



A two-step enzymatic modification method to reduce immuno-reactivity of milk proteins



Srinivasan Damodaran*, Yan Li

Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706, USA

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ABSTRACT

A two-step enzymatic approach to reduce immuno-reactivity of whey protein isolate and casein has been studied. The method involves partial hydrolysis of proteins with proteases, followed by repolymerization with microbial transglutaminase. Whey protein isolate partially hydrolyzed with chymotrypsin, trypsin, or thermolysin retained about 80%, 30%, and 20% of the original immuno-reactivity, respectively. Upon repolymerization the immuno-reactivity decreased to 45%, 35%, and 5%, respectively. The immuno-reactivity of hydrolyzed and repolymerized casein was negligible compared to native casein. The repolymerized products were partially resistant to *in vitro* digestion. Peptides released during digestion of repolymerized thermolysin-whey protein hydrolysate had less than 5% immuno-reactivity, whereas those of whey protein control exhibited a sinusoidal immuno-reactivity ranging from 5 to 20%. Peptides released during digestion of repolymerized thermolysin-casein hydrolysates had no immuno-reactivity. These results indicated that it is possible to produce hypoallergenic milk protein products using the two-step enzymatic modification method involving thermolysin and transglutaminase.

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1. Introduction

Cow's milk allergy is very prevalent among children under the age of three. A significant number of infants allergic to cow's milk protein also develop allergy to other food proteins (Ahn et al., 2003; Burks, Casteel, Fiedorek, Williams, & Pumphrey, 1994; Zeiger et al., 1999). Protein allergy in children is mainly attributed to IgE-mediated hypersensitivity (Exl & Fritsché, 2001). Elicitation of allergenic response of a protein could be either due to a sequence-specific epitope or conformational epitope. Studies have shown that partial enzymatic hydrolysis significantly reduced the allergenicity of proteins (Bindels & Boerma, 1994; Businco et al., 1993; Halken et al., 2000; Oldaeus, Anjou, Bjorksten, Moran, & Kjellman, 1997; Pahud, Monti, & Jos, 1985; Rosendal & Barkholt, 2000). However, the definition of 'partial' hydrolysis is quite arbitrary in terms of the peptide length profile. As a consequence, some partially hydrolyzed protein products have been shown to cause severe allergic reactions in some children (Ragno, Giampietro, Bruno, & Businco, 1993; Rosendal & Barkholt, 2000) due to presence of intact linear epitopes in hydrolysates. Furthermore, protein hydrolysates generally taste bitter (Matoba & Hata, 1972; Ney, 1971; Wróblewska et al., 2004) and lack desirable functional prop-

erties, particularly gelation and texturization, essential for improving sensorial properties of hypoallergenic food products.

A promising enzymatic approach to reduce the allergenicity of proteins is enzymatic cross-linking using enzymes such as transglutaminase (Buchert et al., 2008; Li & Damodaran, 2016; Malandain, 2005; O'Sullivan & Fitzgerald, 2012; Pedersen et al., 2004). Transglutaminase (TGase) catalyzes a cross-linking reaction between glutamine and lysine residues in proteins (Agyare & Damodaran, 2010; Dejong & Koppelman, 2002; Han & Damodaran, 1996). This cross-linking reaction could be inter-molecular and intra-molecular. When inter-molecular cross-linking occurs, which is the predominant route, the reaction produces branched-chain protein polymers (Han & Damodaran, 1996). It is likely that this cross-linking reaction might alter both conformational and linear epitopes and thus render the modified protein to be less responsive to IgE binding. However, studies have shown that polymerization of milk proteins, viz., β -casein and β -lactoglobulin using microbial transglutaminase resulted only in marginal decrease in their immuno-reactivity (Li & Damodaran, 2016; Olivier, Villas-Boas, Netto, & Zollner, 2012; Stanic et al., 2010). On the other hand, transglutaminase crosslinking of wheat flour caused a 70% reduction in glutenin immune reactivity, whereas that of ω -gliadin actually increased in non-competitive ELISA (Leszczyńska, Łacka, & Bryszewska, 2006; Palosuo et al., 2003). In a recent study, Li and Damodaran (2016) reported that

* Corresponding author.

E-mail address: sdamodar@wisc.edu (S. Damodaran).

immuno-reactivity of protein components in heterologous cross-linked protein polymers was significantly lower than in homologous cross-linked protein polymers. However, a significant fraction of conformational and linear epitopes were still intact and were readily accessible to antibody binding in some heterologous protein conjugates, such as cross-linked whey protein-casein, soy protein-casein, and soy protein-whey protein polymers.

The hypothesis of the present study is that a combination of initial partial hydrolysis of protein using a suitable protease followed by TGase-catalyzed re-polymerization of the hydrolysate could produce hypo-immune-reactive protein polymers. This two-step enzymatic approach would completely disrupt both the integrity and accessibility of allergenic epitopes to IgE in proteins. This might also eliminate the bitter flavor often associated with protein hydrolysates. To test this hypothesis, we used trypsin, chymotrypsin, and thermolysin to partially hydrolyze whey protein isolate (WPI) and sodium caseinate (CN) under controlled conditions and repolymerized the resulting hydrolysate using TGase to produce branched chain polymers. The immuno-reactivity of these hydrolysates and polymers were investigated using protein specific ELISA. The digestibility of these branched chain polymers under simulated gastric-duodenal conditions and the immuno-reactivity of the digests were also studied.

2. Materials and methods

2.1. Materials

Commercial sodium caseinate was from Agropur Ingredients (Product No. SR:9710; La Crosse, WI, USA) and whey protein isolate (WPI) was from Davisco Foods International Inc., (BiPRO, Lot No. JE045-5-420, Eden Prairie, MN, USA). Thermolysin (Type X, 30–175 units/mg protein), trypsin (from porcine pancreas, type 2, 1600 BAEE units/mg solid; 1800 ATEE units/mg solid), α -chymotrypsin (from bovine pancreas, 83.9 units/mg solid; 96 units/mg protein), porcine stomach mucosa pepsin (EC 3.4.23.1, activity: 2100 units/mg solid, 2600 units/mg protein), and porcine pancreatin (P7545) were from Sigma-Aldrich (St. Louis, MO, USA). Microbial TGase (Activa-TI, 99% maltodextrin and 1% TGase; 100 units/g of solid) was from Ajinomoto Food Ingredients (Eddyville, IA, USA). The enzyme was used without further purification.

