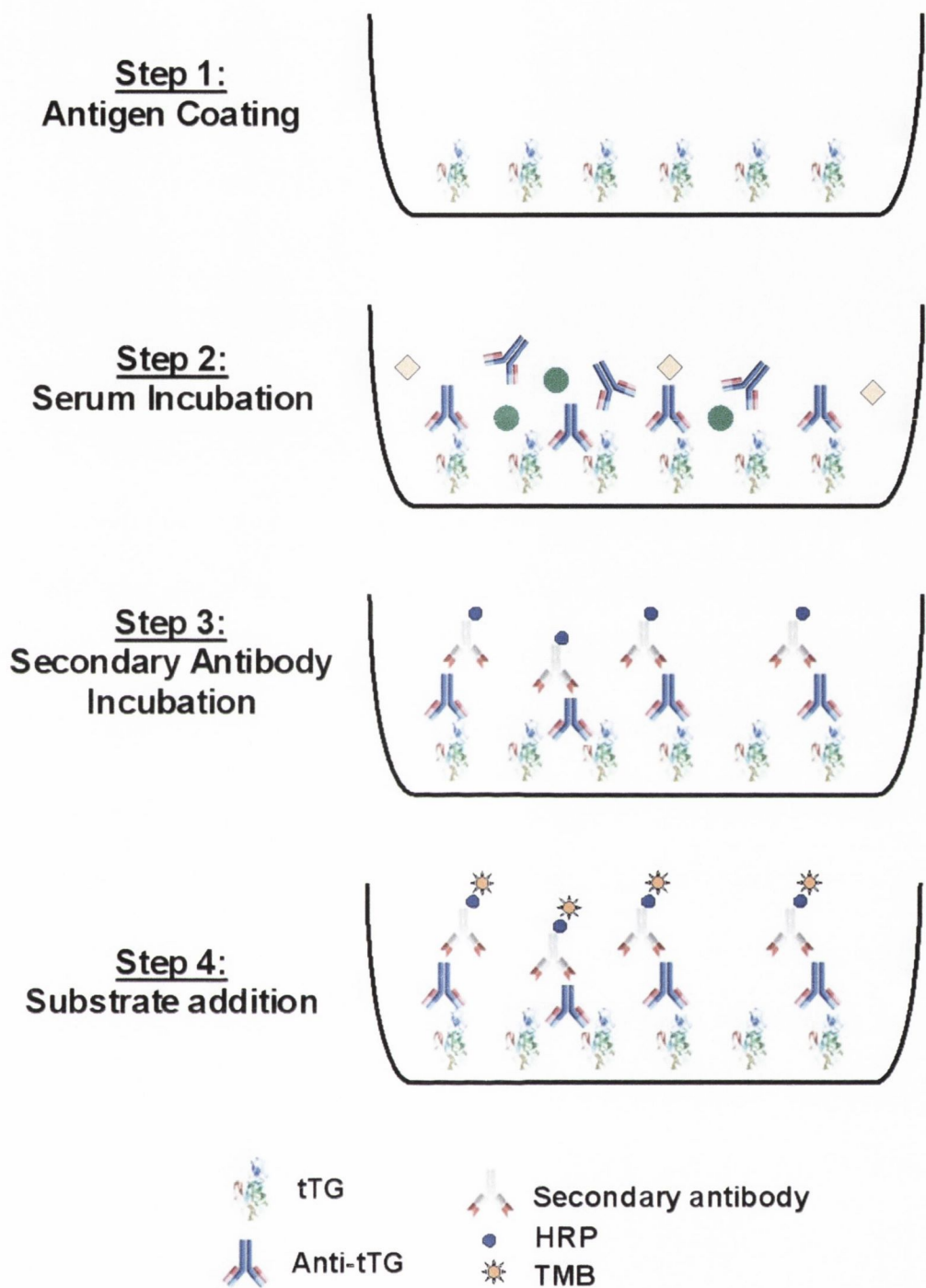


Figure 3.1: Non-competitive indirect solid phase tTG ELISA assay.

tTG ELISA is a four-step process involving antigen binding, test serum application, secondary antibody incubation and substrate addition. The plate is washed thoroughly between each step.

The development of an in-house ELISA assay involves the optimisation of several variables. Coating of ELISA plates with antigen is a crucial variable. Most antigens bind plastic plates through hydrophobic interactions, and are coated at concentrations from 1-50µg/ml. When establishing an assay, checkerboard experiments are often used to optimise antigen coating and secondary antibody concentrations. A high pH buffer is often used to facilitate antigen coating. Physical properties of the antigen can have a profound effect on antigen coating (Ryall, *et al.* 1982).

When deciding upon a substrate for ELISA assays, several factors must be taken into account. First, the substrate must produce a measurable reaction product. For most assays this is a coloured product but fluorescent substrates can be used (Blake and Weimer 1997). Other factors that must be taken into account are sensitivity, background absorbance, stability of the compound and toxicity.

One of the most commonly used enzyme reporters is horseradish peroxidase (HRP), which reduces hydrogen peroxide (H₂O₂) and oxidises a second substrate, which in turn produces a coloured product. The most common substrates are o-phenylenediamine, 5-aminosalicylic acid, 2,2'-azino-di-(3-ethyl-benzthiozoline sulfonate-6 and 3,3',5,5'-tetramethyl-benzidine (TMB) (Carpenter 2002).

There are two ways to handle data generated from ELISA assays, quantification of amount of analyte present in absolute amounts (nanograms to micrograms per millilitre) or the reporting of actual absorbance values in arbitrary ELISA units. In the latter method, a strongly positive sample can be used to create a standard curve by doubling dilution (Diamandis and Christopoulos 1996). Although a standard curve can be generated by simply plotting the absorbance against a log of concentration and performing linear regression, spline methods for curve generation are often more appropriate for ELISA assays. A spline is a special curve defined piecewise by polynomials and can be constructed using software such as Multicalc (Wallac).

Testing a bank of sera from control subjects for reactivity to the antigen is a method used to generate cut-off points for positivity. The cut-off point is usually set as the mean + 2 standard deviations of the control population response (Carpenter 2002).

Various strategies are often employed to ensure OD values for negative controls are <0.2, positive controls are >1.0 and blank wells are <0.1 (Carpenter 2002). Including a blocking step using an unrelated protein such as BSA is a good method to remove non-specific attachments of antibodies to ELISA wells. A detergent such as Tween-20 is also a common additive to ensure efficient plate washing between antibody incubations. Troubleshooting procedures for optimisation of ELISA assays are outlined in Table 3.1.

Table 3.1: Troubleshooting ELISA optimisation

Problem	Solution(s)
OD of blank >0.1.....	Increase number of washes
OD of negative control >0.2.....	Include/lengthen blocking step Increase conjugate dilution Consider alternate solid phase
OD of positive control <1.0.....	Increase amount of antigen Increase purity of antigen Increase incubation time/temp
Overall assay gives low values.....	Check substrate, buffer, pH

Adapted from (Carpenter 2002)

3.1.3 Western Blot Analysis

Western blot analysis is based on a protein-protein hybridisation technique that is used for immunodetection of specific antigens of interest (Cseke, *et al.* 2003). It is a simple, sensitive and effective technology that has been applied in immunology, molecular biology and protein chemistry. The principle of Western blotting is as follows: (1) A protein mixture is first separated based on molecular weight on an SDS-PAGE gel (as per Section 2.2.16). (2) The separated proteins are immobilised on a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. (3) The protein of interest is detected by incubation with a primary antibody raised against an epitope on it. (4) The antigen-antibody complex is visualised by incubation with a second antibody that is either fluorescently labelled, bound to an enzyme with a colorimetric substrate, or is radioactively labelled (Towbin, *et al.* 1979, Fido, *et al.* 1995).

Immobilisation of proteins is carried out electrophoretically and can be carried out under wet or semi-dry conditions. In this study, the semi-dry method was used and consists of soaked filter paper on either side of polyacrylamide gel/PVDF sheets that are separated by dialysis membranes and stacked between graphite plate electrodes. In this horizontal method, the only buffer reservoirs are the transfer buffer-soaked filter papers (Kyhse-Andersen 1984).

3.1.4 Chapter aims

The purpose of this chapter was to compare the antigenicity of full-length recombinant wild type and the catalytic triad mutant tTG using coeliac disease and dermatitis herpetiformis sera. These results indicate the importance of the Cys-His-Asp catalytic triad as an epitope in tTG-autoantibody interactions. Commercially available monoclonal and polyclonal antibodies were used to characterise recombinant tTGs in Western blots and ELISA assays. These experiments tested the antigenicity of wild type tTG and the conservation of epitopes on the mutant tTG.

3.2 Materials and Methods

3.2.1 Serum samples

5 serum samples from coeliac disease patients with very strong anti-tTG serology (>100 AU by Celikey™ assay) were pooled, stored in aliquots at -70°C and used to generate standard curves for ELISA assays. 76 serum samples from 61 coeliac disease patients (46 females, 15 males; age 15-76 years, median = 50) were used to characterise tTG binding. Diagnosis of coeliac disease was based upon histological analysis of duodenal biopsy as well as positivity for anti-tTG and anti-endomysial antibodies. Sera from 10 dermatitis herpetiformis patients (3 females, 7 males; age 19-64 years, median = 53) were also included in the study. 49 sera (29 females, 20 males; age 17-84, median = 53) from individuals found to be negative for tTG reactivity using the Celikey® ELISA system were used to establish cut-off points for ELISA assays. Ethical approval for this study was obtained from the joint ethics committee of St. James's and Tallaght hospitals.

3.2.2 Western Blotting

The Western blotting protocol was taken from *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.* 1989). Briefly, SDS-PAGE was carried out as described in Section 2.2.16 and protein was blotted onto PVDF membranes (0.8mA/cm²) using a semi-dry apparatus (Apollo). The membrane was blocked at 4°C overnight with 5% non-fat dried milk including 0.5% Tween. Primary antibodies used were CUB 7402/TG100, a commercially available anti-tTG mouse monoclonal antibody cocktail (Labvision), GST01, a commercial mouse anti-GST antibody (Labvision) and polyclonal rabbit anti-tTG (Roboscreen). All antibodies were diluted 1:1000 in 5% non-fat dried milk including 0.5% Tween. After 2 hours of incubation at room temperature and thorough washing with PBS (Appendix II) containing 0.1% Tween, the membranes were incubated with 1:1000 dilution of either rabbit anti-mouse conjugated to horseradish peroxidase (HRP) or swine anti-rabbit conjugated to HRP (Dako). Blots

were visualised using chromagenic substrate DAB (3,3'-Diaminobenzidine) and hydrogen peroxide.

3.2.3 Tissue transglutaminase ELISA Optimisation

To maximise data generated from ELISA assays using recombinant tTGs, it was necessary to optimise several parameters. Optimisation of tTG coating concentration and secondary antibody (Rabbit anti-human IgA HRP, Dako) concentration were carried out concurrently in checkerboard experiments. On one Nunc Maxisorp™ plate, antigen concentration was varied from left to right while secondary antibody dilution was varied from top to bottom. Antigen coating concentration ranged from 0.1 to 0.8µg/well and secondary antibody dilution ranged from 1:500 to 1:5000. These antigen-antibody concentrations were used to test two sera in triplicate. The two sera were a strongly anti-tTG positive serum sample and an anti-tTG negative serum (previously characterised by the Celikey™ assay). An example of such a plate layout can be seen in Figure 3.2.

	0.8µg/well			0.5µg/well			0.2µg/well			0.1µg/well		
2°	+ve	-ve	B	+ve	-ve	B	+ve	-ve	B	+ve	-ve	B
1:500												
2°												
1:1000												
2°												
1:2500												
2°												
1:5000												

Figure 3.2: ELISA checkerboard experiment: Checkerboard experiments were carried out in order to optimise antigen coating concentration and secondary antibody dilution. (+ve = known anti-tTG positive serum, -ve = known anti-tTG -ve serum, B = blank)

3.2.4 Standard curve generation

Once antigen coating concentration and secondary antibody dilution were established, standard curves were generated using pooled high-titre anti-tTG positive sera. Pooled positive samples were diluted from 1:160 to 1:10,240 in order to generate a 7-point standard curve in every ELISA assay.

3.2.5 Final ELISA protocol

ELISAs were performed by coating certified 96 well Maxisorp™ plates (Nunc) with 0.3µg of either purified recombinant tTG or mutant tTG for 16 hours at 4°C in coating buffer (50mM Tris-HCL, 150mM NaCl, 5mM CaCl₂, pH 7.5). Wells were blocked with 1% BSA in PBS plus 0.1% Tween (Appendix II) for one hour and washed four times with PBS plus 0.1% Tween between each step. Antibodies used in Western blots were also used as primary antibodies in control ELISAs. Antibodies were diluted as follows: CUB 7402/TG100 and GST01 were diluted 1:1000 with PBS plus 0.1% Tween followed by a 1:1000 dilution of rabbit anti-mouse HRP (Dako). Polyclonal anti-tTG was diluted 1:1000 with PBS plus 0.1% Tween followed by 1:1000 swine anti-rabbit conjugated with HRP (Dako). For IgA assays, human sera were diluted 1:20 with PBS plus 0.1% Tween followed by 1:1000 rabbit anti-human IgA conjugated to HRP (Dako). For IgG assays, human sera were diluted 1:500 with PBS plus 0.1% Tween followed by 1:500 rabbit anti-human IgG conjugated to HRP (Dako). All antibody incubations were carried out at room temperature for 1 hour. ELISAs were developed using TMB liquid substrate system (Sigma) for 7 minutes at room temperature. Assays were stopped by addition of 0.5M H₂SO₄ and were read at A₄₅₀ in a Wallac Victor² multilabel counter.

Cut-off points were established by incubating 49 serum samples found to be negative for anti-tTG responses with wild type and mutant tTGs. Cut off points were calculated as mean +2SD. The finalised plate layout for tTG binding experiments is shown in Figure 3.3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	-ve 1	-ve 1	1	1	9	9	1	1	9	9
B	1:160	1:160	-ve 2	-ve 2	2	2	10	10	2	2	10	10
C	1:320	1:320	+ve 1	+ve 1	3	3	11	11	3	3	11	11
D	1:640	1:640	+ve 2	+ve 2	4	4	12	12	4	4	12	12
E	1:1280	1:1280	Poly	Poly	5	5	13	13	5	5	13	13
F	1:2560	1:2560	Poly	Poly	6	6	14	14	6	6	14	14
G	1:5120	1:5120	mut -ve1	mut -ve1	7	7	15	15	7	7	15	15
H	1:10240	1:10240	mut -ve2	mut -ve2	8	8	16	16	8	8	16	16

Figure 3.3: tTG binding ELISA plate layout. White wells were coated with wild type tTG while shaded wells were coated with mutant tTG. Columns 1 and 2 represent blank and standard curve wells. Columns 3 and 4 represent a series of controls including polyclonal (Poly) anti-tTG (Roboscreen). Columns 5-12 represent test sera, which were analysed for binding to both wild type and mutant tTGs simultaneously in each experiment.

3.2.6 Inter and intra assay variability

Intra-plate assay variability was determined by calculating the percentage co-efficient of variation (%CV) when aliquots of each of two serum samples were distributed about one plate coated with tTG antigen and probed with rabbit anti-human IgA-HRP (Dako). %CV was calculated using the following formula. An acceptable %CV is <15 (Steiner, *et al.* 2003).

$$\%CV = \frac{\text{Standard Deviation} \times 100}{\text{Mean}}$$

Inter-plate assay variability was calculated by including several control sera in each ELISA assay. The %CV was calculated using the above formula.

3.3 Results

3.3.1 Tissue transglutaminase ELISA optimisation

To maximise data generated from ELISA assays using recombinant tTGs, it was necessary to optimise the antigen coating concentration. The optimal choice for an ELISA assay is the combination of antigen coating concentration and secondary antibody dilution that yields positive control optical density (OD)₄₅₀ >1.0, negative control OD₄₅₀ <0.2 and blank OD₄₅₀ of <0.1. Checkerboard ELISA experiments were carried out to optimise these parameters.

In initial checkerboard experiments, antigen coating concentrations of 0.8, 0.5, 0.2 and 0.1µg/well were assessed. Secondary antibody dilutions considered were 1:500, 1:1000, 1:2500 and 1:5000. Test sera were samples of known anti-tTG positivity. Positive control (+ve) was of medium positivity, negative control (-ve) was biopsy proven and blank well (B) was incubated with 1% BSA in PBS. All sera were diluted 1:100. The OD₄₅₀ results of this experiment are shown in Table 3.2.

Table 3.2: Antigen coating concentration and secondary antibody dilution optimisation

+ve Control (AU: 55.7)				
2° Dilution\Antigen Conc.	0.5µg	0.3µg	0.2µg	0.1µg
1:500	1.206	0.955	0.405	0.293
1:1000	1.075	0.823	0.343	0.245
-ve Control (AU: 1.2)				
2° Dilution\Antigen Conc.	0.5µg	0.3µg	0.2µg	0.1µg
1:500	0.139	0.126	0.122	0.122
1:1000	0.117	0.111	0.165	0.105
Blank				
2° Dilution\Antigen Conc.	0.5µg	0.3µg	0.2µg	0.1µg
1:500	0.066	0.06	0.055	0.056
1:1000	0.062	0.059	0.053	0.051

Antigen: wild type tTG, secondary antibody (2°): RAH IgA-HRP, primary antibody was diluted 1:100. AU values were generated using the Celikey™ tTG ELISA assay

From this experiment it was concluded that 0.5µg/well with secondary antibody at 1:1000 gave acceptable OD₄₅₀ values. However, since recombinant tTGs were purified in such low quantities and were the limiting reagent for these experiments, it was decided to examine the efficacy of lower antigen concentrations and serum dilutions. In this experiment, the antigen was coated at 0.3µg/well with test sera diluted 1:20. The results are shown in Table 3.3.

Table 3.3: OD₄₅₀ results of positive, negative and blank samples with antigen concentration at 0.3µg/well, serum dilution at 1:20 and secondary antibody dilution at 1:1000.

Sample	+ve Control	-ve Control	Blank
OD ₄₅₀	1.2976	0.08013	0.054

Test samples were as per Table 3.2

Since these conditions created even more discriminatory OD₄₅₀ values and were in addition more economical with recombinant tTG, these antigen coating and secondary antibody dilution conditions were used for all subsequent ELISA assays.

3.3.2 Serum dilution optimisation

Serum dilutions of 1:20 yielded satisfactory OD₄₅₀ values for positive and negative controls in checkerboard experiments but in order to maximise data generated from available serum, experiments were carried out to investigate whether lower dilutions of patient serum would yield equally satisfactory results. Four serum samples were applied to ELISA assays with antigen coating at 0.3µg/well wild type tTG and secondary antibody (rabbit anti-human IgA-HRP) dilution at 1:1000. Since lower serum dilutions did not yield OD₄₅₀ values >1 for positive control, serum dilution was set at 1:20 for all subsequent ELISA assays in order to maintain clear and reproducible differentiation between samples. Results are shown in Table 3.4.

Table 3.4: Serum dilution optimisation			
Serum dilution\Sample	-ve	wp	+ve
1:50	0.049	0.461	0.908
1:40	0.052	0.506	0.980
1:30	0.066	0.603	1.053
1:20	0.080	0.838	1.225

Results are OD₄₅₀ values. -ve: negative control (AU: 1.2), wp: weak positive control (AU:9.8), +ve: positive control (AU: 55.7). AU values were generated using the Celikey ELISA system.

3.3.3 ELISA standard curve generation

A standard curve was generated using optimised ELISA conditions. ELISA plate wells coated with wild type tTG were probed with doubling dilutions of pooled, strongly anti-tTG positive sera and rabbit anti-human IgA-HRP as secondary antibody. OD₄₅₀ values generated were used to generate a standard curve using Multicalc software (Wallac). An example of a standard curve is shown in Figure 3.4.

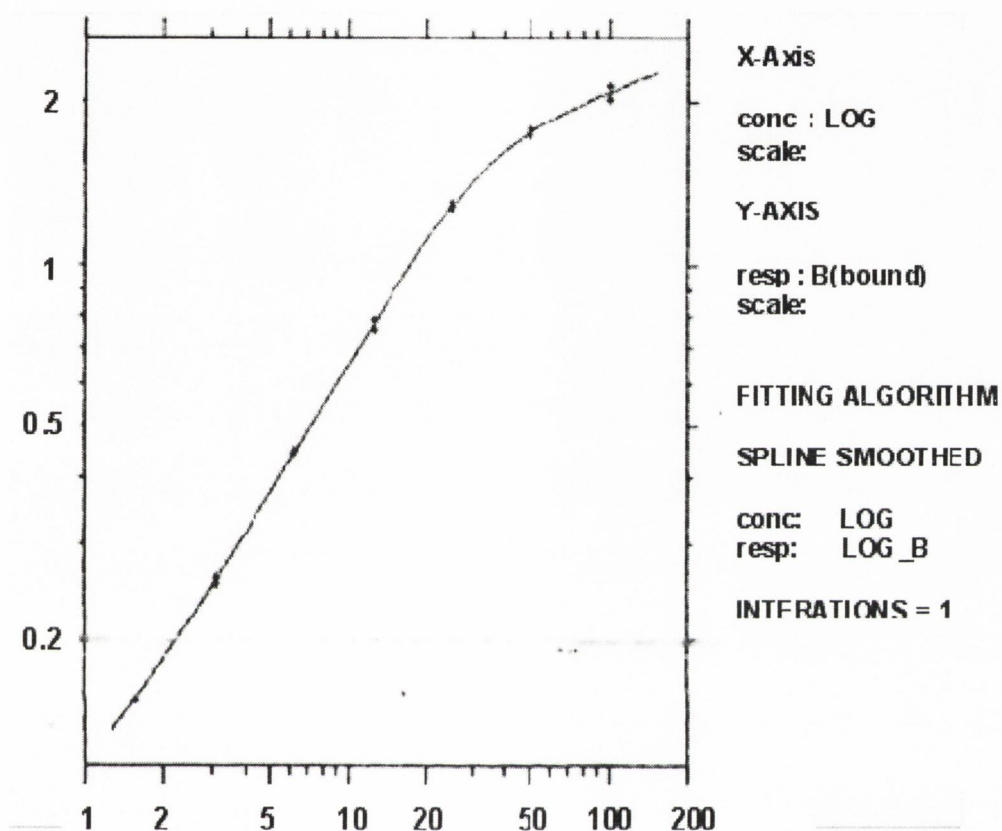


Figure 3.4: Seven point ELISA standard curve. A standard curve was generated by incubating doubling dilutions of pooled, strongly tTG-positive sera with wild type tTG. These curves were used to assign AU values to test sera. The curve was generated using Multicalc (Wallac) software.

3.3.4 Intra-assay variability

The percentage co-efficient of variation (%CV) was generated by distributing 10 aliquots of 2 serum samples about one plate coated with wild type tTG. %CV was calculated on the basis of AU values determined from the standard curve. %CVs for both samples were within acceptable limits. The results of intra-assay variability are shown in Table 3.5.

Table 3.5: Intra-assay variability for IgA anti-tTG ELISA

Replicate	-ve control serum (AU)	+ve control serum (AU)
1	3.155	46.068
2	3.602	44.339
3	3.184	49.045
4	2.918	48.978
5	3.482	44.460
6	3.130	46.318
7	3.573	44.115
8	3.423	45.943
9	3.423	47.751
10	3.543	48.140
Mean	3.3433	46.5157
Standard Deviation	0.2306	1.5738
%CV	6.89833	3.38337

3.3.5 Inter-assay variability

Inter-assay variability was determined by including various controls in each ELISA plate. The %CVs were calculated as per the intra-assay variability. Controls included 2 samples negative for anti-tTG by Celikey™ ELISA, 2 samples positive by Celikey™ ELISA, 2 samples negative for mutant tTG reactivity by our ELISA system and polyclonal anti-tTG (Roboscreen) as a positive control for mutant tTG reactivity. The results of inter-assay variability are shown in Table 3.6. Although inter-assay variabilities for controls wt-ve1, mut-ve1 and mut-ve2 were greater than 15%, this was not considered to be a poor reflection of the assays

reproducibility since negative control samples are much more sensitive to small inter-assay variations.

