The impact of transglutaminase on soy proteins and tofu texture

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Abstract

The enzyme transglutaminase was investigated for its cross-linking effect on the soy proteins of tofu. In vitro incubations confirmed that soy proteins are excellent substrates for transglutaminase, especially when denatured. The macroscopic effects resulting from the addition of transglutaminase were compared to changes at the microstructural and molecular level. Treatment produced a firmer tofu, with a significantly increased fracture force. Examination by SEM showed a change in the matrix structure, with transglutaminase resulting in a finer-stranded, uniform network that accounted for the increase in fracture force. At the molecular level, little, if any, cross-linking occurred within the tofu matrix in situ. This suggests that the change in functional properties afforded by addition of transglutaminase to tofu is due to a side reaction of the enzyme, for example hydrolysis of glutamine residues, rather than its cross-linking activity. These ideas are further explored in the accompanying paper.
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1. Introduction

In many foods, proteins play a major role in product quality and determine many of the functional properties of these systems (Gerrard, 2002). Our research has previously demonstrated that cross-linking of wheat proteins by the enzyme transglutaminase can have a dramatic influence on the properties of bread (Gerrard et al., 1998) and croissants (Gerrard et al., 2000), which could be attributed to cross-linking of specific proteins (Gerrard et al., 2001; Gerrard & Sutton, 2005). In this research, we sought to establish whether similar changes of functional properties in other food products could be correlated with specific changes in cross-linking patterns.

Tofu was selected as our model because its functional properties are determined by denaturation of soy proteins to form a gel. Soy proteins, in their native state, do not form a gel; they must be heat-denatured and then coagulated to form the tofu (Liu, 1997). Furthermore, the sub-unit composition of tofu has been shown to correlate with tofu quality (Poysa, Woodrow, & Yu, 2006); thus, we were optimistic that manipulation of specific proteins within the tofu matrix may lead to new methods to improve product quality. By adding a cross-linking reagent, in this case an enzyme, transglutaminase (and, in the accompanying paper (Yasir, Sutton, Newberrry, Andrews, & Gerrard, in press), Maillard cross-linking agents, glutaraldehyde, formaldehyde and glyceraldehyde), before and after the soy proteins are denatured during tofu manufacture, it was anticipated that the textural properties of the tofu would change to various degrees. Thus, the relationship between the degree of cross-linking of particular sub-units and tofu texture could be established.

The consumption of soy foods in the human diet has increased because of their beneficial effects on nutrition and health (Friedman & Brandon, 2001). This is due to the presence of a near perfect balance of all the essential amino acids, making soy a valuable protein source. Recently, the US Food and Drug Administration (FDA) approved a health claim that soy protein reduces the risk of coronary heart disease (Stewart, 2005). From a product

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development point of view, soy protein has been used in a wide range of food applications, mainly due to its functional properties. Products, such as texturised vegetable protein, soy cheese, soy yogurt and meat analogues, are available as alternative foods. All of these products are manufactured by manipulating soy proteins under various conditions to meet specific functional properties of foods. In order to achieve this, numerous studies have been undertaken with a view to manipulating the physicochemical properties of soy proteins (Stewart, 2005), including the use of transglutaminase as a cross-linking agent.

Soy proteins are known to be good substrates for transglutaminase (Babiker, 2000; Ikura, Kometani, Sasaki, & Chiba, 1980; Tang, Wu, Chen, & Yang, 2006). Kwan and Easa (2003) demonstrated that transglutaminase improved the quality of retort tofu. Addition of transglutaminase was found to suppress retort-induced water release, producing a harder product. In this research, we sought to extend understanding of the mechanism by which transglutaminase improves tofu texture by extracting proteins from treated tofu samples and comparing the cross-linking that takes place within the food matrix to that which takes place in in vitro studies. We aimed to correlate the cross-linking of specific soy proteins with specific attributes of the gelled product. In this way, we hope to assist tofu manufacturers in designing specialist tofu with particular textural properties, perhaps by varying the point of addition of the transglutaminase to the processing regime.

We describe, herein, the macroscopic and microscopic changes that take place on addition of transglutaminase to tofu at different points during tofu manufacture and seek to correlate these with specific cross-linking patterns.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals, reagents and solvents were obtained from Sigma–Aldrich New Zealand Ltd. (Auckland, New Zealand) or BDH Chemicals New Zealand, Ltd. (Palmerston North, New Zealand) and were of analytical grade. Transglutaminase was obtained from Amcor Trading Pty. Ltd. (Sydney, Australia). The commercial preparation consisted of 20% protein and 80% dextrin filler. Defatted soy flour (52% protein content), bovine serum albumin and bromophenol blue were purchased from Sigma Chemical Company Ltd. (MO, USA). Soybeans of Chinese origin were purchased in a single batch from the Asian Food Warehouse, Christchurch, New Zealand and were stored at 20 °C. Anti-foaming agent (BDH 1510 Silicone Antifoam) was purchased from BDH Laboratory Supplies (Poole, England).

2.2. Protein extraction from defatted soy flour

Proteins were fractionated into glycinin and β-conglycinin fractions, according to the method of Peterson and Wolf (1992). Extractions were routinely carried out on a 10 g scale. All extractions were carried out in duplicate.

2.3. In vitro incubations

Defatted soy flour (6 mg in 600 μl of distilled water), glycinin and β-conglycinin (2 mg in 600 μl of distilled water) solutions were prepared in Eppendorf tubes. To these, an appropriate volume of transglutaminase from a 5000 ppm stock solution was added to obtain final concentrations of 500 ppm and 1000 ppm. The final volume was 1 ml. Incubation was carried out at 20 °C. Samples were removed at required intervals. A 20 μl aliquot was pipetted out at each interval and immediately cooled in ice water. All treatments were carried out in duplicate.