AgraQuant® F.A.S.T Casein ELISA test kit was purchased from Romer Labs Inc. (Union, MO, USA). RIDASCREEN® β -lactoglobulin test kit was purchased from R-Biopharm AG (Darmstadt, Germany).

2.2. WPI hydrolysis and re-polymerization

For digestion with thermolysin, a 10% (w/w) solution of WPI at pH 7.0 was treated with thermolysin at enzyme-to-substrate weight ratios of 1/400, 1/1500 and 1/2000 and incubated at 37 °C for 5 min. The hydrolysis was stopped by heating in a boiling water bath for 8 min.

It is known that whey proteins are partly resistant to proteolysis by trypsin and chymotrypsin. Therefore, to facilitate proteolysis with chymotrypsin and trypsin, first a 1% (w/w) solution of WPI was heat denatured in a boiling water bath for 30 min, cooled and lyophilized. A 5% (w/w) solution of this denatured WPI in water at pH 7.0 was treated with trypsin or chymotrypsin at various enzyme-to-substrate ratios and incubation time combinations, i.e., 1:200 for 30 min; 1:100 for 30 min, and 1:100 for 1 h at 37 °C. The hydrolysis reaction was stopped by heating in boiling water bath for 8 min and then lyophilized.

Repolymerization of the WPI hydrolysates using TGase was performed as follows: A 10% thermolysin hydrolysate, and 5% trypsin and chymotrypsin hydrolysates were incubated separately with TGase (100 units/g of substrate) in the presence of 5 mM β -mercaptoethanol for 24 h at 37 °C. Heating the mixture in a boiling water bath for 8 min stopped the reaction.

2.3. Casein hydrolysis and re-polymerization

Aliquots of a 5% w/w solution of sodium caseinate in deionized water at pH 7.0 were incubated with thermolysin at an enzyme to substrate weight ratio of 1:1500 for 5 min, 10 min, and 15 min at 37 °C and hydrolysis was terminated by heating in a boiling water bath for 8 min and then lyophilized. Casein was hydrolyzed with chymotrypsin and trypsin at an enzyme-to-substrate weight ratio of 1:200 under conditions similar to thermolysin hydrolysis. Time-point samples were taken after 5 min, 10 min, and 15 min followed by heat inactivation of the enzyme in boiling water for 8 min and then the samples were lyophilized. TGase-catalyzed re-polymerization of the above hydrolysates was performed as described for WPI hydrolysate.

2.4. In vitro digestion of re-polymerized protein polymers

In vitro digestion of repolymerized-protein polymers under simulated gastro-intestinal conditions was performed as described elsewhere (Dupont et al., 2010; Fu, Abbott, & Hatzos, 2002; Li & Damodaran, 2016). Briefly, protein samples were dissolved (2%) in 0.15 M NaCl and the pH was adjusted to 2.5 with 1 N HCl to simulate the gastric fluid conditions. Porcine stomach mucosa pepsin was added at a 1:20 enzyme-to-protein weight ratio and the mixtures were incubated at 37 °C for 1 h. To create the intestinal condition, the pH of the gastric digest was raised to 6.5 using 1 N NaOH and bile salt and bile-Tris were added so that the final concentrations of these components were 1.85 mM and 26.1 mM, respectively. To this mixture was added porcine pancreatin at a 1:20 enzyme-to-protein ratio and the mixture was incubated at 37 °C. Samples withdrawn at various time intervals during the intestinal-phase of digestion were placed in a boiling water bath for 10 min to terminate digestion. The immuno-reactivity of the digests was analyzed using protein-specific ELISA kits.

2.5. Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples under reducing conditions was carried out as described previously (Agyare & Damodaran, 2010) using a 12.5% acrylamide separating and a 4.5% acrylamide stacking slab gel in a Mini-PROTEAN 3 apparatus (Bio-Rad Laboratories, Hercules, CA, USA). A mixture of molecular weight standards (11–170 kDa) (Sigma-Aldrich Co., St. Louis, MO, USA) was also run along with the samples.

To better resolve low molecular weight peptides in some cases, tris/tricine polyacrylamide gel electrophoresis was also performed according to Schagger and Von Jagow (1987) using a 16.5% acrylamide separating and a 4% acrylamide stacking slab gel. A mixture of molecular weight standards (6–180 kDa) (NOVEX, Life technologies, CA, USA) was also run along with the samples.

2.6. ELISA

Immuno-reactivity of control and treated protein samples were studied using commercially available protein-specific ELISA kits. The experiments were performed as described elsewhere (Li & Damodaran, 2016). In the case of WPI, which is a mixture of β -lactoglobulin and α -lactalbumin, the immune-reactivity of WPI

was determined using a β -lactoglobulin-specific competitive ELISA kit. In all ELISA experiments, the intensity of color products released in the titer wells was measured at 450 nm in an automated Spectra MAX plus ELISA plate reader (Molecular Devices Inc., Sunnyvale, CA, USA). These experiments were done in duplicate and statistical analyses (analysis of variance) of the results were performed using Excel.

3. Results and discussion

3.1. WPI hydrolysis

β -LG is known to be very resistant to digestive enzymes, especially trypsin and chymotrypsin, but its susceptibility improves upon thermal denaturation (O'Loughlin, Murray, Kelly, FitzGerald, & Brodtkorb, 2012; Reddy, Kella, & Kinsella, 1988). Therefore, a pre-heat treatment (see methods) was employed to improve the

susceptibility of WPI to proteolysis. Hydrolysis of WPI by trypsin, chymotrypsin, and thermolysin under various conditions is shown in Fig. 1. It should be noted that even after thermal denaturation in a boiling water bath for 30 min, β -LG in WPI was not completely hydrolyzed by trypsin and chymotrypsin even at an enzyme-to-substrate ratio of 1:100 for 1 h, whereas α -LA was completely hydrolyzed to smaller peptides (Fig. 1A, Lanes 7 and 10). On the other hand, thermolysin was very effective in hydrolyzing both β -LG and α -LA in native WPI to about 3–12 kDa peptides even at an enzyme-to-substrate ratio of 1:2000 for 5 min (Fig. 1A, lane 2). WPI was completely hydrolyzed to small peptides at a thermolysin-to-substrate weight ratio of 1:400, as is evident from faint staining of bands in the SDS-PAGE profile (Fig. 1A, lane 4). Extensive hydrolysis of WPI may release significant amounts of short peptides with no glutamine residue. Such peptides would not participate in TGase-mediated repolymerization reaction. Thus, limited proteolysis that results in release of peptides in the