3.3.6 Cut-off point determination

Forty-nine sera found to be negative for IgA anti-tTG reactivity by Celikey[®] ELISA were used to establish cut-off points for wild type and mutant tTG in IgA and IgG anti-tTG ELISAs. While the control sera reactivity to wild type tTG resulted in cut-off points of that detected 88.16% of anti-tTG positive sera, the cut-off point calculated for IgG assays resulted in poor sensitivity (10.94%). The higher cut-off point in the IgG anti-tTG assay was a result of greater standard deviation in control serum binding to wild type tTG. The reason for this variability is unknown. Since coeliac disease serum IgG bound mutant tTG at levels significantly higher than control sera (Mann-Whitney U test), the mutant tTG was deemed to be antigenic but the ELISA was not sensitive.

Table 3.6: Inter-assay variability

Date	wt –ve1	wt –ve2	wt +ve1	wt +ve2	mut –ve1	mut –ve2	Polyclonal anti tTG
100205 (01)	7.549	4.781	50.275	66.806	nd	nd	20.019
100205 (02)	5.500	4.708	39.267	57.557	nd	nd	19.201
120205	6.718	5.813	41.913	57.607	nd	nd	16.900
160205 (1)	6.518	5.72	40.278	64.339	4.891	6.004	17.576
160205 (2)	6.373	5.359	46.555	77.638	3.904	5.975	18.242
220205 (1)	5.5663	4.097	43.438	66.453	2.746	3.971	19.828
220205 (2)	4.743	4.367	54.436	73.488	2.158	3.991	19.644
240205 (1)	4.825	4.934	57.802	71.096	2.11	4.825	19.416
240205 (2)	5.057	5.219	47.476	74.917	2.234	4.534	20.228
010305	5.081	4.849	52.103	75.207	2.569	5.247	20.129
040305	5.854	5.614	44.74	57.445	3.489	6.664	22.411
240305 (02)	3.841	4.643	36.495	56.212	2.756	2.705	26.933
Mean	5.635	5.009	46.232	66.564	2.984	4.880	20.044
SD	1.024	0.542	6.474	7.933	0.937	1.237	2.587
%CV	18.177	10.829	14.004	11.918	31.408	25.359	12.908

mut: negative controls for mutant tTG binding, wt: wild type tTG, nd: not done, SD: standard deviation. All values are AU.

3.3.7 Correlation with Celikey™ anti-tTG ELISA assay

When the IgA anti-tTG ELISA results from 76 coeliac disease sera were compared to results generated for the same samples using the Celikey™ tissue transglutaminase IgA antibody assay, a correlation co-efficient of 0.805 was obtained. Correlation was calculated using the following formula.

$$\rho_{X,Y} = \frac{\text{Cov}(X,Y)}{\sigma_X \cdot \sigma_Y}$$

Where:

σ = Standard deviation

Cov = Covariance

3.3.8 Characterisation of wild type and mutant tTGs

Wild type and mutant tTG reactivity to commercially available antibodies was assessed in ELISA assays. Antibodies were applied to either Maxisorp™ plates (Nunc) coated with either wild type or mutant tTG at 0.3µg/well. Mouse monoclonal anti-GST antibody (GST01, Neomarkers) was used as a control to ensure that equivalent amounts of GST-fusion protein were being coated to wild type tTG and mutant tTG wells. Mouse monoclonal anti-tTG antibody cocktail (CUB 7402/TG100, Neomarkers) showed slightly decreased binding to the mutant tTG compared to the wild type tTG but this finding did not reach statistical significance. Polyclonal anti-tTG (Roboscreen) bound wild type and mutant tTGs equally (Figure 3.5a).

Wild type and mutant tTGs were also tested for their reactivity to the commercially available antibodies in Western blots. Both wild type and mutant tTGs reacted with GST01, CUB 7402/TG100 and rabbit polyclonal anti-tTG. These Western blots were used to confirm the identity of the purified recombinant tTG and to show that the epitopes targeted by the antibodies are conserved in the mutant tTG. The results of Western blots are shown in Figure 3.5b.

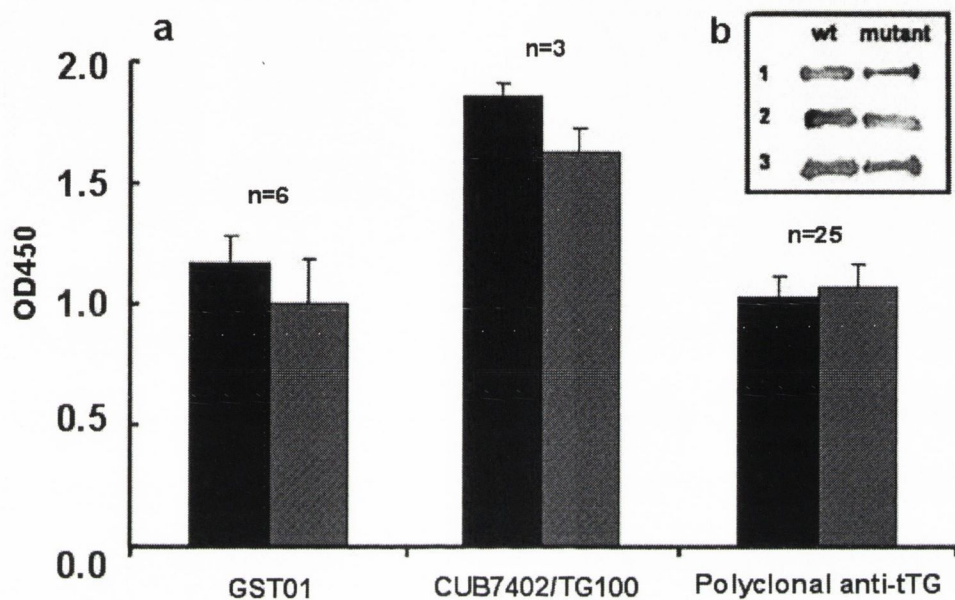


Figure 3.5: (a) ELISA comparison of commercial antibody binding to wild-type (black bars) and mutant tTG (grey bars). **(b)** Western blots of wild type and mutant tTG with GST01 (1), CUB 7402/TG100 (2), and polyclonal anti-tTG (3) are inset.

3.3.9 Wild-type and mutant tTG recognition by coeliac disease serum autoantibodies

Coeliac disease serum IgA reacted with wild type tTG at a range of values that correlated well with their reactivity to tTG in the commercial Celikey® tTG ELISA system ($r=0.805$). However, coeliac disease serum IgA reactivity to mutant tTG was dramatically reduced in comparison to wild type tTG (Fig: 3.6a). In fact, while 88.16% of coeliac disease sera were found to be positive in the wild type antigen assay, only 18.42% of sera exceeded the cut-off point when tested for IgA reactivity to the mutant tTG. There was, on average, a 79% reduction in IgA class autoantibody binding to mutant tTG when compared to wild type tTG.

In IgG ELISA assays, sera were used at a higher dilution than in IgA assays and, as a result, AU values were lower. Coeliac disease IgG bound wild type tTG at significantly higher levels than control sera ($p<0.001$, Mann-Whitney test). Interestingly, IgG reactivity did not mirror the pattern observed in IgA assays (Fig. 3.6b), as there was no significant difference between coeliac disease IgG recognition of wild type or mutant tTG (Wilcoxon signed ranks test).

3.3.10 Wild-type and mutant tTG recognition by dermatitis herpetiformis serum autoantibodies

The IgA anti-tTG assay successfully identified 6 of 10 dermatitis herpetiformis sera as anti-tTG positive (Fig: 3.6c). In a similar pattern to the results with coeliac disease patient sera, dermatitis herpetiformis patient serum IgA showed a dramatically reduced binding to mutant tTG. All samples tested for IgA binding to mutant tTG bound the antigen at a level below the threshold for positivity, with a mean percentage reduction in binding of 58% compared to wild type tTG. Once again, IgG responses did not mirror IgA results. IgG antibodies showed no difference in binding to wild type or mutant tTGs (Fig: 3.6d).

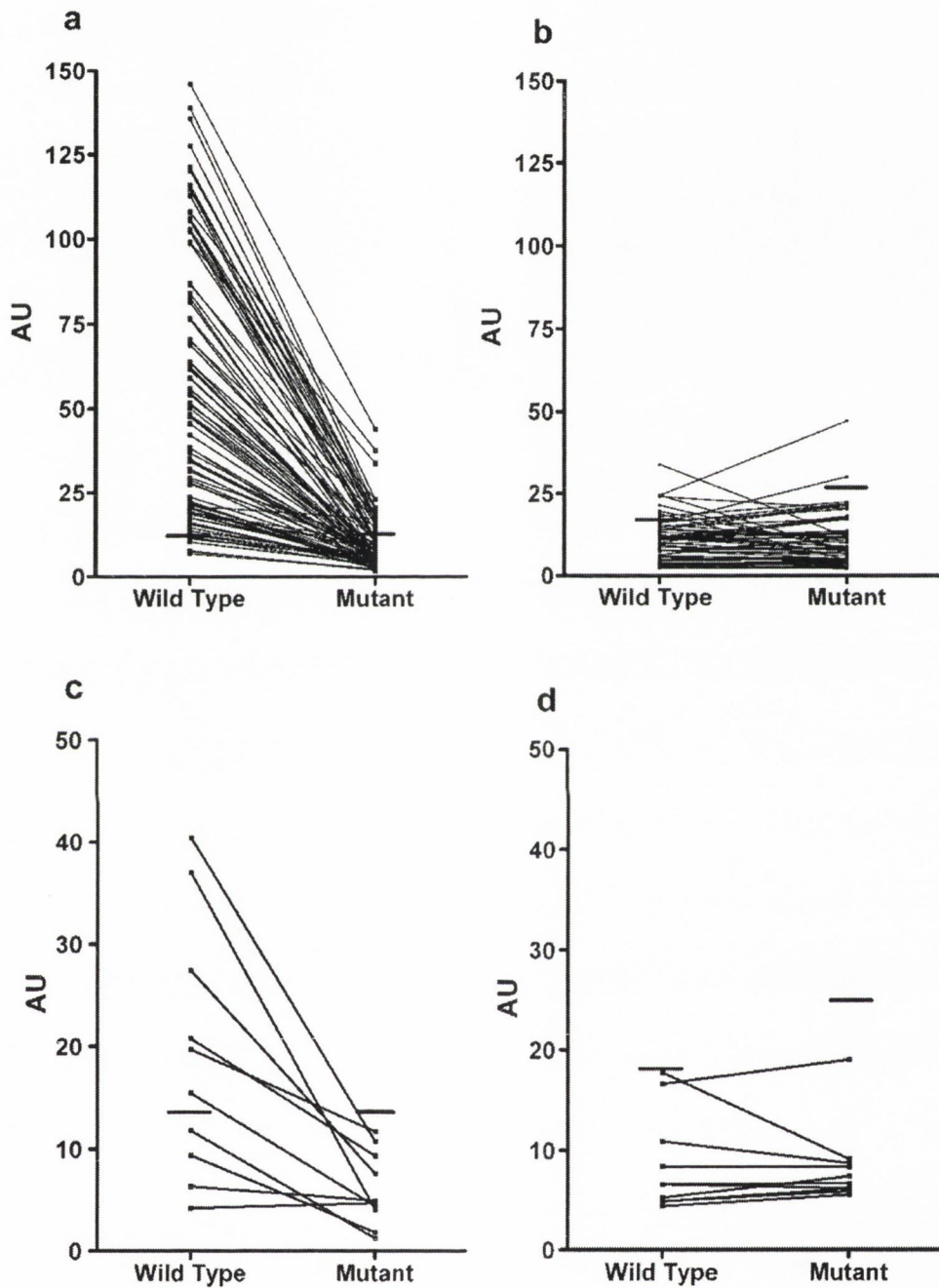


Figure 3.6: Results of ELISA assays. coeliac disease serum IgA (n=76) (a) and IgG (n=64) (b) reactivity were analysed for their ability to bind wild-type and mutant tTGs. dermatitis herpetiformis serum IgA (c) and IgG (d) were also analysed. Cut-off points for positivity are represented by bold lines.

3.4 Discussion

3.4.1 Epitope mapping analysis of wild type and mutant tTGs

In this chapter, the antigenicity of the recombinant wild type and mutant tTGs generated in Chapter 2 was compared in ELISA assays with sera from coeliac disease and dermatitis herpetiformis patients. The mutant tTG was engineered to have the elements of the catalytic triad (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) replaced with alanine residues. This is the first time site-directed mutagenesis has been used to investigate tTG epitopes. The results in this chapter suggest that the IgA response directed against tTG in coeliac disease and dermatitis herpetiformis is very specifically targeted towards the region of the protein responsible for the transamidation and deamidation reactions catalysed by the enzyme.

In recent years, several studies have attempted to map out epitopes on tTG in an attempt to understand the origin and role of the enigmatic autoantibody response in coeliac disease. In 2001 Seissler and co-workers attempted to determine which domains of tTG are targeted by IgA autoantibodies by production of radiolabelled tTG fragments and immunoprecipitation with coeliac disease patient sera (Seissler, *et al.* 2001). This study concluded that the N-terminal and C-terminal third of tTG, comprising amino acids 1-281 and 473-687 harboured the dominant epitopes, while the core region seemed to be of minor antigenicity. However, it should be noted, that 3 of the 4 tTG fragments with the highest levels of bound radioactivity (N₁, N₂ & C₁) contained two or three amino acids of the catalytic triad and included either the amino or carboxy terminus.

In 2003, Tiberti and colleagues compared the antigenicity of full-length recombinant tTG and two truncated constructs (amino acids 227-687 and 473-687). A serum ³⁵S-radioimmunoassay was used to compare the antigenicity of the constructs with different age groups of male and female coeliac disease patients. This study concluded that female patients of less than 4 years expressed IgA tTG (227-687) antibodies in

significantly higher percentage and mean autoantibody titers than all other groups investigated, while IgA tTG (473–687) antibodies were found to be in significantly higher titers in adult female patients (Tiberti, *et al.* 2003). Other investigators have not reported this effect of age and sex on the anti-tTG response although it is possible that the specificity of the response changes over time as a result of affinity maturation.

Nakachi and co-workers (2004) carried out a similar study using nine ³⁵S-labelled tTG constructs produced in a transcription/translation system to characterise epitopes targeted by IgA autoantibodies in coeliac disease. Their study concluded that the N-terminal region of tTG (amino acids 1-89) were important for binding in all coeliac disease sera studied while the central residues (amino acids 401-491) were important for the majority of sera tested (Nakachi, *et al.* 2004).

In 2002, Sblattero and colleagues published a study that investigated tTG epitopes by producing twelve truncated tTG constructs and measuring their antigenicity with coeliac disease sera and single-chain antibody fragments. The twelve tTG fragments comprehensively covered the entire length of the 687 amino acid enzyme. This study identified the core region of tTG (amino acids 140-376) as the region containing the dominant epitopes for coeliac disease autoantibodies as evidenced by ELISA assays with patient sera and with single-chain antibody fragments generated from phage display libraries (Sblattero, *et al.* 2002). This study did, however, acknowledge the limitations of assays using truncated proteins. Shortened fragments of tTG are unlikely to fold in a similar fashion to the full-length protein and could therefore generate misleading results. Sblattero and co-workers suggested that an intact amino or carboxy terminus was necessary to bring about correct folding of tTG fragments. The domain implicated as antigenic by Sblattero *et al.* contains calcium and GTP binding regions (Nakanishi, *et al.* 1991, Liu, *et al.* 2002) and the catalytic triad (Murthy, *et al.* 2002).

Given these results (Sblattero, *et al.* 2002) and the recently elucidated role for the active site of tTG in the deamidation of gliadin peptides (Vader, *et al.* 2002), the catalytic triad represented an interesting target for epitope mapping studies. In order to avoid the conformational issues that may be responsible for the disagreement in the aforementioned protein truncation studies (Seissler, *et al.* 2001, Sblattero, *et al.* 2002, Tiberti, *et al.* 2003, Nakachi, *et al.* 2004), site-directed mutagenesis was the method of choice for this study. The specific mutagenesis of the three amino acids of the catalytic triad should have altered the properties of the region of tTG without affecting the overall conformation of the enzyme. For this reason, this method represents a superior approach for epitope mapping of the protein.

The majority of recombinant tTG ELISA systems use antigen concentrations of 1 µg/well (Biagi, *et al.* 1999, Sardy, *et al.* 1999, Baldas, *et al.* 2004). In our ELISA system, anti-tTG autoantibodies can be detected in a reproducible manner using coating concentrations of just 0.3 µg/well, suggesting that the wild type tTG is both antigenic and highly pure. The IgA anti-tTG ELISA that was established had a sensitivity of 84.21% which is less than most commercially available anti-tTG assays but is, in fact, better than the published sensitivity of the Aesku Diagnostics anti-tTG ELISA kit (Wong, *et al.* 2002). When IgA anti-tTG ELISA results from the current study were compared with Celikey[®] anti-tTG ELISA results for the same samples, a correlation co-efficient of 0.805 was obtained. This correlation co-efficient measures the relationship between two data sets that are scaled to be independent of the unit of measurement. This result indicates that the assay placed samples in a similar order of positivity when compared to the commercial test.

Our results demonstrated that the IgA anti-tTG response in coeliac disease sera is very specifically targeted towards the region of the enzyme responsible for its transamidation and deamidation reactions. In fact, 88.16% of coeliac disease sera found to have IgA reactivity to the wild-type tTG showed no reactivity to mutant tTG (Figure 3.6a). Thus,

mutagenesis of the catalytic triad almost completely abrogated IgA anti-tTG binding to the 687 amino acid enzyme.

In experiments investigating dermatitis herpetiformis patient sera, 6 out of 10 samples were found to react to wild type tTG. This is in keeping with reports that a lower proportion of dermatitis herpetiformis patient sera show positive anti-tTG serology compared to coeliac disease sera (Koop, *et al.* 2000). In our ELISA studies, IgA antibodies from dermatitis herpetiformis patients bound wild-type tTG much more strongly than mutant tTG with an overall reduction in binding to the mutant tTG of 58% (Figure 3.6c).

3.4.2 Conformational integrity of mutant tTG

It is unclear whether the three amino acids of the catalytic triad are intimately involved in IgA anti-tTG binding in coeliac disease and dermatitis herpetiformis or whether a general conformational change in the core region is responsible for the reduction in autoantibody binding. While mutagenesis of three amino acids in the core region of tTG could have resulted in misfolding of the protein and, hence, affected autoantibody binding, there are three reasons that this does not seem to be the case in these experiments. First, a polyclonal antibody raised against recombinant human tTG bound wild type and mutant tTGs equally well in ELISA assays (Figure 3.5a). Second, *in silico* mutation of the amino acids using the 3D-PSSM program (Kelley, *et al.* 2000) showed no notable difference in secondary structure as a result of the mutagenesis (Appendix III). Cys²⁷⁷ represents the beginning of a helical structure in the core of tTG that seems to be maintained in the mutant tTG. Third, unlike IgA anti-tTG responses, there was no significant difference between IgG binding of wild type or mutant tTGs (Figure 3.6b & d).

3.4.3 The specific nature of the IgA anti-tTG response

While in IgA anti-tTG ELISA assays there was a marked difference in autoantibody binding to wild type and mutant tTGs, in experiments investigating IgG anti-tTG responses in coeliac disease and dermatitis herpetiformis sera, there was no significant difference in reactivity to wild type or mutant tTGs (Figure 3.6b & d). Although the majority of coeliac disease IgG bound wild type tTG at levels below the cut off point for positivity, binding was still significantly higher than control sera ($p < 0.001$, Mann-Whitney test), indicating that coeliac disease patients did have an elevated level of IgG anti-tTG. It has been reported that IgG anti-tTG ELISA assays are considerably less sensitive than IgA assays with sensitivity as low as 13% being reported previously (Feighery, *et al.* 2003).

The results suggest a difference in specificity between IgA and IgG class anti-tTG responses in coeliac disease. The IgA anti-tTG response seems focused on the active region of the enzyme while the IgG antibodies appear to recognise other sites on the protein and are largely unaffected by mutation of the catalytic triad. The targeting of the active core of tTG by IgA antibodies suggests the possibility that these autoantibodies may affect the enzyme's function. It could be argued that inhibition of such a multifunctional and ubiquitous enzyme would result in some degree of systemic pathology but we suggest that inhibition of tTG may be limited to the gut and the effect may be primarily mediated by the mucosal immunoglobulin class, IgA. These antibodies could, therefore, contribute to the local coeliac disease lesion. Using phage antibody libraries Marzari and colleagues (2001) demonstrated that unlike those against gliadin, IgA antibodies directed against tTG can be isolated from intestinal libraries but not from peripheral blood libraries. These results suggest that the anti-tTG response is generated at a local level whereas anti-gliadin responses are systemic (Marzari, *et al.* 2001).

The development of IgA⁺ B cells in the Peyer's patches seems to depend upon antigenic stimulation and the induction of germinal centres (Butcher, *et al.* 1982). The specialised environment in germinal centres facilitates strong interactions between B cells, antigens (in the context of class II molecules) and local CD4⁺ T cells. It has been suggested that Peyer's patches germinal centres are intrinsically different from other germinal centres owing to constant antigenic stimulation and the presence of special regulatory T cells and dendritic cells that promote preferential class-switching to IgA (Lycke 1998). Somatic hypermutation is another process that occurs in the germinal centres of the Peyer's patches. Multiple rounds of mutation of variable (V), diversity (D) and joining (J) exons, followed by the selection of B cells with enhanced binding to antigens results in a process often referred to as "affinity maturation". Somatic hypermutation is likely to be more frequent at mucosal sites (Gonzalez-Fernandez and Milstein 1993) in order to deal with the highly diverse intestinal flora. These highly specialised functions of intestinal IgA responses may explain a possible difference between IgA and IgG anti-tTG responses against tTG in coeliac disease. IgG responses may undergo less affinity maturation and therefore remain less specific than IgA responses.

3.4.4 The autoantibody response in dermatitis herpetiformis

Epidermal transglutaminase has recently been identified as the autoantigen of dermatitis herpetiformis. It has been suggested that dermatitis herpetiformis patients may produce populations of antibodies that react with tTG, epidermal transglutaminase and cross-reactive antibodies that target epitopes common to both enzymes (Sardy, *et al.* 2002). Our results suggest that IgA anti-tTG antibodies in dermatitis herpetiformis have a similar specificity to those found in coeliac disease. It is possible that the autoimmune response in dermatitis herpetiformis is initiated in the gut against tTG with subsequent intermolecular epitope spreading to epidermal transglutaminase.

Although serum antibodies directed against tTG and epidermal transglutaminase are characteristic for dermatitis herpetiformis, they do not bind to papillary skin structures (Kadunce, *et al.* 1989, Sardy, *et al.* 2002). This suggests that the circulating antibodies are not directed against dermal targets and that the IgA/epidermal transglutaminase aggregates in the dermis of these patients may represent immune complexes (Preis, *et al.* 2005). It has been suggested that the dermal IgA forms immune complexes in the intestine, binds to the skin and activates complement with subsequent inflammation (van der Meer 1969, Seah, *et al.* 1973). It has been demonstrated that IgA and C3 are present in the skin of dermatitis herpetiformis patients (Reitamo, *et al.* 1981) and can remain there for many years, even after the adoption of a gluten-free diet (Fry 1995). However, the IgA deposits found in the papillary dermis of dermatitis herpetiformis patients are exclusively IgA₁, which is not the IgA class predominantly produced in the GALT (Hall and Lawley 1985). This suggests that either the IgA deposits may not be gut-derived or that IgA₁ is preferentially bound to dermatitis herpetiformis skin.

