INFLUENCE OF TRANSGLUTAMINASE-INDUCED CROSS-LINKING ON *IN VITRO* DIGESTIBILITY OF SOY PROTEIN ISOLATE

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ABSTRACT

The influence of covalent cross-linking by microbial transglutaminase (MTGase) on the sequential in vitro *pepsin and trypsin digestion process and the digestibility of soy protein isolate (SPI), was investigated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nitrogen release analyses. Various subunits of β-conglycinin and acidic subunits of glycinin were cross-linked by MTGase to form high molecular weight (MW) biopolymers, while basic subunits of glycinin were unaffected. SDS-PAGE analysis indicated that the cross-linking mainly affected* in vitro *pepsin digestion pattern of various subunits of β-conglycinin, while the trypsin digestion pattern of native SPI was nearly unaffected. Nitrogen release analysis showed that the* in vitro *pepsin or/and trypsin digestibility of native SPI (at the end of pepsin or trypsin ingestion) was significantly decreased (*$P \leq 0.01$*) by the MTGase treatment (for more than 2 h). The cross-linking by MTGase also significantly decreased the* in vitro *digestibility of preheated SPI. These results suggest that the cross-linking by means of transglutaminase may negatively affect the nutritional properties of food proteins.*

INTRODUCTION

Soy proteins have been widely used as a food ingredient, due to their highly nutritious and some desirable functional properties. During the production of soy proteins, a diversity of physical, chemical and even enzymatic treatments or processes may be applied. Such treatments are likely to affect their nutritional properties. The nutritive value of a protein can be evaluated by

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various *in vitro* and *in vivo* methods. Due to their simplicity and speed, the *in vitro* digestibility methods have been more widely used than *in vivo* methods.

It is well known that legumes are of poor nutritional value unless subjected to heat treatment. Thus, the influence of the thermal treatment on the *in vitro* digestibility of legume proteins has been widely investigated (Adeyeye 1997; Clemente *et al*. 1998; El-moniem 1999; Elsheikh *et al*. 2000; Kiers *et al*. 2000). Generally, cooking or other heat treatments can affect *in vitro* digestibility (and *in vivo* digestibility) of legume proteins. The digestibility of legume proteins was not only affected by the presence of other seed components, such as protease inhibitors and lectins, but also affected by the structure of the protein (Nielsen 1991). Thus, those processes or treatments affecting the structure of the protein will be expected to result in changes in the digestibility of legume proteins. However, except the thermal treatment, there is a paucity of information on the influence of other treatments on the nutritional or *in vitro* digestibility of legume proteins.

Transglutaminase (TGase; EC 2.3.2.13), especially from microbial sources, has been widely applied to modify some functional properties of food proteins or its products, including emulsifying properties and gel properties, through covalent cross-linking (Færgemand *et al*. 1998; Babiker 2000; Babin and Dickinson 2001; Siu *et al*. 2002; Flanagan *et al*. 2003; Tang *et al*. 2005a). This kind of enzyme has also been used to induce the gelation of food proteins (Nio *et al*. 1985; Chanyongvorakul *et al*. 1994, 1995; Schorsch *et al*. 2000; Babin and Dickinson 2001; Tang *et al*. 2006a,b), including soymilk (Tang *et al*., in press), as a substitute for glucono-δ-lactone and $CaSO_4$. Furthermore, this enzyme has also been shown to effectively improve the properties of protein or protein-based cast films, especially the mechanical strength and the film surface hydrophobicity (Mariniello *et al*. 2003; Tang *et al*. 2005b). All these studies suggest that this kind of enzyme has good potential to be commercially applied in the food industry. However, there are few literatures about the influence of TGase-induced cross-linking on the nutritional properties of food proteins, particularly soy proteins.

Thus, the objective of this work was to investigate the effect of microbial transglutaminase (MTGase)-induced cross-linking on the digestive process and *in vitro* digestibility of soy proteins, using a model. The effect of thermal pretreatment on *in vitro* digestibility of soy proteins was also investigated.