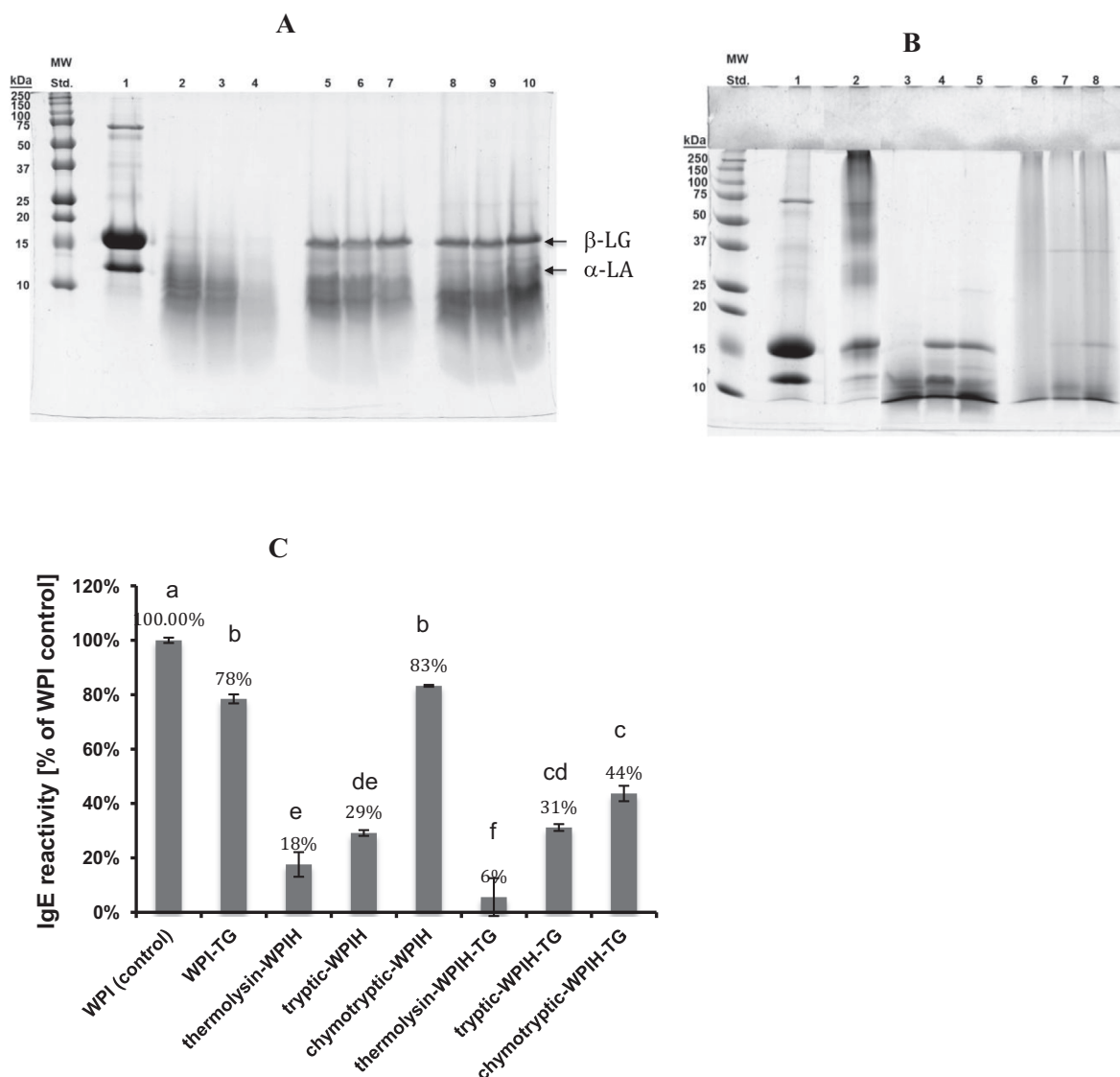


Fig. 1. (A) Tricine-SDS-PAGE of WPI and WPI hydrolysates produced under various substrate-to-enzyme ratios and hydrolysis times: Lane 1, WPI control; Lane 2, WPI + thermolysin, 2000:1, 5 min; Lane 3, WPI + thermolysin, 1500:1, 5 min; Lane 4, WPI + thermolysin, 400:1, 5 min; Lane 5, WPI + trypsin, 200:1, 30 min; Lane 6, WPI + trypsin, 100:1, 30 min; Lane 7, WPI + trypsin, 100:1, 60 min; Lane 8, WPI + chymotrypsin, 200:1, 30 min; Lane 9, WPI + chymotrypsin, 100:1, 30 min; Lane 10, WPI + chymotrypsin, 100:1, 60 min. (B) SDS-PAGE of WPI, WPI hydrolysates produced under specified optimum substrate-to-enzyme ratios and hydrolysis times, and WPI hydrolysates repolymerized using TGase. Lane 1, WPI control; Lane 2, WPI + TGase; Lane 3, WPI + thermolysin, 1500:1, 5 min; Lane 4, WPI + trypsin, 100:1, 30 min; Lane 5, WPI + chymotrypsin, 100:1, 30 min; Lane 6, WPI + thermolysin + TGase; Lane 7, WPI + trypsin + TGase; Lane 8, WPI + chymotrypsin + TGase. (C) IgE reactivity of various enzyme-treated WPI products expressed on the basis of the immune-reactivity of WPI control. Statistical analysis was performed using Tukey HSD test. a–e: Mean values of columns with different letters imply that they are significantly different ($p > 0.05$).

