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The impact of Maillard cross-linking on soy proteins and tofu texture

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Abstract

In this paper, we explore the use of Maillard cross-linking agents (formaldehyde, glyceraldehyde and glutaraldehyde) in tofu, to probe the relationship between cross-linking of soy proteins and tofu texture. 1–2 mM glutaraldehyde treatment resulted in an acceptable product with altered microstructure. At 2 mM glutaraldehyde, the fracture force was increased if the reagent was added before soymilk boiling, but decreased if it was added after boiling, when more cross-linking was observed due to the denaturation of the proteins. The fracture forces and microstructures are hard to rationalise in terms of cross-linking, suggesting that other factors, such as non-cross-linking protein glycation, may be responsible for the observed changes. Taken together with the results reported in the previous paper, these findings suggest that protein cross-linking agents may change the functional properties of tofu via non-cross-linking modifications of the sidechains of the amino acid residues, changing their isoelectric point and their gelation properties.
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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). In the previous paper (Yasir, Sutton, Newberry, Andrews, & Gerrard, this issue), we explored the use of transglutaminase, a well known cross-linking enzyme, in tofu, and sought to correlate specific changes in protein cross-linking with changes in functional properties. Surprisingly, although the soy proteins were cross-linked *in vitro* and transglutaminase treatment *in situ* resulted in a firmer tofu with an altered microstructure, very little protein cross-linking was found within the food matrix. The impact of transglutaminase was therefore postulated to be due to a side reaction of the enzyme, hydrolysis of glutamine residues to glutamate, altering the isoelectric point of the soy proteins and changing their gelation properties.

In this parallel study, we further investigate the relationship between protein cross-linking and tofu texture, using three molecules that have been established to cross-link food proteins via Maillard-type chemistry: formaldehyde, glyceraldehyde and glutaraldehyde. These molecules, available in sufficient quantities to use in real food systems, have been extensively explored in our previous work with wheat proteins (Gerrard, Brown, & Fayle, 2002a, 2002b, 2002c) and shown to provide a useful range of cross-linking activity. They have also been reported to cross-link other proteins (Acharya, Cho, & Manjula, 1988; Galembeck, Ryan, Whitaker, & Feeney, 1977; Marquie, 2001; Marquie, Tessier, Aymard, & Guilbert, 1997; Silva, Sousa, Gubitz, & Cavaco-Paulo, 2004). Whilst unlikely to be employed as food ingredients themselves (although glutaraldehyde is approved for use in food in the USA), these molecules provide a simple means to probe the potential utility of Maillard chemistry to alter the texture of processed food. To our knowledge, they have not previously been employed within a tofu matrix. However, Kwan and Easa (2003) employed glucose as a Maillard active molecule in the

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preparation of retort tofu, which resulted in a firmer product. Thus we were optimistic that the addition of more reactive carbonyl reagents would lead to an alteration in the firmness of treated tofu samples. We report, herein, a study of the reaction of soy proteins with formaldehyde, glyceraldehyde and glutaraldehyde, both *in vitro* and *in situ* within the tofu matrix.

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. Defatted soy flour (52% protein content), bovine serum albumin and bromophenol blue were purchased from Sigma Chemical Company Ltd. (MO, USA). Soybeans were purchased in a single batch from the Asian Food Warehouse, Christchurch, New Zealand and were of Chinese origin and stored in the dark at room temperature. Antifoaming 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), as described in the previous paper. Extractions were routinely carried out on a 10 g batch size. 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 formaldehyde, glyceraldehyde or glutaraldehyde stock solution was added to obtain the desired final concentration, which varied according to the molecule employed. The final volume was 1 ml. Incubation was carried out at 20 °C and 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), using a mould design adapted from Byun, Kang, and Mori (1995), and is described in detail in the previous paper (Yasir et al., in press).

2.5. Preparation of glutaraldehyde-treated tofu

For glutaraldehyde-treated tofu, the glutaraldehyde was added either before or after the soymilk was boiled. When added before soymilk boiling, the appropriate volume of glutaraldehyde was 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 standard tofu. For treatment after soymilk boiling, soymilk (740 ml) was heated to 97 °C with constant stirring. Upon reaching 97 °C, the temperature was maintained for 5 min and the soymilk was allowed to cool. Once the soymilk reached 87 °C, 720 ml were measured out (if necessary the volume was made up with hot water) and poured, simultaneously with 30 ml of glutaraldehyde solution of the appropriate concentration and 50 ml coagulant, into a beaker. The mixture was allowed to coagulate for 8 min to form a tofu gel.