3.4.5 Conclusions

To summarise, the results described in this chapter suggest that the domain of tTG that contains the catalytic triad of the enzyme (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) is specifically targeted by IgA class autoantibodies in coeliac disease and dermatitis herpetiformis sera. This was demonstrated by the almost complete abrogation of IgA anti-tTG binding when the three amino acids of the catalytic triad (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) were mutated to alanine residues. In similar experiments testing IgG anti-tTG reactivity, although most samples were found to be negative, no such pattern of reduced binding to the mutant tTG was observed. This result suggested that IgG antibodies produced against tTG in coeliac disease have a different specificity to IgA class antibodies (Byrne, *et al.* 2006).

Future work in this area could involve the production of tTG mutants with single amino acids of the catalytic triad mutated in order to investigate whether the effects observed in this study were attributable to one of the three amino acids targeted. The observed difference in IgG anti-tTG specificity could be further confirmed by examining serum from coeliac disease patients with high anti-tTG IgG levels, possibly from IgA-deficient patients (Lenhardt, *et al.* 2004) or young patients, since IgG1 anti-tTG has been reported as more common at a young age of diagnosis (Agardh, *et al.* 2003).

The discovery that tTG is intimately involved in coeliac disease pathogenesis has led to speculation about a role for the anti-tTG autoantibody component of this multifactorial disease. In this study we have described evidence for the highly specific targeting of the active site of tTG by IgA autoantibodies while IgG autoantibodies seem to target other regions of the enzyme. These results confirm and extend observations made by Sblattero and colleagues using truncated tTG fragments (Sblattero, *et al.* 2002). These findings suggest that autoantibodies of mucosal origin could possibly have a negative effect on tTG activity. These results may help further dissect the role of the autoantibody response against tTG in coeliac disease.

Chapter 4

Inhibition of Tissue Transglutaminase by Coeliac Disease Autoantibodies

4.1 Introduction

4.1.1 Autoantibody responses to enzymes

Experimental evidence suggests that the production of antibodies against self-components in autoimmune diseases is the result of a conventional immune response that is driven by self-components. That is, the responses proceed in a manner comparable to the immune response to foreign antigen: class-switch from IgM to IgG; mutation of the antibody gene over time; a high level of specificity (stronger reactions to the human target protein when compared to a homologous protein from another species); and affinity maturation (Miller, *et al.* 1990, Shlomchik, *et al.* 1990). A remarkable fact is that of the estimated 20,000-60,000 human proteins, only 1-2% are the targets of autoimmunity (Plotz 2003). This has led to speculation that structural properties, catabolism, location and immunological properties may predispose particular proteins to become autoantigens.

As a result of affinity maturation, autoantibody responses directed against self-proteins often target specific domains, rather than polyclonal responses across the whole protein. For example, the development of inhibitory antibodies against Factor VIII is a severe complication in haemophilia A patients undergoing Factor VIII replacement therapy. Anti-Factor VIII antibodies may also develop in individuals without congenital Factor VIII deficiency in a rare condition named acquired haemophilia A. Although both conditions target the same protein, the epitope specificity differs between the two conditions. Most inhibitors in haemophilia A patients recognise epitopes within the A2, A3 and C2 domains while Inhibitory antibodies in acquired haemophilia A are directed against A2 or C2, but not both (Lollar 1999), and they may recognise different epitopes (Prescott, *et al.* 1997). As a result of these differing specificities, these distinct antibody populations have different effects on the interaction of Factor VIII with thrombin, phospholipids and von Willebrand Factor (Bayry, *et al.* 2003).

Enzymes occur as autoantigen in several disorders and the autoantibody response directed against these enzymes often results in loss of function. Examples include the inhibition of proteinase 3 by anti-neutrophil cytoplasmic antibodies in Wegener's granulomatosis (van der Geld, *et al.* 2002) and inhibition of the H^+K^+ -ATPase proton pump by autoantibodies in pernicious anaemia (Burman, *et al.* 1989).

Given the apparent specific targeting of the catalytic triad of tTG by IgA class autoantibodies (described in Chapter 3), the aim of this chapter was to determine whether these anti-tTG autoantibodies mediate inhibition of the transamidation reaction catalysed by recombinant wild type tTG.

4.1.2 Transglutaminase assays

Many assays have been developed for measuring the cross-linking activity of transglutaminase enzymes. The hydroxaminolysis assay involves the measurement of the ferric complex of hydroxamate formed by the tTG catalysed attack of hydroxylamine (Folk and Cole 1966, Folk and Finlayson 1977, Folk and Chung 1985). tTG activity can also be detected by measuring the incorporation of various radiolabelled amines (e.g. putrescine, histamine, spermine, cadaverine) into glutaminy substrates such as casein, *N,N*-dimethylcasein or the acetylated B chain of oxidised insulin (Curtis and Lorand 1976, Folk and Finlayson 1977, Esposito, *et al.* 2002). In addition, several fluorescence-based assays have been developed (Lorand, *et al.* 1968, Lorand, Parameswaran, *et al.* 1979, Lorand, Siefring, *et al.* 1979, Lorand, *et al.* 1983, Jeitner, *et al.* 2001, Jeitner, *et al.* 2005). A more recent assay developed for measuring transglutaminase activity involves the use of 5-(biotinamido) pentylamine as substrate (Slaughter, *et al.* 1992). The biotinylated product is detected by streptavidin-alkaline phosphatase and is quantified by measuring absorbance at 405nm following addition of *p*-nitrophenylphosphate.

Each of these assays has advantages and disadvantages. The hydroxaminolysis assay is relatively simple to carry out but insensitive since the extinction co-efficient at $A_{\max}$ (525nm) of the hydroxamate product is only 850. As a result, the lower limit of detection is 0.6mU of

transglutaminase enzyme. Radiometric assays are very sensitive but labour-intensive and involve the use of radioactive substrates. The fluorometric tTG assay described by Lorand *et al.* measuring the formation of fluorescent amide from dansylcadaverine and (β -phenylpropionyl)thiocholine iodide requires the continuous extraction of the coupled product into *n*-hexane followed by fluorescence measurement (Lorand, Parameswaran, *et al.* 1979). Therefore, although this method is very sensitive, it is labor-intensive. The 5-(biotinamido) pentylamine assay developed by Slaughter and co-workers is a sensitive method but involves several manipulations (Slaughter, *et al.* 1992).

4.1.3 Continuous fluorescence-based assay for transglutaminase activity

In order to quantify the cross-linking activity of tTG in this study, a continuous fluorescence-based assay was developed based on previous publications. In 2001, Jeitner *et al.* published a method to detect tTG activity in real time by measuring the rate of fluorescence enhancement (λ_{ex} 260nm, λ_{em} 538) when 1-*N*-(carbobenzoxymethyl-L-glutaminyglycyl)-5-*N*-(5'-*N,N'*-dimethylaminonaphthalenesulfonyl) diamidopentane (CGG-DNS) is cross-linked to dansylcadaverine (Figure 4.1). In this reaction, CGG-DNS acts as glutaminy substrate while dansylcadaverine acts as amine substrate. This assay can be carried out in a 96-well plate format and can detect as little as 60 μ U (4.2ng) of guinea pig liver tissue transglutaminase activity (Jeitner, *et al.* 2001). This method is simple, fast, sensitive and does not require the use of radioisotopes.

In 2005, Jeitner *et al.* published a modified version of the above method replacing CGG-DNS with *N,N*-dimethylcasein (DMC) as glutaminy substrate (Jeitner, *et al.* 2005). The reasons for the change in substrate were three-fold. First, unlike CGG-DNS, DMC is soluble at saturating levels, making the assay amenable to Michaelis-Menton kinetic analysis (Jeitner, *et al.* 2005). Second, CGG-DNS is a costly substrate. Third, the binding of dansylcadaverine to DMC by tTG reduces the motion of the dansyl group causing it to fluoresce in a more measurable manner

(Jeitner 2005, Personal communication). Dimethylation of casein blocks the ϵ -lysine residues of the substrate, making self cross-linking impossible (Curtis and Lorand 1976). This makes DMC a superior tTG substrate when compared to casein.

In the transglutaminase assays described by Jeitner *et al*, transglutaminase was added to the assays in unit measurements rather than microgram quantities. This avoids inter-batch variation of enzyme purity confounding results and allows for specific activity calculations. In this study, the hydroxaminolysis assay (Cooper, *et al*. 1997) was used to establish the enzyme unit concentrations of each batch of recombinant wild type tTG generated in this study.

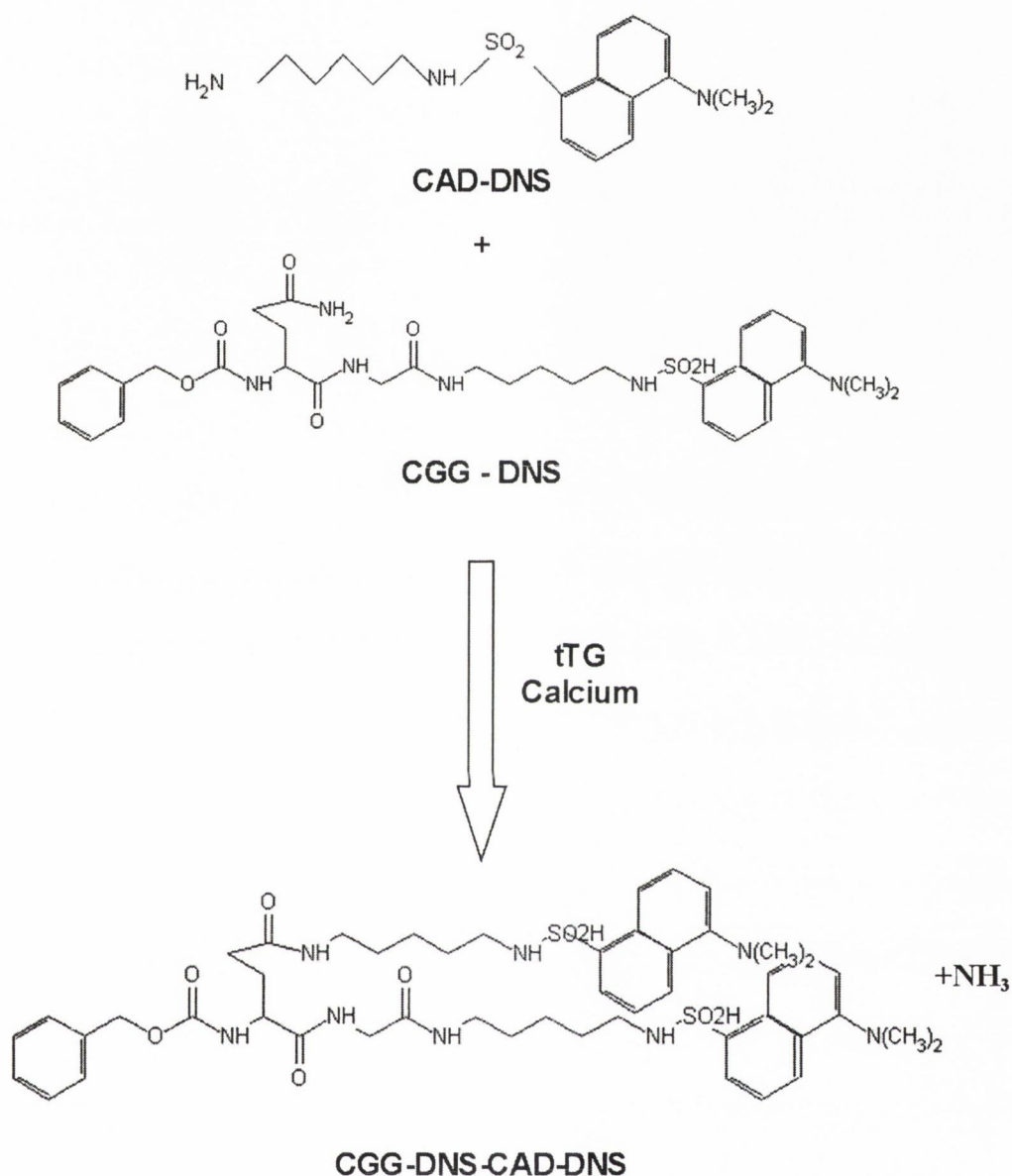


Figure 4.1: Structures of glutaminyl and amine substrates adapted from Jeitner and co-workers (Jeitner, *et al.* 2001). Amine substrate: dansylcadaverine (CAD-DNS). Glutaminyl substrate: CGG-DNS. The lowermost structure is the postulated product formed by the tTG-catalysed attack of the free amine of CAD-DNS by the carboxamide moiety of CGG-DNS. Note the stacking of the dansyl groups.

4.1.4 Transglutaminase activity assays and coeliac disease

There is considerable debate as to the ability of anti-tTG antibodies to inhibit the activity of the enzyme (Esposito, *et al.* 2002, Dieterich, *et al.* 2003, Roth, *et al.* 2003). If anti-tTG autoantibodies from coeliac disease patients do, in fact, have an inhibitory effect on the enzyme, it could be mediated either by blocking the catalytic triad of the enzyme, prevention of substrate access to the catalytic triad or by blockade of calcium mediated enzyme activation. Several studies have attempted to determine whether coeliac disease autoantibodies are capable of inhibiting tTG *in vitro* with varying results (Esposito, *et al.* 2002, Dieterich, *et al.* 2003, Roth, *et al.* 2003).

Esposito *et al.* showed that serum IgA and IgG anti-tTG antibodies were capable of inhibiting the cross-linking activity of a tTG-containing cell lysate in a dose-dependent manner by measuring the incorporation of [³H] spermidine into DMC. Furthermore, this study demonstrated that coeliac immunoglobulin could inhibit tTG *in situ* by measuring the tTG-catalysed incorporation of biotinylated monodansylcadaverine into human umbilical cord sections using a streptavidin fluorescein isothiocyanate conjugate (Esposito, *et al.* 2002). In this study, the source of tTG was transfected Madin-Darby canine kidney cell lysates. The obvious disadvantage of a cell lysate is the presence of confounding protein inhibitors/enhancers as well as potential proteases.

In studies by Dieterich *et al.*, a fluorescence-based activity assay was used to show that coeliac disease serum IgA and IgG could inhibit tTG activity in a significant fashion. These experiments did not demonstrate complete blockade of tTG activity and assays could measure tTG activity for only 2 minutes due to the instability of their recombinant tTG preparation. This limitation prohibited the preincubation of tTG with potential inhibitors prior to assay.

Roth *et al.* used crossed immune electrophoresis and staining for guinea pig transglutaminase activity to demonstrate that after incubation with coeliac disease serum, transglutaminase activity was still detectable (Roth, *et al.* 2003). However, this method is a qualitative one and would be insensitive to low levels inhibition. Furthermore, although guinea pig and human transglutaminase are similar in sequence (Dahele, *et al.* 2001), they may be targeted differently by coeliac disease serum autoantibodies.

Given our results from Chapter 3, it seems logical to expect that if IgA anti-transglutaminase antibodies from coeliac disease patient sera target the core region of tTG, the activity of the enzyme could be affected. This notion is supported by previous publications using quantitative tTG assays (Esposito, *et al.* 2002, Dieterich, *et al.* 2003). The complete blockade of tTG seems unlikely given that the enzyme has evolved to have a high affinity for its natural substrates and IgA autoantibodies may compete with substrates for access to the active site of tTG. In order to cause complete blockade of tTG activity, autoantibodies would have to have a greater affinity for the tTG active core than the affinity of the core for its substrate. Even if such an affinity was present, since antibody-antigen interactions are non-covalent and reversible (Roitt, *et al.* 2001) the complete blockade of tTG activity seems unlikely. However, since significant levels of inhibition may have biological significance *in vivo* by dampening down tTG activity, clarification of the role for anti-tTG autoantibodies in coeliac disease is required.

4.1.5 Immunoglobulin purification

Jacalin is a 40kDa, tetrameric two-chain lectin from jack fruit (*Artocarpus integrifolia*) seeds (Kabir 1998). It is highly specific for the α -O-glycoside of the disaccharide Thomsen-Freidenreich antigen (Gal β 1-3GalNAc). Jacalin has the unique property of binding IgA (Chatterjee, *et al.* 1979, Roque-Barreira and Campos-Neto 1985) with specificity for the IgA1 subclass (Kondoh, *et al.* 1986).

The Jacalin protein can be easily immobilised on agarose beads and used to selectively purify IgA1 (monomeric and dimeric) and secretory IgA (Kondoh, *et al.* 1986). The IgA-Jacalin interaction is multivalent and strong because 10 O-linked glycans per IgA1 molecule are accessible for binding with Jacalin. The specificity for IgA1 is based on a 13 amino acid deletion (223-235) in the hinge region of IgA2 allotypes (Kabir 1998). IgA bound to Jacalin beads can be eluted under very gentle conditions by using a solution containing α -O-galactose or melibiose.

Jacalin immobilised to cross-linked agarose can be used to purify IgA1 from human serum (Chui, *et al.* 1990). A simple column-based affinity chromatography method can yield >90% pure human IgA1 in a short period of time. Due to the increased amount of O-linked glycans in polymeric IgA molecules, they tend to elute from the column slower and can be differentially eluted using different concentrations of galactose (Kabir 1998).

In this study, immobilised Jacalin was used to purify IgA from human serum in order to test its ability to inhibit cross-linking by tTG. Although the targeting of the catalytic triad of tTG by serum IgA was described in Chapter 3, it should be remembered that while ~90% of circulating IgA is of the IgA1 subclass, in secretions, IgA2 may make up as much as 50% of total IgA (Yoo and Morrison 2005).

4.2 Materials and Methods

4.2.1 Hydroxaminolysis assay

In order to determine the enzyme unit concentration of the various batches of recombinant wild type tTG generated in Chapter 2, hydroxaminolysis assays were performed. One unit of tissue transglutaminase activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol of hydroxamate per minute at 37°C in a reaction mixture containing 50mM carbobenzoxy-L-glutaminyglycine (CGG) as glutaminy substrate and 100mM hydroxylamine as amine substrate. The method is based upon methods devised by Folk and Cole (Folk and Cole 1966) and modified by Cooper *et al.* for well plate analysis (Cooper, *et al.* 1997).

CGG (168.65mg, Nzyme Biotec) was dissolved in a 6 ml solution consisting of 2mls 1M tris, pH 8.0 (Sigma), 2mls 500mM hydroxylamine, pH 8.0 (Sigma) and 2mls 50mM CaCl₂/DTT, pH 8.0 (Sigma). Thirty microlitres of this reaction mixture was dispensed into microcentrifuge tubes, which were incubated at 37°C for 10 minutes. The reaction was initiated by addition of 20 μ l transglutaminase (final molarity of reaction mixture was 50mM CGG, 200mM tris, 100mM hydroxylamine, 10mM CaCl₂, 10mM DTT). Since the volume of tTG added to each reaction was fixed, the microgram quantities of tTG depended upon batch concentration. Reaction tubes included one blank containing no tTG and one tube containing tTG for each time point being analysed. The assay could not be carried out in duplicate due to limited amounts of enzyme. Reaction was stopped at each time point by addition of 0.2mls of stop solution (0.37M ferric chloride, 0.67 M HCL, 0.2M TCA). The solution was centrifuged at 10,000g for 2 minutes and the absorbance of the supernatant was measured at 525nm using a Genosys™ 5 spectrophotometer (Spectronic) and an ultra micro absorption cuvette (Hellma) to accommodate the small volume.

The slope generated by the increase in A_{525} over time was used to calculate the tTG units present using Beer's law (The molar extinction coefficient of the hydroxamate product is 850 (Cooper, *et al.* 1997)).

$$A = \epsilon l c$$

Where A = absorbance ($M^{-1} \text{ cm}^{-1}$), l = pathlength of cell (cm), c = molar concentration of the absorbing species (M) and ϵ = the molar extinction coefficient. When assaying enzyme activity we use ΔA so $A = \epsilon l \Delta c$ since as the concentration of the chromophore changes, so will the absorbance.

$$\begin{aligned}\Delta A/\text{min} &= \epsilon l (\Delta c/\text{min}) \\ \Delta c/\text{min} &= (\Delta A/\text{min}) / \epsilon l = M/\text{min} \\ &\text{Convert to } \mu M/\text{ml}/\text{min}\end{aligned}$$

4.2.2 Continuous fluorescence tTG assay: CGG-DNS as substrate

Initial fluorescent tTG activity assays were carried out using substrates CGG-DNS and dansylcadaverine as per Jeitner and colleagues (Jeitner, *et al.* 2001). Reactions were carried out according to the published protocol. Working solutions of both substrates were created and stored at -20°C . 100mg CGG-DNS (Nzyme Biotec) was dissolved in 3.818mls dimethylsulphoxide (DMSO) and diluted 10-fold in 10mM HCL to give a final concentration of 4mM CGG-DNS in 10% DMSO/10mM HCL. A 50mg/ml dansylcadaverine was dissolved in glacial acetic acid before being buffered with 1M tris-HCL, pH 8.0 in a 1:28 ratio (Dansylcadaverine:buffer) to give a final solution of 5.14mM dansylcadaverine, pH 8.0.

The 200 μ l reaction solution was created by adding 100 μ l reaction buffer (200mM tris-HCL, 20mM CaCl₂, 20mM DTT, pH 8.0), 10 μ l working solution CGG-DNS, 3.9 μ l dansylcadaverine working solution and dH₂O to 200 μ l (Note: solution was warmed to at least 20°C before CGG-DNS addition since lower temperatures cause precipitation). The final concentrations of reagents were 100mM tris-HCL, 10mM CaCl₂, 10mM DTT, 0.2mM CGG-DNS and 100 μ M dansylcadaverine. After warming the plate for 10 minutes at 37°C, the reaction was started by addition of 5-10mU recombinant wild type transglutaminase (a blank lacked enzyme). All analytes were measured in duplicate. The fluorescence was measured ($\lambda_{\text{ex}}275$ $\lambda_{\text{em}}535$ or $\lambda_{\text{ex}}275$ $\lambda_{\text{em}}595$) every two minutes for at least ten minutes in a Spectrafluor Plus™ instrument (Tecan) at 37°C with 2 seconds orbital shaking before each measurement. Full instrument settings are described in Appendix IV. Output was arbitrary fluorescence units (AU). Preliminary experiments used guinea pig liver tissue transglutaminase (Sigma) for optimisation of gain etc.

4.2.3 Continuous fluorescence tTG assay: *N'*N-dimethylcasein as substrate

The final tTG activity assay protocol was adapted from an improved version of the CGG-DNS protocol published by Jeitner and colleagues during the course of this study (Jeitner, *et al.* 2005). Briefly, 100 μ l of a reaction buffer (200mM tris, 20mM CaCl₂/DTT, pH 8.0), 0.1-5mU wild type recombinant transglutaminase, 0.5-30 μ g antibody inhibitor and dH₂O to make a final volume of 188.75 μ l were added to wells of a 96 well plate (Nunc). This reaction solution was incubated at 37°C for 10 minutes to allow interaction between tTG and the inhibitor being tested. In some assays measuring inhibition of tTG by CUB 7402/TG100, enzyme and inhibitor were pre-incubated at 37°C separate from reaction buffer to avoid the influence of Ca²⁺.

