

# Modification of the nitrogen solubility properties of soy protein isolate following proteolysis and transglutaminase cross-linking

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## Abstract

The effect of (a) limited hydrolysis [0.5–2.0% degree of hydrolysis (DH)] with Alcalase<sup>TM</sup>, (b) cross-linking with transglutaminase (TGase) and (c) combinations of these modifications on the nitrogen solubility (pH 3–8) of soy protein isolate (SPI) was investigated. Between pH 3.0 and 5.0, SPI hydrolysates, hydrolysates of cross-linked SPI and the cross-linked products of SPI hydrolysates displayed significant ( $P < 0.05$ ) increases in solubility compared to unmodified SPI. Cross-linking pre- or post hydrolysis did not alter the overall trend of increased ( $P < 0.05$ ) solubility relative to the unmodified control at low pH. At 2% DH, cross-linking pre- or post-hydrolysis resulted in greater solubility ( $P < 0.05$ ) than that observed in hydrolysates per se at low pH. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) indicated that the 22 kDa 11S basic polypeptide was relatively resistant to Alcalase hydrolysis and that the 18 and 22 kDa 11S basic polypeptides were not susceptible to TGase cross-linking. The results demonstrate that a combination of enzymatic treatments and the order in which they are applied may have potential for creating novel food ingredients with improved functional properties, especially those properties that are dependant on high solubility at low pH.

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## 1. Introduction

Soy proteins are used in foods as functional and nutritional ingredients and as a substitute for animal-derived proteins from milk, meat and eggs (Qi, Hettiarachchy, & Kalapathy, 1997; Rhee, 1994). Soy protein isolate (SPI) has good gelation, emulsifying, foaming and water absorption properties (Utsumi, Matsumura, & Mori, 1997), however, it exhibits poor solubility within the acidic pH regions. Soy protein has found widespread use as a fat and protein replacer in dairy (Chronakis & Kasapis, 1993), meat (Lusas & Riaz, 1995) and bakery products (Brewer, Potter, Sprouls, & Reinhard, 1992) as well as in infant formula (Kolar, Richert, Decker, Steinke, & Vander Zanden, 1985).

Enzymatic hydrolysis of proteins yields products that are smaller in molecular mass, with less secondary structure and in some cases improved functional properties compared to the intact protein (Adler-Nissen &

Olsen, 1979). Alcalase<sup>TM</sup> (Subtilisin Carlsberg) is a serine protease produced by *Bacillus licheniformis*. It has a broad specificity but mainly cleaves on the carboxyl side of hydrophobic amino acids (Adler-Nissen, 1986). Alcalase hydrolysis has been employed to modify the functional properties of a range of protein substrates: these include SPI (Adler-Nissen & Olsen, 1979), dairy protein (Mietsch, Fehér, & Halász, 1989), gluten (Chobert, Briand, Guéguen, Popineau, Larré, & Haertlé, 1996), chickpea protein (Clemente, Vioque, Sánchez-Vioque, Pedroche, Bautista, & Millán, 1999), blood plasma (Hyun & Shin, 2000) and fish protein (Kristinsson & Rasco, 2000). Transglutaminase, (TGase, glutaminyl-peptide:amine  $\lambda$ -glutamyltransferase, E.C. 2.3.2.13) is a widely distributed enzyme in nature. *Streptovorticillium* spp. is currently the primary source for the production of calcium-independent TGase (Ando et al., 1989). TGase can modify proteins by catalysing acyl transfer between a  $\lambda$ -carboxamide of a peptide/protein bound glutamine and lysine forming an  $\epsilon$ -( $\lambda$ -glutamyl) lysine [ $\epsilon$ -( $\lambda$ -Glu)Lys] cross-link (Kuraishi, Yamazaki, & Susa, 2001). This cross-linking results in the polymerisation of protein/peptide molecules with a

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subsequent increase in molecular mass. TGase is used in the processing of dairy, seafood (surimi), meat (sausages, hams), noodles, soy (tofu, kamaboko) and bakery (dough, cakes) products (Kuraishi et al., 2001; Lorenzen & Schlimme, 1998; Motoki & Seguro, 1998; Sharma, Lorenzen, & Qvist, 2001).