2.6. Assessment of product quality

An Instron Universal Testing Machine (Model 4444, Canton, MA, USA) was used to assess tofu quality, as described in detail in the previous paper (Yasir et al., this issue).

2.7. Protein extraction from tofu, analysis of soy proteins by SDS-PAGE and scanning electron microscopy (SEM)

All analyses of proteins and tofu microstructure were carried out as described in the previous paper (Yasir et al., this issue).

2.8. Statistical analysis

The significance of differences between means was determined by paired sample *t*-test, using the SPSS statistical package (SPSS, 2003). The level of significance used was 95%.

3. Results and discussion

3.1. Maillard cross-linking of soy proteins in vitro

Following the methods of the previous paper, formaldehyde, glyceraldehyde and glutaraldehyde were each incubated with samples of defatted soy flour, as well as glycinin and β-conglycinin fractions extracted from soy flour according to the method of Peterson and Wolf (1992). Preliminary trials were undertaken over a range of temperatures and confirmed that, at a given concentration, glutaraldehyde was by far the most reactive cross-linking agent, with formaldehyde and glyceraldehyde requiring over 50 times the concentration to achieve a cross-linking rate comparable to glutaraldehyde. Concentrations of 1 and 2 mM glutaraldehyde and 50 mM formaldehyde and glyceraldehyde were therefore adopted as

standard conditions (Gerrard et al., 2002a, Gerrard, Brown, & Fayle, 2002b).

SDS-PAGE profiles of defatted soy flour incubated with formaldehyde, glyceraldehyde and glutaraldehyde are presented in Figs. 1 and 2. The order of cross-linking reactivity was as expected: glutaraldehyde was found to be the most reactive, followed by formaldehyde, and the least reactive molecule was glyceraldehyde. In the glutaraldehyde incubation (Fig. 1), the intensities of the α′, α, β, A and B subunits were gradually reduced as multimeric proteins (Multimer *a*) and aggregates were formed, as evidenced by a smearing toward the top of the gel. This indicates that most of the protein subunits were cross-linked. A similar pattern was observed during the formaldehyde experiment (Fig. 2a) and glyceraldehyde experiment (Fig. 2b), but at slower rates. The A subunits of glycinin had the highest

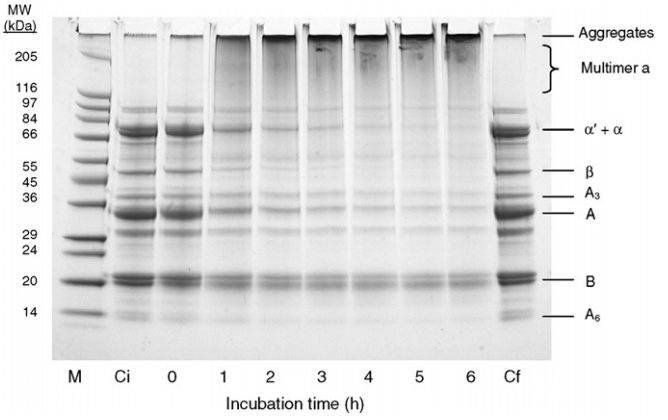

Fig. 1. A typical SDS-PAGE profile of defatted soy flour incubated with 1 mM glutaraldehyde. Six milligram of defatted soy flour were incubated with 1 mM glutaraldehyde at 30 °C in 1 ml of solution. M = Marker; C_i = Control at the beginning of the incubation; C_f = Control at final incubation time. The image is a representative of duplicate gels.