The substrate solution was prepared by combining 400 μ l DMC (10mg/ml) and 50 μ l dansylcadaverine (5.14mM). The reaction was started by addition of 11.25 μ l substrate solution to the pre-heated reaction volume giving a final volume of 200 μ l. All analytes were measured in duplicate. Final reaction buffer concentrations were 100mM tris-HCL, 10mM CaCl₂, 10mM DTT, 0.5mg/ml DMC and 32 μ M dansylcadaverine. The fluorescence was measured (λ_{ex} 275 λ_{em} 535 or λ_{ex} 275 λ_{em} 595) every two minutes for at least forty minutes in a Spectrafluor Plus™ instrument (Tecan) at 37°C. The assay output was arbitrary fluorescence units (AU).

4.2.4 Purification of IgA from human serum

IgA purification was carried out using Immobilised Jacalin agarose (Pierce) as per the manufacturer's protocol. Briefly, Jacalin agarose was equilibrated to room temperature before adding 5mls 50% gel slurry to an 8ml capacity reusable glass column (Pierce). The column was equilibrated with 5 column volumes of PBS. IgA was isolated from ~4ml pools of human serum (anti-tTG negative serum, n=5; anti-tTG positive coeliac disease sera, n=20). Pooled human serum was mixed 1:1 with PBS and added to the column. In order to remove unwanted protein, the column was washed with ten column volumes PBS. The final column wash was retained for A₂₈₀ analysis. Bound IgA was eluted by addition of 100mM melibiose (Sigma) in PBS. IgA was collected in 5x 2ml aliquots and tested for A₂₈₀ using a Genosys™ 5 spectrophotometer (Spectronic). The aliquot with the highest A₂₈₀ value was visualised by SDS-PAGE and stored for tTG inhibition studies. The Jacalin agarose column was stored at 4°C with 0.02% sodium azide and was regenerated with 20 column volumes of PBS before re-use. Melibiose was removed from purified IgA preparations prior to inhibition assays by passing them through Zeba™ desalt columns (Pierce).

4.2.5 Depletion of anti-tTG from purified IgA

Anti-tTG antibodies were depleted from purified IgA using tTG-bound Affi-Gel affinity medium (Bio-Rad). Since tris buffers interfere with Affi-Gel binding, ~3mg pooled recombinant wild type tTG generated in Chapter 2 was desalted (x2) using Zeba™ desalt columns (Pierce). Recombinant tTG was stored in 50% glycerol as a cryoprotective agent and to facilitate passage through Zeba™ columns, samples were diluted from 50% to 25% glycerol using dH₂O. After desalting, a 1/20 volume of 1M hepes buffer (Gibco) was added to the tTG solution. The final pH of the hepes buffered tTG solution was 7.25. Since estimated pI of GST-tTG is 5.135 (calculated using the EMBL WWW Gateway to Isoelectric Point Service: <http://www.embl-heidelberg.de/cgi/pi-wrapper.pl>) Affi-Gel 15 was the solid support chosen since it accommodates proteins with Pi <6.5. One millilitre Affi-Gel 15 was washed x3 with ice-cold dH₂O and incubated with the entire tTG solution (~14mls) overnight at 4°C with mixing. Pooled recombinant tTG before and after desalting, supernatant post Affi-Gel incubation and an aliquot of tTG-bound Affi-Gel were analysed by SDS-PAGE. For some experiments, human serum albumin (Pharmacia & Upjohn) was bound to Affi-Gel 15 for sham-treatment of coeliac disease IgA aliquots.

In anti-tTG depletion experiments, purified IgA antibodies from pooled coeliac disease sera were diluted 1:1 with PBS and incubated with tTG-bound Affi-Gel for 1 hour at 4°C with agitation. After incubation, the tTG-bound Affi-Gel was pelleted and the anti-tTG depleted supernatant was retained. Fluorescence-based tTG activity assays using these IgA preparations as inhibitors failed due to the formation of a white precipitate in each well containing IgA. The levels of precipitation seem to be proportional to the amount of PBS present in each well. We speculated that the precipitate forming was calcium phosphate (CaPO₄) resulting from interaction between the phosphate in PBS and calcium in the tTG reaction buffer. Once the IgA preparations were de-salted (x2) using Zeba™ columns, the problem was resolved. Depletion of anti-tTG was confirmed by Celikey™ tissue transglutaminase IgA antibody assay.

Sham-treatment of IgA with human serum albumin (Pharmacia & Upjohn)-bound Affi-Gel 15 was carried out in a similar fashion.

4.2.6 Bradford assay protein quantification

Purified immunoglobulin preparations were quantified using the method developed by the Bradford (Bradford 1976). The principle of the assay is based upon the fact that the absorbance maximum for an acid solution of Coomassie Brilliant Blue G-250 dye shifts from 465nm to 595nm when binding to protein occurs (Reisner, *et al.* 1975). Protein quantification was carried out using Bradford protein assay dye reagent (Bio-rad) as per the manufacturer's recommendations. Briefly, the dye reagent was prepared by diluting one part dye with four parts dH₂O. This working solution was used to generate a BSA standard curve ranging from concentrations of 0.312 to 20µg/ml. Unknown analytes were diluted 200-fold in the dye reagent working solution and one tube served as a blank with dH₂O added in place of protein sample. After incubation of the tubes at room temperature for at least five minutes, 300µl of each sample was loaded in triplicate in a 96 well plate (Nunc). Absorbance at 595nm was measured using a Spectrafluor Plus instrument (Tecan). Values generated by the standard curve were used to estimate protein concentration in unknown samples.

4.3 Results

4.3.1 Hydroxaminolysis assay

The tTG catalysed cross-linking of CGG to hydroxylamine was monitored spectrophotometrically and the slopes generated were used to establish enzyme unit concentrations of tTG in various batches of recombinant wild type tTG. The concentration of tTG added to assays was limited by the dilute nature of recombinant wild type tTG purifications but was sufficient to generate curves and calculate enzyme units (Figure 4.2). Our results were in agreement with reports by Cooper *et al.* that state the hydroxaminolysis assay should be linear for at least one hour if the final A_{525} is <0.5 (Cooper, *et al.* 1997). The following is an example of how tTG unit concentration was calculated.

wild type tTG 28/6/05 slope = 0.00267

Since.....

$$\Delta c/\text{min} = (\Delta A/\text{min}) / \epsilon \text{ l mol/L/min}$$

$$\Delta c/\text{min} = 0.00267/850 \times 1 \text{ mol/L/min}$$

$$\Delta c/\text{min} = 3.139494 \times 10^{-6} \text{ mol/L/min}$$

$$\Delta c/\text{min} = 0.003139 \text{ } \mu\text{mol/ml/min}$$

Therefore, 20 μ l tTG from 28/6/05 contains 3.14mU tTG.

Since 20 μ l wild type tTG from 28/6/05 contains 2.56 μ g tTG,

$$1\text{mU tTG 28/06/05} = 0.815\mu\text{g}$$

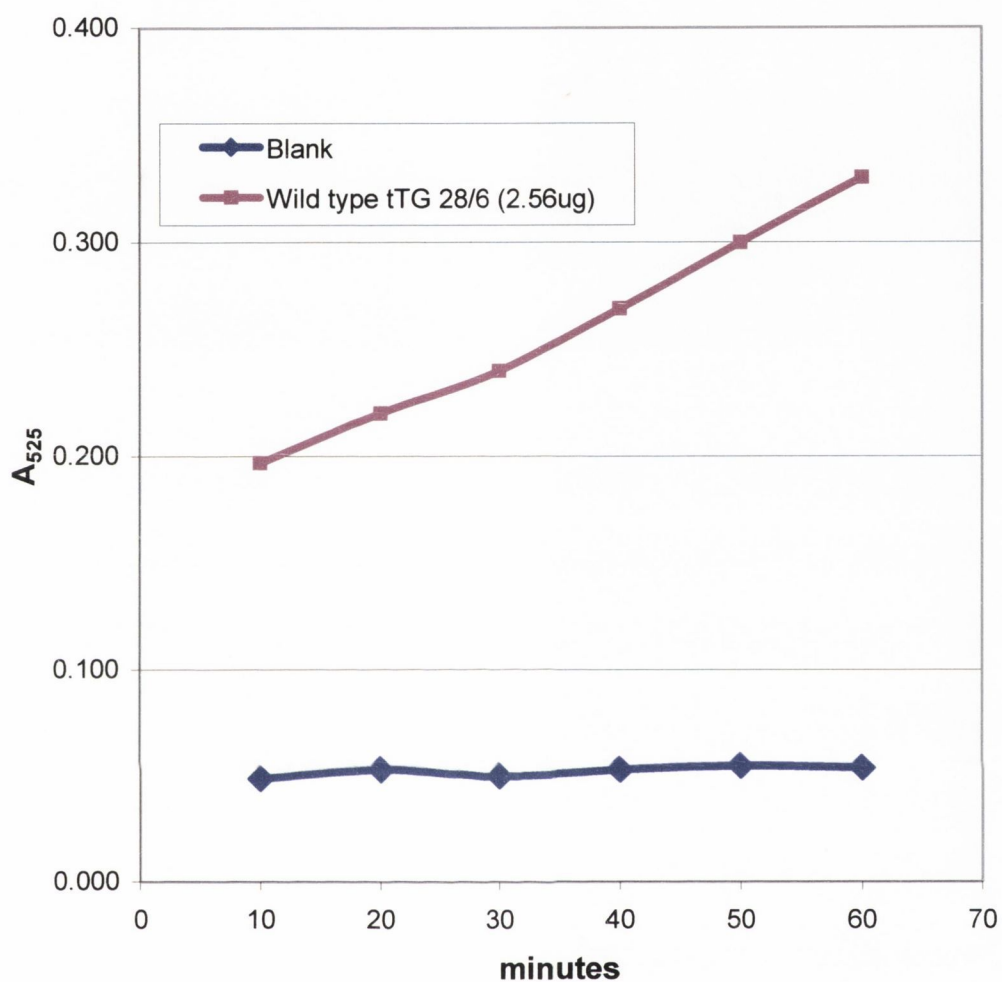


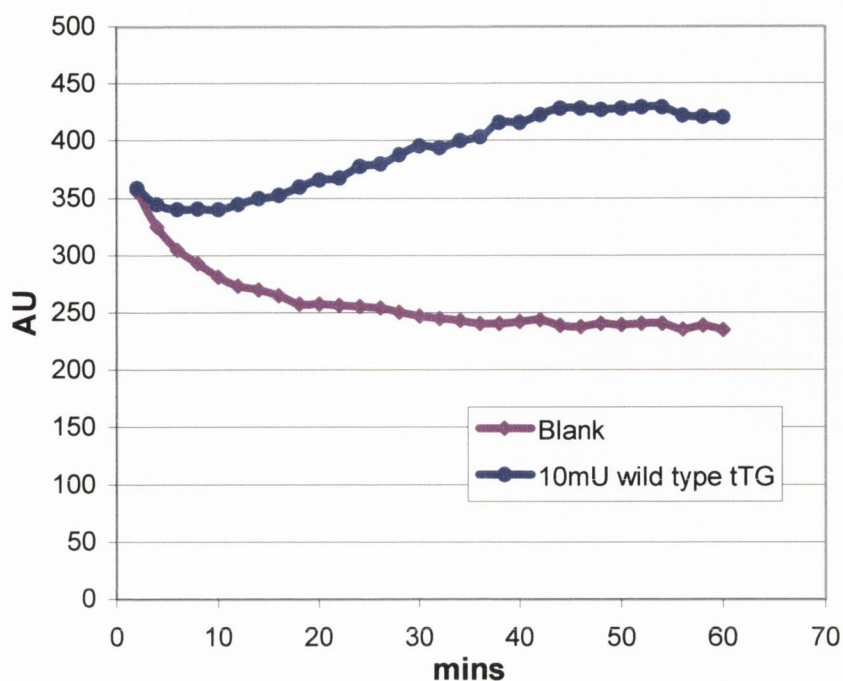
Figure 4.2: A representative example of hydroxaminolysis reaction curve. A linear increase in A_{525} was detectable after addition of recombinant wild type tTG to the reaction mixture. Slope was calculated and unit concentration of tTG was determined using Beer's law ($A = \epsilon l c$).

4.3.2 Continuous fluorescence tTG assay: CGG-DNS as substrate

A continuous fluorescence-based assay for detecting tTG activity using CGG-DNS and dansylcadaverine as substrates was successfully established. Cross-linking activity was measured by detecting the increase in fluorescence intensity when CGG-DNS was cross-linked to dansylcadaverine. Initial experiments used an emission wavelength of 535nm as previous publications reported 538nm as optimal for this assay (Jeitner, *et al.* 2001). During assay optimisation, an emission wavelength of 595nm was found to give much better differentiation between samples (data not shown). All subsequent assays were performed with $\lambda_{\text{ex}}275$ and $\lambda_{\text{em}}595$.

It was necessary to subtract blank values from analyte values at every time point since the wells lacking tTG were found to decrease in fluorescence as time progressed (Figure 4.3). Once blank values were subtracted at all time points, a sigmoidal curve was generated.

a.



b.

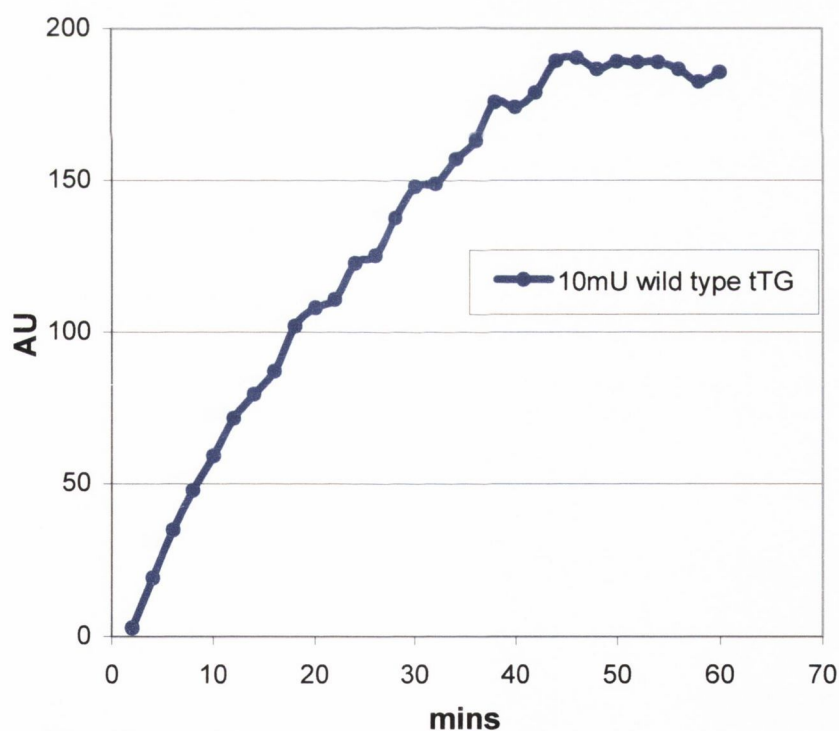


Figure 4.3: Subtraction of blank values was necessary to generate a tTG activity curve. a. Results of a CGG-DNS fluorescent tTG assay. Fluorescence values from wells lacking tTG (Blank) decreased in fluorescence over time. b. Subtraction of these blank values at each time point yielded a tTG activity curve that was sigmoidal in nature.

4.3.3 Comparison of recombinant wild type tTG activity with guinea pig liver tTG

Guinea pig liver tissue transglutaminase (gptTG) is available commercially in a lyophilised form (Sigma). gptTG unit concentration is defined by the manufacturer (2U/vial) using the hydroxaminolysis assay and was determined to be 2.56U/vial when measured using our hydroxaminolysis assay. gptTG was repeatedly found to be considerably more active than recombinant wild type tTG preparations. As described in Chapter 2, recombinant tTG preparations were stored in 50% glycerol in order to avoid damage caused by repeated freeze-thaw cycles. It was determined that glycerol in recombinant wild type tTG preparations was inhibitory to the reaction by reconstituting gptTG in 50% glycerol and comparing to gptTG without glycerol (Figure 4.4). However, even taking this glycerol inhibition into account, the cross-linking of CGG-DNS to dansylcadaverine by recombinant wild type tTG was never comparable to gptTG (Table 4.1).

Table 4.1: Activity of 10mU wild type recombinant tTG compared to commercially available gptTG with or without 50% glycerol

Enzyme	Slope
Wild type tTG 23/6 (50% glycerol)	3.57
gptTG	16.06
gptTG (+50% glycerol)	10.52

gptTG was reconstituted with 10mM DTT with or without 50% glycerol. Slopes were generated using arbitrary fluorescence units generated from 20-40 minutes.

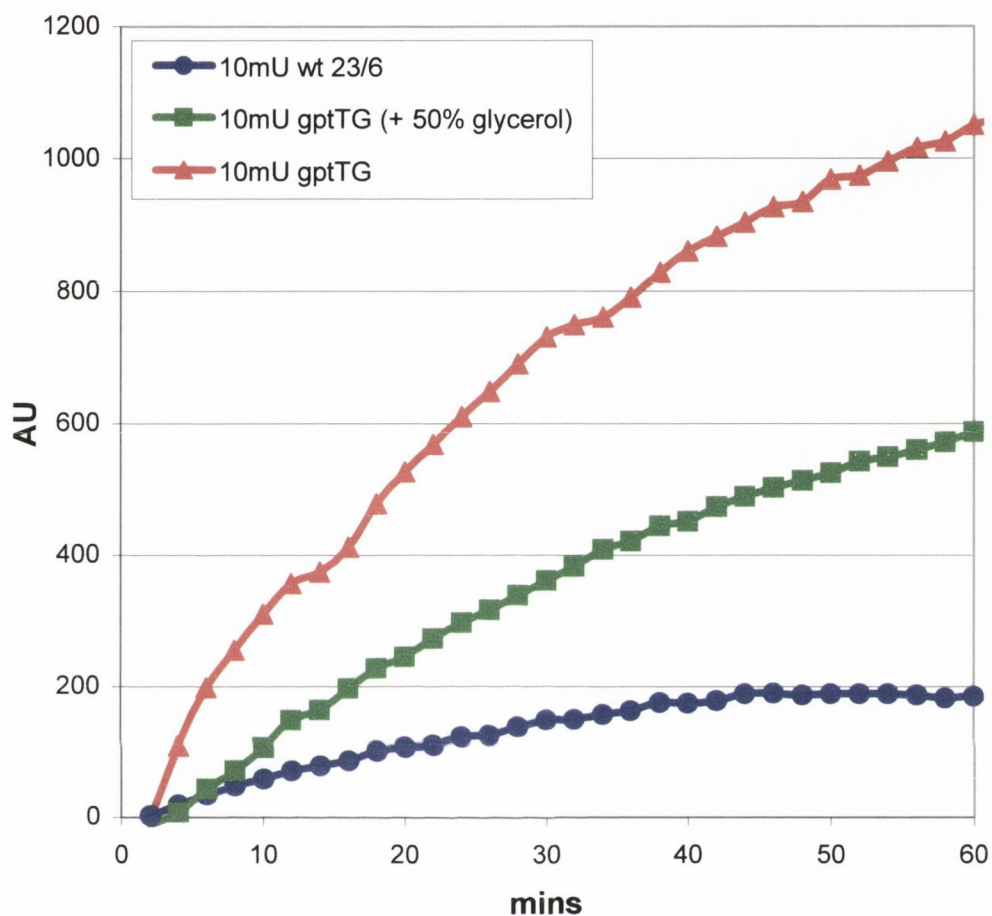


Figure 4.4: 50% Glycerol had an inhibitory effect on tTG activity. gptTG activity was reduced by reconstitution of the lyophilised enzyme in 50% glycerol. Recombinant wild type tTG had lower enzyme activity than gptTG.

4.3.4 Stability of recombinant wild type tTG

Since recombinant human tTG has been shown to be a very labile enzyme (Dieterich, *et al.* 2003), the activity of enzyme preparations that had been stored for ~10 months at -20°C was assessed. Although enzyme activity was greatly diminished, some activity was still detectable (Figure 4.5). Fresh recombinant tTG was also shown to retain >92% of its cross-linking activity after incubation at 37°C for 10 minutes (data not shown).

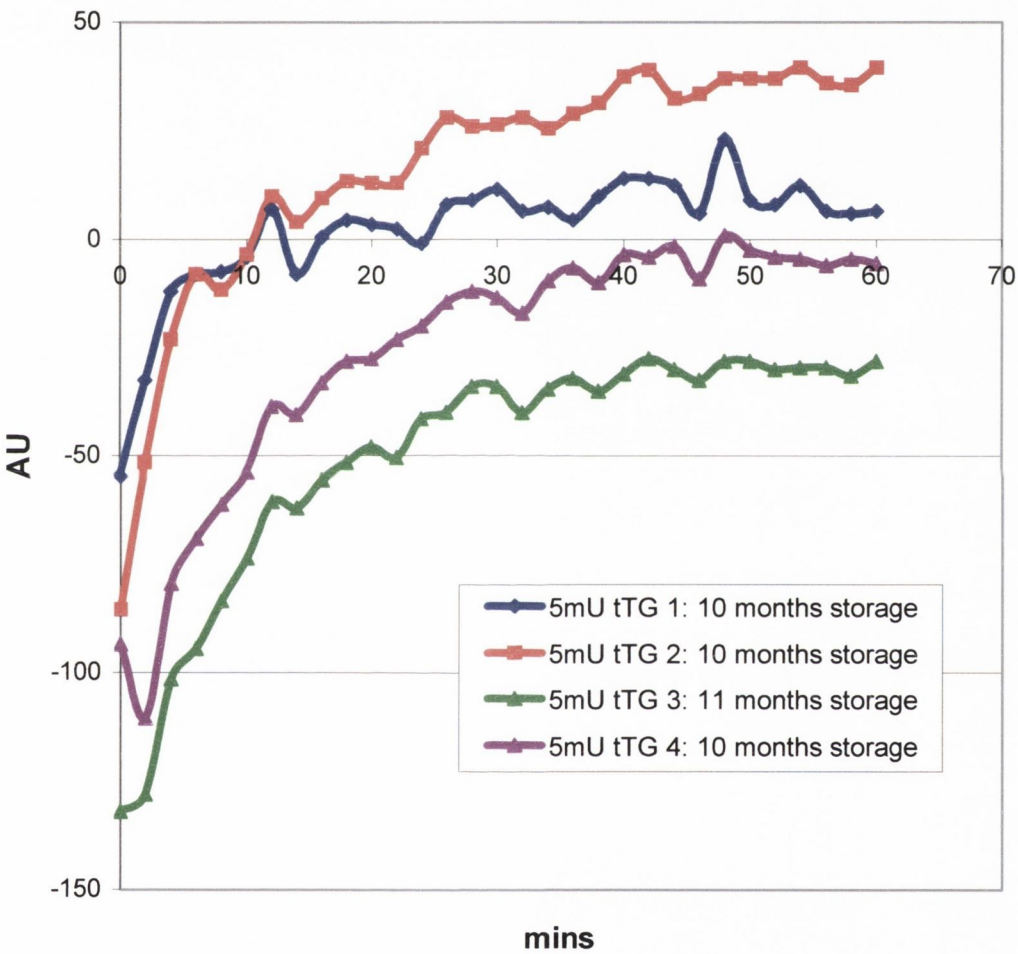


Figure 4.5: Stability of recombinant wild type tTG. Even after storage at -20°C for 10-11 months recombinant wild type tTG preparations still had some cross-linking activity, albeit at greatly diminished levels. tTGs 1-4 represent different batches of recombinant wild type tTG.

4.3.5 Continuous fluorescence transglutaminase assay: N’N-dimethylcasein as substrate

The switch to using DMC as substrate in the fluorometric tTG assays was prompted by a publication by Jeitner *et al.* during the course of this study (Jeitner, *et al.* 2005). The reasons for the change in protocol were outlined in section 4.1.2. Assays with DMC as glutaminy substrate generated sigmoidal enzyme activity curves at AU values higher than those produced by CGG-DNS assays. Although values for wells containing no tTG were still subtracted from analyte readings, these wells did not decrease in fluorescence as profoundly as in CGG-DNS assays. Once again $\lambda_{em}595$ was found to give better differentiation between samples than $\lambda_{em}535$ (Figure 4.6).