MATERIALS AND METHODS

Materials

The whole soybean seed powder was provided by Henan HEBEI Co. (Hebei City, China). Commercial enzyme of MTGase was obtained from

Chanshou Biological Co. Ltd. (Chanshou City, China). Pepsin (catalog no. P7000, 600–1,000 units/mg) and trypsin powder (from porcine pancreas; catalog no. T4799, 1,000–5,000 BAEE units/mg solid) were purchased from Sigma Chemical Co (St. Louis, MO). The wide molecular weight (MW) standard protein markers (M4038) were purchased from Sigma Chemical Co., composed of the rabbit muscle myosin (205.0 kDa), β-galactosidase (*Escherichia coli*) (116.0 kDa), rabbit muscle phosphorylase *b* (97.0 kDa), rabbit muscle fructose-6-phosphate kinase (84.0 kDa), bovine serum albumin (66.0 kDa), bovine liver glutamic dehydrogenase (55.0 kDa), chicken egg ovalbumin (45.0 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), bovine erythrocytes carbonic anhydrase (29.0 kDa), bovine pancrease trypsinogen (24.0 kDa), soybean trypsin inhibitor (20.0 kDa), bovine milk α-lactalbumin (14.2 kDa) and bovine lung aprotinin (6.5 kDa). All other chemicals were of analytical reagent or better grade.

The purification of MTGase and its activity determination were according to the method of Tang *et al*. (2005a). The activity of this enzyme was measured by the colorimetric procedure using N_{α}-CBZ-GLN-GLY as the substrate. One unit of this enzyme activity is defined as the formation of 1 μmol L-glutamic acid γ-monohydroxamate per min at 37C.

Preparation of Soy Protein Isolate (SPI)

SPI was prepared according to the method of Iwabuchi and Yamauchi (1987), with a minor modification. The following procedures were performed at room temperature. Defatted soybean meal was prepared from ground soybean seed powder by solvent extraction with *n*-hexane. Defatted soybean meal was then extracted with 20-fold 0.03 mol/L Tris-HCl buffer (pH 8.0) containing 10 mmol/L β-mecaptoethanol (2-ME), and centrifuged to remove the insoluble material. The pH of the extract was adjusted to pH 4.8 at 4C using 2-N HCl, and the precipitate or curd was collected by centrifugation. The obtained curd was dissolved in water at 4C, and adjusted to pH 7.5 with 2-N NaOH, and then centrifuged at 4C, yielding the SPI supernatant. The supernatant was dialyzed three times at 4C against desalted water (1:100, three times), and then lyophilized to yield the SPI product. The protein content of this SPI was 97.0% (dry basis) (determined by Kjeldahl method, $N \times 6.25$).

Thermal Pretreatment of SPI

The 2% (w/v) SPI solution in 0.05-M Tris-HCl buffer (pH 7.0, 25C) was prepared, and preheated at 80C for 45 min. After this treatment, this solution was immediately cooled to room temperature, then dialyzed and lyophilized to obtain the preheated SPI, or mixed with MTGase to start the enzymatic reaction.

Cross-linking Reactions Induced by MTGase

The 2% (w/v) native and preheated SPI solutions in 0.05-M Tris-HCl buffer (pH 7.0) were preincubated at 37C, then mixed with 20 units of MTGase per gram of substrate protein and incubated at 37C for various times. At various times, the reactions were stopped by directly mixing with the sample buffer (×2) of electrophoresis, for the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. As for the preparation of MTGase-polymerized SPIs, the reaction mixtures were immediately cooled in ice bath to 4C after being incubated for 2 or 6 h, and then dialyzed and lyophilized.