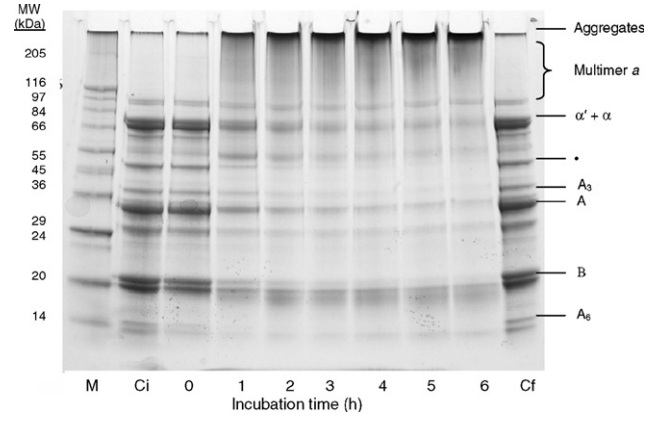

Fig. 2a. A typical SDS-PAGE profile of defatted soy flour incubated with 50 mM formaldehyde. Six milligram of defatted soy flour were incubated with 50 mM glyceraldehyde at 30 °C in 1 ml of solution. M = Marker; C_i = Control at the beginning of the incubation; C_f = Control at final incubation time. The image is a representative of duplicate gels.

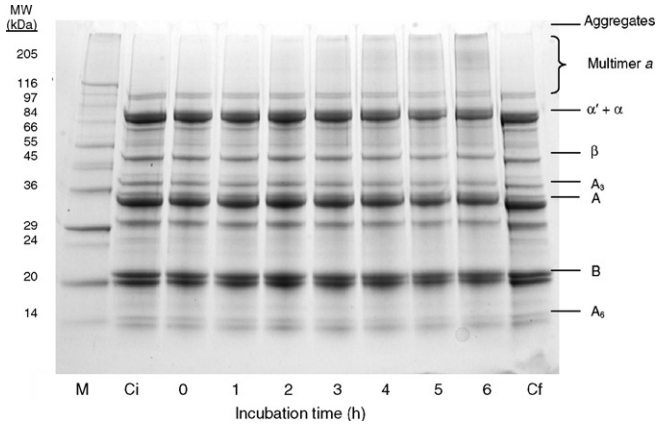

Fig. 2b. A typical SDS-PAGE profile of defatted soy flour incubated with 50 mM glyceraldehyde. Six milligram of defatted soy flour were incubated with 50 mM glyceraldehyde at 30 °C in 1 ml of solution. M = Marker; C_i = Control at the beginning of the incubation; C_f = Control at final incubation time. The image is a representative of duplicate gels.

reaction rate, followed by α′ + α and β subunits of β-conglycinin. The B subunits were the least reactive.

The cross-linking experiments were repeated on fractionated soy proteins, glycinin and β-conglycinin. The results were consistent with those described above for the incubations with defatted soy flour already described. However, when using fractionated glycinin, the reaction rates of the B subunits approached those of the A subunits. It is likely that Maillard-reactive lysine and arginine residues are made more available for Maillard chemistry by the extraction process. This change in reactivity upon denaturation, which parallels that described for transglutaminase treatment in the previous paper (Yasir et al., this issue), has implications for the reactivity of Maillard reactive agents within the tofu matrix, where the denaturation of the native proteins will vary according to the point in processing at which the cross-linking agent is added.

3.2. Comparison of fracture force and microstructure in standard tofu and glutaraldehyde-treated tofu

Preliminary experiments, introducing glyceraldehyde and formaldehyde to a food matrix, suggested that their low reactivity led to no measurable change in the properties, as had been noted previously with wheat proteins (Gerrard, Brown, & Fayle, 2002c). Thus, *in situ* studies focussed on the introduction of the most reactive reagent, glutaraldehyde, to tofu. High concentrations (15 mM and above) led to an undesirable tofu product, with significant browning due to Maillard chemistry, extensive cross-linking and a substantially reduced fracture force. However, lower concentrations (1–2 mM) led to an acceptably coloured product, and this treatment was therefore examined in detail.

As described in the previous paper (Yasir et al., this issue), the microstructure of the control tofu showed small pores distributed uniformly in a dense, well-connected

network. The microstructures of control and glutaraldehyde-treated tofu are shown in Fig. 3. The microstructure resulting from treatment with 1 mM glutaraldehyde tofu shows a honeycomb-like structure with relatively large pore sizes and a thick-stranded network. The network is mostly regular and the pores are uniformly distributed. When the concentration of glutaraldehyde was increased to 2 mM, the microstructure comprised a smaller pore size with thick strands and a denser and uniformly compact network. Whilst there was no significant increase in fracture force for the 1 mM treatment, the 2 mM treatment resulted in a fracture force that was significantly higher than the control.