2.4. Standard tofu preparation

The standard procedure of tofu manufacture was based on the method of Cai, Chang, Shih, Hou, and Ji (1997). Dried soybeans (150 g) were soaked with water for 10 h at room temperature. After soaking, the wet beans were rinsed. The wet beans were weighed (typically 326 g) and subsequently blended with 730 ml of warm water (25–30 °C) using a kitchen blender (Kambrook) at high speed for 2 min. After blending, the slurry was juiced with a juice extractor (Braun) and then filtered through a muslin cloth to obtain the soymilk. The volume of soymilk was measured and found to be in the range 740–750 ml. To the soymilk, 20–30 ml of water were added to make the volume up to 770 ml and one drop of anti-foaming agent was also added. Subsequently, this soymilk was heated in a beaker, with constant stirring, to 97 °C, on a Chiltern magnetic stirrer hotplate (Chiltern, Auckland, New Zealand) with the heat set at 7 °C. During heating, the beaker was covered with aluminium foil to minimise evaporation. When the temperature of the soymilk reached 97 °C, the temperature was held for 5 min before the beaker was removed from the hot plate, and allowed to cool to 87 °C. Upon reaching 87 °C, 750 ml of hot soymilk were measured (if the sample was less than 750 ml, hot water was added to make the volume up to 750 ml) and poured in to a beaker simultaneously with 50 ml of coagulant (containing 6.7 g $CaSO_4 \cdot 2H_2O$ in 50 ml of water). The coagulation was left for 8 min to allow the soymilk to form a gel. This gel was then transferred into the mould (Section 2.4) and compressed with a 600 g (or 900 g) load for 1 h. After removing the load, the tofu was allowed to remain standing in the mould and left overnight at room temperature, prior to further testing. All experiments were carried out in duplicate.

2.5. Mould design

The prototype tofu mould design was adapted from Byun, Kang, and Mori (1995) but modified to include a lower drain hole, which improved the consistency of the product along the height of the column. The volume,

weight, water and protein contents in soymilk, tofu and whey were monitored and it was verified that the mass balance was preserved. The moisture content of the sample was determined by a gravimetric method (AOAC, 1984). Protein contents of samples were determined by the Dumas Method, using a LECO CNS-2000 Analyser (Laboratory Equipment Corporation Ltd., MI, USA) calibrated with EDTA for % nitrogen. Instrument output was in % nitrogen and was converted to % protein by using conversion factor of 6.25 (AOAC, 1996). Protein content was calculated on a dry weight basis. The density of tofu was determined by measuring water displacement using a measuring cylinder.

2.6. Preparation of transglutaminase-treated tofu

The standard tofu recipe required modification to test the impact of transglutaminase, since the enzyme is not active above 70 °C (Motoki & Seguro, 1998). Control tofu for transglutaminase treatments (tga-control tofu) was prepared as for standard tofu (Section 2.3) with the following modifications: (i) 770 ml of soymilk were heated with constant stirring to 50 °C, held at this temperature for 8 min before resuming heating to 97 °C, with constant stirring, and being held at this temperature for 5 min; (ii) after heating, the hot soymilk was allowed to cool to 50 °C. At this temperature, 750 ml of soymilk were measured (if necessary, the volume was made up with water) and poured simultaneously into a beaker with 50 ml of coagulant.

For transglutaminase-treated tofu, the enzyme was added, either before or after the soy milk was boiled. When added before soymilk boiling, 32 mg of transglutaminase (1000 ppm) or 160 mg transglutaminase (5000 ppm) (weight transglutaminase per weight soy proteins) were added to 770 ml of soymilk at 50 °C and held for 8 min. Subsequently, the heating, coagulating, compressing and storing were as for the tga-control tofu. For the transglutaminase added after soymilk boiling, the enzyme was added when the soymilk had cooled to 50 °C, together with 50 ml of coagulant and held for 8 min. The control samples had identical thermal histories to the treated samples in each case.

2.7. Assessment of product quality

Changes in tofu texture were used to determine the affect of TGA on tofu product quality. Tofu texture was measured, using an Instron Universal Testing Machine (Model 4444, Canton, MA, USA) interfaced to a PC. A programme written in LabVIEW Version 7.11 (National Instruments, Austin, TX, USA), running on the interfaced PC, was used to control the UTM and to collect the force and time data during tofu compression. A flat circular probe of diameter 65 mm, attached to the UTM crosshead, fitted with a 50 N load cell, was used to compress a tofu sample 36 mm in diameter and 30 mm thick. The probe was driven down at 100 mm/min until the tofu sample

had been compressed by 50% (corresponding to 15 mm compression of tofu sample). The force–distance data were analysed in MS Excel, using a specifically developed Visual Basic for Applications (Microsoft, Seattle, WA, USA) macro to determine the maximum force, compression distance and maximum compression modulus (Sharma, Mulvaney, & Rizvi, 2000). The compression probe and UTM base plate were lubricated with glycerine. Three tofu samples were cut from each of the tofu columns obtained from the mould (see Section 2.5) by cutting the column into top, middle and bottom portions. A sample was cut from the centre of each portion, using a 36 mm diameter circular cutter, and then compressed on the UTM.

2.8. Protein extraction from tofu

Approximately 1 g of soybean powder, soymilk or tofu sample was placed in an extraction thimble (30 mm × 100 mm (Whatman, Maidstone, UK)) and defatted using 100 ml of *n*-hexane in a standard laboratory Soxhlet apparatus for about 4 h (Lusas & Riaz, 1995). The remaining samples were dried overnight under aeration in a fume cupboard prior to electrophoresis. Four milligrams of each sample (defatted soybean, tofu and soymilk, and whey) were dissolved in a 500 μl urea solution, containing 8 M urea, 0.05 M Tris, 0.05 M dithiothrietol (DTT) at pH 10, to extract the proteins (Woods & Orwin, 1987). The solution was then vortexed (Heidolph, Reax top, Germany), sonicated (Sonics, Vibra cell, USA) for 15 s and centrifuged at 11,000 rpm at room temperature for 5 min.