TGase cross-linking has been reported to reduce the bitterness of zein, skim milk protein and gluten hydrolysates (Watanabe, Arai, Tanimoto & Seguro, 1992). TGase cross-linking has also been used to improve the emulsifying and foaming properties of pronase, papain, chymotrypsin and acid hydrolysates of soy protein (Babiker, 2000; Babiker, Khan, Matsudomi, & Kato, 1996) and of gluten (Babiker, Fujisawa, Matsudomi, & Kato, 1996).

High solubility, while not an absolute requirement for all functional properties of food proteins, plays an important role in their effective functional performance (Zayas, 1997). The study of Babiker, Khan et al. (1996), demonstrated that TGase cross-linking improved the solubility, as measured by turbidity at 500 nm, of protease and acid hydrolysates of acid precipitated soy protein. However, Babiker, Khan et al. (1996) did not report the effect of cross-linking pre-hydrolysis on the nitrogen solubility. Flanagan and FitzGerald (2002), found that the order of TGase cross-linking, i.e., pre- or post-enzymatic hydrolysis had a significant effect on the nitrogen solubility of sodium caseinate. No information is currently available on the combined contribution of hydrolysis with Alcalase and cross-linking with TGase on the nitrogen solubility of SPI. The objective of this study was therefore to determine the nitrogen solubility properties of Alcalase hydrolysates of SPI following cross-linking with TGase pre- and post-hydrolysis.

## 2. Materials and methods

### 2.1. Materials

Alcalase 2.4 l was obtained from Novo Nordisc A/S (Bagsvaerd, Denmark). Calcium independent microbial TGase (1 unit  $\text{mg}^{-1}$  powder) from *Streptovercillium* spp. was obtained from Forum Products (Redhill, Surrey, England). Soy protein isolate (Supro 530) was obtained from National Food Ingredients Ltd. (Limerick, Ireland). Low molecular mass standards for polyacrylamide gel electrophoresis (PAGE) were from Pharmacia (Amersham-Pharmacia, Amersham, UK). All other reagents were of analytical grade.

### 2.2. Alcalase hydrolysis of SPI

Soy protein isolate (2.4 l, 5% (w/w) protein) was hydrolysed with Alcalase at 50 °C. The pH was maintained constant at 8.0 by constant addition of 0.5 N

NaOH. Sodium azide (0.02% w/v) was added as an anti-bacterial agent. The degree of hydrolysis (DH), defined as the percentage of peptide bonds hydrolysed, was calculated from the volume and molarity of NaOH required to maintain constant pH (Adler-Nissen, 1986). The enzyme-to-substrate ratio (E:S) used, (0.003% w/w), was calculated on the basis of weight of protein in SPI solution and the weight of protein in the Alcalase preparation. The protein content in the SPI was determined using the macro-Kjeldahl procedure (IDF, 1993). The nitrogen-to-protein correction factor used was 6.25. At 3.3, 7.0, 12.4 and 19.4 min, corresponding to DH values of 0.5, 1.0, 1.5 and 2.0%, samples (400 ml) of hydrolysate were removed and heated at 80 °C for 20 min to terminate enzyme activity. Hydrolysates (400 ml) were divided into two (200 ml) aliquots. One aliquot was designated “Hyd” SPI and stored at 8 °C until further analysis. The second aliquot was later subjected to TGase cross-linking. A control SPI sample without added Alcalase, was subjected to similar incubation and heat inactivation conditions as the test samples.

### 2.3. TGase cross-linking of SPI

Soy protein isolate (1.2 l, 5% (w/w) protein), was cross-linked with TGase at 40 °C, pH 7.0 for 2.5 h. The protein solution was pre-incubated at 40 °C for 30 min and adjusted to pH 7.0 prior to cross-linking. Sodium azide (0.02% w/v) was added as an anti-bacterial agent. The enzyme-to-substrate ratio (E:S) used for cross-linking, (0.0013% w/w), was calculated on the basis of weight of protein/protein equivalent in SPI and the weight of protein in the TGase preparation. TGase was inactivated by heating at 60 °C for 15 min. This sample was designated “Cr” SPI.

