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Physicochemical and Nitrogen Solubility Properties of *Bacillus* Proteinase Hydrolysates of Sodium Caseinate Incubated with Transglutaminase Pre- and Post-hydrolysis

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Sodium caseinate (NaCN), hydrolyzed with Protamex, a *Bacillus* proteinase preparation, to 0.5, 1.3, and 17.5% degrees of hydrolysis, was incubated with transglutaminase (TGase) for 3, 42, and 290 min at enzyme/substrate ratios of 1, 1, and 10% (w/w), respectively, pre- and post-hydrolysis. The electrophoretic, reversed-phase high-performance liquid chromatography (RP-HPLC) and nitrogen solubility profiles of the modified products were investigated. Combinations of hydrolysis and incubation with TGase generated products displaying novel physicochemical and nitrogen solubility properties. Significant changes in sodium dodecyl sulfate (SDS) and urea polyacrylamide gel electrophoresis profiles were apparent in the modified caseinate samples. Extensive TGase cross-linking resulted in polymers that were unable to enter the resolving gel during SDS polyacrylamide gradient gel electrophoresis. Extensive combined enzymatic modification resulted in peptides eluting earlier on RP-HPLC than limited combined enzymatic modification or limited hydrolysis. Combination of enzymatic treatments resulted in significantly ($P < 0.005$) improved solubility around pH 4.6, compared to incubation with TGase or hydrolysis of NaCN alone.

KEYWORDS: Transglutaminase; casein hydrolysates; nitrogen solubility index

INTRODUCTION

The application of sodium caseinate (NaCN) as a functional ingredient in formulated food products such as salad dressings, cream liqueurs, and soufflés may be improved following enzymatic modification. Commercially available crude proteinase preparations (Alcalase, Neutrase, and Protamex) and pancreatic enzymes such as trypsin or individually isolated proteases from microbial sources have been used in the hydrolysis of NaCN. Hydrolysis results in a reduction in molecular mass and an increase in the number of ionizable groups (*1*) and is generally associated with improved solubility and enhanced surface activity properties (*2*−*4*).

Limited hydrolysis of sodium caseinate with Protamex, a *Bacillus* subtilisin-like proteinase (*5*), resulted in improved functional properties compared to unmodified NaCN (*6*, *7*). Furthermore, Protamex is claimed to produce nonbitter hydrolysates (*8*).

Transglutaminase (TGase; EC 2.3.2.13) may also be used to modify the functional properties of sodium caseinate. TGase (protein-glutamine:amine γ-glutamyltransferase) catalyzes acyl group transfer between the γ-carboxyamide group of peptide-bound glutamine residues (an acyl donor) and a variety of acyl acceptors. The acceptor can be the ϵ-amino group of lysine, either as free lysine or as peptide/protein bound lysine, resulting in the formation of ϵ-(γ-glutamyl)lysine isopeptide bonds. Water molecules may also act as acyl acceptors, resulting in the deamidation of glutamine to glutamic acid (*9*). Early studies on modification of the functional properties of proteins with TGase were performed with a Ca^{2+}-dependent enzyme isolated from animal or plant sources. However, the discovery and subsequent large-scale production of a Ca^{2+}-independent TGase from *Streptoverticillium* (*10*) has increased interest in TGase modification of food proteins.

Motoki, Nio, and Takinami (*11*) reported improved nitrogen solubility of α_{s1}-casein at pH 5 and 6 compared to native α_{s1}-casein on incubation with guinea pig liver TGase. Deamidation of α_{s1}-casein after citraconylation, using soluble guinea pig liver (*9*) and immobilized microbial TGases (*12*), resulted in increased solubility at pH 5 and 5.5, compared to the unmodified protein. Incubation with TGase has been reported to reduce the bitterness of proteolytic digests (*13*) and to improve the emulsifying properties (*14*−*16*) and the gel strength of milk proteins (*17*). Lorenzen (*18*) did not observe any change in the nitrogen solubility of microbial TGase-treated NaCN at pH 7; however, the water-binding capacity of NaCN was greatly improved following incubation with TGase. The heat stability of reconstituted skim milk powder (9.0% total solids) was improved between pH 6.6 and 7.3 following TGase treatment (*19*).