MW range of 3–10 kDa is desirable to increase the yield of cross-linked polymers during TGase-catalyzed repolymerization of hydrolysates. Accordingly, the following hydrolysis conditions were selected for the three proteases: An enzyme-to-substrate ratio of 1:1500 and 5 min was used for thermolysin and an enzyme-to-substrate ratio of 1:100 and 30 min was used for trypsin and chymotrypsin.

3.2. Immuno-reactivity of WPI hydrolysates and their re-polymerized products

The native WPI and hydrolyzed WPI formed large polymers when treated with TGase (Fig. 1B). The polymerized native WPI showed new smeared bands in the molecular weight range of ~30 to ~400 kDa (Fig. 1B, lane 2). However, some (~10%) monomeric β -LG and α -LA were present in the polymerized sample even after 24 h of treatment with TGase. A similar observation had been made in previous studies (Han & Damodaran, 1996; Li & Damodaran, 2016). In the case of hydrolysates, thermolysin was very effective in hydrolyzing β -LG and α -LA (Fig. 1B, lane 3), whereas a small amount of intact β -LG and α -LA was left unhydrolyzed in tryptic and chymotryptic hydrolysates (Fig. 1B, lanes 4 and 5). When these hydrolysates were treated with TGase, the intensities of residual β -LG and α -LA bands were greatly reduced, indicating that they along with other peptides in the hydrolysates were copolymerized to high molecular weight polymers by TGase (Fig. 1B, lanes 7 and 8); however, it has to be noted that some residual amount of β -LG was still present along with appearance of a β -LG dimer band (Fig. 1B, lanes 7 and 8). The molecular weight of the heterogeneous cross-linked polymers ranged from approximately 6 kDa to well over 400 kDa as evident from the continuous smeared bands in SDS-PAGE. This wide molecular size distribution might be the result of limited availability of glutamine and lysine residues in peptides of the hydrolysates. Whey proteins contain a low glutamine-to-lysine ratio. For instance, β -LG contains 10 glutamine and 15 lysine residues, whereas α -LA contains 5 glutamine and 12 lysine residues. As a result, depending on their distribution in the primary sequence and the sites of cleavage by the proteases, it is theoretically possible that some of the peptides released in the hydrolysates might be devoid of glutamine residues. It would not be possible for such peptides to take part in the TGase-catalyzed polymerization reaction. The low molecular weight polymers in Fig. 1B, Lanes 6–8 might be such polypeptides.

The immuno-reactivity of β -LG in WPI and its fragments containing allergenic epitopes in various enzyme treated WPI samples is shown in Fig. 1C. TGase polymerization of native WPI (WPI-TG) decreased its immuno-reactivity to 78% of that of native WPI control. Since according to the SDS-PAGE profile only a small amount (~10%) of intact β -LG was present in the WPI-TG sample (Fig. 1B, lane 2), only a 22% reduction in immuno-reactivity of the WPI-TG polymers suggests that most of the antigenic epitopes of native β -LG were not disrupted during the polymerization reaction and they were still easily accessible to antibody binding. Among the WPI hydrolysates, the immuno-reactivity of the chymotryptic hydrolysate (chymotryptic-WPIH) was 83% and that of the tryptic-WPIH was about 29% of the WPI control.

The high immune-reactivity of chymotryptic-WPIH cannot be totally due to residual β -LG in the sample, because this residual amount was less than 10% of the control WPI whereas the immuno-reactivity of the sample was 83% (Fig. 1B, lanes 1 and 5). This is also true of the tryptic-WPIH. Therefore, these greater than expected of tryptic and chymotryptic hydrolysates might be due to presence of intact linear epitopes in the peptide fragments, especially in chymotryptic-WPIH. On the other hand, the thermolysin hydrolysate (thermolysin-WPIH) exhibited the lowest (18%) immuno-reactivity, suggesting that among the three pro-

teases thermolysin was more effective in disrupting allergenic epitopes in WPI. Miller, Meredith, Selo, and Wal (1999) reported that the IgE binding epitopes of β -LG are found in segments 21–40, 41–60, 107–117, and 148–162 of the protein. The amino acid sequences of these segments are SLAMAASDISLLDAQSAPLR, VYVEELKPTPEGDLEILLQK, MENSAEPEQS, and RLSFNPTQLEEQCHI, respectively. It should be noted that the interior parts of segments 21–40, 107–117, and 148–162 do not contain lysine, arginine, tyrosine, tryptophan, and phenylalanine residues and therefore it is more likely that these IgE binding epitopes might remain intact in a partially hydrolyzed tryptic-WPIH and chymotryptic-WPIH hydrolysates. On the other hand, however, since thermolysin cleaves on the N-side of peptide bonds of hydrophobic residues, the segments 21–40, 41–60, and 148–162, which contain several hydrophobic residues, would be disrupted by thermolysin hydrolysis and thus peptides containing these allergenic epitopes would not be present in the thermolysin-WPIH hydrolysate. The low immuno-reactivity of thermolysin-WPIH compared to tryptic-WPIH and chymotryptic-WPIH hydrolysates (Fig. 1C) substantiates these arguments.

Upon re-polymerization using TGase, the immuno-reactivity of the TG polymers of thermolysin WPI hydrolysate (thermolysin-WPIH-TG) decreased from 18% to 6%, and that of the TG polymers of chymotryptic WPI hydrolysate (chymotryptic-WPIH-TG) decreased from 83% to 44%, indicating that the immuno-reactive epitopes were further disrupted and/or have become masked by the cross-linking reaction. In contrast, re-polymerization of tryptic hydrolysate (tryptic-WPIH-TG) did not significantly change its immuno-reactivity, which remained at 29–31%. (Fig. 1C), suggesting that the configuration of the branched-chain polymers in tryptic-WPIH-TG was different from those of the thermolysin-WPIH-TG and chymotryptic-WPIH-TG polymers and the epitopes were as easily accessible in the re-polymerized sample as in the hydrolysate to antibody binding. Since trypsin cleaves proteins at the C-end of lysine (and arginine) residues, lysine would be at the C-terminal of the peptides in the hydrolysate. As a result, when treated with TGase, the cross-linking would occur between the amine group of C-terminal (exo) lysine residues (or the N-terminal amino group of peptides) and the amide group of *endo*-glutamine residues, resulting in an *exo*-*endo* type only cross-linking between peptides. In contrast, in the cases of chymotryptic and thermolysin hydrolysates, which do not cleave at lysine and glutamine residues, *endo*-*endo* type would be the major cross-linking between *endo*-lysine and *endo*-glutamine residues along with some *exo*-*endo* cross-linking between *exo* N-terminal amine and *endo* glutamine residues of peptides. These differences in the mode of cross-linking might create branched-chain polymer structures in which accessibility of allergenic epitopes to antibody binding may vary. Nevertheless, the data in Fig. 1C clearly indicate that initial hydrolysis of WPI with thermolysin, followed by repolymerization of the hydrolysate with TGase drastically reduces the immuno-reactivity of the neo-protein polymers.