The microstructures of tofu treated with 1 and 2 mM glutaraldehyde after soymilk boiling are also presented in Fig. 3. The microstructures at both concentrations show honeycomb-like pores with a continuous network and regular strands. The distribution of pores was uniform. However, the pores are larger in the 2 mM glutaraldehyde-treated tofu than they are in the 1 mM glutaraldehyde-treated sample. The resulting fracture forces of 1 and 2 mM glutaraldehyde-treated tofu were significantly lower than those of the control.

As in the transglutaminase-treated tofu (Yasir et al., this issue), the compression modulus of the samples was also examined and corroborated the fracture force measurements. Additionally, no correlation was found between fracture force and moisture content.

3.3. SDS-PAGE analysis of protein extracts from glutaraldehyde-treated tofu

Since glutaraldehyde 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. Fig. 4 shows an SDS-PAGE analysis of proteins extracted from soymilk and tofu samples that had been treated with glutaraldehyde before boiling, and Table 1 shows the results of the densitometric analysis of these gels. A small degree of cross-linking was observed, although this was barely

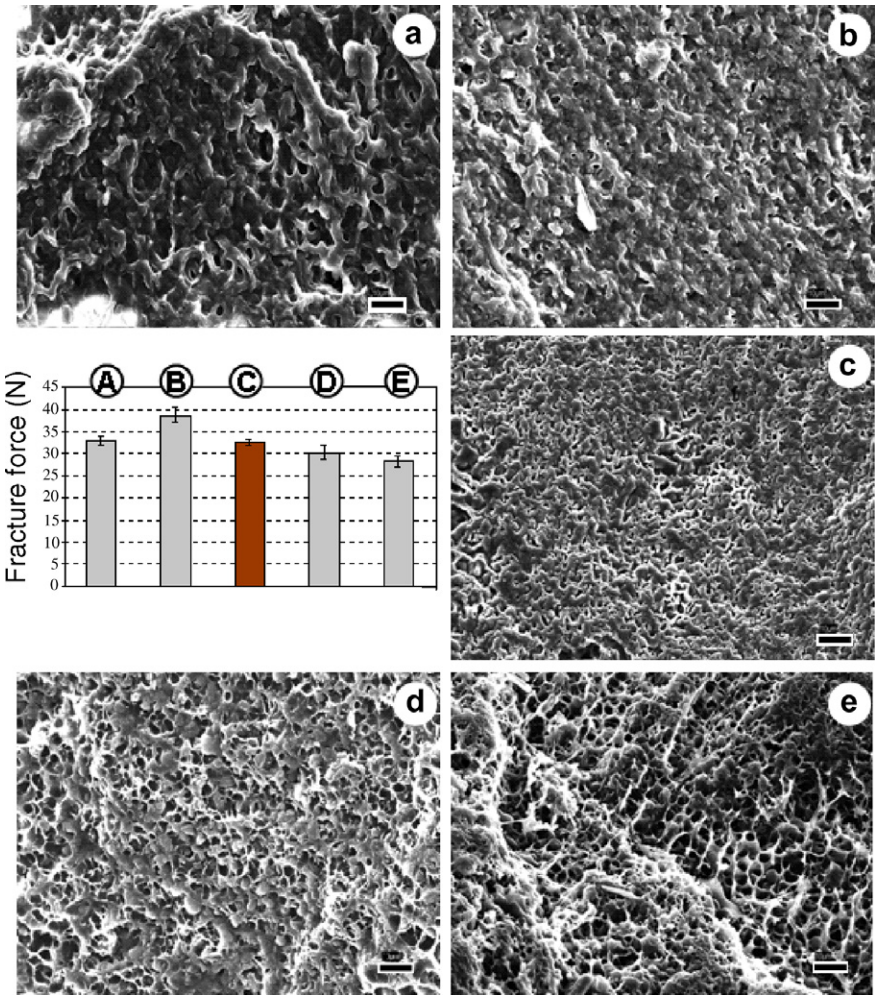

Fig. 3. The SEM micrographs of lower level of glutaraldehyde-treated tofu and their respective fracture forces. a and b represent micrographs of 1 and 2 mM glutaraldehyde added before soymilk boiling. c represents control. d and e represent micrographs of 1 and 2 mM glutaraldehyde added after soymilk boiling. The images are representative of five replicate experiments. The scale bar represents 10 μm.