Sequential *In Vitro* Digestion

Sequential *in vitro* pepsin and trypsin digestion experiment was carried out according to the method of Njingtang *et al*. (2001) and Nunes *et al*. (2004), with some modifications. One gram of SPI samples (1%, w/v) was dispersed in 100 mL of 0.1-N HCl (pH 1.5), and preincubated in a bath at 37C for 3–5 min. Then, 10 mg of pepsin powder was added and mixed, and the mixture was incubated at 37C to start the pepsin digestion reaction (0–120 min). At various times, the digestion mixtures were adjusted with 1.0-N NaOH to pH 7.0 to stop the pepsin digestion reaction. At the same temperature, the neutralized digestion mixtures were mixed with 30 mg of porcine trypsin to start the further digestion for various times (0–120 min). At appropriate intervals, samples were taken during digestion by pepsin and by trypsin and precipitated by trichloroacetic acid (TCA).

SDS-PAGE

SDS-PAGE electrophoresis was performed on a discontinuous buffered system according to the method of Laemmli (1970) using 12% separating gel and 4% stacking gel. The pepsin or/and trypsin digestion mixtures were directly mixed with the same volume of electrophoresis sample buffer with pH 6.8, containing 0.125-M Tris-HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol (2-ME), 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue. These mixtures were heated for 5 min in boiling water before electrophoresis. Every sample (10 μL) was applied to each lane. Before the sample entering the separating gel, electrophoresis was performed at 10 mA, and the other was at 20 mA. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% TCA, and destained in 7% acetic acid (methanol : acetic : water, 227:37:236 [v/v/v]).

Determination of Nitrogen Release during Digestion

The TCA-soluble nitrogen fraction of the pepsin or/and trypsin digest was obtained by directly mixing the digest with the same volume of 10% TCA

and centrifugation at 8,000 × g for 15 min. The nitrogen content of the corresponding precipitates or other SPI samples was measured by the Kjeldahl method (N × 6.25). The % nitrogen release was defined as follows:

$$\frac{N_0 - N_t}{N_{tot}} \times 100,$$

where N_t represents the TCA-precipitated nitrogen content after digestion for t min (mg), N_0 the TCA-precipitated nitrogen content in SPI before the digestion (mg), and N_{tot} the total nitrogen content in the SPI samples (mg).

Statistics

An analysis of variance of the data was performed, and a least significant difference test with a confidence interval of 99% was used to compare the means.

RESULTS AND DISCUSSION

Cross-linking of SPI Induced by MTGase

The SPIs prepared in this study were mainly composed of glycinin and β-conglycinin, as evaluated by SDS-PAGE (Fig. 1, lane 1). When incubated with MTGase (20 U/g) at 37C, a majority of protein constituents of β-conglycinin and acidic subunits (AS) of glycinin declined continuously with increasing incubation time from 0 to 360 min, and correspondingly, new high MW biopolymers gradually increased on the top of separating and stacking gel, while the basic subunits (BS) of glycinin were almost intact during the entire period (Fig. 1, lanes 2–8).

In the subunits of β-conglycinin, the susceptibility of β-subunit to MTGase was lower than that of α′- and α-subunits. Similar results have been reported in TGase or MTGase-induced cross-linking reactions of soy globulins and pea legumin (Nonaka *et al*. 1989; Larré *et al*. 1992; Kang *et al*. 1994; Zhang *et al*. 2003). The different reactivity of individual proteins of SPI with MTGase is related to the native structures of glycinin and β-conglycinin. Usually, the BS of glycinin are buried in the interior of hexamer structure of glycinin, and the relative hydrophobicity of β-subunit of β-conglycinin is higher than that of α′- and α-subunits (Thanh and Shibasaki 1976, 1977). Therefore, the active site of this enzyme (MTGase) is not easily accessible for BS of glycinin and β-subunit of β-conglycinin.