TGase has also been used to improve the functional properties of Pronase, papain, pepsin, and acid hydrolysates of soy protein (*20*, *21*) and gluten (*22*).

Gel filtration analysis of tryptic hydrolysates of TGase cross-linked sodium caseinate revealed the presence of nonhydroylzed

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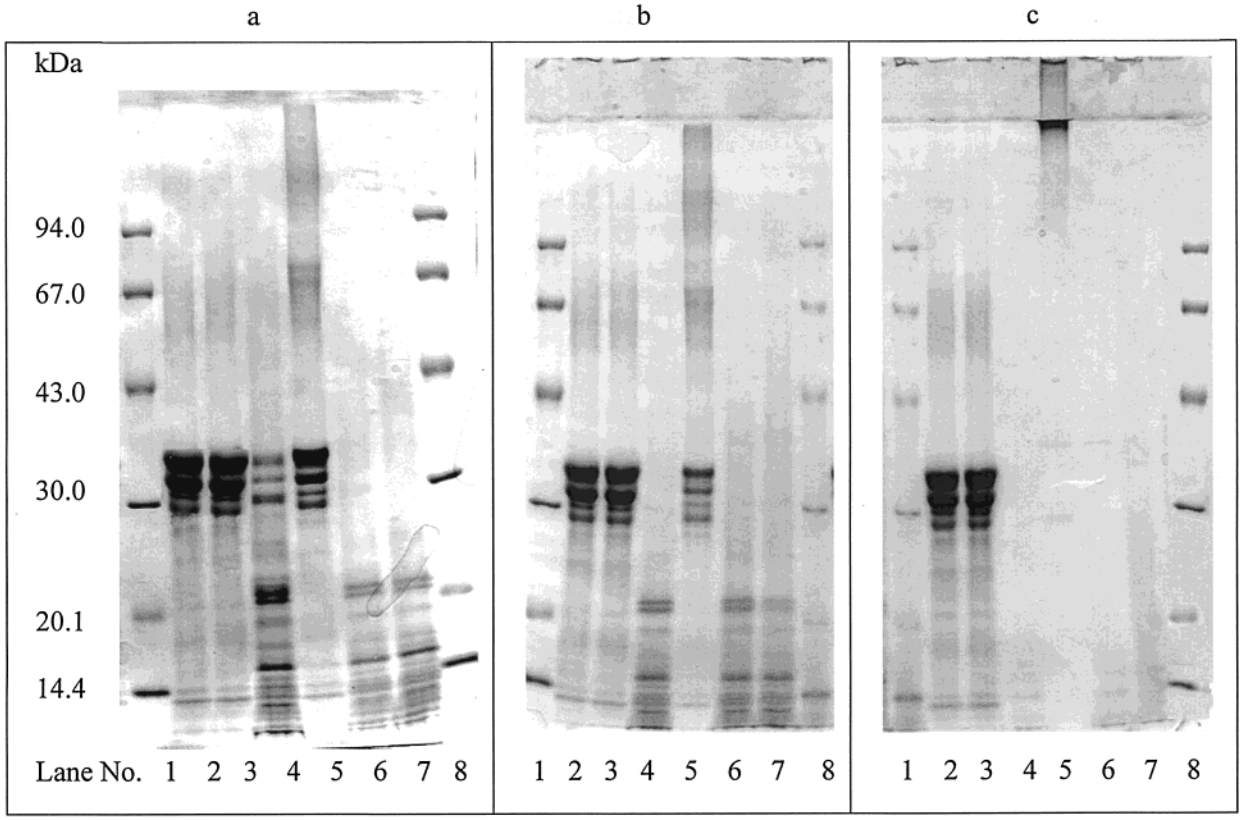