2.9. Analysis of soy proteins

Protein concentrations were assessed using the Bradford (1976) method. Samples were analysed by SDS–PAGE, as described previously for wheat proteins (Gerrard et al., 2001). Three milligrams of defatted soy flour or 1 mg of fractionated glycinin and β-conglycinin, with or without transglutaminase treatment, were dissolved in 500 μl of distilled water. The solutions were vortexed and centrifuged at 14,000 rpm for 5 min at 20 °C. All SDS–PAGE analyses were carried out in triplicate. SDS–PAGE was carried out under reducing conditions for each extract, using precast Tris-glycine *i*-gels (Gradipore, NSW) at 4–20% acrylamide concentration. The gel image was analysed by Phoretix 1D Plus Software (NonLinear Dynamics Ltd., Newcastle-upon-Tyne, UK) for quantification of protein subunits. The areas of protein subunits were marked and the interference backgrounds were subtracted from the image. All measurements were carried out in triplicate.

2.10. Scanning electron microscopy (SEM)

A Leica S440 electron microscope (Wetzlar, Germany) was used for microscopic scanning of tofu samples, following Kang et al. (1994). A freeze-fracture technique was employed to observe the internal microstructure (Goldstein

et al., 1992). A small piece of tofu sample (<2 mm cube) was cut with a razor blade, and immediately immersed in liquid nitrogen for freeze-fracture. This fractured sample was subsequently freeze-dried overnight. After freeze-drying, the sample was mounted on an aluminium stub with double sided carbon tabs. The surface of the fractured tofu was positioned facing up. Then, the sample was earthed with conductive carbon paint. The sample was put in the Polaron, sputter-coated at 1.2 kV and 20 mA for 2 min, placed under the microscope at 10 kV and 50 pA, and at 20 mm working distance. The examination was carried out in five replications for each treatment.

2.11. Statistical analysis

The significance of differences between means was determined by paired sample t-test using the SPSS statistical package (Minitab Inc.). The level of significance used was 5%.

3. Results and discussion

3.1. Transglutaminase-catalysed cross-linking of soy proteins in vitro

3.1.1. General

Before transglutaminase was introduced into the tofu system, its effects on extracted soy protein fractions incubated in vitro were examined. Transglutaminase was incubated with samples of defatted soy flour and isolated glycinin and β-conglycinin fractions, extracted from soy flour according to the method of Peterson and Wolf (1992). Preliminary trials were undertaken to establish standard conditions; these confirmed that soy proteins are good substrates for transglutaminase (Ikura et al., 1980; Tang et al., 2006).

3.1.2. Defatted soy flour

The SDS–PAGE profile of defatted soy flour incubated with 1000 ppm TGA is presented in Fig. 1. Cross-links between soy proteins were seen to form and the intensity of the α′, α and β subunits of β-conglycinin dropped rapidly at 0 h incubation. The intensities of A subunits were also reduced, followed by the B subunits, which were least reactive.

Within the B subunits, the B_3 polypeptide was cross-linked at 1000 ppm transglutaminase, but the B_1, B_2 and B_4 polypeptides remained throughout the incubation. This was in agreement with the results reported by Tang et al. (2006). The changes of intensities were quantified densitometrically. Reaction was sufficiently rapid that, a time zero measurement was hard to make, with 10% and 27% of proteins cross-linked before a measurement could be taken. The concentration of unreacted protein, for all treatments, was analysed in order to provide a point of comparison for proteins extracted from transglutaminase-treated tofu. The protein concentration followed an exponential decline.

The differences in reactivities of the subunits relate to the amino acid sequence specificity of transglutaminase (Grootjans, Groenen, & deJong, 1995; Kamiya, Takazawa, Tanaka, Ueda, & Nagamune, 2003) and the native fold of the proteins (Tang et al., 2006).
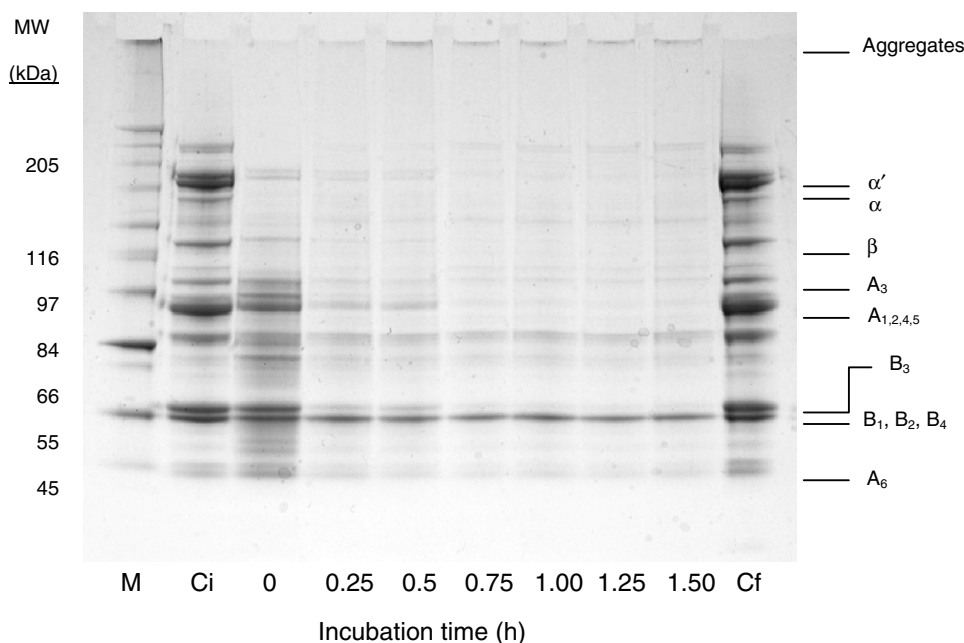