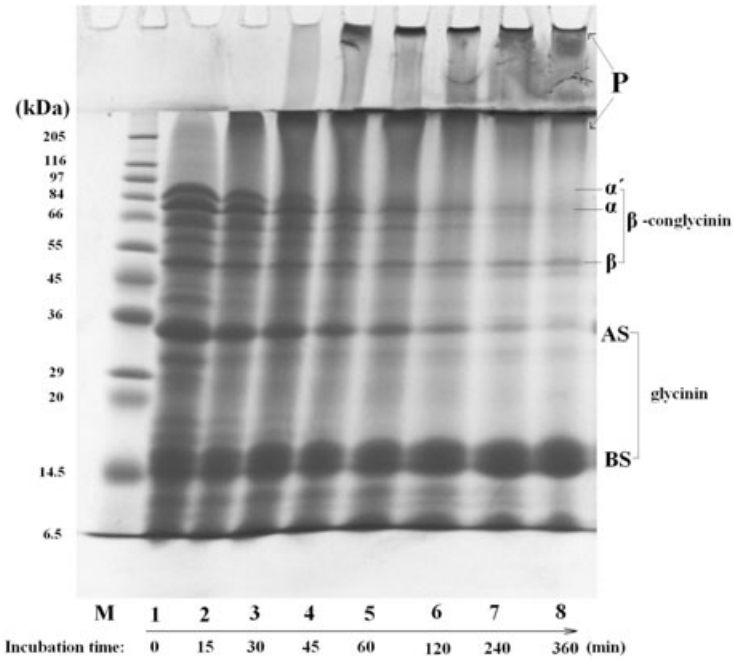

FIG. 1. SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS OF NATIVE SOY PROTEIN ISOLATE (SPI) INCUBATED WITH MICROBIAL TRANSGLUTAMINASE (MTGase) AT 37C FOR VARIOUS TIMES
The concentration of SPI solution was 2% (w/v), and the enzyme amount applied was 20 units per gram of protein. M and P indicate the protein markers and the cross-linked biopolymers, respectively. Lanes 1–8 indicate the SPI incubated with MTGase for 0, 15, 30, 45, 60, 120, 240 and 360 min, respectively. AS, acidic subunits; BS, basic subunits.

SDS-PAGE Analysis of *In Vitro* Digest

The sequential *in vitro* pepsin and trypsin digestion process of native and MTGase-polymerized SPIs (1%, w/v), as analyzed by SDS-PAGE, is shown in Fig. 2. During the pepsin digestion of native SPI, the protein constituents of glycinin (including the AS and BS) were rapidly digested by pepsin within about 1 min (Fig. 2A, lane 2), and those of β-conglycinin (including α′-, α- and β-subunits) were much less prone to digestion by pepsin. The susceptibility of α-subunit to pepsin was much higher than that of α′- and β-subunits, and α′-subunit was almost intact during the pepsin digestion (Fig. 2A, lanes 2–6). Usually, the structure of glycinin, with the BS buried in the interior and the AS surrounded peripherally, is more unstable and easily destroyed in extreme acid medium than that of β-conglycinin. It is reasonable to imply that the BS as well as the AS will be exposed to the exterior of glycinin component