3.3. Immuno-reactivity of in vitro digested TG-polymers of WPI hydrolysate

Since trypsin is site specific for lysine and arginine residues, and chymotrypsin is specific for tryptophan, tyrosine, and phenylalanine, the repolymerized tryptic-WPIH-TG and chymotryptic-WPIH-TG samples would not contain cleavage sites for trypsin and chymotrypsin, respectively, during the duodenal phase of digestion. Therefore, we limited our studies only to examining the immuno-reactivity of the thermolysin-WPIH-TG polymers.

The time course of hydrolysis of native WPI and thermolysin-WPIH-TG polymers under simulated gastric and duodenal conditions is shown in Fig. 2. The native WPI was rapidly hydrolyzed

to small peptides with only a small amount of β -LG left undigested after the first 60 min of gastric phase digestion. The digest contained a ~ 10 kDa polypeptide as the major component (Fig. 2A). Digestion of polypeptides continued during the duodenal phase digestion, however this ~ 10 kDa polypeptide appeared to be resistant to digestion, as evidenced from its very slow disappearance during 180 min of duodenal phase digestion. In contrast, although most of the thermolysin-WPIH-TG polymers were digested during 60 min of gastric phase digestion, the digest still contained some

high molecular weight polymers, as evidenced from a smeared band in the molecular weight range of 5–100 kDa after 60 min of gastric digestion (Fig. 2B). During the duodenal phase of digestion, these remaining high molecular weight polymers were very slowly digested down to lower molecular weight polymers. However, a smeared band in the range of 5–10 kDa molecular weight was still present even after 180 min of hydrolysis (Fig. 2B). These seemingly un-digestible peptides might be remnants of branched-chain polymers that could not be further digested because of blocked lysine

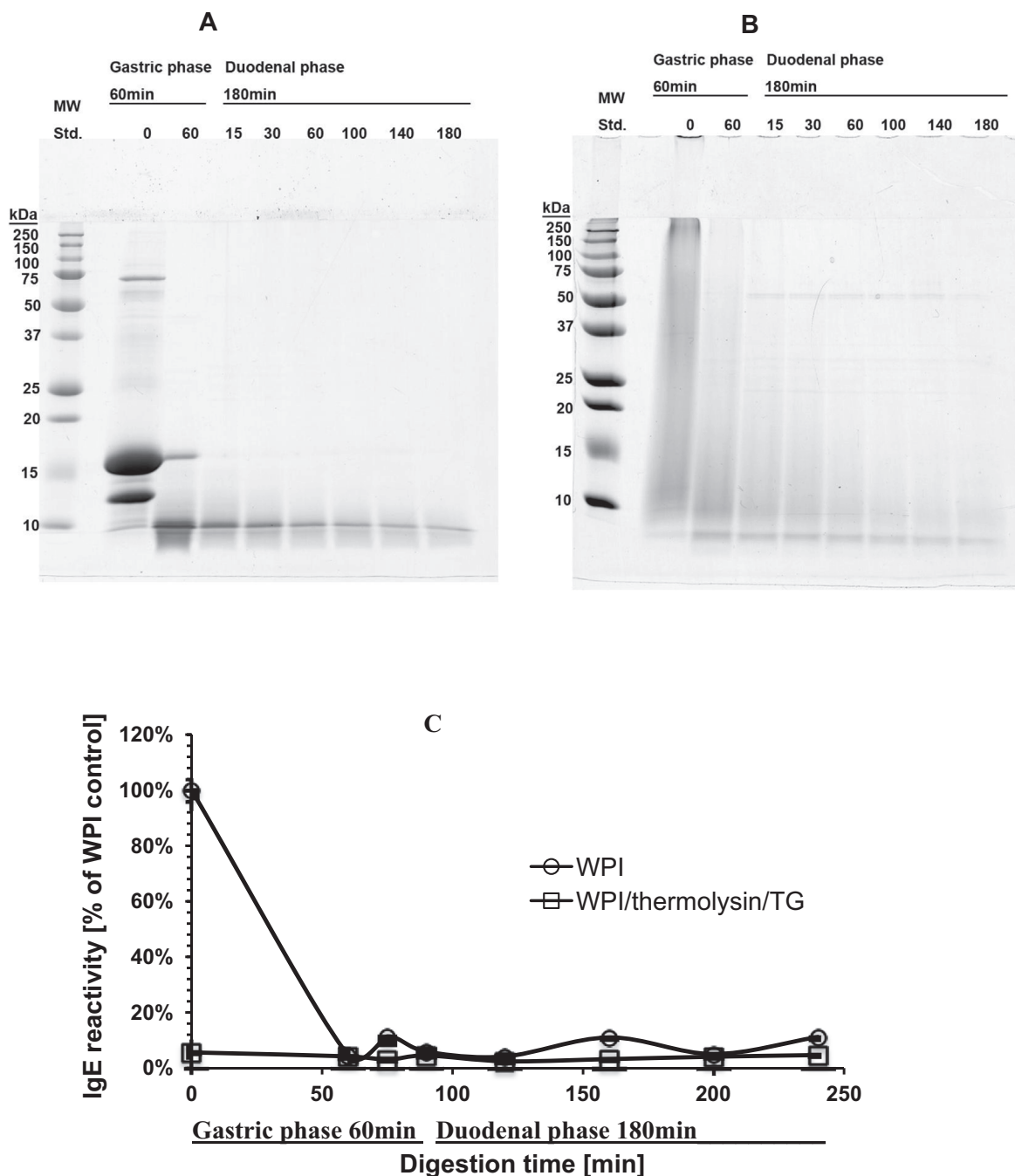


Fig. 2. (A) SDS-PAGE profiles of peptides released from native WPI during time course of *in vitro* digestion under simulated gastric and duodenal digestion conditions. The gastric digestion was performed for 60 min, followed by 180 min of duodenal phase digestion. (B) SDS-PAGE profiles of peptides released from thermolysin-WPIH-TG polymers during time course of *in vitro* digestion under simulated gastric and duodenal digestion conditions. (C) Immuno-reactivity of peptides released during time course of simulated gastric and duodenal phase digestion of thermolysin-WPIH-TG polymers.

residues and/or inaccessibility of the remaining peptide bonds to proteases and peptidases of pancreatin due to steric constraints imposed by branched chains.