Fig. 1. A typical SDS–PAGE profile of defatted soy flour incubated with 1000 ppm TGA; M, marker; Ci, control initial; Cf, control final; incubation conditions: 6 mg of defatted soy flour were dissolved in 1 ml of water and incubated with 1000 ppm of TGA at 20 °C. The image is a representative of duplicate gels.
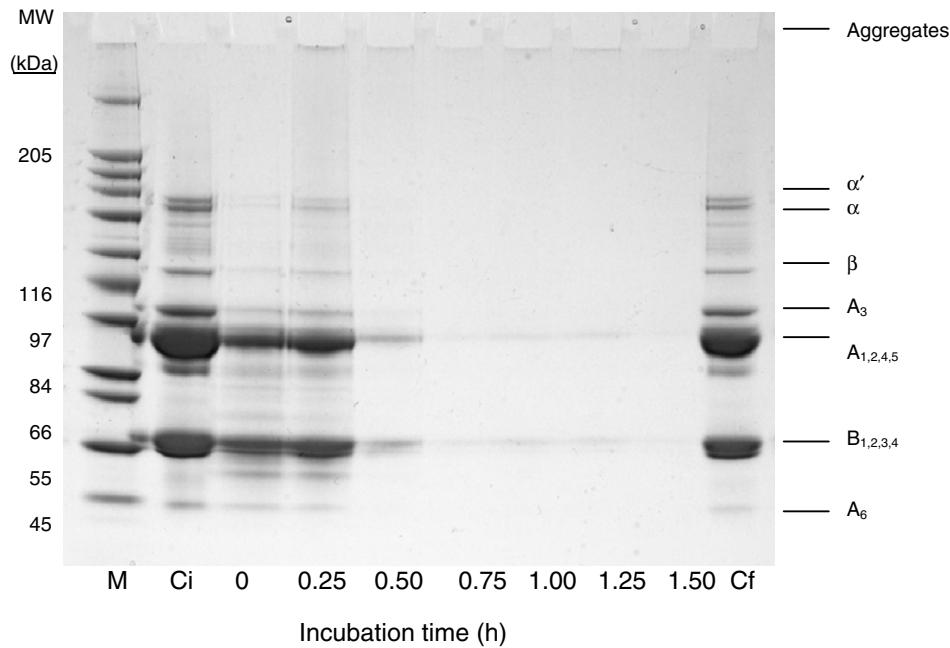
Fig. 2. A typical SDS–PAGE profile of glycinin incubated with 1000 ppm TGA; M, marker; Ci, control initial; Cf, control final; incubation condition: 2 mg glycinin were dissolved in 1 ml of water and incubated with 1000 ppm of TGA at 20 °C. The image is a representative of duplicate gels.

3.1.3. Glycinin

Parallel analyses were carried out on an isolated fraction of glycinin protein. After 15 min of incubation, a rapid cross-linking of A and B subunits was observed. In the 1000 ppm incubation (Fig. 2), A and B subunits were completely cross-linked after 30 min of incubation. This suggests that denaturation of the proteins, during extraction from the defatted soy flour (enhanced by the 2-mercaptoethanol present in the extraction buffer), made the B sub-units more accessible to transglutaminase-catalysed cross-linking than had been the case in defatted soy flour. The formation of cross-linked proteins was not observed on the top of the gel, due to the formation of very large aggregates that were unable to penetrate the matrix.

3.1.4. β-Conglycinin

Analogous experiments, using the β-conglycinin fraction extracted from defatted soy flour incubated with 500 and 1000 ppm TGA, showed similar results. At 1000 ppm TGA, especially, the intensities of α', α and β subunits were rapidly reduced (Fig. 3). After 30 min of incubation, these subunits were mostly cross-linked, in agreement with the results reported by Tang et al. (2006). Since the β-conglycinin fraction was cross-contaminated with A and B subunits of glycinin, the A subunits were also rapidly cross-linked. Within the B subunits, the B_3 polypeptide rapidly cross-linked, but the B_1, B_2 and B_4 polypeptides remained unreacted. This profile is similar to that observed in defatted soy flour (Fig. 1). The gels were analysed densitometrically in order to provide a point of comparison for the transglutaminase-treated tofu.

The change in reactivity with transglutaminase upon denaturation has implications for the reactivity of transglutaminase within the tofu matrix, where the denaturation of the native proteins will vary according to the point in processing at which the enzyme is added.

3.2. Comparison of fracture force and microstructure for standard tofu to tga-control tofu

3.2.1. Fracture force of control tofus

The standard tofu manufacturing process (Cai et al., 1997) gave a standard tofu with properties comparable to commercial Chinese tofu, purchased from a local supermarket. In order to assess the impact of transglutaminase on the texture of tofu, it was necessary to modify the standard process in order to ensure that the enzyme was added to the soy milk at a temperature cool enough to preserve its activity. This resulted in a tga-control tofu with a significantly lower fracture force than the standard preparation ($P < 0.05$). Thus the standard tofu preparation had a fracture force over 30 N, whereas the tga-control tofu had a fracture force of less than 25 N. The differing thermal histories of the standard and tga-control tofus may account for the differing fracture behaviours of these otherwise identical tofus.