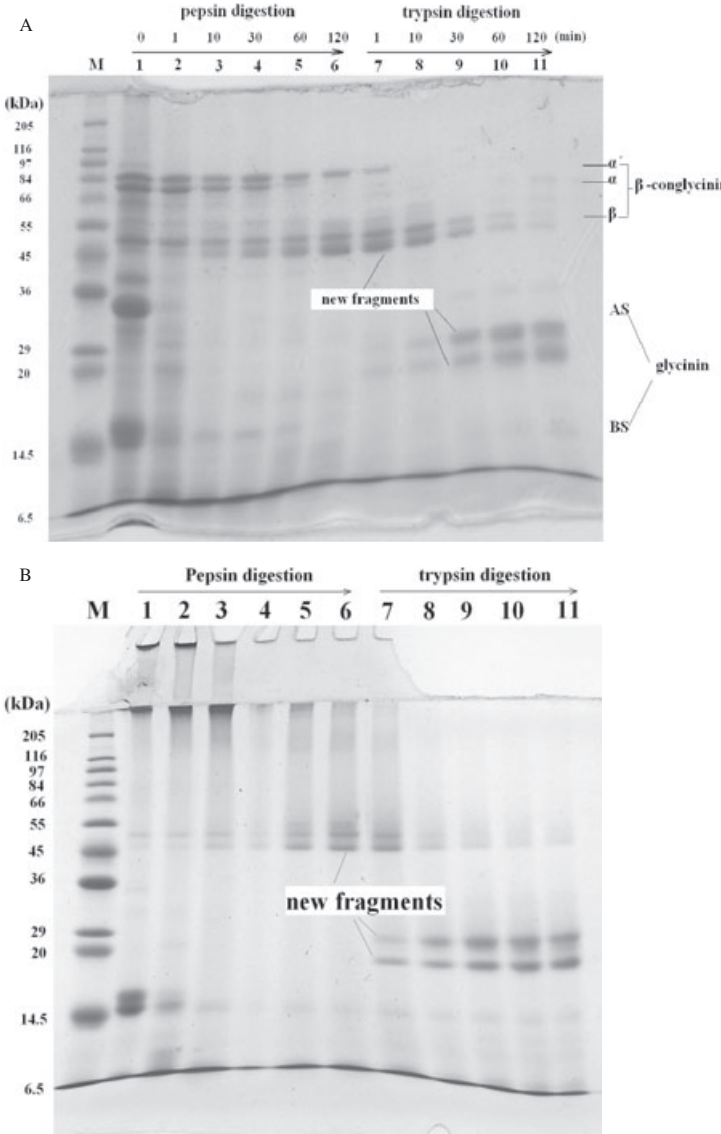

FIG. 2. SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSES OF *IN VITRO* PEPSIN AND TRYPSIN DIGESTION OF NATIVE (A) AND POLYMERIZED (B) SOY PROTEIN ISOLATES (SPIs)

Panel A and panel B: lanes 1–6 indicate native or polymerized SPIs, digested by pepsin after 0, 1, 10, 30, 60 and 120 min, respectively; lanes 7–11, the pepsin hydrolysates digested further by trypsin for 0, 1, 10, 30, 60 and 120 min, respectively; M, the protein markers; AS, acidic subunits; BS, basic subunits.

after the acid-induced dissociation. Thus, the susceptibility difference of different subunits of glycinin and β-conglycinin may be attributed to the difference of structure stability of these two protein components in about pH 2.0 medium.

After incubation with pepsin for more than 60 min, α-subunit (MW ~72 kDa) was completely digested, and consequently, a new peptide fragment with a MW of ~48 kDa was produced. After the pepsin digestion, the undigested protein constituents of SPI were further digested by trypsin (Fig. 2A, lanes 7–11). The α-subunit was most susceptible to digestion by trypsin, which was completely digested within several minutes. The β-subunit and the fragment from α-subunit were digested gradually by trypsin, and transformed to two new fragments with MW close to 20 and 29 kDa.

The polymerized SPI, obtained after incubation with MTGase for 6 h, mainly consisted of BS of glycinin and high MW (>205 kDa) of biopolymers (which could not enter the separating and stacking gels) (Fig. 2B, lane 1). Undoubtedly, those biopolymers were the covalently cross-linked products from AS of glycinin and α- and α′-subunits of β-conglycinin. As with the digestion of native SPI, the BS included in the polymerized SPI was easily digested by pepsin (Fig. 2B, lanes 2–3). The biopolymers were gradually digested upon incubation with pepsin, to release a series of protein fragments with MW higher than 50 kDa. Interestingly, two fragments with MW of about 48 and 50 kDa, corresponding to β-subunit of β-conglycinin and the fragment from α-subunit in native SPI case (Fig. 2A), continually accumulated and became obvious after 60 min during the pepsin digestion (Fig. 2B, lanes 5–6). By contrast, α′-subunit of β-conglycinin, most resistant to pepsin digestion in native SPI case, was not released during pepsin digestion of the polymerized SPI. These results suggest that the *in vitro* pepsin digestion pattern of various subunits of β-conglycinin is affected by the MTGase-induced cross-linking.