### 2.4. Hydrolysis post-cross-linking of SPI

The SPI previously cross-linked with TGase was hydrolysed with Alcalase. Cross-linked SPI (1 l, 5% (w/w) protein) was incubated at 50 °C and the pH adjusted to 8.0. During the Alcalase hydrolysis reaction the pH was maintained constant at 8.0 by addition of 0.5 N NaOH. The E:S ratio used (0.003% w/w) was as detailed previously. At defined time intervals, samples (200 ml) of hydrolysate corresponding to DH values of 0.5, 1.0, 1.5 and 2.0% were removed and heated to 80 °C for 20 min to terminate enzyme activity. The cross-linked and unhydrolysed control SPI samples were similarly subjected to these deactivation conditions. These samples were designated cross-linked-hydrolysed SPI or “CrH”.

### 2.5. Hydrolysis pre-cross-linking of SPI

Aliquots (200 ml, 5% protein w/w) of SPI at 0.5, 1.0, 1.5 and 2.0 DH% from the Alcalase hydrolysis reaction

were subjected to cross-linking with TGase after pre-incubation at 40 °C and pH adjustment to 7.0. The E:S ratio used, (0.0013% w/w), was identical to that in the cross-linking reaction described previously. Cross-linking was allowed to proceed for 2.5 h at 40 °C, pH 7.0, before deactivation by heating at 60 °C for 15 min. These samples were designated as hydrolysed-cross-linked-SPI or “HCr”.

## 2.6. Nitrogen Solubility Index

The solubility of 1% (w/w) soy protein dispersions was determined in duplicate between (pH 3 to pH 8). Soy protein solutions (0.3 g protein) were weighed into pre-weighed 50 ml plastic bottles. Twenty-five grams of distilled deionised water was added. Samples were stirred on an orbital stirrer (Gerhardt Schuttelmaschine RO 10, Bonn, Germany), at speed-setting 5 for 1 h. The dispersions were allowed to remain undisturbed for at least 1 h after mixing to allow for hydration of the dispersed protein. The pH of each sample was adjusted to a pH value between pH 3.0 and 8.0 while stirring using 0.1 M NaOH or 0.1 M HCl and water was added to adjust the final weight to 30 g. Samples were left to stand for 1 h. Protein samples were then mixed using a magnetic stirrer and an aliquot (9 ml) was removed in duplicate for estimation of total nitrogen content by macro-Kjeldahl. The remaining solutions were centrifuged at 1300×g for 30 min using a Sorvall RC 5C Plus Centrifuge (Sorvall Products, Newtown, CT, USA). The supernatant was decanted from the pellet and filtered through Whatman No. 1 filter paper (Whatman International, Maidstone, England). Soluble nitrogen in the supernatant was determined in duplicate using the macro-Kjeldahl procedure (IDF, 1993). Solubility was expressed as the percentage nitrogen content of supernatant divided by the overall nitrogen content in the starting solution.

## 2.7. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of SPI samples was carried out by the method of Laemmli (1970). The discontinuous system used consisted of a 4% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide separating gel. Samples (1 mg/ml protein) were dissolved in Tris/Glycine (pH 8.9) containing 20 g l<sup>-1</sup> SDS and 50 g l<sup>-1</sup> 2-mercaptoethanol. Electrophoresis (Protean II xi Electrophoresis System, Bio-Rad Laboratories, Herts, UK) was carried out at 60 mA until the tracker dye reached the bottom of each gel. After electrophoresis, the gels were stained with 0.2% (w/v) Coomassie brilliant blue R-250 [in 10% (v/v) acetic acid: 40% (v/v) methanol] and destained with 10% (v/v) acetic acid containing 40% (v/v) methanol for 16 h.

## 2.8. Statistical analysis

One-way analysis of variance (ANOVA) (using Fisher's least squares differences as a post-hoc comparison) was performed on all functionality data, comparing duplicate samples of the control and modified SPI at a confidence level of 95% using SPSS, Version 9.0 (Coakes, 1999). A significant difference in results infers a significant difference at  $P < 0.05$ .