3.2.2. Microstructure of tofu

Scanning electron microscopy (SEM) has been variously employed to examine the microstructure of food, and relate the structure to texture (Wilkinson, Dijksterhuis, & Minekus, 2000), with uniform continuous networks showing the firmest textures (Kao, Su, & Lee, 2003). Freeze-fracture SEM was used to capture high magnification images of the internal microstructure of standard and tga-control tofu.
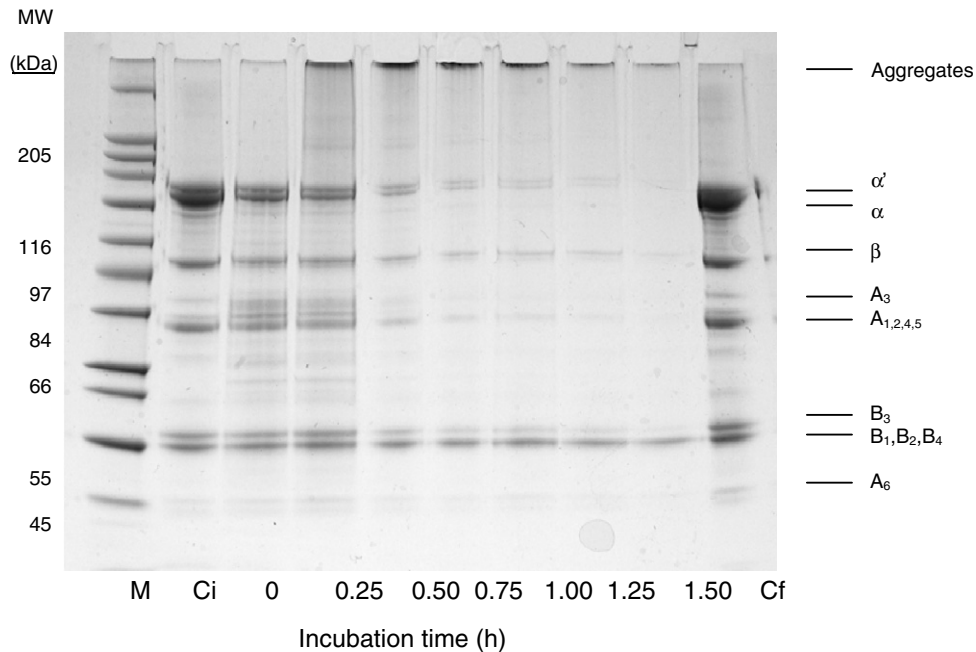
Fig. 3. A typical SDS–PAGE profile of β-conglycinin incubated with 1000 ppm TGA at 20 °C; M, marker; Ci, control initial; Cf, control final; α′, α and β are subunits of β-conglycinin. A and B are the cross-contaminated acidic and basic subunits of glycinin. The image is a representative of duplicate gels.

The microstructure of standard tofu (Fig. 4A) showed small pores distributed uniformly in a dense, well connected network. In contrast, the tga-control tofu (Fig. 4B) had larger pores, and consisted of a "flakey", discontinuous, loose network, with wide pores and a diffuse structure. The appearance of the tofu gel microstructure was consistent with the observed changes in fracture force.

3.3. Analysis of microstructure and fracture force for transglutaminase-treated tofu

Preliminary experiments suggested that 1000–5000 ppm of transglutaminase were required in order to change the firmness of tofu. Samples of tofu were prepared, with transglutaminase added either before the soy milk was boiled, or after the soy milk had been boiled and cooled to the appropriate temperature. The microstructures of tofu treated with 1000 ppm and 5000 ppm transglutaminase, before and after boiling, are shown in Fig. 4. The corresponding fracture forces are illustrated in Fig. 5.

Compared to the control, the fracture force at both transglutaminase concentrations, whether added before or after soymilk boiling, increased. Further, with the increase in transglutaminase concentration, both for treatment before and after boiling, the fracture force significantly increased ($P < 0.05$). However, the fracture force for the tofu treated after boiling was lower than that for those treated before boiling, perhaps reflecting the limited access of the enzyme to its substrates, which had already begun to form a gel. To corroborate the fracture force measurements, the compression modulus of the samples was also examined. The compression modulus followed the same pattern as the fracture force: for all transglutaminase concentrations, it was significantly increased ($P < 0.05$) over the tga-control sample. Moisture content was measured for all samples, but no correlation was found with the firmness of tofu.

Using 1000 ppm of transglutaminase before boiling (Fig. 4C), the microstructure showed a fine-stranded network with small, uniformly distributed pores. However, when the concentration of transglutaminase was increased to 5000 ppm (Fig. 4D), the microstructure did not show this 'honey comb' type structure, and the aggregates seemed more rigid. With transformation from a loose network, in the tga-control tofu, to a fine-stranded network, to a more rigid structure, the fracture force of tofu increased. The fracture force of 1000 ppm transglutaminase-treated tofu was similar to the standard tofu, reflecting their similar microstructures.

A similar trend in microstructure was observed with treatment after boiling. With 1000 ppm transglutaminase (Fig. 4E), the microstructure showed a "sponge-like" structure. This resulted in the lowest fracture force among all transglutaminase treatments. When the concentration of TGA was increased to 5000 ppm (Fig. 4F), a fine-stranded network was also formed, but was 'tighter' in appearance. This structure corresponded to a tofu with a greater fracture force, although not significantly different from the sample treated with the same concentration of enzyme before boiling.