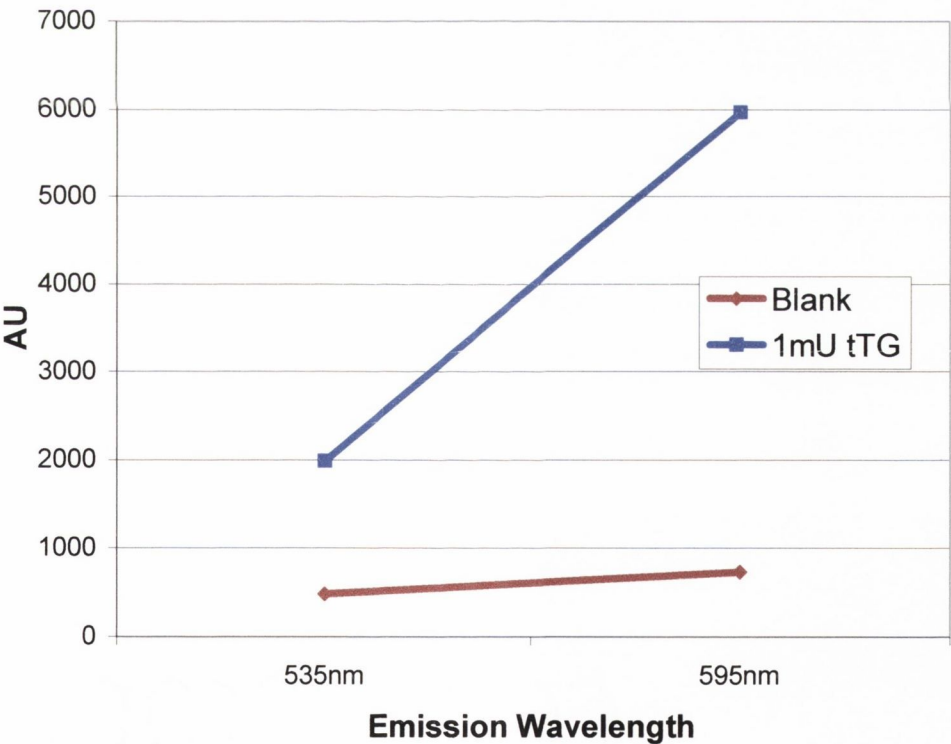


Figure 4.6: Endpoint (96 minutes) of DMC fluorescent tTG activity assay at $\lambda_{em}535$ and $\lambda_{em}595$. Differentiation between blank and tTG containing wells is more clearly distinguished at $\lambda_{em}595$.

4.3.6 Recombinant mutant tTG showed no cross-linking activity

Cross-linking activity of wild type and mutant tTGs was assessed in a fluorescent tTG activity assay with DMC as substrate. 5mU wild type tTG and an equivalent microgram amount of mutant tTG were tested for duration of 80 minutes at 37°C (mutant tTG cannot be measured in enzyme units since it has no activity in the hydroxaminolysis assay). While wild type tTG generated a sigmoidal activity curve, mutant tTG showed no increase in fluorescence, confirming that mutagenesis of the catalytic triad results in loss of transamidation activity (Figure 4.7).

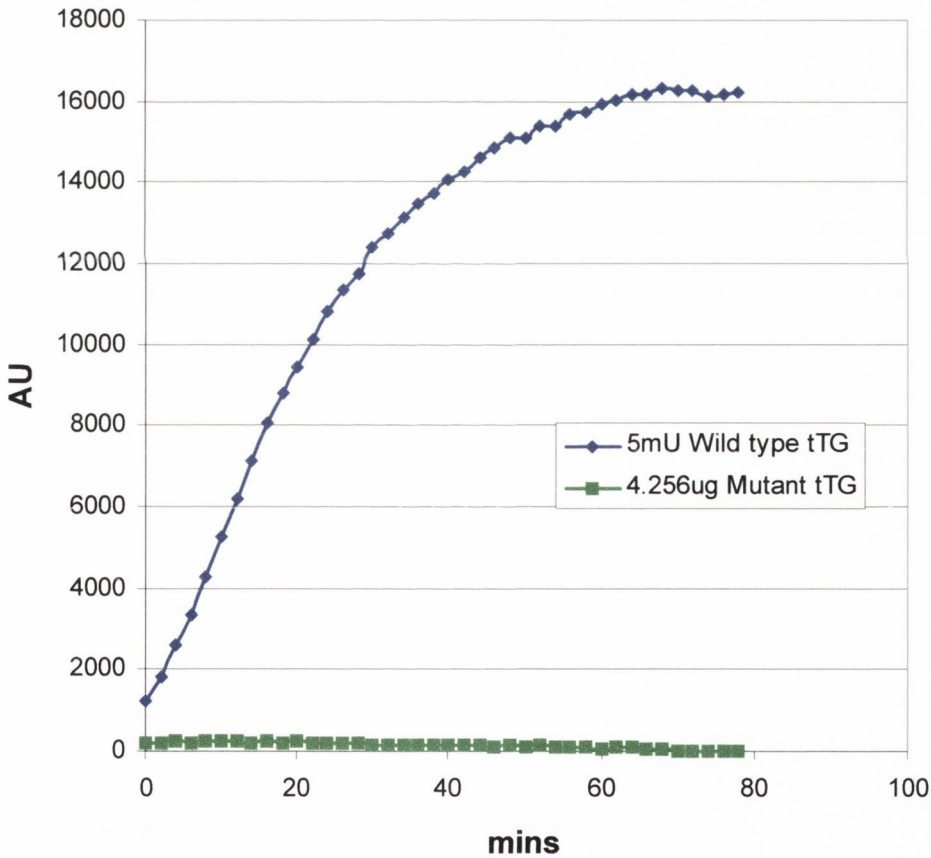


Figure 4.7: Mutant tTG shows no transamidation activity. A fluorescent tTG assay show that a microgram equivalent amount of mutant tTG shows no cross-linking activity when compared to wild type tTG.

4.3.7 tTG activity is dependent upon calcium and inhibited by zinc

It has been well documented that Ca^{2+} is required for the cross-linking activity of tTG (Griffin, *et al.* 2002) and that Zn^{2+} inhibits cross-linking (Lorand and Conrad 1984). In order to further characterise the recombinant wild type tTG generated in this study, the effect of these divalent cations was analysed. Tissue transglutaminase activity assays were carried out in the presence and absence of the divalent cations Ca^{2+} and Zn^{2+} in order to determine whether recombinant wild type tTG behaved in a similar fashion to published reports suggesting Ca^{2+} is an absolute requirement for tTG activity and Zn^{2+} is capable of tTG inhibition (Stenberg 1976, Aeschlimann and Paulsson 1994, Chen and Mehta 1999, Roth, *et al.* 2003). When 1mU tTG was added to a reaction buffer lacking Ca^{2+} , cross-linking activity was completely absent. Micromolar amounts of Zn^{2+} inhibited tTG activity in a dose dependent fashion (Figure 4.8). Experiments examining whether higher molar concentrations of zinc would inhibit tTG further were not carried out because ZnCl_2 had a low solubility in the enzyme assay reaction buffer.

a.

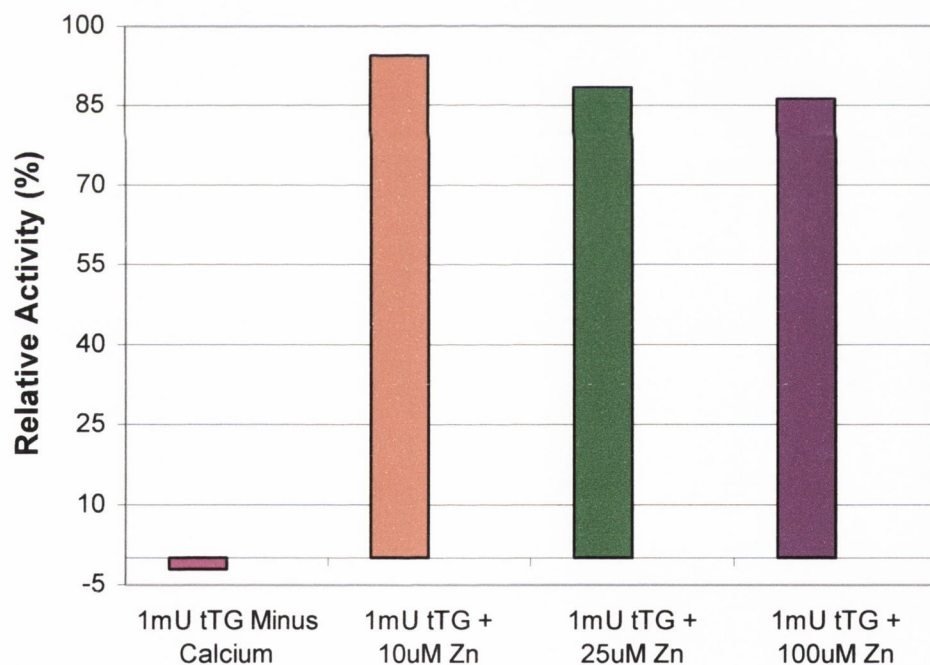
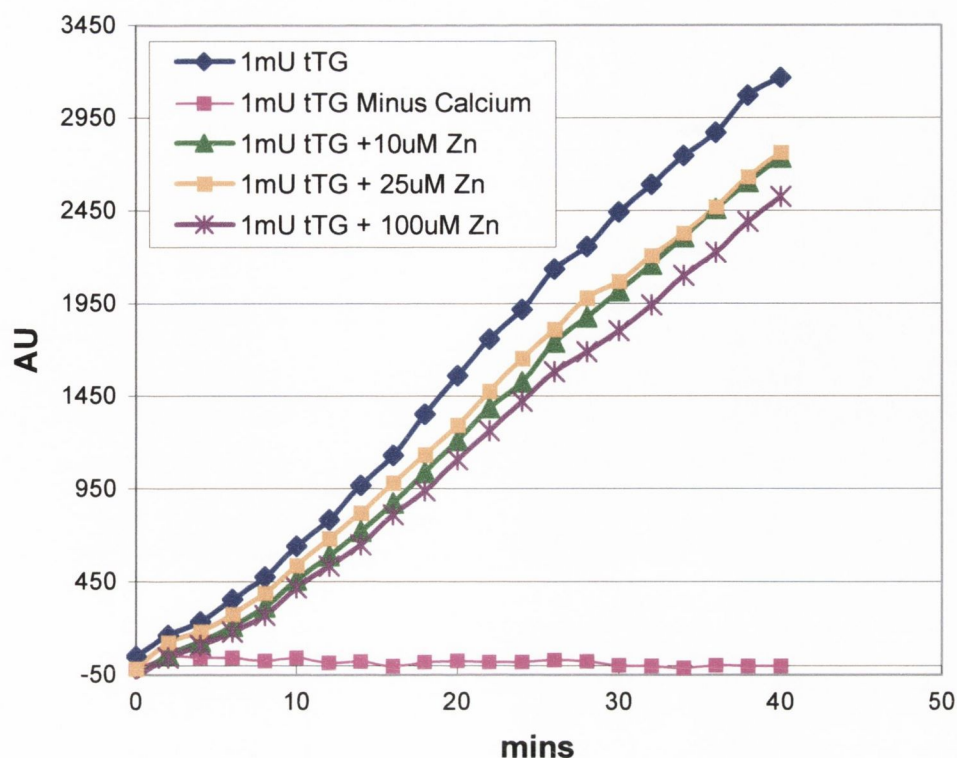


Figure 4.8: Calcium is an absolute requirement for tTG activity while zinc inhibits tTG activity. A. Curves generated by activity assays. **B.** Relative activities calculated by expressing slope for 20-40 minutes as a percentage of the slope of uninhibited tTG. When tTG activity assays were run in the absence of Ca_{2+} , no cross-linking activity was observed. When Zn^{2+} was added, a dose dependent inhibition was observed.

4.3.8 Inhibition of tTG activity by CUB 7402/TG100

The inhibition of tTG cross-linking activity by the antibody CUB 7402 has been previously published (Esposito, *et al.* 2002, Dieterich, *et al.* 2003). Our fluorescent tTG activity assays with DMC as substrate confirmed this finding but the effect could be overcome by the addition of higher amounts of enzyme. The cross-linking activity of 2.5mU tTG was not inhibited by incubation with 0.5µg CUB 7402/TG100. However, incubation of 0.5µg CUB 7402/TG100 with 1mU or less of tTG resulted in clear inhibition except in the case of 0.1mU tTG since this amount of enzyme was insufficient to generate a good curve (Figure 4.9). Relative activities are shown in Table 4.2.

Table 4.2: Inhibition of tTG by CUB 7402/TG100

Reagents	Relative activity
2.5mU tTG + 0.5µg CUB 7402/TG100	96.7%
1mU tTG + 0.5µg CUB 7402/TG100	62.89%
0.5mU tTG + 0.5µg CUB 7402/TG100	51.71%

Relative activities are calculated by expressing the slope from 20-40 minutes of the reaction as a percentage of the slope of the uninhibited enzyme.

AU values generated in these experiments were proportional to the amount of tTG enzyme added to the reaction. For future tTG inhibition experiments a 1mU concentration of enzyme was decided upon since higher levels are difficult to inhibit and lower levels might generate more ambiguous results.

The inhibition of tTG by CUB 7402/TG100 was only observed when enzyme and inhibitor were pre-incubated in a Ca²⁺ free reaction buffer. When tTG was pre-incubated with CUB 7402 /TG100 in the presence of Ca²⁺, >95% of tTG activity remained (data not shown). This is in keeping with reports by Esposito and colleagues (Esposito, *et al.* 2002).

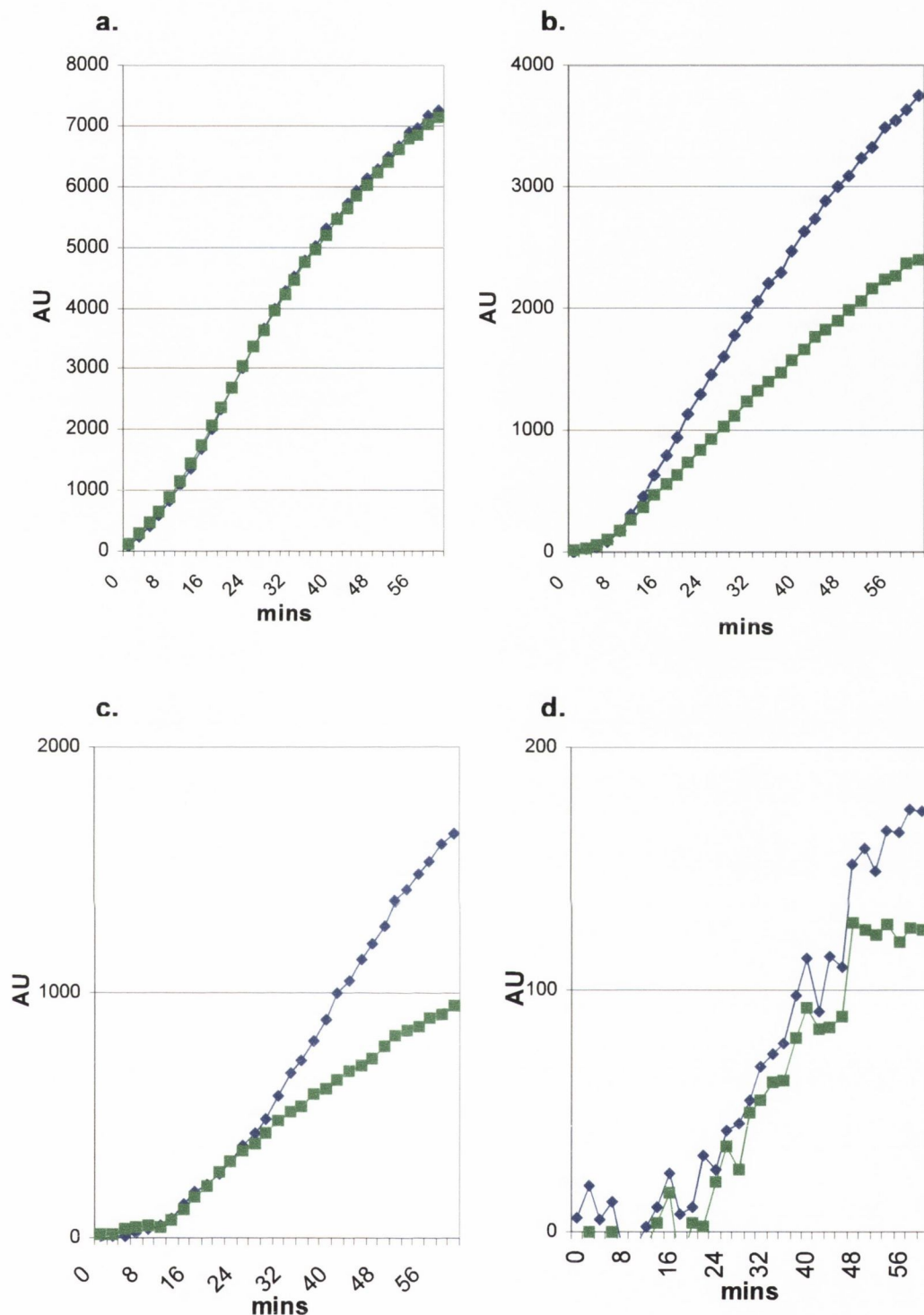


Figure 4.9: CUB 7402/TG100 monoclonal antibody cocktail is capable of inhibiting tTG but only at lower mU concentrations. The inhibitory effect of 0.5 μ g CUB 7402/TG100 was tested at **a.** 2.5mU tTG, **b.** 1mU tTG, **c.** 0.5mU tTG and **d.** 0.1mU tTG. $\blacklozenge$ = Uninhibited tTG. $\blacksquare$ = tTG + CUB 7402/TG100. The inhibitory effect of CUB 7402/TG100 was demonstrated at tTG levels lower than 2.5mU. 0.1mU tTG (D) is insufficient tTG to generate a good curve and investigate tTG inhibition.

4.3.9 Purification of IgA from human serum

IgA purification was carried out using Jacalin agarose (Pierce). λ_{280} analysis of the column eluates from the Jacalin column indicated that the majority of IgA invariably desorbed from the column in the second 2ml aliquot recovered (Figure 4.10). A sample of the final column wash was also examined spectrophotometrically and the mean λ_{280} of the final wash was 0.011 indicating that the majority of serum protein had been effectively washed away before IgA elution.

Visualisation of purified IgA by reducing SDS-PAGE clearly showed heavy and light chains of the purified immunoglobulin. An example of an SDS-PAGE of purified IgA is shown in Figure 4.11. Although some contaminating bands are visible the purity of IgA appeared consistent with the expected purity (>90%). Isoelectric focusing confirmed the presence of some contaminating IgG (data not shown). Purified IgA was quantified using the Bradford method. Yields of IgA from ~4mls of control and coeliac disease sera were 3.579mg and 4.228mg respectively.

Reference ranges for human IgA levels in serum vary from 1-3 grams per litre (Berth, *et al.* 1999). Since the concentrations of protein in IgA purifications fell within this range, anti-tTG levels of the purified IgA could be quantified using the Celikey[®] ELISA assay. Anti-tTG levels for control and coeliac disease IgA were 1.2 AU and 53.9 AU respectively (cut-off point for positivity in the Celikey[®] assay is 1.9 AU).

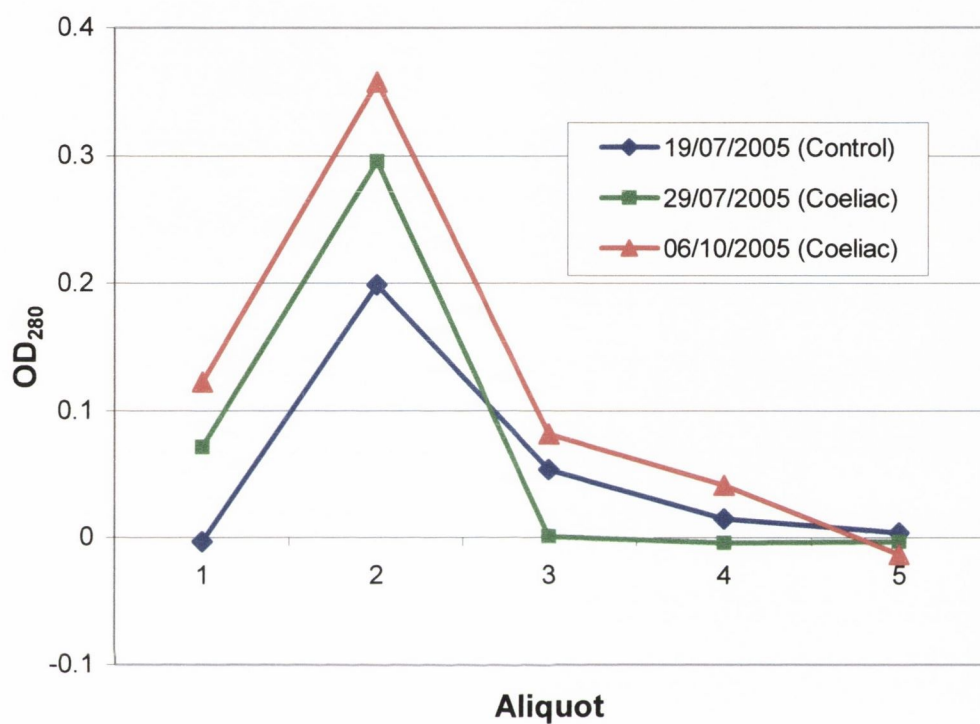


Figure 4.10: λ_{280} analyses of Jacalin column eluates. The results of three separate IgA purification experiments. The majority of purified IgA was present in the second 2ml aliquot retrieved from the Jacalin agarose column.

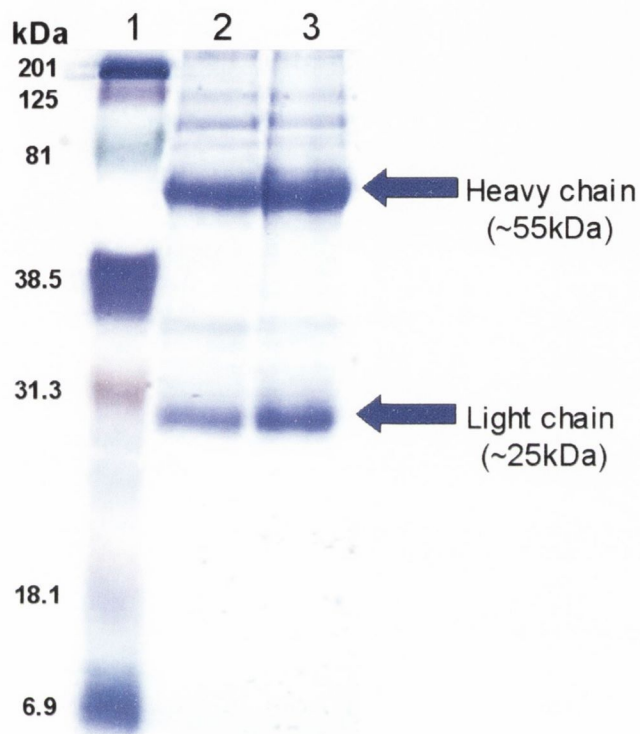


Figure 4.11: SDS-PAGE of purified IgA. 5 μ g IgA from control (Lane 2) and coeliac disease sera (lane 3) were separated by SDS-PAGE. Heavy and light chains at the correct molecular weight were observed. Higher molecular weight bands were also visible but since λ_{280} values of column wash were low these bands may represent intact IgA monomers and dimers that were not fully reduced.