## 3. Results and discussion

The nitrogen solubility profile of SPI was altered following incubation with TGase. From Fig. 1a, it is seen that cross-linking resulted in a significant increase in solubility at pH 3.0 relative to control. Cross-linked SPI was significantly less soluble than the control SPI at pH 5.0 and 5.5. Cross-linked SPI was significantly more soluble than the control at pH 6.0. The observed differences in solubility may be due to changes in molecular mass, overall charge and surface hydrophobicity following incubation with TGase (Babiker, 2000; Motoki, Nio, & Takinami, 1984; Yildirim, Hettiarchy, & Kalapathy, 1996). In general, a high degree of solubility is observed in proteins/polypeptides when their molecular masses are low, the overall net charge is high and the content of exposed hydrophobic groups is low (Nielsen, 1997). No differences in solubility were observed between pH 4.0–4.5 and 6.5–8.0 (Fig. 1a). The results of Babiker (2000), which were obtained using a turbidometric based analysis, displayed a trend of increased solubility between pH 2.0 and 12.0 for a TGase cross-linked acid precipitated soy protein sample compared to a non cross-linked control. The discrepancy in the effects of cross-linking on solubility between the findings of Babiker (2000), and the present study may be due to differences in soy protein sample, cross-linking procedure and method used for solubility analysis. Motoki et al. (1984), reported a reduction in solubility of isolated 11S and 7S globulins following cross-linking with the calcium-dependent TGase from guinea pig liver.

At low pH, i.e. between pH 3.0 and 5.0, all the Hyd, HCr and CrH samples displayed significant increases in solubility compared to the SPI control (Fig. 1b–e). No direct correlation was apparent between DH (ranging from 0.5 to 2.0%) and solubility for the Hyd, HCr and CrH samples at these pHs. Apart from the 2% DH hydrolysate at pH 6.5, Alcalase hydrolysis along with combinations of hydrolysis and cross-linking yielded hydrolysates that were significantly less soluble than unhydrolysed SPI between pH 5.5 and 8.0. Excluding CrH samples at 1.0, 1.5 and 2.0% DH at pH 6.5, the combined use of cross-linking pre- or post-hydrolysis resulted in improved solubility between pH 2.0 and 5.0