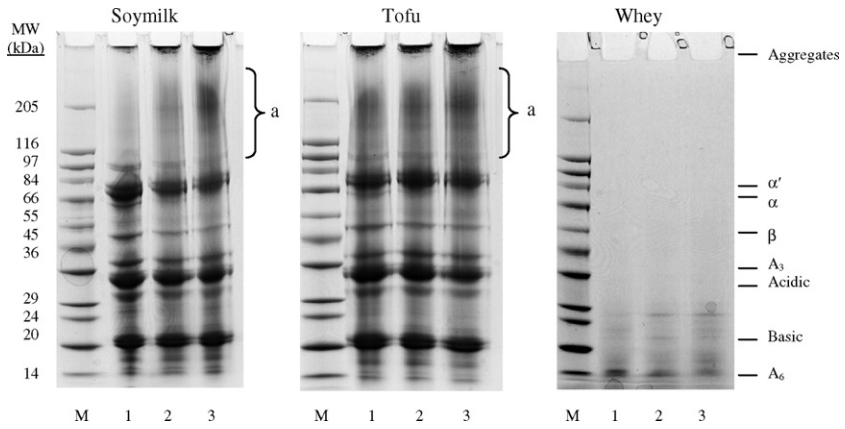

Fig. 4. SDS-PAGE profile of proteins in soymilk, tofu and whey obtained when glutaraldehyde is added before soymilk boiling. M = marker; 1 = control; 2 and 3 are 1 and 2 mM glutaraldehyde; the electrophoretic profiles are representative of duplicate gels.

Table 1
The changes of protein composition in aggregates and subunits following treatment with glutaraldehyde added before boiling the soy milk

	Soymilk (% of control)[a]		Tofu (% of control)[a]	
	1 mM	2 mM	1 mM	2 mM
Aggregates	180 ± 11	251 ± 22	106 ± 18	134 ± 14
α′ + α	94 ± 2	64 ± 1	115 ± 3	96 ± 1
β	94 ± 1	36 ± 2	101 ± 6	74 ± 3
A_3	106 ± 2	50 ± 3	116 ± 4	97 ± 2
A	89 ± 1	61 ± 2	111 ± 11	96 ± 7
B	97 ± 1	76 ± 1	122 ± 10	105 ± 5
A_6	98 ± 16	71 ± 4	96 ± 2	54 ± 4

[a] Values are the means ± standard errors of the mean of duplicate measurements. The percentage was based on the control soymilk and tofu, respectively.

significant for the 1 mM treatment. At 2 mM, a significant decrease was only observed for the β and A_6 subunits in the final tofu. The whey of the control tofu (Fig. 4) showed a group of lower molecular weight proteins present around 14 kDa, which is consistent with the profiles reported by Kao, Su, and Lee (2003). There was no significant difference in the whey proteins upon treatment with 1–2 mM glutaraldehyde.

The effect of 1 and 2 mM glutaraldehyde added to the tofu processing after soymilk boiling on the SDS-PAGE profiles of soymilk, tofu and whey is presented in Fig. 5 and Table 2. Since glutaraldehyde was not added to the soymilk during boiling, no changes were observed in the soymilk analysis. The electrophoretic pattern in tofu treated with 1 mM glutaraldehyde shows that the relative concentration of cross-linked protein was increased to 130%, whilst the relative concentration of subunits decreased to 80–90%. Increasing the concentration to 2 mM resulted in a further increase in aggregated protein concentration to 178% with a concurrent decrease in subunit concentration to 50–80%. Among the subunits, α′, α and β subunits were the most reactive, consistent with earlier results from *in vitro* incubation (Section 3.1). Compared with glutaraldehyde treatment before boiling (Fig. 4), α′, α and β subunits were more reactive when treated with glutaralde-