Thus transglutaminase treatment promoted the formation of fine-stranded networks and gave a more homogeneous structure with a higher fracture strength. In all cases, a firmer tofu was obtained, especially for the samples treated before boiling. This suggests that transglutaminase treatment may offer a means to increase the firmness of
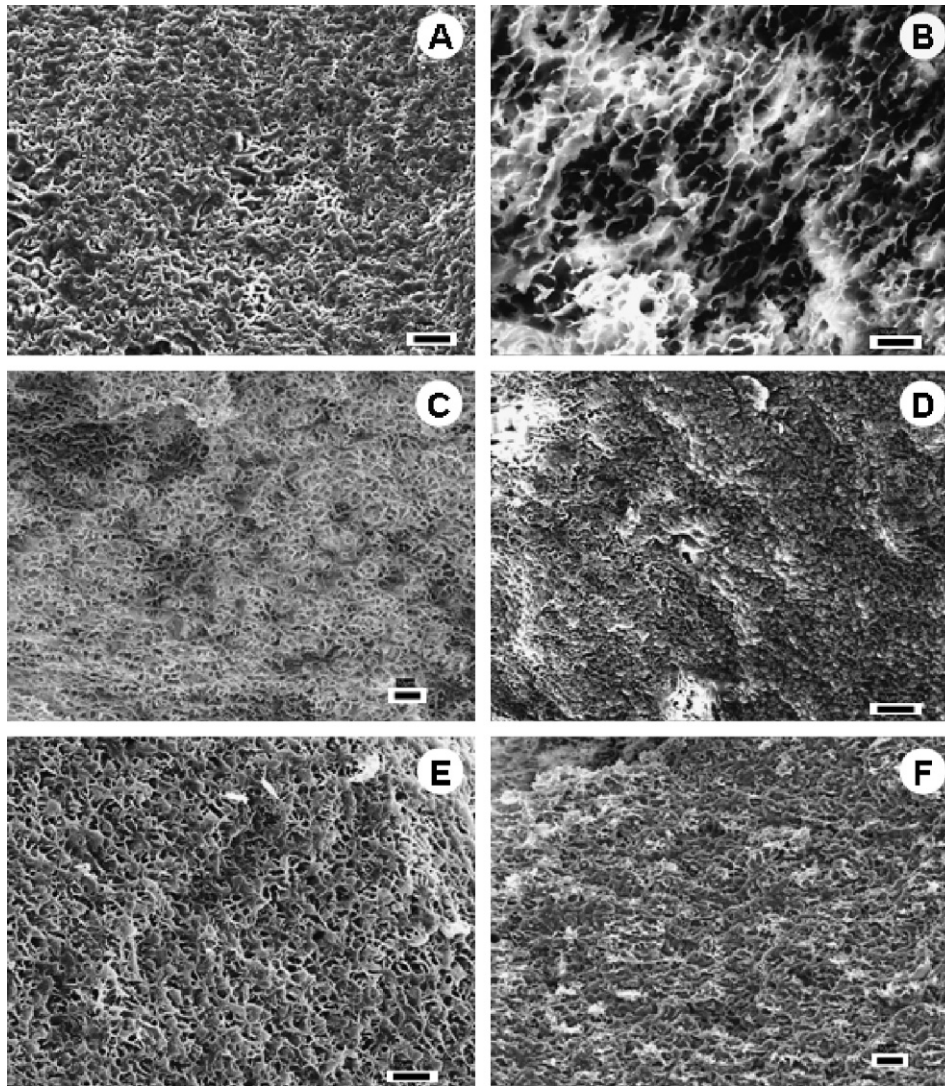
Fig. 4. Freeze-fracture SEM micrographs of standard and TGA-treated tofu. Both control tofus, the standard control tofu (A), and the TGA-control (B) are shown. Tofu was treated with TGA added either before boiling (at 1000 ppm) (C) and 5000 ppm (D) or after boiling (at 1000 ppm (E) and 5000 ppm (F)). The images are representative of five observations of replicate experiments. The black scale bar represents 10 μm.

tofu, to a degree that can be influenced by enzyme dose and addition point.

3.4. SDS–PAGE analysis of protein extracts from transglutaminase-treated tofu

Since transglutaminase had been shown to cross-link soy proteins in vitro and to alter the texture of tofu in situ, we expected to find that cross-linking had occurred, within the tofu matrix, to varying degrees, according to the degree of denaturation of the soy proteins. Proteins were thus extracted from control and transglutaminase-treated tofu in order to establish whether specific cross-linking patterns might account for the observed changes in fracture force and microstructure. To our surprise, when the extracted proteins were analysed by SDS–PAGE, only very limited cross-linking occurred in tofu that had been treated with transglutaminase, either before or after boiling.
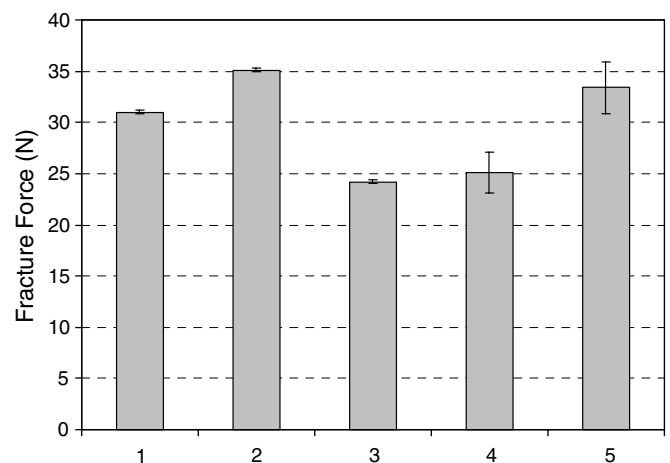