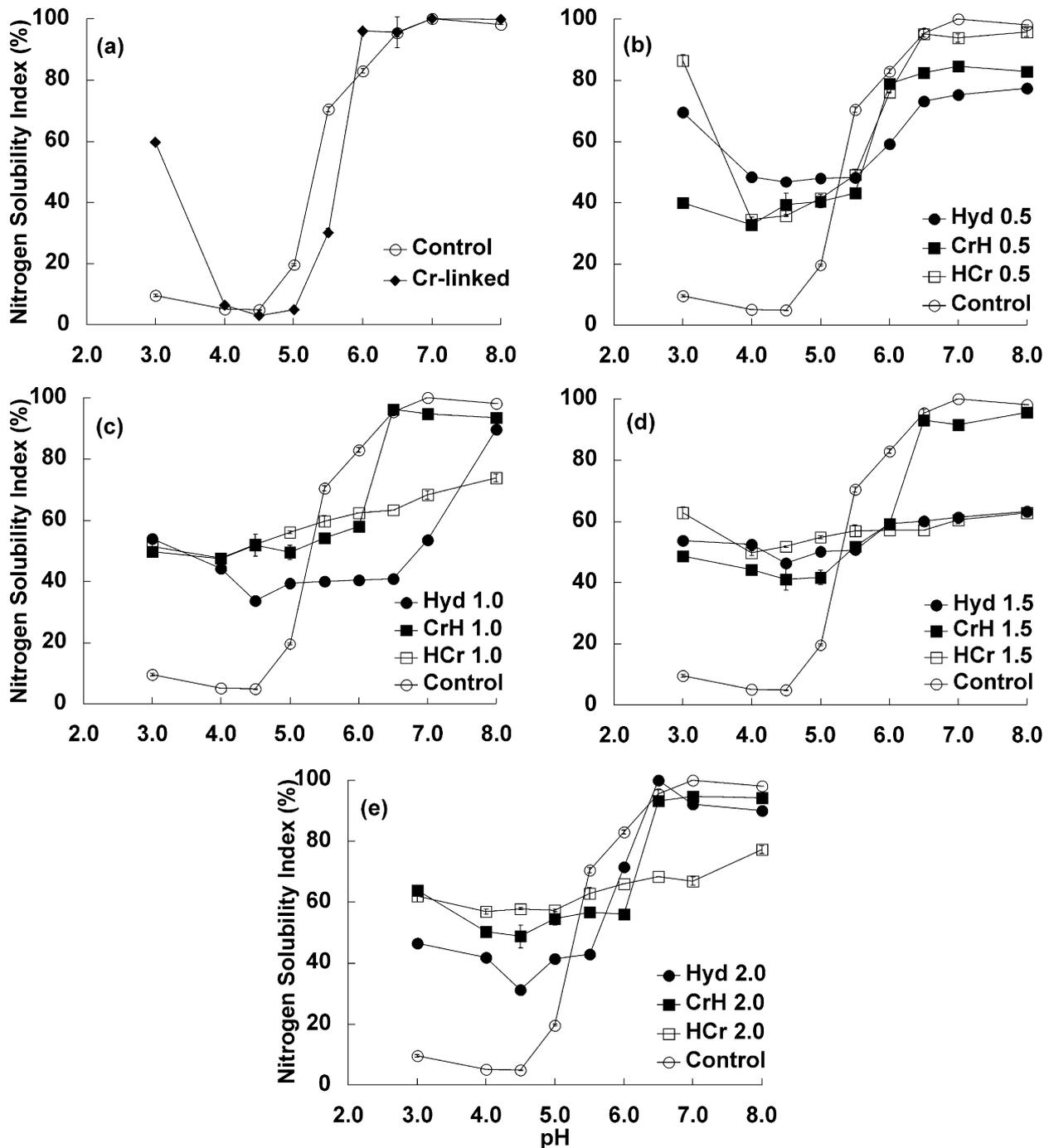


Fig. 1. Effect of pH on the nitrogen solubility profiles of unmodified (control), transglutaminase cross-linked (Cr-linked), Alcalase hydrolysed (Hyd), cross-linked hydrolysed (CrH) and hydrolysed-cross-linked (HCr) soy protein isolate. Hydrolysis was allowed to proceed to defined degree of hydrolysis (DH) values, i.e., (b) 0.5, (c) 1.0, (d) 1.5, (e) 2.0% DH. Data points are means ± standard deviation of duplicate analyses.

and decreased solubility between pH 5.5 and 8.0. Interestingly, the pH-dependant trend in these nitrogen solubility results agrees with those of Flanagan and FitzGerald (2002), where sodium caseinate was incubated with combinations of a *Bacillus* proteinase and with TGase. Significant differences in NSI were observed depending on the sequence with which SPI was subjected to cross-linking with TGase or hydrolysis

with Alcalase. For example, at low DH (0.5%) and low pH, apart from HCr at pH 3.0, sample solubilities had the following trend: Hyd > CrH/HCr (Fig. 1b). However, the 2% DH hydrolysates had the following solubility trend: CrH/HCr > Hyd in the same pH region (Fig. 1e). On the other hand, at high pH the solubility trend for the 0.5% DH samples was as follows: HCr > CrH > Hyd while the solubility trend in the 2%

DH samples was Hyd/CrH > HCr (Fig. 1b and e). The increased solubility of hydrolysed SPI at low pH is in agreement with previous findings (Adler-Nissen & Olsen, 1979; Kim, Park, & Rhee, 1990). From pH 6.0 to 8.0 hydrolysates had significantly lower solubility than control. Significantly improved solubility at the pI and decreased solubility between pH 6.0 and 8.0 agree with previous reports on casein hydrolysates (Flanagan & FitzGerald, 2002). To our knowledge no previous study reports on the effect of TGase cross-linking pre- or post-hydrolysis on the nitrogen solubility properties of SPI.