The immuno-reactivity of peptides released during the time course of *in vitro* digestion is shown in Fig. 2C. While the immuno-reactivity of the native WPI digests exhibited a sinusoidal pattern, i.e., rising and falling with digestion time, within the range of 5%–20% reactivity, the immuno-reactivity of the thermolysin-WPIH-TG polymer digest was constant at ~5% throughout the

240 min digestion period, indicating that gastro-duodenal digestion of thermolysin-WPIH-TG polymers did not release any immune reactive peptides. Previously, it has been shown that when WPI-TG polymers (i.e., intact WPI polymerized with TGase) were subjected to simulated gastro-duodenal digestion, its digest also exhibited a sinusoidal immuno-reactivity pattern similar to native WPI ranging from 5% to 20% as a function of digestion time (Li & Damodaran, 2016). The absence of this pattern with the thermolysin-WPIH-TG polymers indicates that all potential linear

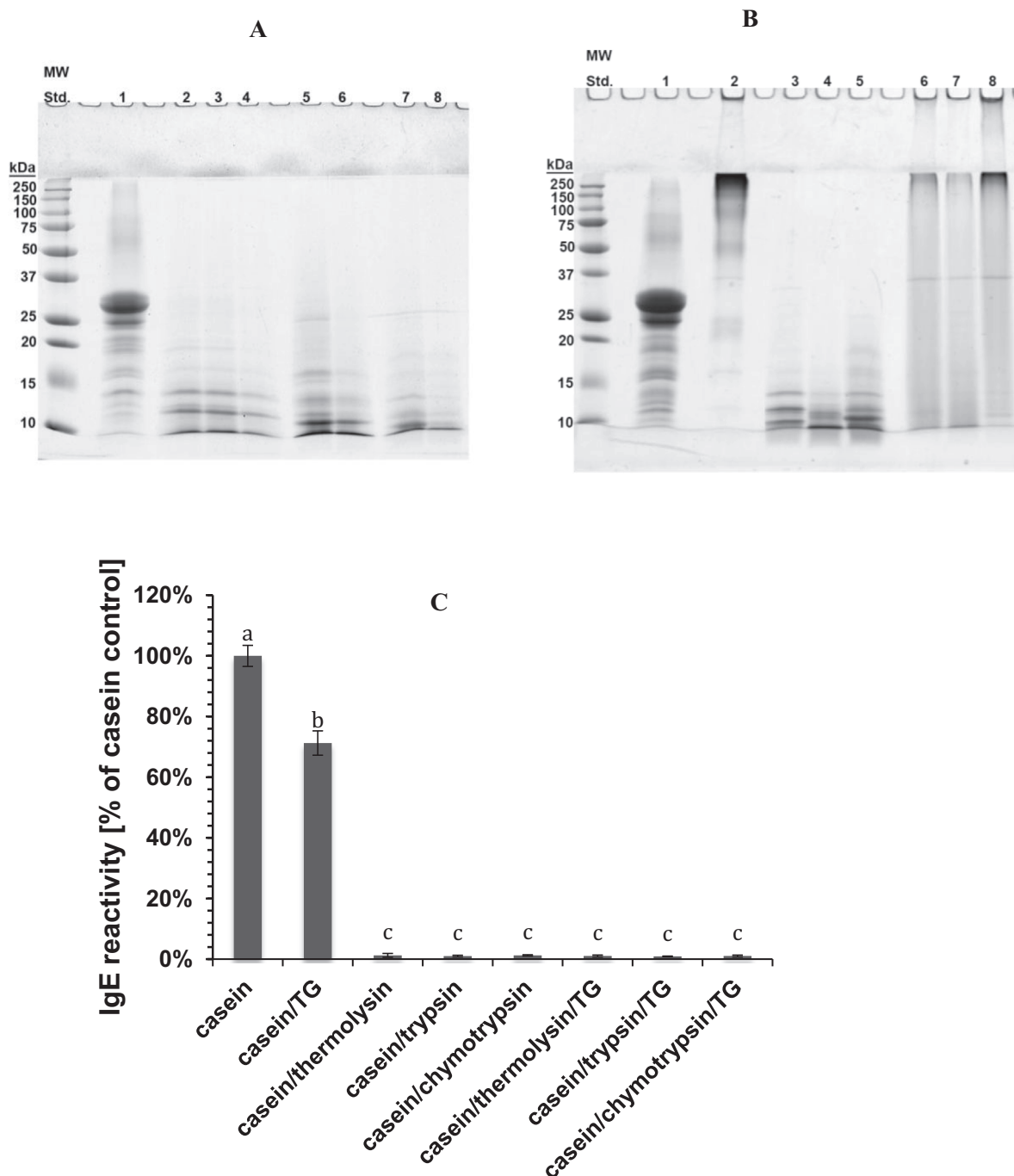


Fig. 3. (A) SDS-PAGE of casein and casein hydrolysates produced under various substrate-to-enzyme ratios and hydrolysis times: Lane 1, Casein control; Lane 2, casein + thermolysin, 2000:1, 5 min; Lane 3, casein + thermolysin, 1500:1, 5 min; Lane 4, casein + thermolysin, 800:1, 5 min; Lane 5, casein + trypsin, 400:1, 10 min; Lane 6, casein + trypsin, 200:1, 10 min; Lane 7, casein + chymotrypsin, 200:1, 10 min; Lane 8, casein + chymotrypsin, 100:1, 10 min. (B) SDS-PAGE of casein, casein hydrolysates produced under specified optimum substrate-to-enzyme ratios and hydrolysis times, and casein hydrolysates repolymerized using TGase. Lanes 1, casein control; Lane 2, casein + TGase; Lane 3, casein + thermolysin, 1500:1, 5 min; Lane 4, casein + trypsin, 400:1, 10 min; Lane 5, 5% casein + chymotrypsin, 200:1, 10 min; Lane 6, casein + thermolysin + TGase; Lane 7, casein + trypsin + TGase; Lane 8, casein + chymotrypsin + TGase. (C) Immuno-reactivity of enzyme-treated caseinate products. Statistical analysis was performed using Tukey HSD test. a–c: Mean values of columns with different letters imply that they are statistically significantly different ($p > 0.05$).