Fig. 5. Fracture forces of transglutaminase-treated tofu: 1 – 1000 ppm transglutaminase added before boiling; 2 – 1000 ppm of transglutaminase added before boiling; 3 – tga-control; 4 – 1000 ppm of transglutaminase added after boiling; and 5 – 5000 ppm of transglutaminase added after boiling.
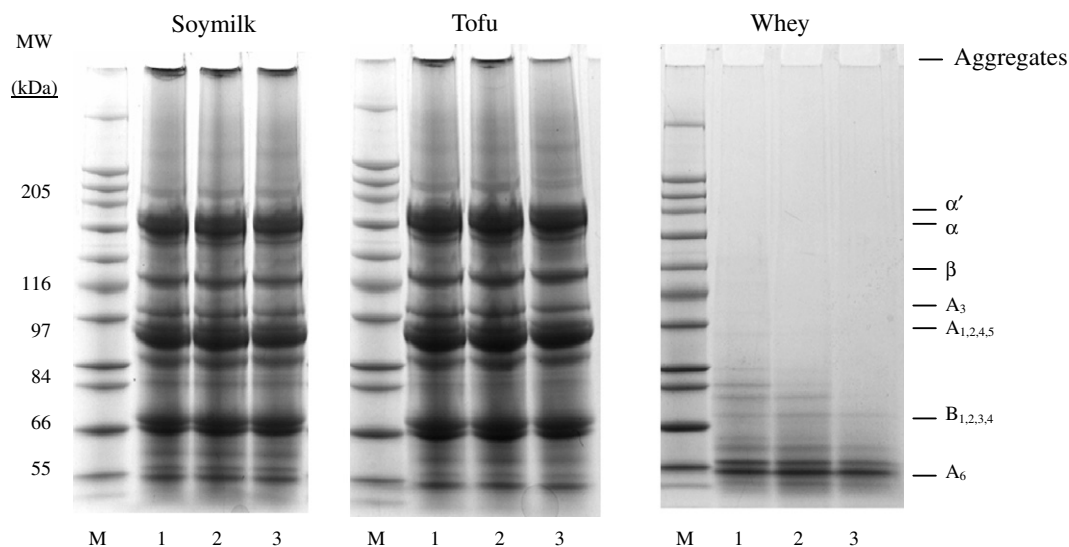
Fig. 6. SDS–PAGE profile of proteins in soymilk, tofu and whey under reducing conditions in the treatment before boiling. M = marker; 1 – tga-control; 2 and 3 are 1000 and 5000 ppm of TGA. The electrophoretic profiles are representative of duplicate gels.

Fig. 6 shows the SDS–PAGE analysis of proteins extracted from soy milk and tofu samples that had been treated with transglutaminase before boiling, and Table 1 shows the densitometric analysis of these gels. No significant loss of any sub-unit was apparent at either 1000 or 5000 ppm transglutaminase treatment levels. A decrease in aggregated material was recorded for the tofu treated with 5000 ppm of transglutaminase, perhaps reflecting the further aggregation of already aggregated material, to a size that could no longer enter the gel.

In the corresponding whey samples, lower molecular weight protein bands were observed in a range of 14–24 kDa. As the concentration increased from 1000 to 5000 ppm, a number of bands started to disappear, suggesting that lower molecular weight proteins were the only proteins to be cross-linked by the enzyme in tofu. Densi-

tometry revealed that the relative concentration of proteins decreased to 77% of the control value at 1000 ppm, and 52% at 5000 ppm. However, the total concentration of these proteins was very low, and did not significantly impact on the protein content of the treated tofu.

The SDS–PAGE analysis of the proteins of soymilk, tofu and whey, for samples treated with transglutaminase after boiling, are presented in Fig. 7 and Table 2. Since the soymilk sample had not been treated with transglutaminase, no change in protein profile was expected. In the transglutaminase-treated tofu samples, no loss of protein subunits was seen at 1000 ppm, but, at 5000 ppm TGA, the intensities of the subunit bands decreased slightly, to 68–98% of the control, suggesting that small proportions of proteins were cross-linked. These results suggest that the cross-linking activity of the enzyme was increased, when added after boiling the soymilk, than when added before boiling, consistent with greater substrate availability in the denatured protein. The cross-linking reaction appeared to favour the smaller subunits, again hinting at a preferential availability of smaller substrates to the enzyme in the protein mixture.

In the corresponding whey, the electrophoretic profile was in contrast to those obtained for treatment before boiling. As the transglutaminase concentration was increased, the number of low molecular weight proteins present in the whey *increased* to 130% of the control for the 5000 ppm transglutaminase treatment. This perhaps indicates that transglutaminase had increased the solubility of the whey proteins, as has been previously noted for soy proteins (Babiker, 2000) and postulated to be due to the known side activity of transglutaminase, hydrolysis of glutamine residues to glutamic acid (Motoki, Seguro, Nio, & Takinami, 1986). Why this should be different to the before boiling treatment is not clear.

Table 1
The changes of protein concentration in the cross-linked proteins and subunits in the transglutaminase treatment, with the enzyme added before soymilk boiling

Aggregates and subunits	Relative protein concentration (% of tga-control)			
	Soymilk		Tofu	
	1000 ppm	5000 ppm	1000 ppm	5000 ppm
Aggregates	100 ± 2	96 ± 1	95 ± 3	57 ± 4
α′ + α	95 ± 9	85 ± 16	95 ± 5	97 ± 1
β	92 ± 8	84 ± 14	92 ± 9	95 ± 15
A$_3$	86 ± 12	72 ± 21	93 ± 2	83 ± 3
A$_{1,2,4,5}$	91 ± 5	79 ± 13	95 ± 4	100 ± 1
B	88 ± 1	79 ± 16	95 ± 4	98 ± 11
A$_6$	89 ± 5	69 ± 26	81 ± 4	93 ± 18