After pepsin digestion, polymerized SPI had almost the same pattern of the subsequent trypsin digestion as that in the native SPI case. However, the two new fragments (MW, about 20 and 29 kDa) were seemingly released more rapidly as compared to that in native SPI digestion case. Thus, it is possibly suggested that the cross-linking treatment to some extent can improve the susceptibility of β-subunit or fragments of β-conglycinin to trypsin digestion.

The Nitrogen Release Analysis of *In Vitro* Digestion

During the pepsin digestion, both native SPI and its polymerized ones (including 2 and 6 h) showed a similar trend of the % nitrogen release: increased fast and linearly at the initial stage (0–20 min), and then slowly and gradually reached the maximum with further digestion (Fig. 3). During the initial 20 min of digestion, the change pattern of nitrogen release for the

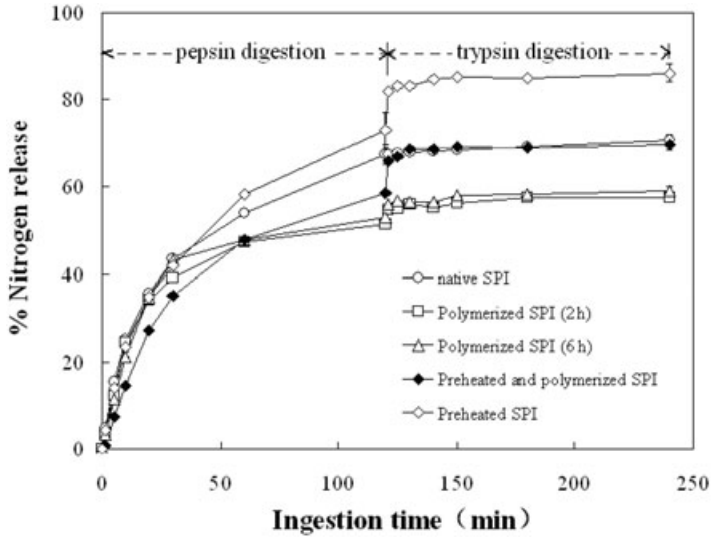

FIG. 3. THE % NITROGEN RELEASE DURING *IN VITRO* PEPSIN AND TRYPSIN DIGESTION PROCESS OF NATIVE AND MICROBIAL TRANSGLUTAMINASE-POLYMERIZED SOY PROTEIN ISOLATES (SPIs)
Error bars indicate the mean values ± SDs ($n = 3$).

polymerized SPIs was almost identical with that of native SPI. However, after the pepsin incubation for more than 30 min, the % nitrogen release of polymerized SPIs became much slower than that of native SPI. At the end of pepsin digestion (120 min), the nitrogen release (~50%) of polymerized SPIs (including 2 and 6 h) was significantly ($P \leq 0.01$) lower than that of native SPI (68%). These results are consistent with that of SDS-PAGE analysis (Fig. 2), confirming that the decline in the pepsin digestibility by the MTGase treatment results from the covalent cross-linking of the protein constituents of SPI (e.g., AS of glycinin and subunits of β-conglycinin). The influence on the digestibility of native SPI by the MTGase treatment may be in part attributed to the occurrence of the MTGase-induced aggregation of SPI. In some *in vivo* studies, the low digestibility of legume globulins caused by thermal treatment has been also attributed to the protein aggregation (Carbonaro *et al*. 2000, 2005). In a previous study, we had confirmed the coagulation of SPI induced by MTGase, and the corresponding coagula were mainly composed of BS of glycinin and high MW biopolymers (Tang *et al*. 2006b).