4.3.10 Coeliac disease IgA is a significantly stronger inhibitor of tTG than control IgA at certain concentrations

In order to investigate the inhibitory capacity of coeliac disease IgA on the cross-linking activity of tTG, equivalent microgram amounts of control or coeliac disease IgA were added to fluorescent tTG activity assays. The effect of 10, 20 and 30 μ g of IgA on the cross-linking activity was assessed by comparing the slope generated during the linear phase of the reaction (20-40 minutes) to the slope of the uninhibited enzyme. The results generated were derived from five independent experiments.

At 10 μ g inhibitor concentration, coeliac disease IgA had the effect of reducing relative activity of tTG to 88.6% while control IgA had no significant effect on tTG activity with the mean relative activity of tTG being 97%. This finding was statistically significant ($p=0.0077$). At 20 μ g inhibitor, once again there was a statistically significant difference between the effect of control and coeliac disease IgA ($p=0.0072$) but control IgA had the effect of reducing tTG activity to 84.8%. Finally, at 30 μ g inhibitor, there was no significant difference between the effect of control and coeliac disease IgA with both resulting in ~70% relative activity (Figure 4.12).

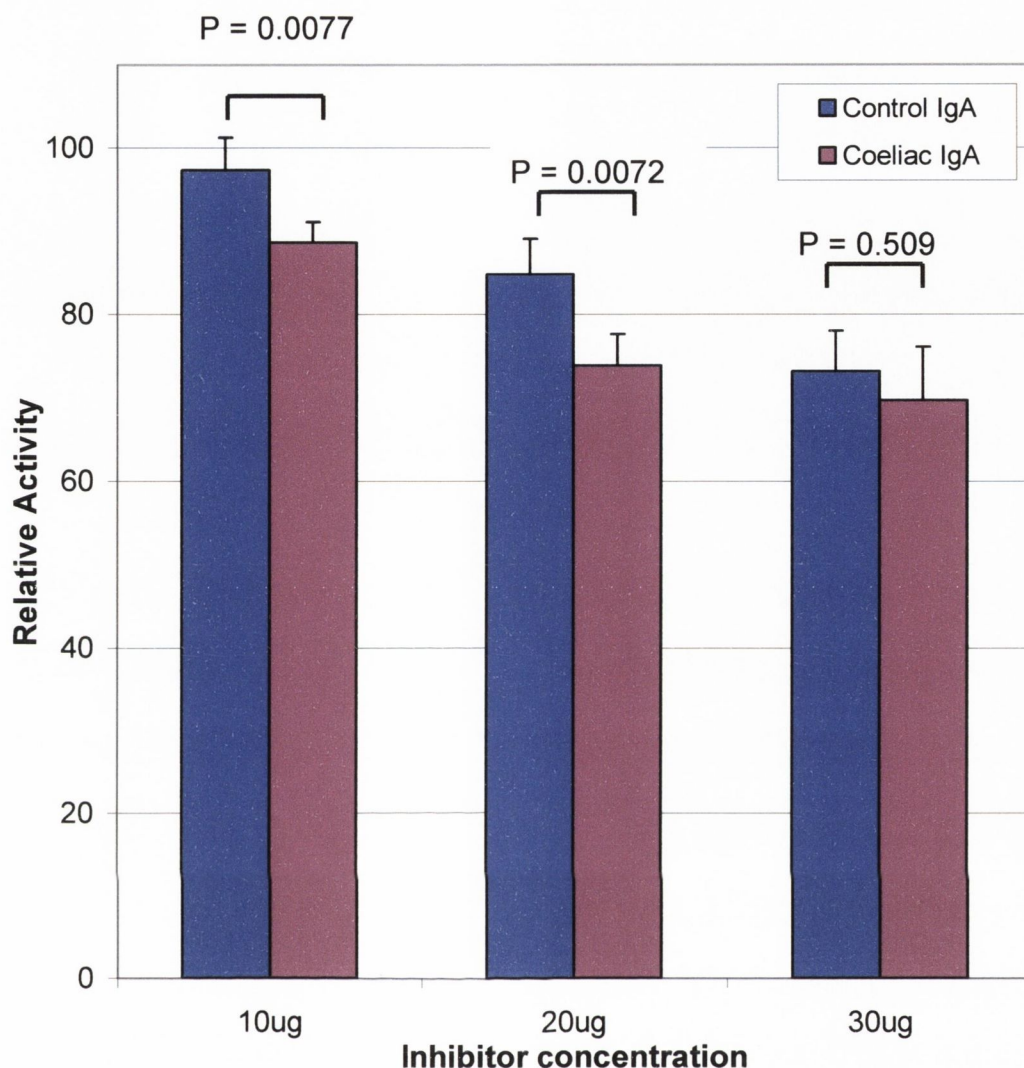


Figure 4.12: The effect of control and coeliac disease IgA on tTG activity (n=5). When 10 and 20 μ g IgA were added to activity assays, coeliac disease IgA inhibited tTG significantly more than control IgA. The effect was not replicated at 30 μ g IgA. Although coeliac disease IgA inhibited tTG more significantly than control IgA (Students t-Test), a concentration dependent inhibition of tTG by control IgA was also observed.

4.3.11 Unrelated antibody inhibition of tTG

In order to determine whether the concentration dependent inhibition of tTG by anti-tTG negative IgA observed was a result of low affinity non-specific antibody interactions, two commercially available, unrelated antibodies were tested for their capacity to inhibit tTG in the fluorescence-based cross-linking assay.

The inhibition of 10, 20 and 30 μ g of polyclonal swine anti-rabbit (Dako) and polyclonal rabbit anti-human IgA (Dako) was tested. A concentration dependent inhibition of tTG similar to that seen with control IgA was observed (Figure 4.13). Since the pattern of inhibition mirrored that observed with control IgA, it was concluded that this assay is very sensitive to low-affinity antibody binding and that some degree of inhibition of tTG can be observed when sufficient quantities of unrelated antibody are added.

The concentration dependent inhibition of tTG by control antibodies was not a non-specific protein effect since the addition of BSA was shown to increase tTG activity in separate experiments (data not shown).

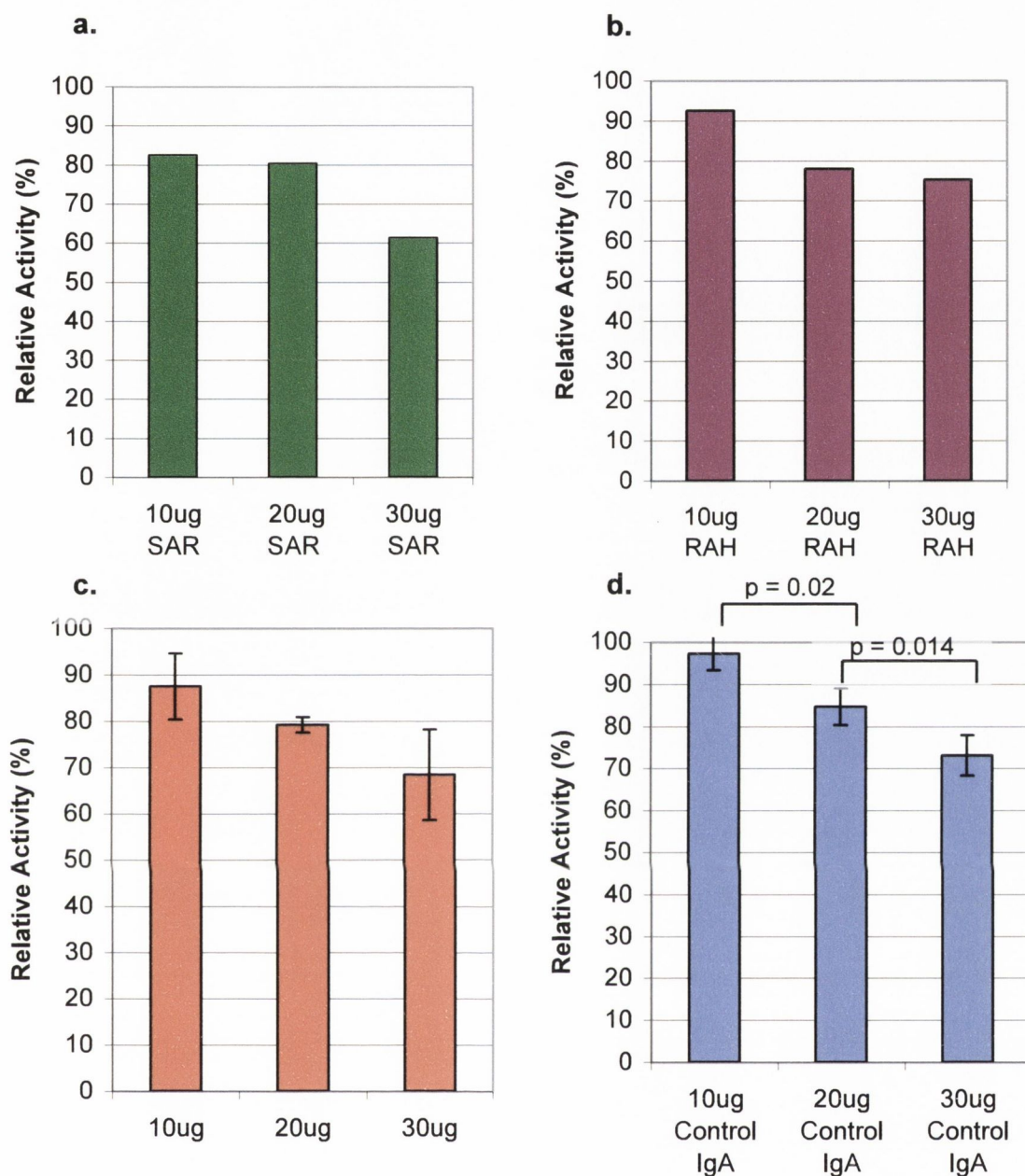


Figure 4.13: Inhibition of tTG by unrelated antibodies. A concentration dependent inhibition of tTG was observed at 10, 20 and 30 µg swine anti-rabbit (SAR) (a) and rabbit anti-human (RAH) IgA (b). The mean relative activities of both unrelated antibodies (c) closely mirrors the inhibition observed in control IgA experiments (d) (see section 4.3.10).

4.3.12 Attachment of recombinant wild type tTG to Affi-Gel solid support

In order to determine whether the inhibition of tTG by coeliac disease serum IgA was a result of anti-tTG antibodies, experiments were carried out to deplete anti-tTG from Jacalin agarose purified coeliac disease IgA.

Recombinant tTG was coupled to Affi-Gel 15 (Pierce), an activated immunoaffinity support. Since any primary amino group is automatically bound to Affi-Gel supports by displacement of the N-hydroxysuccinimide group and formation of a stable amide bond, the ligand of choice cannot be in a tris or glycine buffer. Recombinant wild type tTG (generated in Chapter 2) was desalted in order to remove tris buffer before incubation with the Affi-gel solid support.

The effect of de-salting on the concentration of tTG and the efficiency of coupling of tTG to the Affi gel beads was analysed by SDS-PAGE (Figure 4.14). De-salting had no visible effect on tTG concentration (Figure 4.14, lanes 2 & 3). Wild type tTG in solution was clearly depleted by incubation with Affi-Gel 15 (Figure 4.14, lane 4). Electrophoresis of a small sample of Affi-Gel beads showed that tTG was attached to Affi-Gel 15 (Figure 4.14, lane 5) and tTG was not washed off the beads subsequent to attachment (Figure 4.14, lane 6). Human serum albumin (HSA) was also attached to Affi-gel 15 for control purposes and SDS-PAGE analysis was carried out in a similar fashion to confirm attachment (data not shown).

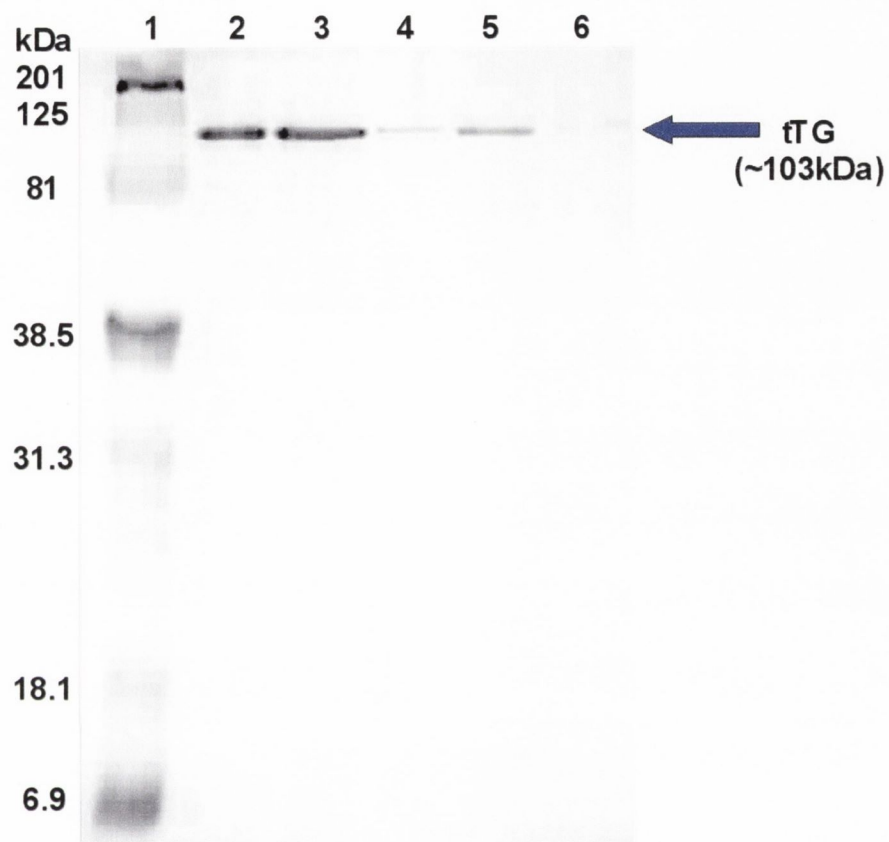


Figure 4.14: SDS-PAGE analysis of tTG binding to Affi-Gel 15. Lane 1: kaleidoscope marker. Lane 2: pooled wild type tTG pre-desalt. Lane 3: pooled wild type tTG post-desalt. Lane 4: supernatant post Affi-Gel binding. Lane 5: 5µl tTG-bound Affi-Gel. Lane 6: final wash of tTG-bound Affi-Gel.

4.3.13 The inhibition of tTG is mediated by anti-tTG IgA

Coeliac disease IgA was purified from pooled coeliac disease sera as per section 4.2.4. Anti-tTG autoantibodies were depleted from coeliac disease IgA by incubation with tTG-bound Affi-Gel 15 for one hour at 4°C. The anti-tTG level of the Affi-gel eluate was measured by Celikey® tTG ELISA assay. Since the concentration of anti-tTG depleted IgA was ~5-fold more dilute than human serum IgA, anti-tTG reactivity was measured at a dilution of 1:20 rather than the usual 1:100. The Celikey® IgA anti-tTG ELISA results for purified coeliac disease IgA and anti-tTG depleted coeliac disease IgA were 37.86 and 0.876 respectively (cut-off point for positivity in the Celikey® assay is 1.9 AU).

It was concluded that tTG-bound Affi-Gel 15 treatment successfully depleted anti-tTG autoantibodies from purified coeliac disease IgA. Treatment of purified coeliac disease IgA with tTG-bound Affi-Gel 15 resulted in a 46% reduction in IgA concentration. A second aliquot of purified coeliac disease IgA was sham-treated with HSA-bound Affi-gel 15 beads as a control. The two supernatants were then used as inhibitor in a series of fluorescent tTG assays. A schematic representation of these experiments is shown in Figure 4.18.

When the inhibitory capacity of 50µl anti-tTG depleted and 50µl sham-treated coeliac disease IgA were compared, it was found that while coeliac disease IgA sham-treated with HSA-bound Affi Gel inhibited tTG to a relative activity of 65.7%, anti-tTG depleted coeliac disease IgA inhibited tTG significantly less ($p < 0.001$, student's T test), to a relative activity of 82% (Figure 4.19).

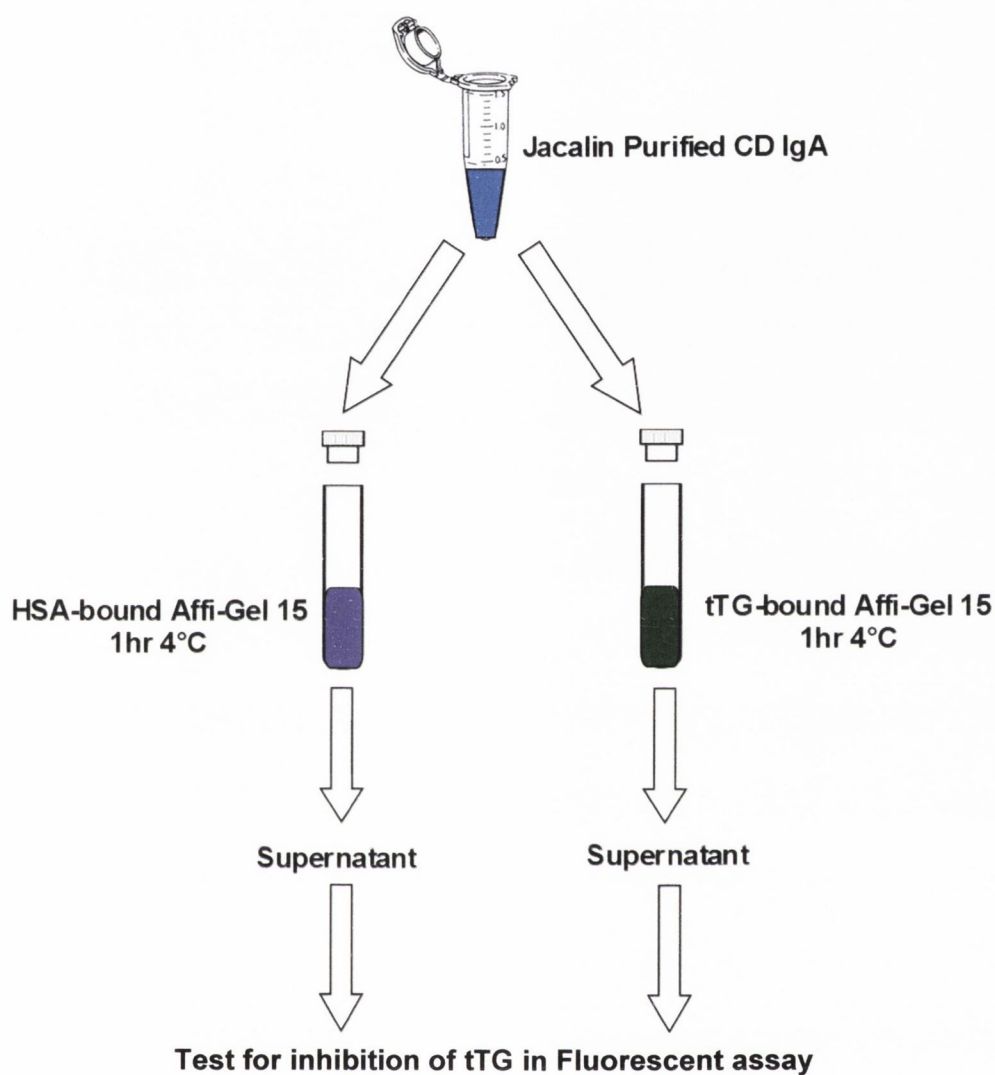


Figure 4.18: Schematic representation of anti-tTG depletion experiment 2. In this experiment, one aliquot of Jacalin agarose purified coeliac disease IgA was incubated with tTG-bound Affi-Gel 15 while a second aliquot was incubated with HSA-bound Affi-Gel 15. The two unmanipulated supernatants were tested for inhibition in fluorescent tTG assays.

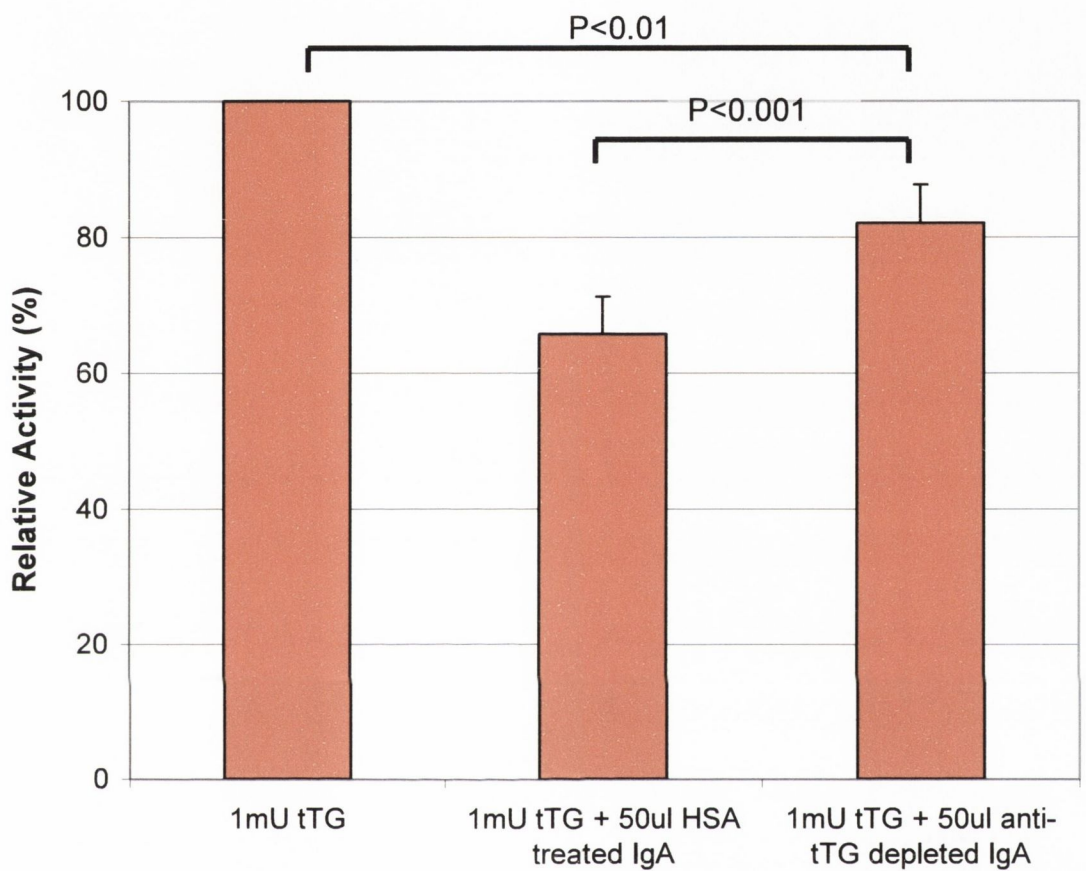


Figure 4.19: HSA sham-treated coeliac disease IgA has significantly greater inhibitory capacity than anti-tTG depleted coeliac disease IgA (n=4).

4.4 Discussion

4.4.1 Transglutaminase assay development

The purpose of this chapter was to develop a sensitive transglutaminase cross-linking assay to investigate whether the specific targeting of the catalytic triad of tTG (described in Chapter 3) had any effect on the activity of recombinant wild type tTG (generated in Chapter 2).