The generation of peptide fragments having low molecular mass, the increased exposure of hydrophilic amino acid residues and the exposure of amino and carboxyl groups on hydrolysis may account for the observed increase in solubility of the hydrolysed samples at pH values between 2.0 and 5.0 (Hayakawa & Nakai, 1985; Panyam & Kilara, 1996; Nielsen, 1997). Cross-linking pre- or post-hydrolysis did not alter the overall trend of increased solubility relative to the control at pH values less than 5.0. While TGase cross-linking increases the molecular masses of many of the products generated during hydrolysis, these products retain good solubility presumably due to increased electrostatic

repulsion and interaction with water from the hydrophilic/charged groups exposed during hydrolysis at these pHs (Fig. 1b–e). Adler-Nissen and Olsen (1979) reported that hydrolysis of SPI with Alcalase to a DH value of 7.7% resulted in an increase in solubility to 75% between pH 2.0 and 8.0. The reason for the observed decrease in solubility of the Hyd, HCr and CrH samples relative to unhydrolysed control at high pH is unclear. The loss in solubility may arise from a pH-dependent reduction in ionisable groups and an associated increase in exposed hydrophobic residues leading to decreased electrostatic repulsion and increased hydrophobic interactions between peptides at high pH values. Flanagan and FitzGerald (2002), attributed the decrease in solubility of sodium caseinate hydrolysates at high pH values to a possible shift in the pI of hydrolysed peptides compared to intact protein. It is interesting to note that Adler-Nissen and Olsen (1979) also reported a decrease in solubility for a 1.0% DH Alcalase hydrolysate of SPI at pH 4.2. This was attributed to a loss in solubility as a consequence of the heating step used to inactivate the enzyme activity. However, the loss in solubility at high pH observed in the present study (Fig. 1b–e) only occurs in hydrolysed