Figure 1. SDS-PAGGE profiles of enzymatically modified NaCN: (lanes 1 and 8) molecular weight markers, (lane 2) unheated NaCN, and (lane 3) heat-treated NaCN (80 °C for 20 min); (a) (lane 4) hydrolyzed with Protamex to 0.5% DH, (lane 5) cross-linked with TGase for 3 min, [E:S] 1% (w/w), (lane 6) cross-linked with TGase for 3 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 0.5% DH, and (lane 7) hydrolyzed with Protamex to 0.5% DH after cross-linking with TGase for 3 min, [E:S] 1% (w/w); (b) (lane 4) hydrolyzed with Protamex to 1.3% DH, (lane 5) cross-linked TGase for 42 min, [E:S] 1% (w/w), (lane 6) cross-linked with TGase for 42 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 1.3% DH, and (lane 7) hydrolyzed with Protamex to 1.3% DH after cross-linking with TGase for 42 min, [E:S] 1% (w/w); (c) (lane 4) hydrolyzed with Protamex to 17.5% DH, (lane 5) cross-linked with TGase for 290 min, [E:S] 10% (w/w), (lane 6) cross-linked with TGase for 290 min, [E:S] 10% (w/w) after hydrolysis with Protamex to 17.5% DH, and (lane 7) hydrolyzed with Protamex to 17.5% DH after cross-linking with TGase for 290 min, [E:S] 10% (w/w). Gels were loaded with 30 μg of protein equivalent for all samples.

and partly hydrolyzed polymers. Furthermore, the gel filtration pattern of the TGase cross-linked 120 min tryptic proteolysate of NaCN showed a very small increase in molecular size of the cross-linked peptides in relation to the non-cross-linked proteolysate (*23*). Apart from Lorenzen et al. (*23*), no research appears to have been carried out on the effects of incubation of hydrolysates with TGase pre- and post-hydrolysis on the functional properties of NaCN hydrolysates. The unique and novel peptides that may be obtained may have much improved physicochemical and functional properties. The objective of this work was to determine the physicochemical and solubility changes of *Bacillus* proteinase hydrolysates of NaCN incubated with TGase pre- and post-hydrolysis.

EXPERIMENTAL PROCEDURES

Materials. Sodium caseinate (85.35% protein) was obtained from Armor Protéines (Saint-Brice-en Coglès, France). Glycine, trizma base, sodium dodecyl sulfate (SDS), glycerol, trichloroacetic acid (TCA), ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), methylenebis(acrylamide), and acrylamide (all electrophoretic grade), Coomassie Blue G, Coomassie Brilliant Blue R-250, low and high molecular weight marker proteins for SDS−polyacrylamide gradient gel electrophoresis (PAGGE), and α-, β-, and κ-casein standards for urea-PAGE were from Sigma Chemical Co. (Poole, Dorset, U.K.). Kjeldahl catalyst tablets, urea (GPR grade), and trifluoroacetic acid (TFA) were from BDH (Leicestershire, U.K.). Acetonitrile (HPLC grade) and HPLC grade water were from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Protamex was kindly provided by Novo Nordisk (Bagsvaerd, Denmark), and calcium-independent microbial TGase from *Streptoverticillium* spp. was kindly supplied by Forum Products Ltd. (Brighton Rd., Redhill, Surrey, U.K.).

Methods. *Cross-Linking after Hydrolysis (CH).* An 8% (w/v) aqueous solution of sodium caseinate (500 mL), fully hydrated at 40 °C, was adjusted to pH 7.0. Four milliliters of a 5% (w/v) Protamex solution was added to the reaction vessel to give an enzyme/substrate ([E:S]) ratio of 0.5% (w/w). The pH was kept constant at pH 7.0 using a pH stat (718 STAT Titrino, Metrohm, Herisau, Switzerland), and the extent of the reaction was monitored by the consumption of 2.2 N NaOH. Degree of hydroylsis (DH, percent) was determined according to the method of Adler-Nissen (*24*)

$$\mathrm{DH}\ (\%) = BN_b(1/\alpha)(1/\mathrm{MP})(1/h_{tot}) \times 100$$

where B = base consumption in mL, N_b = normality of the base, $1/\alpha$ = average degree of dissociation of the α-NH_2 group, MP = mass of protein in g, and h_{tot} = total number of peptide bonds in the protein substrate (mequiv g^{-1} of protein). Values taken for $1/\alpha$ and h_{tot} were 3.00 and 8.2, respectively. When a DH value of 0.5% had been reached, the hydrolysate was held at 80 °C for 20 min to inactivate the enzyme. The hydrolysate sample was subsequently cooled to 20 °C and diluted to 4% (w/v) protein equivalent with distilled water. The hydrolysate solution was then incubated with TGase at an [E:S] of 1% (w/w) for 3 min at 20 °C, pH 7.0. TGase was inactivated by heating at 80 °C for 1 min, and 0.02% (w/v) sodium azide was added on cooling to inhibit microbial growth. This sample was referred to as cross-linked after hydrolysis and labeled CH (a). Similarly, CH (b) was obtained by