The hydroxaminolysis assay, as described by Cooper and co-workers (Cooper, *et al.* 1997) was used to standardise tTG enzyme unit concentrations. Wild type recombinant tTG was shown to cross-link CGG to hydroxylamine in a measurable manner, generating curves that were linear for up to one hour. In subsequent fluorescence-based assays, recombinant wild type tTG produced slopes that were considerably less than those generated by equivalent unit concentrations of commercially available guinea pig tTG. This was unexpected since the hydroxaminolysis assay should have standardised enzyme activity levels. As a result of the low concentrations of recombinant wild type tTG preparations, the microgram concentration of recombinant tTG added to the hydroxaminolysis reaction (mean=3.34 μ g) was significantly less than that of gptTG added (10 μ g). It is possible that the hydroxaminolysis assay is less sensitive at lower concentrations of enzyme. The long-term storage of recombinant tTG at -20°C could also have had adverse effects on cross-linking activity, meaning that the unit concentrations generated in the hydroxaminolysis assay became less correct as storage time progressed. These factors could possibly account for the discrepancy between hydroxaminolysis and fluorescent assay results. Since the fluorescence-based tTG assays were based upon the relative activities of inhibited and uninhibited enzyme, discrepancies in the unit concentration of tTG added to the reaction would have no effect on the interpretation of the results.

A fluorescence-based assay for detecting cross-linking by tTG was developed in order to assay enzyme inhibition by coeliac disease serum autoantibodies. The assay was developed based upon publications by Jeitner and colleagues (Jeitner, *et al.* 2001, Jeitner, *et al.* 2005). Initially, substrates chosen were CCG-DNS as glutaminy substrate and dansylcadaverine as amine substrate. These assays generated sigmoidal activity curves, albeit only after subtraction of blank values at each time-point. The decrease in fluorescence in the wells containing no enzyme is probably attributable to photochemical burnout of the fluorochrome due to exposure to light. An emission wavelength of 595nm gave superior results when compared 535nm, which is closer to the published optimum of 538nm (Jeitner, *et al.* 2001).

During the course of this study, Jeitner and co-workers published an improved protocol for their fluorescence-based tTG cross-linking assay which replaced CCG-DNS with DMC as glutaminy substrate (Jeitner, *et al.* 2005). In our hands, this assay was found to produce higher levels of fluorescence and wells containing no enzyme did not decrease in fluorescence over time as significantly as in the previous assay. This result suggests that CCG-DNS was the compound in the earlier experiments that was more susceptible to photochemical burnout. The use of DMC as glutaminy substrate also avoided the tendency for CCG-DNS to precipitate in solution at room temperature. Once again, an emission wavelength of 595nm (Figure 4.6) was shown to be superior to the wavelength optimised by Jeitner and colleagues (Jeitner, *et al.* 2005).

The reason for this disparity in optimal emission wavelengths is unknown but the fluorescence of the dansyl group is strongly dependant on the polarity of its surroundings. While dansylcadaverine-lysine shows an emission maximum of 578nm which is close to the wavelength measured in our tTG assay, a lipophilic environment can result in a hypsochromic shift to an emission maximum of ~500nm (Schiller 1972). It is possible that the microtitre plates used by Jeitner *et al.* are more lipophilic than the Nunc Maxisorp™ plates used in these experiments. In fact, according to a Nunc bulletin concerning principles of adsorption to polystyrene,

Maxisorp™ plates are considerably less lipophilic than other Nunc plates (www.nuncbrand.com).

4.4.2 Characterisation of the activity of recombinant tTGs

The fluorescence-based cross-linking assay using CGG-DNS as substrate was used to compare the cross-linking activity of 5mU wild type tTG and an equivalent microgram amount of mutant tTG. This experiment confirmed that mutant recombinant tTG showed no cross-linking activity (Figure 4.7). It is well documented that cysteine 277 is necessary for the transamidation reactions catalysed by tTG (Fesus and Piacentini 2002). Several publications have shown that recombinant enzymes or transfection constructs of tTG with cysteine 277 replaced by either alanine or serine residues show no cross-linking activity (Lee, *et al.* 1993, Murthy, *et al.* 2002, Milakovic, *et al.* 2004). The recombinant mutant tTG generated in this study is the first engineered to have all elements of the papain-like catalytic triad (Cys²⁷⁷, His³³⁵, Asp³⁵⁸) altered. The rationale for targeting all three amino acids of the catalytic triad was to induce a significant alteration of the chemical and steric properties of the substrate binding pocket of tTG.

The cross-linking assay using CGG-DNS as substrate was also used to demonstrate that, even after 10-11 months of storage at -20°C, wild type tTG showed cross-linking activity (Figure 4.5), albeit at lower levels and for reduced time (<30 minutes). The considerable stability of the recombinant tTG generated in this study makes it a very useful tool in tTG inhibition analysis. A previous study that produced recombinant tTG for experiments investigating inhibition of tTG by coeliac disease serum autoantibodies reported that their enzyme preparations were very unstable and that measurement of cross-linking could be carried out for only 2 minutes. The instability of this tTG preparation also prevented the preincubation of the enzyme with potential inhibitors at 37°C prior to assay (Dieterich, *et al.* 2003).

In experiments investigating the effect of divalent cations on the recombinant wild type tTG generated in the study, it was demonstrated that Ca^{2+} is an absolute requirement for wild type tTG activity. In similar experiments investigating the effect of micromolar levels of Zn^{2+} , a dose dependant inhibition of tTG was observed (Figure 4.8). The role of zinc as a tTG inhibitor has been previously documented (Lorand and Conrad 1984). In fact, physiological levels of zinc block calcium induced activation of tTG (Credo, *et al.* 1976). It has been suggested that the low levels of zinc in the intestine facilitate the activation of tTG and gliadin deamidation. Malabsorption secondary to villous atrophy could then contribute to hypozincaemia and facilitate further tTG activation (Roth, *et al.* 2003). Whether supplementation of coeliac disease patient diet with zinc would help the condition by inhibiting deamidation of gliadin peptides has not been investigated.

In experiments investigating the effect of a commercially available mouse anti-human tTG antibody cocktail (CUB 7402/TG100), it was shown that in agreement with the literature (Esposito, *et al.* 2002), CUB 7402 is capable of inhibiting the cross-linking activity of tTG. However, this incubation could be overcome by addition of more enzyme (Figure 4.9a). Inhibition by CUB 7402 was only apparent when the enzyme was pre-incubated with antibody in the absence of Ca^{2+} . This result suggests that CUB 7402 binds tTG in its closed conformation and possibly interferes with the Ca^{2+} induced activation of the enzyme. In fact, the region targeted by CUB 7402 (amino acids 447-478) contains two potential Ca^{2+} binding sites at amino acids 450 and 470 (Ichinose, *et al.* 1990). Immunohistochemical studies have shown that CUB 7402 does not bind cell surface tTG, which should be in the active conformation due to extracellular calcium concentrations (Esposito, *et al.* 2003). A new antibody called 6B9 targets tTG in its active conformation and has been used to demonstrate tTG expression on the cell surface of the T84 intestinal epithelial cell line (Maiuri, *et al.* 2005).

4.4.3 Inhibition of tTG by coeliac disease serum autoantibodies

Section 4.3.9 describes the purification of IgA from coeliac disease and control sera using immobilised Jacalin. SDS-PAGE and Celikey[®] anti-tTG ELISA were used to confirm the presence of IgA in column eluates and yields of IgA were roughly 1mg/ml serum. This purified IgA was used as potential inhibitor in fluorescence-based tTG cross-linking assays. Coeliac disease IgA was found to be a more potent inhibitor of tTG when compared to control IgA at 10µg and 20µg, but not at 30µg (Figure 4.12). The results were confounded by what appeared to be inhibition of tTG by low-affinity interactions with unrelated antibodies.

Experiments showing dose-dependant inhibition of tTG by commercially available, unrelated antibodies supported this assumption. Swine anti-rabbit and rabbit anti-human IgA were found to cause a dose dependent inhibition of tTG (Figure 4.13a & b) that was comparable to the effect mediated by similar concentrations of control IgA (Figure 4.13d) but again was less than inhibition by coeliac disease IgA at 10µg and 20µg. In order to determine whether the inhibitory effect was caused by the addition of any protein, experiments were carried out where bovine serum albumin (BSA) was added in similar concentrations. The addition of BSA enhanced the tTG-catalysed cross-linking in a dose-dependant manner (data not shown) suggesting that the inhibition by control antibodies was not a non-specific protein effect.

In order to show that the inhibition of tTG was caused by anti-tTG antibodies, wild type tTG was bound to a solid support and was used to remove anti-tTG from purified coeliac serum IgA. Recombinant wild type tTG was bound well by Affi Gel as evidenced by SDS-PAGE (Figure 4.14) and depletion of anti-tTG was successful as evidenced by Celikey[®] IgA anti-tTG ELISA assay. The depletion of anti-tTG antibodies from purified coeliac serum IgA was found to significantly reduce its capacity as an inhibitor of tTG while sham-treatment of a second aliquot of coeliac serum IgA with HSA-bound Affi-gel had no effect on inhibition of tTG

(Figure 4.19). We can conclude from these experiments that the inhibition of tTG by coeliac serum IgA is mediated primarily by anti-tTG antibodies.

We can speculate from these results that the partial inhibition of tTG in the gut is mediated by the mucosal immunoglobulin class, IgA. Given the increased prevalence of IgA deficiency in coeliac disease, it seems unlikely that these inhibitory autoantibodies are involved in disease aetiology but they may contribute to the gut lesion associated with coeliac disease. Although the inhibition of tTG appears to be only partial, it may be of biological significance. The anti-tTG IgA detected in the periphery is considered “spillover” from the gut compartment and may only represent a small fraction of anti-tTG IgA being produced. In fact, at least 80% of human plasma cells are located in the intestinal lamina propria, and together, they produce more IgA ($40\text{--}60\text{mg kg}^{-1}\text{ day}^{-1}$) than all other immunoglobulins combined (Fagarasan and Honjo 2003). Large amounts of anti-tTG IgA could have a significant effect on the activity of the enzyme *within the small intestine*. Marzari and colleagues (2001) used phage antibody libraries to demonstrate that unlike anti-gliadin responses, antibodies directed against tTG could only be isolated from gut-derived lymphocyte libraries (Marzari, *et al.* 2001).

Partial inhibition of enzyme activity by autoantibodies has been assigned a significant role in some diseases. For example, in Wegener’s granulomatosis inhibition of proteinase 3 by anti-neutrophil cytoplasmic antibodies (ANCA) has been well characterised. It has been shown that although total blockade of proteinase 3 by autoantibodies is rarely observed, ANCA from patients with active disease have a higher inhibitory capacity towards the enzymatic activity of proteinase 3 than from patients in remission and may therefore contribute to the disorder (van der Geld, *et al.* 2002).

In order to confirm these observations regarding the highly specific nature of anti-tTG IgA targeting of tTG, future work could include the isolation of IgA anti-tTG antibodies and analysis of their potency as tTG inhibitors in isolation. Measurement of tTG inhibition by coeliac disease serum IgG

autoantibodies would help confirm that IgG responses are less specific and possibly less inhibitory. A further dissection of IgA₁/IgA₂ classes would also further our understanding of the effect of these autoantibodies. The analysis of dermatitis herpetiformis serum antibodies as tTG inhibitors would also generate useful information, although these patients are less numerous.

4.4.4 Conclusions

To summarise, in this chapter we have performed hydroxaminolysis assays to determine enzyme unit concentrations of recombinant wild type tTG and established a fast, simple and highly sensitive fluorescence-based assay for measuring tTG catalysed cross-linking. This fluorescence-based assay was used to demonstrate that the recombinant wild type tTG generated in this project is active, robust and has metal ion requirements in agreement with the literature. In contrast, the catalytic triad mutant tTG shows no cross-linking activity whatsoever.

Serum IgA from coeliac disease patients was found to be a more potent inhibitor of tTG than control IgA at certain concentrations. This result was confounded by what appeared to be the dose-dependent inhibition of tTG by low-affinity unrelated antibody interactions. The depletion of anti-tTG antibodies from purified coeliac disease serum IgA was used to demonstrate that anti-tTG antibodies mediate the partial inhibition of tTG. The *in vivo* significance of this finding is unknown but may contribute to the gut lesion associated with coeliac disease. This will be further discussed in Chapter 5.

Future work in this area could involve characterising the inhibitory effect of IgA anti-tTG purified from coeliac disease serum. Furthermore, the demonstration that coeliac disease serum IgG is less inhibitory to tTG than coeliac serum IgA would also support observations made in Chapter 3 regarding the specificity of IgG anti-tTG responses.

Chapter 5

General Discussion

5.1 Review of results

The detection of autoantibodies directed against tTG has become an integral part of coeliac disease diagnosis (Schuppan, *et al.* 2005). Furthermore, it has been shown that tTG catalyses the site specific deamidation of glutamine residues in gliadin peptides, introducing negative charges and favouring binding to the HLA-DQ2 and DQ8 molecules (van de Wal, Kooy, van Veelen, Pena, Mearin, Papadopoulos, *et al.* 1998, Arentz-Hansen, *et al.* 2000, Fleckenstein, *et al.* 2002, Vader, *et al.* 2002). The advent of a specific role for tTG in amplifying immune responses against gliadin peptides in coeliac disease has generated interest in a possible role for the autoantibody response directed against the enzyme. Several studies have shown partial inhibition of tTG cross-linking activity mediated by coeliac autoantibodies (Esposito, *et al.* 2002, Dieterich, *et al.* 2003), while Freitag and co-workers have demonstrated that the induction of anti-tTG autoantibody responses in mice results in periductal lymphocytic infiltrates in lacrimal glands (Freitag, *et al.* 2004). The focus of this thesis was to determine whether the catalytic triad of tTG (Cys²⁷⁷, His³³⁵ & Asp³⁵⁸) represents a target for coeliac serum autoantibodies and whether these autoantibodies mediate inhibition of the function of the enzyme. Unlike other tTG epitope mapping studies (Seissler, *et al.* 2001, Griffin, *et al.* 2002, Sblattero, *et al.* 2002, Tiberti, *et al.* 2003, Nakachi, *et al.* 2004), site-directed mutagenesis was used as the method of choice in this study in an attempt to maintain overall protein conformation.

In Chapter 2 RT-PCR, recombinant DNA technology and protein purification techniques were used to generate a GST-tagged, full-length, recombinant human tTG in BL21 *E.coli*. In agreement with previous reports (Shi, *et al.* 2002) recombinant tTG was inclined to aggregate in insoluble inclusion bodies and exhaustive optimisation of protein induction and purification conditions was necessary in order to obtain significant yields of highly pure tTG. Site directed mutagenesis was performed in order to generate a second novel tTG with all three amino acids of the catalytic triad replaced with alanine residues. Given the dilute

nature of batches of wild type and mutant tTG and the necessity for accurate measurement of protein concentrations, NanoOrange[®] (Molecular Probes), a sensitive, fluorescence-based protein quantification reagent was used.

In chapter 3, the antigenicity of wild type tTG and mutant tTG was compared in ELISA assays. The assay was optimised to a high standard and only 0.3µg of antigen per well was required indicating that the wild type protein was highly pure and antigenic. While coeliac disease serum IgA bound the wild type antigen at values that correlated well with the values generated by the commercially available Celikey[®] ELISA system, coeliac disease serum IgA showed a marked reduction in binding to the mutant tTG. In fact, while 88.16% of coeliac disease sera were found to be positive in the wild type antigen assay, only 18.42% of sera exceeded the cut-off point when tested for IgA reactivity to the mutant tTG. There was, on average, a 79% reduction in IgA class autoantibody binding to mutant tTG when compared to wild type tTG.

In similar experiments investigating IgG anti-tTG antibodies, there was no significant difference in IgG reactivity to wild type or mutant tTGs. Although the assay showed low sensitivity, IgG anti-tTG binding was still significantly higher in coeliac disease than in control sera. Several controls were used in order to assess the conformation of the mutant tTG. First, a polyclonal antibody raised against recombinant human tTG bound both wild type and mutant tTG equally in ELISA assays. Second, *in silico* mutation using the 3D-PSSM program (Kelley, *et al.* 2000) showed that the mutagenesis caused no profound change in secondary structure in the region. Finally, IgG anti-tTG antibodies bound wild type and mutant tTGs equally, albeit at low AU values.

In ELISA assays investigating dermatitis herpetiformis patient serum anti-tTG binding to wild type and mutant tTG, similar patterns of IgA and IgG binding to those of coeliac disease patients were discovered. Briefly, 60% of dermatitis herpetiformis sera reacted with wild type tTG while none

reacted to the mutant tTG. In IgG ELISA studies, there was no overall difference in IgG binding to the wild type or mutant tTGs.

These ELISA results suggest a possible difference in epitope specificity between the IgA and IgG anti-tTG responses in both coeliac disease and dermatitis herpetiformis. It has been previously reported that unlike anti-gliadin responses, the anti-tTG response is initiated in the gut, a major site of IgA synthesis (Marzari, *et al.* 2001). It is possible that IgA anti-tTG antibodies mediate inhibition of the enzyme locally in the gut by targeting the active site, while IgG responses may be secondary and target the enzyme with a less restricted specificity.

It was concluded from these results that the IgA response directed against tTG in coeliac disease and dermatitis herpetiformis specifically targets the region of the enzyme responsible for its transamidation and deamidation reactions. The IgG anti-tTG response in these patients was not affected in a similar fashion by the mutagenesis and therefore seems to target other regions of the enzyme.

In chapter 4, a sensitive fluorescence-based enzyme assay was used to investigate the activity of the recombinant tTGs and assess the ability of IgA anti-tTG from coeliac disease patient sera to inhibit the cross-linking activity of tTG. A hydroxaminolysis assay (Cooper, *et al.* 1997) was used to determine the unit concentration of purified recombinant tTG. The fluorescence-based tTG cross-linking assay was developed based on publications by Jeitner and colleagues (Jeitner, *et al.* 2001, Jeitner, *et al.* 2005) and was used to demonstrate that the wild type tTG was influenced by the divalent cation ligands calcium and zinc in a similar fashion to previous reports (Lorand and Conrad 1984, Griffin, *et al.* 2002).

It was demonstrated that the recombinant wild type tTG enzyme is active and stable. While other recombinant tTGs have been described as being labile and retain their activity for only 2 minutes at 37°C (Dieterich, *et al.* 2003), our wild type tTG was shown to be active at 37°C for up to 1 hour. The stability of the enzyme was also shown by the detection of measurable cross-linking activity in enzyme preparations that had been

stored at -20°C for 10-11 months. We have also demonstrated that recombinant mutant tTG lacks any measurable cross-linking activity.

In experiments investigating the inhibitory capacity of coeliac disease vs. control IgA, we have shown that, at certain concentrations, coeliac disease serum IgA caused significantly higher inhibition of cross-linking by tTG. This result was confounded by a concentration dependent inhibition of tTG by control IgA. This effect seemed to be mediated by low-affinity, non-specific antibody-tTG interactions since commercially available, unrelated antibodies mediated the same effect. The effect did not seem to be protein related since addition of BSA to the reaction in similar concentrations actually enhanced tTG-catalysed cross-linking.

In order to establish whether inhibition of tTG was mediated by anti-tTG IgA, depletion studies were performed. Recombinant wild type tTG (generated in Chapter 2) was attached to Affi-gel 15 solid support (Bio-Rad). While one aliquot of purified coeliac disease patient serum IgA was incubated with tTG-bound Affi-gel, another aliquot was sham-treated by incubation with HSA-bound Affi-gel. Anti-tTG depleted coeliac disease serum IgA was found to be a significantly less potent inhibitor of tTG when compared to the sham-treated aliquot of purified IgA.

It was concluded from this chapter that the wild-type tTG is a robust, stable enzyme that requires calcium ions for activity and is inhibited by zinc ions. The mutant tTG has no cross-linking activity whatsoever. It was also concluded that the activity of tTG is, at least, partly inhibited by coeliac disease serum IgA and that anti-tTG antibodies mediate this inhibition.

5.2 Possible effects of tTG inhibition in coeliac disease

Although the inhibition of tTG by coeliac serum autoantibodies reported in this study is only partial, it may still be of biological significance *in vivo*. If the anti-tTG response detected in the periphery is “spillover” from the gut compartment, the levels of IgA anti-tTG within the gut may exert a

considerable local effect on the activity of the enzyme and have several possible downstream effects.

tTG has been shown to have a role in the wound healing process (Haroon, *et al.* 1999) and its blockade by IgA anti-tTG could interfere with coeliac lesion repair. Nicholas and co-workers demonstrated that following wounding of a monolayer of Swiss 3T3 cells induced to overexpress tTG, a dramatic increase in tTG cross-linking activity could be measured at the edge of the wound bed. Their work also demonstrated that wounding of Swiss 3T3 and ECV 304 cells by electroporation resulted in the formation of highly cross-linked, transglutaminase-dependent proteinaceous shells that entrapped DNA. The prevention of DNA release may be a tTG-dependent mechanism that serves to dampen down subsequent inflammation (Nicholas, *et al.* 2003).

Although there is much evidence to suggest that tTG plays a role in facilitating cell adhesion and motility through interactions with fibronectin and integrins (Gaudry, Verderio, Jones, *et al.* 1999, Akimov, *et al.* 2000, Akimov and Belkin 2001a, Balklava, *et al.* 2002), it should be noted that these functions have been shown to be independent of transamidation and may be retained even in the event of transamidation reaction blockade (Balklava, *et al.* 2002).

A second possible effect of tTG inhibition is the reduction of levels of active TGF- β . tTG has a role in the attachment of the latent TGF- β complex via latent TGF- β binding protein 1 to the ECM (Nunes, *et al.* 1997), a process that is considered essential for TGF- β release through generation of a modified complex susceptible to plasmin activation in cell cultures. TGF- β is a cytokine required for maturation of crypt cells into enterocytes (Barnard, *et al.* 1989). It has been shown that serum IgA from coeliac disease patients can block the differentiation of T84 intestinal crypt cells *in vitro* (Halttunen and Maki 1999). TGF- β is also a potent mediator of tissue repair (Cox and Maurer 1997) and may be an important cytokine in mucosal wound healing (Schuppan, *et al.* 1998). It has been shown in animal models that local or topical application of

recombinant or natural TGF- β 1 or β 2 improves or accelerates cutaneous wound healing in models of normal or impaired wound healing (Roberts 1995).

TGF- β also has a role in the induction of matrix metalloproteinase (MMP) inhibitors (Grande 1997). TGF- β is, therefore, a negative regulator of matrix degradation and lower levels of active TGF- β in the gut could result in increased MMP activity. In coeliac disease and dermatitis herpetiformis the increased expression of mRNA for MMP-1, MMP-3 has been described (Daum, *et al.* 1999) as well as increased enzymatic activity of MMP-12 (Ciccocioppo, Di Sabatino, Bauer, *et al.* 2005). In a recent immunohistochemical study increased expression of MMP-1, -3 and -9 as well as increased expression of tissue inhibitor of metalloproteinase-1 in duodenal biopsies from coeliac disease patients has been demonstrated (Mohamed, *et al.* In press). Interestingly, a TGF- β response element has been characterised in the promoter region of tTG in the mouse and the cytokine has been shown to upregulate tTG expression in human dermal fibroblasts. The reduction in active TGF- β may, therefore, result in decreased tTG expression.

The transgenic dnTGF- β RII mouse has been engineered to express a dominant negative form of TGF- β . This mouse has been shown to have lymphocytic infiltration into tissues and "spontaneous" T cell activation with the most severe inflammation observed in the gut and lungs (Gorelik and Flavell 2000, Lucas, *et al.* 2000). Since the gut and lungs are exposed to many environmental and microbial antigens and are, therefore, major sites of antigenic stimulation, it has been postulated that TGF- β plays a role in the maintenance of T cell homeostasis. The gut normally expresses high levels of TGF- β (Hansson, *et al.* 2002) and in the absence of TGF- β signalling, T cells reactive to environmental and microbial antigens could differentiate into Th1 cells that are capable of producing high levels of TNF- α and IFN- γ , two pro-inflammatory cytokines that have been reported as elevated in the small intestine in coeliac disease (Lahat, *et al.* 1999). High levels of these cytokines in the intraepithelial area of the gut could damage the epithelial cells directly, or cause activation and infiltration of other cells that could produce toxic pro-

inflammatory mediators that cause pathological changes to gut epithelia (Gorelik and Flavell 2002). This represents another mechanism by which reduced TGF- β activity could contribute to the inflammatory lesion that is characteristic of coeliac disease.