antigenic epitopes were effectively eliminated/disrupted in this two-step enzymatically modified WPI. The allergenicity of WPI and partially hydrolyzed WPI often observed in patients might be due to this residual 20% immuno-reactivity of the digested products, and this level of immuno-reactivity might be sufficient enough to trigger a strong gut immune response. However, it is possible that the low (<5%) immuno-reactivity of thermolysin-

WPIH-TG polymers digests might not elicit a strong response from the gut immune system of patients allergic to milk proteins.

3.4. Casein hydrolysate and its re-polymerized products

Caseins are highly flexible proteins and, as a result, are readily digested by proteases. Therefore, optimization of partial hydrolysis

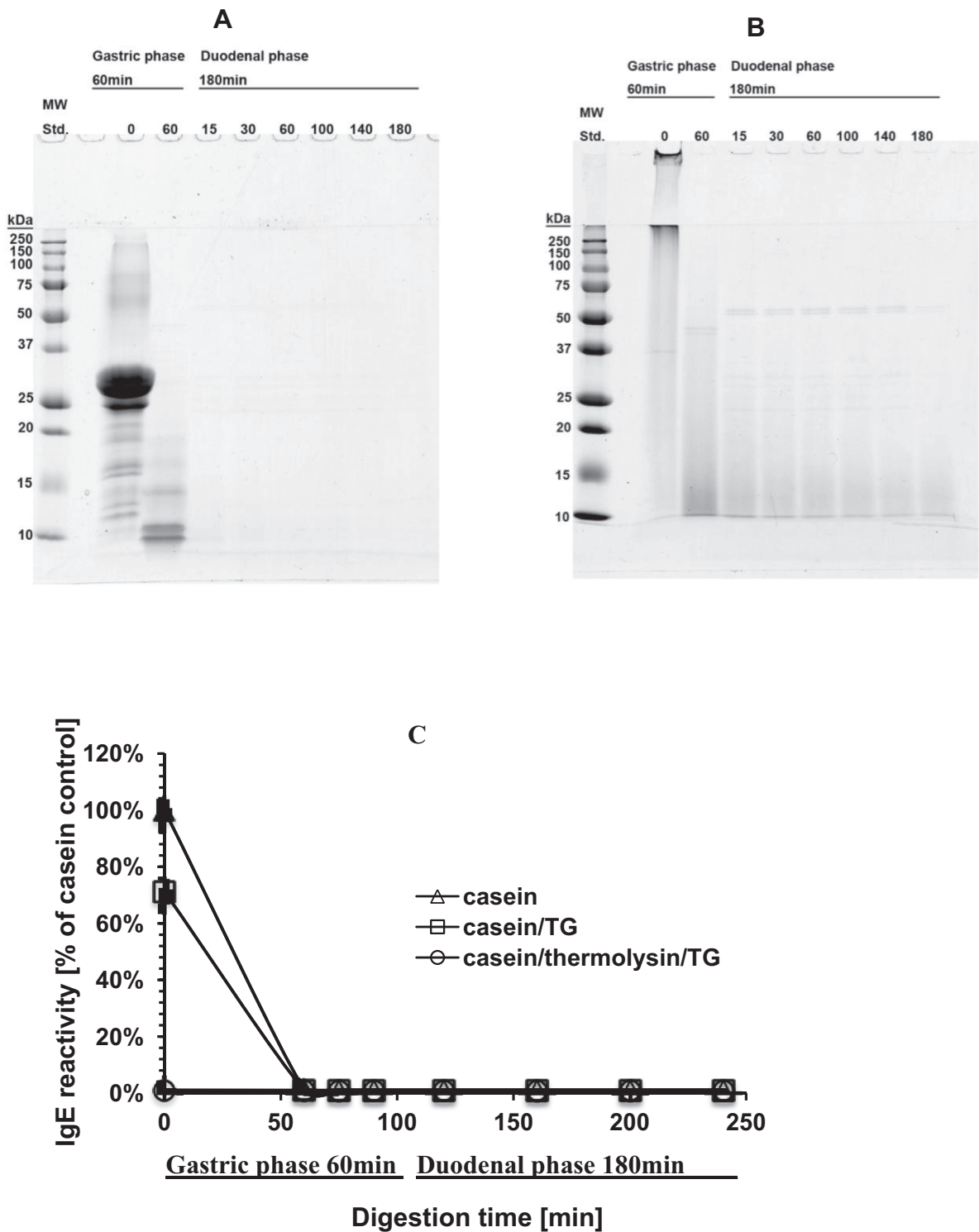


Fig. 4. (A) SDS-PAGE of peptides released from native casein during time course of *in vitro* digestion under simulated gastric and duodenal digestion conditions. The gastric digestion was performed for 60 min, followed by 180 min of duodenal phase digestion. (B) SDS-PAGE of peptides released from thermolysin-CNH-TG polymers during time course of *in vitro* digestion under simulated gastric and duodenal digestion conditions. (C) Immuno-reactivity of peptides released during time course of simulated gastric and duodenal phase digestion of thermolysin-CNH-TG polymers.

conditions to produce peptide fragments in the MW range of 3–10 kDa was necessary for the subsequent TGase-catalyzed repolymerization reaction. Fig. 3A shows the SDS-PAGE profile of casein digested with thermolysin, trypsin, and chymotrypsin at various enzyme-to-substrate ratios and hydrolysis times. Among the three thermolysin hydrolysates, those corresponding to 1:2000 and 1:1500 enzyme-to-substrate ratios showed no noticeable difference in the SDS-PAGE profile, while the 1:800 sample was over hydrolyzed as seen from the reduced intensity of the coomassie blue staining. To choose between 1:2000 and 1:1500 enzyme-to-substrate ratios, the Immuno-reactivity of both these hydrolysates were tested using casein-specific ELISA. The result showed that both these samples had very low Immuno-reactivity (data not shown). However, the one hydrolyzed at 1:1500 enzyme-to-substrate ratio had lower activity than the one at 1:2000 enzyme-to-substrate ratio. Therefore, the 1:1500 enzyme-to-substrate ratio was chosen for subsequent steps. Similarly, the 1:400 enzyme-to-substrate ratio/10 min and the 1:200 enzyme-to-substrate ratio/10 min hydrolysis conditions were chosen as optimum conditions for tryptic and chymotryptic digestions, respectively.