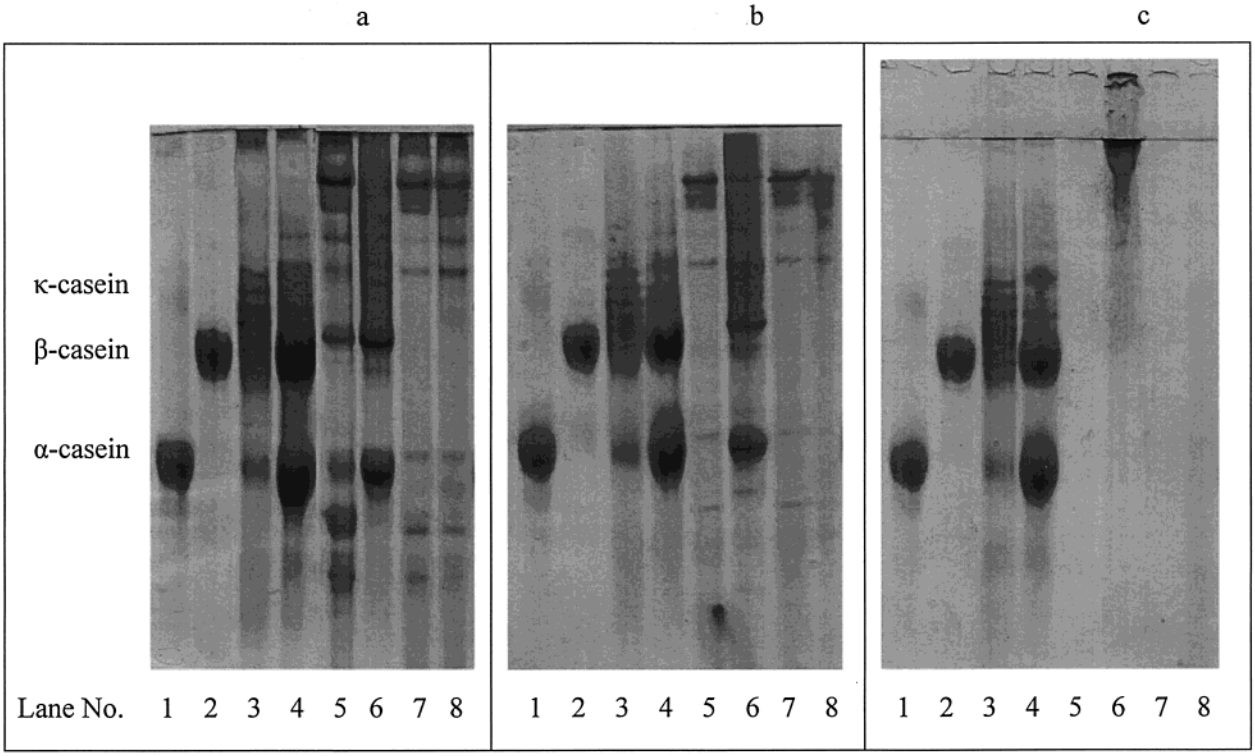

Figure 2. Urea-PAGE profiles of enzymatically modified NaCN: (lanes 1–3) α-, β-, and κ-casein, respectively, and (lane 4) unheated NaCN; (a) (lane 5) hydrolyzed with Protamex to 0.5% DH, (lane 6) cross-linked with TGase for 3 min, [E:S] 1% (w/w), (lane 7) cross-linked with TGase for 3 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 0.5% DH, and (lane 8) hydrolyzed with Protamex to 0.5% DH after cross-linking with TGase for 3 min, [E:S] 1% (w/w); (b) (lane 5) hydrolyzed with Protamex to 1.3% DH, (lane 6) cross-linked TGase for 42 min, [E:S] 1% (w/w), (lane 7) cross-linked with TGase for 42 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 1.3% DH, and (lane 8) hydrolyzed with Protamex to 1.3% DH after cross-linking with TGase for 42 min, [E:S] 1% (w/w); (c) (lane 5) hydrolyzed with Protamex to 17.5% DH, (lane 6) cross-linked with TGase for 290 min, [E:S] 10% (w/w), (lane 7) cross-linked with TGase for 290 min, [E:S] 10% (w/w) after hydrolysis with Protamex to 17.5% DH, and (lane 8) hydrolyzed with Protamex to 17.5% DH after cross-linking with TGase for 290 min, [E:S] 10% (w/w). Gels were loaded with 90, 75, 75, and 60 μg for the standards, unheated NaCN, heat-treated NaCN, and the enzymatically modified samples, respectively.

hydrolysis to 1.3% DH followed by incubation with TGase for 42 min at an [E:S] of 1% (w/w), and CH (c) was obtained by hydrolysis to 17.5% DH followed by incubation with TGase for 290 min at an [E:S] of 10% (w/w). Hydrolysate controls were obtained by hydrolysis to 0.5, 1.3, and 17.5% DH.

Hydrolysis after TGase Cross-Linking (HC). Sodium caseinate (4% (w/v) protein) was incubated with TGase at 20 °C and pH 7.0 for 3 min at an [E:S] of 1% (w/w). TGase was inactivated by heating at 80 °C for 1 min and subsequently brought to 40 °C. The TGase-treated sample was hydrolyzed with Protamex to 0.5% DH ([E:S] 0.5% (w/w)) at pH 7.0 using the pH-stat method. The hydrolysis reaction was inactivated by heating at 80 °C for 20 min, and sodium azide (0.02% w/v) was added. This sample was referred to as hydrolysis after cross-linking and labeled HC (a). Similarly, HC (b) was obtained by incubation with TGase for 42 min at an [E:S] of 1% (w/w), followed by hydrolysis with Protamex to 1.3% DH. Finally, HC (c) was obtained by incubation with TGase for 290 min at an [E:S] of 10% (w/w), followed by hydrolysis with Protamex to 17.5% DH.