Conversely, the inhibition of tTG activity may actually function to limit the damage induced by gliadin ingestion. If deamidation of gliadin peptides occurs extracellularly, anti-tTG autoantibodies could block deamidation and, thus, reduce immune stimulation and inflammation. Since the levels of anti-tTG in patient sera have never been shown to inversely correlate with disease severity, this seems to be an unlikely phenomenon. Also, there is some evidence to suggest that deamidation of gliadin peptides may occur intracellularly, in the early endosomal compartment, where the slightly acidic conditions may favour deamidation, rather than transamidation reactions (Fleckenstein, *et al.* 2002).

Since the prevalence of IgA deficiency in patients with coeliac disease is increased compared to normal individuals (Lenhardt, *et al.* 2004), it seems unlikely that these autoantibodies are involved in coeliac disease aetiology but perhaps contribute to failure to repair the gut lesion. The possible effects of tTG inhibition in coeliac disease are summarised in Figure 5.1.

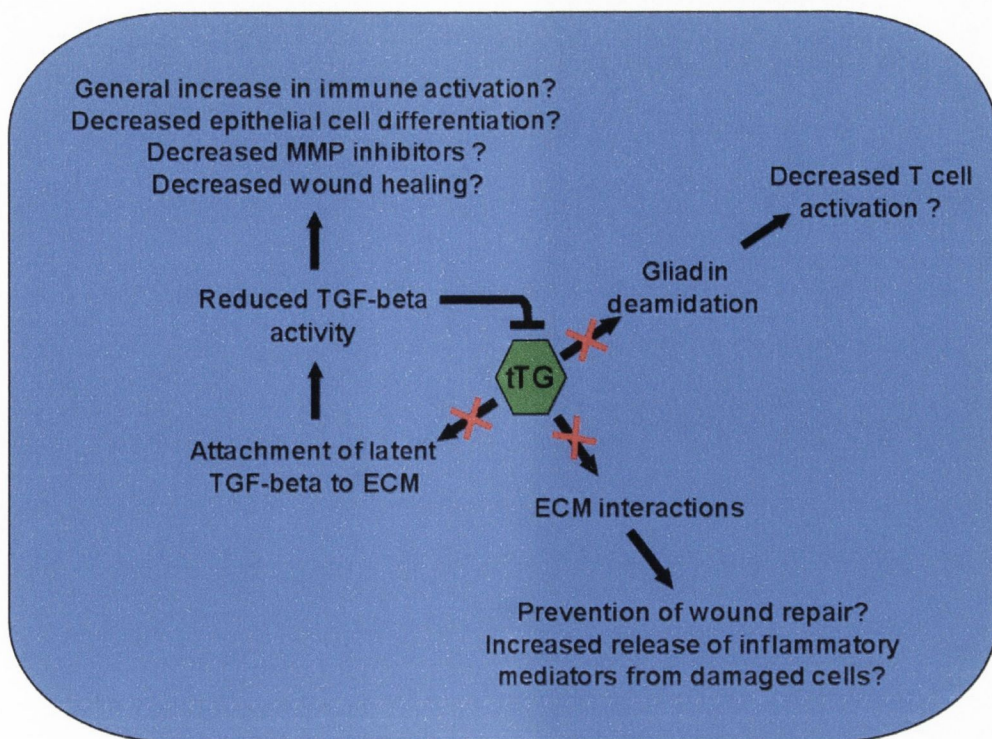


Figure 5.1: Possible effects of tTG inhibition in coeliac disease. The inhibition of tTG may contribute to coeliac disease pathogenesis by blocking ECM interactions and wound healing, blockade of TGF- β activation or interference with gliadin deamidation. Red crosses denote processes inhibited by anti-tTG antibodies.

5.3 The origins of the anti-tTG response in coeliac disease

The currently accepted hypothesis concerning the origin of anti-tTG antibodies in coeliac disease is the hapten-carrier complex theory, as proposed by Sollid (1997). This theory does not require the presence of tTG-reactive T cells. However, unpublished work from our group suggests that tTG-reactive T cells do exist in peripheral blood and that they are elevated in coeliac disease patients compared to controls (Christian Coates, personal communication). It is conceivable that the tTG-reactive T cells detectable in the periphery are regulatory T cells. If this is the case, anti-tTG autoimmunity could represent a breakdown of regulation by CD4⁺CD25⁺ T cells.

An alternate model for the origin of the anti-tTG response could be based upon immune-complex formation. It is well established that tTG can form covalent complexes with gliadin and that these complexes seem instrumental in the origin of the subsequent humoral response against tTG (Fleckenstein, *et al.* 2004). It has been shown that interstitial dendritic cells (DCs) in the gut can take up antigen via Fc α R (CD89) and that cross-linking of CD89 triggers endocytosis (Geissmann, *et al.* 2001). In addition, internalisation of polymeric IgA complexes induced the production of IL-10 and DC activation, as reflected by upregulation in CD86 expression and allostimulatory capacity.

It is possible that tTG-gliadin complexes, bound by anti-gliadin IgA, could be internalised by interstitial DCs. These DCs could be induced to adopt a mature phenotype either by the CD89 ligation itself or by the inflammatory milieu associated with coeliac disease. For example, TNF- α has been shown to mature DCs (Banchereau and Steinman 1998) and is upregulated in the mucosa of coeliac disease patients (Lahat, *et al.* 1999). Furthermore, recent reports have suggested that peptic fragments of gliadin can induce the maturation of DCs through an, as yet, unknown mechanism of innate immunity (Palova-Jelinkova, *et al.* 2005).

The mature DCs loaded with tTG-gliadin complexes could then present tTG fragments to tTG-specific CD4⁺ T cells, which could in turn provide help to tTG-specific B cells for autoantibody production. The potential mechanism is outlined in Figure 5.2.

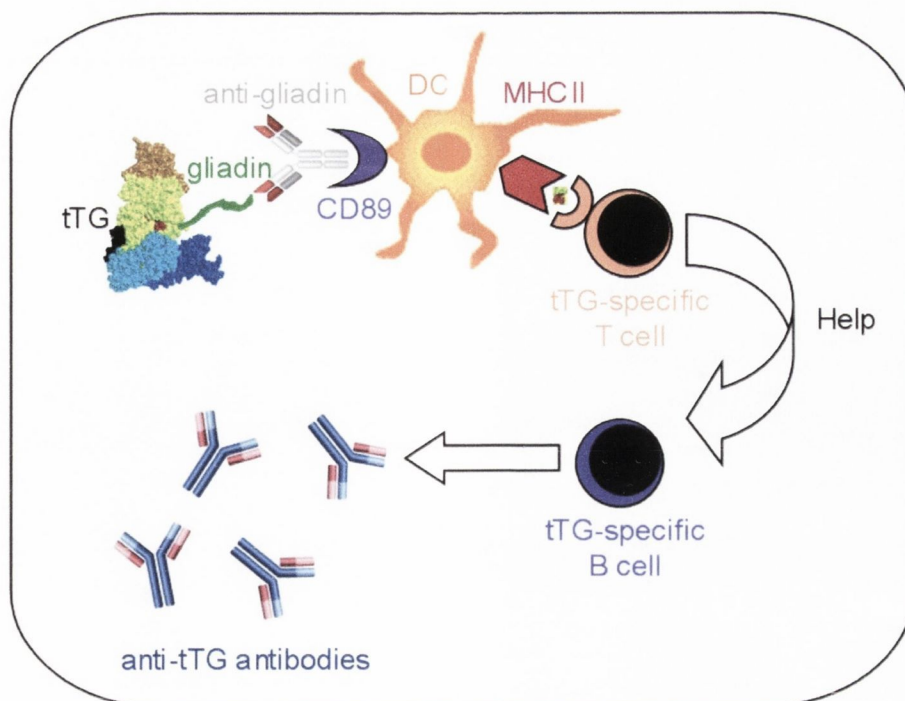


Figure 5.2: Origin of anti-tTG antibodies by immune complex formation. After initial anti-gliadin responses, humoral anti-tTG responses may be initiated by endocytosis of tTG-gliadin complexes via IgA anti-gliadin and CD89 with subsequent presentation of tTG peptides to tTG-specific T cells.

5.4 Tissue transglutaminase as a target for coeliac disease therapy

Given the role for tTG in gliadin deamidation and increased T cell reactivity, the enzyme represents an interesting target for disease intervention. The inhibition of the deamidation activity of tTG represents a possible method to dampen down the increased T cell reactivity associated with deamidated gliadin peptides. Several tTG inhibitors have been well characterised including cystamine (Jeitner, *et al.* 2005) and recombinant peptide inhibitors (Sohn, *et al.* 2003). However, since tTG has many potentially important roles (possibly including wound healing), blockade of the enzyme may yield unwanted side effects. Also, since the early endosomal compartment is a possible location of gliadin deamidation, the efficacy of any ingested tTG blockers in prevention of gliadin deamidation may be poor. Since zinc acts as a competitive inhibitor of tTG (Lorand and Conrad 1984), the supplementation of the coeliac diet with zinc-rich foods may reduce gliadin deamidation by tTG.

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Appendix I

DNA Sequences & Electrophoresis conditions

Nucleotide and amino acid sequence for tissue transglutaminase

Catalytic triad codons are boxed in blue

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      10      20      30      40      50      60
ATGCCGAGGAGCTGCTCTTAGAGAGGTGTGATCTGGAGCTGGAGACCAATGGCCGAGAC
M A E E L V L E R C D L E L E T N G R D

      70      80      90     100     110     120
CACCAACGCGCGACCTGTGCGCGGAGAAAGCTGGTGGTGGACGCGGCCAGCCCTTCTGG
H H T A D L C R E K L V V R R G Q P F W

     130     140     150     160     170     180
CTGACCCCTGCACCTTTGAGGGCGCGCAACTACCAGGCCAGTGTAGACAGTCTCACCTTCAGT
L T L H F E G R N Y Q A S V D S L T F S

     190     200     210     220     230     240
GTCGTGACCGGCGCCAGCCCTAGCCAGGAGGCGCGGACCAAGGCCCGTTTTCCACTAAGA
V V T G P A P S Q E A G T K A R F P L R

     250     260     270     280     290     300
GATGCTGTGGAGGAGGGTGA CTGGACAGCCACCGTGGTGGACCAGCAAGACTGCACCCCTC
D A V E E G D W T A T V V D Q Q D C T L

     310     320     330     340     350     360
TCGCTGCAGCTCAACACCGCGCGCAACCGCCCCATCGGCCTGTATCGCCTCAGCCTGGAG
S L Q L T T P A N A P I G L Y R L S L E

     370     380     390     400     410     420
GCCTCCACTGGCTAACAGGGATCCAGCTTTGTGCTGGGCCACTTCATTTTGCTCTTCAAC
A S T G Y Q G S S F V L G H F I L L F N

     430     440     450     460     470     480
GCCTGGTGCCAGCGGATGCTGTGTACCTGGACTCGGAAGAGGAGCGGCAGGAGTATGTC
A W C P A D A V Y L D S E E E R Q E Y V

     490     500     510     520     530     540
CTCACCCAGCAGGGCTTTATCTAACAGGGCTCGGCCAAGTTCATCAAGAACATACCTTGG
L T Q Q G F I Y Q G S A K F I K N I P W

     550     560     570     580     590     600
AAATTTGGGCAGTTTCAAGATGGGATCCTAGACATCTGCCTGATCCTTCTAGATGTCAAC
N F G Q F Q D G I L D I C L I L L D V N

     610     620     630     640     650     660
CCCAAGTTCCTGAAGAACGCGCGCGCTGACTGCTCCCGGCGCAGCAGCCCGTCTACGTG
P K F L K N A G R D C S R R S S P V Y V
```


670 680 690 700 710 720
 G G C C G G G T G G G T A G T G G C A T G G T C A A C T G C A A C G A T G A C C A G G G T G T G C T G C T G G G A C G C
 G R V G S G M V N C N D D Q G V L L G R

730 740 750 760 770 780
 T G G G A C A A C A A C T A C G G G G A C G G G T C A G C C C C A T G T C T G G A T C G G C A G C G T G G A C A T C
 W D N N Y G D G V S P M S W I G S V D I

790 800 810 820 830 840
 C T G C G G G C T G G A A G A A C C A C G G C T G C C A G C G G T C A A G T A T G C C A C T G T T G G G T C T T C
 L R R W K N H G C Q R V K Y G Q C W V F

850 860 870 880 890 900
 G C C G C G T G G C C T G C A C A G T G C T G A G G T G C C T A G G C A T C C C T A C C C G G T C G T G A C C A A C
 A A V A C T V L R C L G I P T R V V T N

910 920 930 940 950 960
 T A C A A C T C G G C C C A T G A C C A G A A C A G C A A C C T T C T C A T G A G T A C T T C C G C A A T G A G T T T
 Y N S A H D Q N S N L L I E Y F R N E F

970 980 990 1000 1010 1020
 G G G G A G A T C C A G G G T G A C A A G A G C G A G A T G A T C T G G A A C T T C A C T G C T G G G T G G A G T C G
 G E I Q G D K S E M I W N F H C W V E S

1030 1040 1050 1060 1070 1080
 T G G A T G A C C A G G C C G G A C C T G C A G C C G G G G T A C G A G G G C T G G C A G G C C C T G A C C C A A C G
 W M T R P D L Q P G Y E G W Q A L D P T

1090 1100 1110 1120 1130 1140
 C C C C A G G A G A A G A G C G A A G G A A C G T A C T G C T G T G G C C C A G T T C A G T T C G T G C C A T C A A G
 P Q E K S E G T Y C C G P V P V R A I K

1150 1160 1170 1180 1190 1200
 G A G G G C G A C C T G A G C A C C A A G T A C G A T G C G C C C T T T G T C T T T G C G G A G G T C A A T G C C G A C
 E G D L S T K Y D A P F V F A E V N A D

1210 1220 1230 1240 1250 1260
 G T G G T A G A C T G G A T C C A G C A G G A C G A T G G G T C T G T G C A C A A A T C C A T C A A C C G T T C C C T G
 V V D W I Q Q D D G S V H K S I N R S L

1270 1280 1290 1300 1310 1320
 A T C G T T G G G C T G A A G A T C A G C A C T A A G A G C G T G G G C C G A G A C G A C G G G A G G A T A T C A C C
 I V G L K I S T K S V G R D E R E D I T

1330 1340 1350 1360 1370 1380
 C A C A C C T A C A A A T A C C C A G A G G G G T C C T C A G A G G A G A G G G A G G C T T C A C A A G G G C G A A C
 H T Y K Y P E G S S E E R E A F T R A N

1390 1400 1410 1420 1430 1440
 C A C C T G A A C A A A C T G G C C G A G A A G G A G G A G A C A G G G A T G G C C A T G C G G A T C C G T G T G G G C
 H L N K L A E K E E T G M A M R I R V G

1450 1460 1470 1480 1490 1500
 C A G A C C A T G A A C A T G G G C A G T G A C T T T G A C G T C T T T G C C C A C A T C A C C A A C A A C A C C G C T
 Q S M N M G S D F D V F A H I T N N T A

1510 1520 1530 1540 1550 1560
 GAGGAGTACGTCTGCCGCCTCCTGCTCTGTGCCCGCACCGTCAGCTACAATGGGATCTTG
 E E Y V C R L L L C A R T V S Y N G I L

1570 1580 1590 1600 1610 1620
 GGGCCCCAGTGTGGCACCAAGTACCTGCTCAACCTAAACCTGGAGCCTTTCTCTGAGAAAG
 G P E C G T K Y L L N L T L E P F S E K

1630 1640 1650 1660 1670 1680
 AGCGTTCCTCTTTGCATCCTCTATGAGAAATACCGTGACTGCCTTACCGAGTCCAACCTC
 S V P L C I L Y E K Y R D C L T E S N L

1690 1700 1710 1720 1730 1740
 ATCAAGGTGCGGGCCCTCCTCGTGAGCCAGTTATCAACAGCTACCTGCTGGCTGAGAGG
 I K V R A L L V E P V I N S Y L L A E R

1750 1760 1770 1780 1790 1800
 GACCTCTACCTGGAGAAATCCAGAAATCAAGATCCGGATCCTTGGGGAGCCCCAAGCAGAAA
 D L Y L E N P E I K I R I L G E P K Q K

1810 1820 1830 1840 1850 1860
 CGCAAGCTGGTGGCTGAGGTGTCCCTGCAGAAACCCGCTCCCTGTGGGCCCTGGAAGGCTGC
 R K L V A E V S L Q N P L P V A L E G C

1870 1880 1890 1900 1910 1920
 ACCTTCACTGTGGAGGGGGCCGGCCTGACTGAGGAGCAGAAGACCGTGGAGATCCCAGAC
 T F T V E G A G L T E E Q K T V E I P D

1930 1940 1950 1960 1970 1980
 CCCGTGGAGGCAGGGGAGGAAGTTAAGGTGAGAAATGGACCTCGTGCCGCTCCACATGGGC
 P V E A G E E V K V R M D L V P L H M G

1990 2000 2010 2020 2030 2040
 CTCCACAAGCTGGTGGTGAACCTTCGAGAGCGACAAGCTGAAGGCTGTGAAGGGCTTCCGG
 L H K L V V N F E S D K L K A V K G F R

2050 2060
 AATGTCATCATTTGGCCCCGCCTA
 N V I I G P A

LiCor Genereadir™ settings for DNA sequencing

<u>Instrument</u>	Genereadir™ 4200
<u>Prerun</u>	
Focus channel	800
Auto-gain 800	Average 5.0 Noise 2.0
Prerun time	00.08 (HH.MM)
<u>High voltage power supply</u>	
Set voltage (V)	1500
Set current (mA)	35
Set power (W)	35
<u>Miscellaneous</u>	
Set temperature	50°C

Appendix II

All chemicals are from Sigma-Aldrich unless otherwise stated

Reagents for Nucleic Acid Extraction

DEPC water

0.1% DEPC in dH₂O was stirred overnight and autoclaved

Red cell lysis solution

155mM Ammonium chloride
10mM Potassium hydrogen carbonate
1mM EDTA

Denaturing solution

4M Guanidinium thiocyanate
25mM Sodium citrate
0.1M 2-Mercapto-ethanol
0.5% Sarkosyl

White cell lysis solution

25mM EDTA
2% SDS

TE Buffer

10mM Tris-HCL, pH 8.0
1mM EDTA

Reagents for PCR Purification

TAE Buffer

50mM Tris-acetate, pH 8.0
1mM EDTA

Microbiological reagents

LB Broth

10g Tryptone
5g Yeast extract
10g NaCl

Dissolve in 1L dH₂O
Autoclave, 15 minutes, 121°C, 15lb/in²

LB Agar

10g Tryptone
5g Yeast extract
10g NaCl
15g Bacto-agar

Dissolve in 1L dH₂O
Autoclave, 15 minutes, 121°C, 15lb/in²

12% SDS PAGE reagents

Resolving gel

3.3mls	dH ₂ O
4mls	Bis-acrylamide solution (30:0.8) Bio-Rad
2.5mls	1.5M Tris-HCL, pH 8.8
100μl	10% Ammonium persulphate 100μl
10%	SDS
5μl	N,N,N',N'-Tetramethylethylenediamine (TEMED)

Stacking gel

2.1mls	dH ₂ O
0.5mls	Bis-acrylamide solution (30:0.8) Bio-Rad
0.37mls	0.5M Tris-HCL pH 6.8
30μl	10% Ammonium persulphate
30μl	10% SDS
3μl	N,N,N',N'-Tetramethylethylenediamine (TEMED)

Running buffer

25mM Tris-HCL
200mM Glycine
0.1% (w/v) SDS

Loading buffer

62.5mM Tris
10% (v/v) Glycerol
5% (v/v) 2-Mercaptoethanol
2% (w/v) SDS
0.001% (w/v) Bromophenol blue

Coomassie blue stain

1.2g	Coomassie brilliant blue stain
500mls	Methanol
200mls	Glacial acetic acid
500mls	dH ₂ O

Solution was stirred o/n and filtered through Whatman paper No.1 and stored at RT.

Destain solution

12.5% (v/v) Isopropanol
10% (v/v) Glacial acetic acid

Reagents for ELISA assays**Phosphate buffered saline (PBS)**

13.7mM NaCl
0.27mM KCL
0.43mM Na₂HPO₄
0.15mM KH₂PO₄

pH 7.5

ELISA Wash Buffer

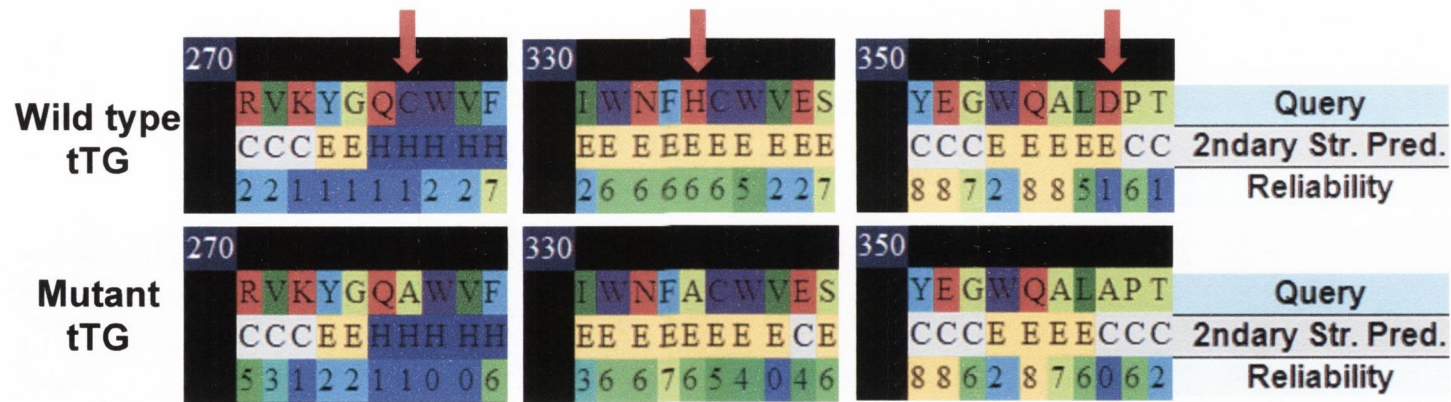
PBS + 0.1% Tween

ELISA Blocking Buffer

PBS + 0.1% Tween + 1% BSA

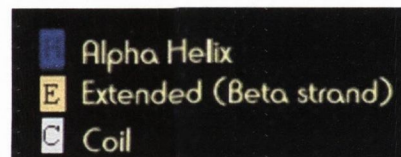
Appendix III

Predicted effect of site-directed mutagenesis on secondary structure of tTG (Calculated using 3D-PSSM software)



Alignment of wild type and mutant tTG 3D-PSSM structure prediction results.

Sites of mutagenesis are denoted by red arrows



Appendix IV

Fluorescent tTG cross-linking assay instrument settings.

Instrument	Spectrafluor Plus (Tecan)
Gain	60
Number of flashes	3
Excitation filter	275nm
Emmission filter	595nm (or 535nm)
Temperature	37°C
Agitation	Orbital shaking before measurement
Measurement	Every 2 minutes