The SDS-PAGE profiles of thermolysin, tryptic, and chymotryptic hydrolysates of casein (CNH) and their repolymerized polymers (CNH-TG) are shown in Fig. 3B. Under the hydrolysis condition used, the molecular weights of peptides in all the three hydrolysates were in the range of about 10–20 kDa (Fig. 3B, Lanes 3–5). Upon treatment with TGase, casein and its hydrolysates were able to form large MW polymers (Fig. 3B, Lanes 2, 6–8). Some of these polymers could not pass through the stacking and separating gels. In the cases of repolymerized hydrolysates, the smeared bands from 10 kDa to top of the separating and stacking gels (Fig. 3B, lanes 6–8) indicated that they contained a heterogeneous mixture of cross-linked polymer with wide ranging molecular weights. As in the case of WPIH-TG polymers (Fig. 1B, Lanes 6–8), this wide molecular size distribution might be the result of limited availability of glutamine and lysine residues in peptides of the hydrolysates. Caseins contain approximately equal amount of glutamine and lysine residues. However, depending on their distribution in the primary structure and the sites of cleavage by the proteases, it is likely that some of the peptides in the hydrolysate might not contain or contain only a limited number of glutamine residues, which would limit their participation in the polymerization reaction. The wide molecular size distribution of CN-TG polymers (Fig. 3B, lanes 6–8) might be a reflection of this phenomenon.

3.5. Immuno-reactivity of casein hydrolysates and their TG-polymers

Polymerization of intact casein (CN) using TGase decreased its immuno-reactivity to about 70% of the control (Fig. 3C), indicating that a majority of antigenic epitopes (both conformational and linear epitopes) was still accessible to antibody binding in CN-TG polymers. On the other hand, the thermolysin, tryptic, and chymotryptic hydrolysates of CN showed no Immuno-reactivity. In addition, the TG-polymers of these CN hydrolysates (CNH-TG) also showed no immuno-reactivity, indicating that almost all antigenic epitopes in casein were disrupted by hydrolysis; repolymerization of the peptides in the hydrolysate to large MW polymers did not result in reformation of any conformational epitopes.

Fig. 4A and B shows the digestibility of native CN and thermolysin-CNH-TG polymers, respectively, in the two-step simulated gastric and duodenal phase digestion system. Native CN was readily digested to 10–15 kDa peptides during the gastric phase digestion and these peptides disappeared during the duodenal phase digestion. In the case of thermolysin-CNH-TG polymers, all the high MW polymers that could not enter the stacking and separating gels were digested completely to <60 kDa polypeptides during the 60 min gastric phase digestion. These polypeptides

were further digested to <15 kDa peptides during the 180 min duodenal phase digestion. However, as in the case of WPI, these <15 kDa polypeptides appeared to be resistant to further break down to dipeptides and amino acids even after 180 min of duodenal phase digestion, indicating that these were the remnants of branched-chain polymers that no site-specific enzyme and endo- and exo-peptidases in pancreatin could digest. It is possible that a longer than 180 min incubation time with pancreatin could have further digested these limit polypeptides. However, since the gastro-intestinal transit time of food after intake is typically 3–4 h, it is unlikely that these limit polypeptides would be digested under normal *in vivo* conditions.

The immuno-reactivity of peptides released during the time course of *in vitro* digestion of thermolysin-CNH-TG polymers is shown in Fig. 4C. The immuno-reactivity of digested native casein and the thermolysin-CNH-TG polymers was almost zero throughout the 240 min digestion period, indicating that gastro-duodenal digestion of thermolysin-CNH-TG polymers did not release any antigenic peptides.

4. Conclusion

Both caseins and whey proteins are known to be strongly allergenic (Baudon, Mougenot, & Didry, 1987; Docena, Fernandez, Chirido, & Fossati, 1996). However, casein hydrolysate has been found to be safe in infants allergic to cow's milk (Sampson, Bemhisel-Broadbent, Yang, & Scanlon, 1991). However, partially hydrolyzed whey protein has been shown to trigger allergic symptoms in children with cow's milk allergy (Ragno et al., 1993). Those findings tentatively suggested that allergenicity of hydrolyzed milk proteins essentially stemmed from partially hydrolyzed whey protein components. The results of the current study, which is based on ELISA, agree with results of those earlier *in vivo* studies.

The results of the present study also strongly indicate that the immuno-reactivity of hydrolyzed milk proteins, viz., whey proteins and casein, can be significantly reduced and/or eliminated using a two-step enzymatic process, namely initial partial hydrolysis using thermolysin, trypsin, or chymotrypsin, followed by repolymerization of the hydrolysate using TGase. Among the proteases, thermolysin is the most effective in disrupting allergenic epitopes in whey proteins. Although the immuno-reactivity of all WPI hydrolysates decreased further upon repolymerization, the immuno-reactivity of thermolysin-WPIH-TG polymers was the lowest (~5% of the control WPI), whereas the immuno-reactivity of tryptic-WPIH-TG and chymotryptic-WPIH-TG polymers were at unacceptable levels of 35% and 45%, respectively. Likewise, the thermolysin-CN-TG polymers also did not have any immuno-reactivity. In addition, digestion of the repolymerized CN and WPI hydrolysates under simulated gastro-duodenal conditions did not release immune reactive peptides during the 240 min time course of digestion. This two-step enzymatic modification method might not only produce hypoallergenic protein polymers, but also help in alleviating undesirable sensory properties, e.g., bitter flavor, associated with protein hydrolysates.

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