Values are the means ± standard error of the means of duplicate measurements. The percentage is based on the control soymilk and tga-control tofu.
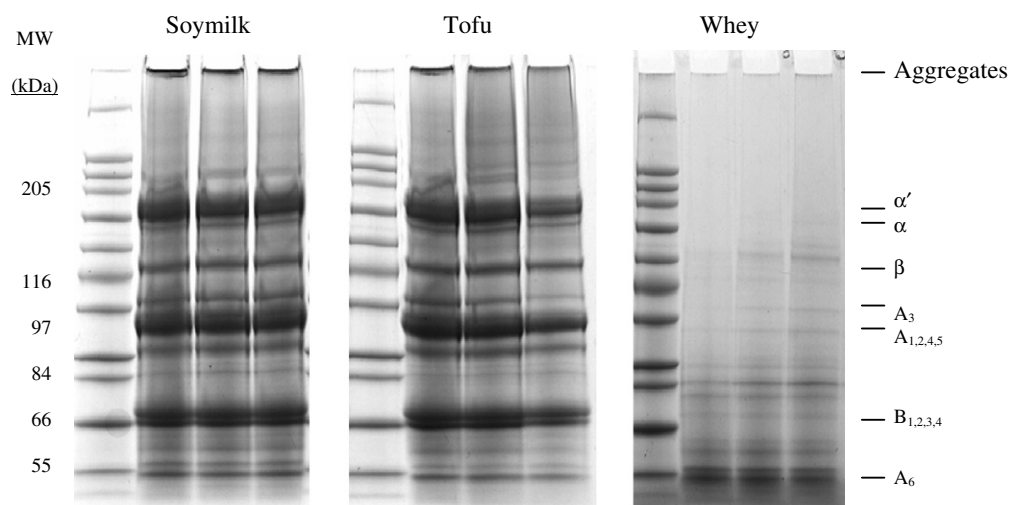
Fig. 7. SDS–PAGE profile of proteins in soymilk, tofu and whey under reducing conditions in the treatment after boiling. M = marker; 1 = control; 2 and 3 are 1000 and 5000 ppm of TGA; soymilk was not treated with TGA. The electrophoretic profiles are representative of duplicate gels.

Table 2
The changes of protein concentration in the cross-linked proteins and subunits in the transglutaminase treatment, with the enzyme added after soymilk boiling

Cross-linked and subunits	Relative protein concentration in tofu (% of tga-control)	
	1000 ppm TGA	5000 ppm TGA
Aggregates	91 ± 6	80 ± 7
$\alpha' + \alpha$	92 ± 3	74 ± 5
β	100 ± 1	88 ± 9
A_3	86 ± 11	68 ± 17
$A_{1,2,4,5}$	100 ± 1	79 ± 8
$B_{1,2,3,4}$	100 ± 1	90 ± 5
A_6	93 ± 8	85 ± 11

Values are the means ± standard error of the means of duplicate measurements. The percentage is based on the control tofu, respectively.

Taken as a whole, these results suggest that, whilst transglutaminase clearly alters the microstructure and texture of tofu, cross-linking does not play a major role in the mechanism of action.

4. Conclusion

Treatment with transglutaminase produced a firmer tofu, with a significantly increased fracture force, suggesting that it may indeed be a useful ingredient for tofu manufacturers. SEM revealed that the microstructures of the samples were consistent with the changes in fracture force. The firmness varied according to both the concentration of enzyme added and the point of addition in the manufacturing process, suggesting opportunities for customising tofu by simple alterations in the timing of transglutaminase addition.

At the molecular level, in vitro cross-linking of soy proteins resulted in efficient cross-linking of most proteins in the extract, confirming earlier research that showed soy proteins to be an excellent substrate for transglutaminase (Tang et al., 2006). However, when proteins were extracted from the tofu matrix after treatment with transglutaminase in situ, little, if any, cross-linking occurred. This observation challenges the tacit assumption made by many
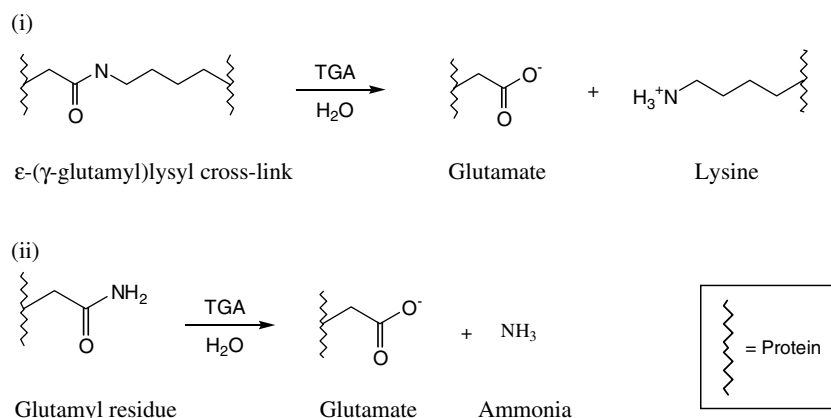

Fig. 8. The hydrolysis of glutamyl residue of the protein by TGA, either by hydrolysis of glutamyl-lysyl cross-links (i), or glutamyl residues (ii) (Motoki et al., 1986).

researchers that the results from in vitro incubations of food components with food enzymes can be assumed to be valid within the food.

These findings suggest that the change in functional properties afforded by addition of transglutaminase to tofu is perhaps due to a side reaction of the enzyme, hydrolysis of glutamine residues to glutamate, as previously postulated by Babiker (2000). Since water is abundant during tofu manufacture, it is likely that transglutaminase-promoted hydrolysis of glutamine residues of the protein to glutamate residues could occur, by one of two mechanisms, as shown in Fig. 8. Either the ε-(γ-glutamyl)lysyl cross-link is formed and subsequently hydrolysed into glutamate and lysine, or the glutamyl residue is hydrolysed directly into glutamate. The resulting change in isoelectric point of the soy proteins would be predicted to change the gelation and microstructure of the proteins (Dickinson, 1997). Further work is needed to test this hypothesis in this, and other, systems in which side reactions of transglutaminase are implicated as important for changing the properties of food (Gerrard & Sutton, 2005).

In order to further probe the relationship between cross-linking and tofu texture, an alternate means of cross-linking of the protein gel matrix was explored: the introduction of Maillard cross-linking reagents. This work is described in the following paper (Yasir et al., in press).

Acknowledgements

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