In the further trypsin digestion of native SPI, the % nitrogen release increased gradually and slowly from the initial value (68%) to a maximum value (73%). The presence of high activity of trypsin inhibitors in beans (Khokar and Chanhan 1986; Marquez *et al*. 1998) may account for the low

susceptibility of the pepsin digest to trypsin. In the polymerized SPIs, the change pattern of nitrogen release during trypsin digestion was almost the same as that of native SPI, except in the initial stage (0–15 min), during which the % nitrogen release increased much faster than that in the native SPI trypsin digestion case (Fig. 3). During the whole trypsin digestion, the nitrogen release of those polymerized SPIs (2 and 6 h) was always significantly lower than that of native SPI ($P \leq 0.01$). At the end of trypsin digestion (after 120 min), the % nitrogen release of native and polymerized SPI (2 h) were 75 and 58%, respectively. The % nitrogen release of polymerized SPI (6 h) was slightly higher than that of polymerized SPI (2 h). This may be a result of the decline in the activity of trypsin inhibitors, caused by the MTGase treatment, because trypsin inhibitors are a kind of peptide, which should be also the catalytic substrate of MTGase. These results confirmed that the covalent cross-linking by MTGase significantly decreased the *in vitro* digestibility of native SPI.

Commercial SPI products, obtained after acid precipitation, alkali solubilization and spray drying, are usually denatured proteins. The effect of thermal treatment (especially wet heating) on *in vitro* digestibility of bean storage proteins has been widely investigated (Romero and Ryan 1978; Geervani and Theophilus 1980; Walker and Kochar 1982; El-moniem 1999). In most of these cases, the thermal treatment significantly improves the protein digestibility, most likely by destroying heat-labile protease inhibitors, and also by denaturing globulins, which are highly resistant to proteases in the native state (Liener 1976; Walker and Kochar 1982).

For the practical consideration, we also investigated the influence of MTGase-induced cross-linking on the *in vitro* digestibility of preheated SPI. As expected, the heat treatment significantly improved the nitrogen release during *in vitro* digestion of native SPI, particularly in the trypsin digestion stage (Fig. 3). SDS-PAGE analysis confirmed that all the subunits of β-conglycinin were rapidly digested by pepsin after heat treatment (data not shown). This distinct increase of *in vitro* trypsin digestibility could be in part attributed to the reduction of trypsin inhibitor activity by thermal treatment. As described earlier, the heat treatment (80C, 30 min) reduced the trypsin inhibitor activity in chickpea albumins by 16% with respect to the initial activity (Clemente *et al*. 2000). Of course, the changes in structural conformation of globulins (particularly the β-conglycinin) caused by the heat treatment (80C, 45 min) also significantly improved the susceptibility of these globulins to proteases, because the denaturation temperature of β-conglycinin in the pH 7.6 buffer was near 80C (Renkema *et al*. 2001).

After incubation with MTGase at 37C for 2 h (under the same condition as in the native SPI case), individual proteins of preheated SPI were cross-linked to a similar extent as that of native SPI (data not shown). This MTGase treatment or cross-linking also remarkably decreased the nitrogen release

during the *in vitro* pepsin and trypsin digestion of preheated SPI (Fig. 3). The digestibility of preheated SPI at the end of pepsin digestion (120 min) declined by 20%, and that at the end of trypsin digestion declined by 19%, after being treated by MTGase for 2 h ($P \leq 0.01$). These results showed that this kind of cross-linking by MTGase also significantly decreased the *in vitro* digestibility of preheated SPI.

Interestingly, the nitrogen release of the polymerized SPI (preheated) was lower in the initial stage of pepsin digestion (0–60 min), while in the remaining digestion course, much higher than that of the polymerized SPI (2 h) (Fig. 3). This may be due to the increase in the cross-linking extent of protein constituents of glycinin to MTGase by thermal treatment, because in the initial phase of pepsin digestion, the nitrogen release mainly results from the digestion of glycinin (Fig. 2).

CONCLUSIONS

The covalent cross-linking by MTGase significantly decreased the *in vitro* digestibility of soy proteins, especially that observed for pepsin digestion. This influence of cross-linking on the digestion pattern and nitrogen release of SPI seems to be related with the extent of protein denaturation or aggregation, and the presence of trypsin inhibitor activity. Further studies are needed to better understand the influence of MTGase-induced cross-linking on the *in vivo* digestibility of food proteins.

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