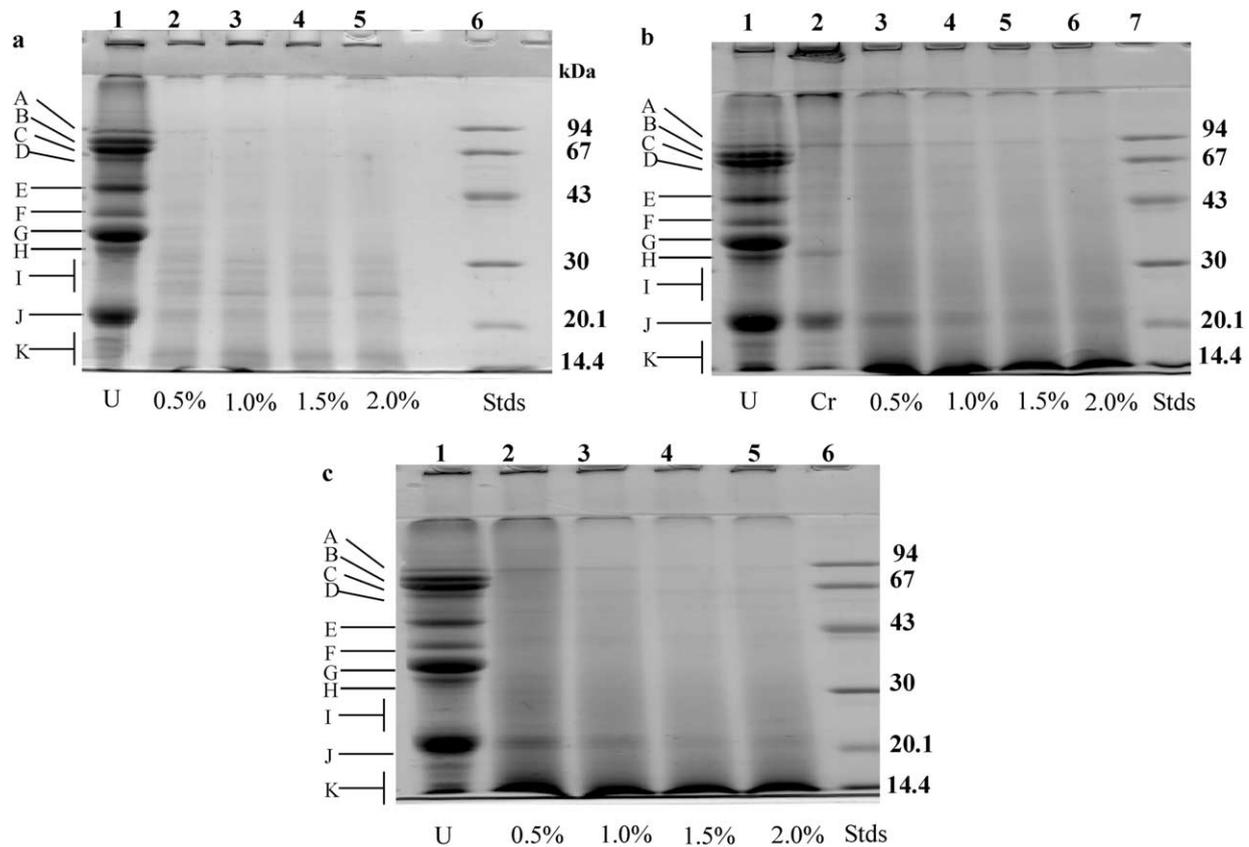


Fig. 2. Effect of (a) Alcalase hydrolysis, (b) cross-linking with transglutaminase (TGase) followed by hydrolysis with Alcalase and (c) hydrolysis with Alcalase followed by TGase cross-linking on the electrophoretic profiles of soy protein isolate: A, lipoxygenase; B,  $\alpha'$ -sub-unit of 7S globulin; C,  $\alpha$ -subunit of 7S globulin; D,  $\beta$ -amylase; E,  $\beta$ -sub-unit of 7S globulin; F, G and H, 11S acidic polypeptides of glycinin; I, J and K, 11S basic polypeptides of glycinin; U, unmodified soy protein isolate; Cr, cross-linked soy protein isolate; 0.0–2.0%, degree of hydrolysis; Stds, low molecular mass protein standards.

samples as the heat-treated control SPI displayed no loss in solubility at high pH (Fig. 1a).

### 3.1. Polyacrylamide gel electrophoresis

Fig. 2 shows the effect of hydrolysis with Alcalase and cross-linking with TGase on the electrophoretic profiles of SPI. As can be seen from Fig. 2a, low levels of hydrolysis, i.e. DH% values ranging from 0.5 to 2.0%, result in a rapid disappearance of bands corresponding to intact soy proteins. It is evident from this gel that the 11S basic polypeptide (22 kDa) is slightly more resistant to Alcalase hydrolysis than the 7S polypeptides (50, 76 and 80 kDa) and the 11S acidic polypeptides (33, 35 and 38 kDa). Incubation of SPI with TGase results in the disappearance of bands corresponding to the 7S globulins and two of the 11S acidic polypeptides (35 and 38 kDa) along with the appearance of high molecular mass material that does not enter the stacking or separating region of the gel. The 11S basic polypeptides (18 and 22 kDa) are still present after incubation with TGase suggesting that these polypeptides are not very susceptible to cross-linking (Fig. 2b, lane 2). This is not unexpected as these basic polypeptides contain relatively low levels of glutamine and lysine, i.e. the preferred substrates of TGase, compared to the acidic polypeptides (Nielsen, 1985). It is also seen that hydrolysis of cross-linked SPI results in a progressive decrease in the amount of high molecular mass material retained in the stacking and separating region of the gel (Fig. 2b, lanes 3–6). While no distinct bands were evident in the samples that were cross-linked post hydrolysis, a high level of smearing was evident on the gel, particularly for the 0.5% DH sample (Fig. 2c, lane 2). This is as expected since hydrolysis to low DH values should lead to higher molecular mass cross-linked products (Fig. 2c, lane 2 compared to lane 5).

## 4. Conclusion

Significant improvements in the NSI of SPI were observed between pH 3.0 and 5.0 using a combination of TGase cross-linking and Alcalase hydrolysis. Furthermore, at a DH of 2%, pre- or post-cross-linking of the hydrolysates with TGase resulted in greater solubilities than hydrolysis per se at low pH. Combined use of TGase cross-linking and hydrolysis has the potential for generating improved soy protein ingredients for use in low pH food and beverage products. No study to date has demonstrated the beneficial role that the combined application of hydrolysis and cross-linking may offer to improve the solubility of SPI at pH values between 3.0 and 5.0. The results demonstrate the possibility of exploiting combinations of enzymatic hydrolysis and cross-linking to modify the solubility of SPI. Furthermore, these changes

in solubility may contribute to the enhancement of other functional properties especially those dependent on good solubility.

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