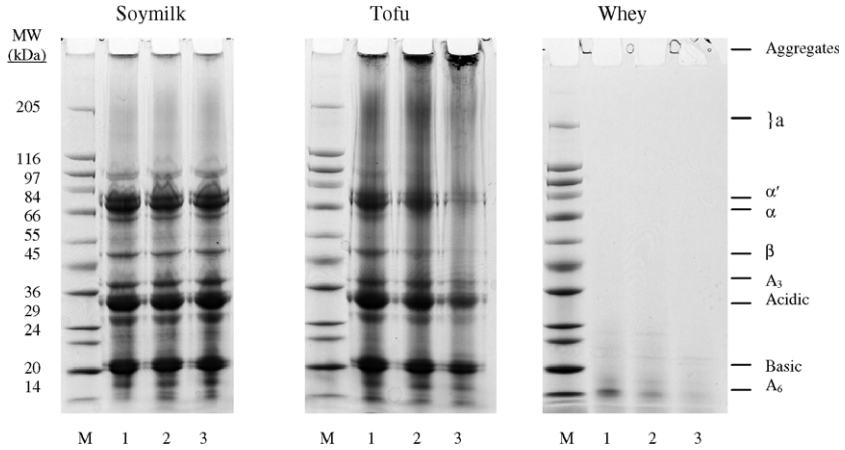

Fig. 5. SDS-PAGE profile of proteins in soymilk, tofu and whey obtained when glutaraldehyde is added after soymilk boiling. M = marker; 1 = control; 2 and 3 are 1 and 2 mM glutaraldehyde; the electrophoretic profiles are representative of duplicate gels.

Table 2
Relative concentration of aggregated proteins and protein subunits following treatment with glutaraldehyde after boiling

	1 mM glutaraldehyde treated (% of control)[a]	2 mM glutaraldehyde treated (% of control)[a]
Aggregates	130 ± 30	173 ± 28
$\alpha' + \alpha$	88 ± 13	51 ± 3
β	78 ± 7	49 ± 3
A_3	84 ± 4	64 ± 4
$A_{1,2,4,5}$	83 ± 3	70 ± 1
$B_{1,2,3,4}$	85 ± 1	79 ± 3
A_6	81 ± 1	70 ± 13

[a] Values are the means ± standard error of the means of duplicate measurements. The percentage was based on the control soymilk and tofu, respectively.

hyde after boiling, suggesting that in the denatured state, the lysine and arginine residues were more available than they were in the native state for Maillard-type cross-linking. Multimeric proteins were also observed on the gel, represented by region a. However, their intensities, which were more intense in the control and 1 mM treatment, were less intense in the 2 mM treatment, indicating that they were cross-linked to form larger aggregates that were unable to enter the gel.

These results suggest that, whilst glutaraldehyde clearly alters the microstructure and texture of tofu, cross-linking is not the only factor in the mechanism of action. Non-cross-linking reaction with glutaraldehyde, i.e. non-enzymatic protein glycation, is also likely to have occurred, which is known to alter the isoelectric point of proteins (Fayle, Healy, Reid, Gerrard, & Ames, 2001). Such a change in isoelectric point would be predicted to have a similar impact on the aggregation and coagulation of the soy proteins, as was described for the deamidation of glutamine residues by transglutaminase, in the previous paper (Yasir et al., this issue).

4. Conclusions

(i) While cross-linking of food proteins *in vitro* occurs in a manner that is readily predictable from results in model proteins, these results do not readily translate to *in situ* experiments within an actual food matrix.
(ii) Cross-linking agents can be used to manipulate the properties of tofu, as evidenced by changes in microstructure and changes in fracture force.
(iii) Other, non-cross-linking, reactions, such as deamidation of amino acids or protein glycation via the Maillard reaction, may also occur and are likely to account for at least some of the changes in properties observed. This is believed to be due to changes in isoelectric point, but more work is required to substantiate this hypothesis.
(iv) Irrespective of the mechanism of action, these reagents can favourably influence the properties of tofu and the point of addition during manufacture has a significant impact on the properties of the final product, suggesting further ways in which tofu manufacturers might customise the texture of their product. Unlike most cross-linking chemicals, transglutaminase is an approved food processing aid, and would be the cross-linking agent of choice for tofu manufacturers at the current time.

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