Cross-linked controls were obtained by incubating 4% (w/v) NaCN solutions with TGase for 3, 42, and 290 min, at [E:S] values of 1, 1, and 10% (w/w), respectively, followed by heat inactivation at 80 °C for 1 min. These samples were referred to as CL-1, -2, and -3, respectively.

Preparation of Heat-Treated Control. A heat-treated control was prepared by holding an 8% (w/v) NaCN solution at 40 °C and pH 7.0 for 60 min followed by heating at 80 °C for 20 min, with an aliquot of water added instead of enzyme. Subsequently, 0.02% (w/v) sodium azide was added.

Solubility analyses were performed on unfrozen samples immediately. Samples for electrophoretic and reversed-phase high-performance liquid chromatographic (RP-HPLC) analyses were stored at −18 °C prior to analysis. As required, the frozen samples were thawed at room temperature. The samples were subsequently brought to 37 °C to resolubilize any precipitated material and returned to room temperature prior to analysis.

Nitrogen solubility, performed in duplicate, and RP-HPLC analyses were carried out as previously described (*7*). SDS-PAGGE was carried out on a Protean II Xi electrophoretic system in vertical slab gels, with a 4% stacking gel and 9−15% gradient separating gel, using the method of Laemmli (*25*). The SDS-PAGGE gels were stained with Coomassie Brilliant Blue R-250, (0.25% (w/v) in 10% (v/v) aqueous glacial acetic acid) for 3 h at room temperature. Destaining was performed with methanol/glacial acetic acid/water (5.0:7.5:87.5). Urea-PAGE was carried out according to the method of Andrews (*26*), with minor modifications (*7*). The urea gels were fixed in 12.5% (w/v) TCA for 10 min. Protein bands were stained for 30 min with 0.25% (w/v) aqueous Coomassie Blue G. Gels were destained in 10% (v/v) aqueous glacial acetic acid.

One-way analysis of variance (ANOVA) (using Fisher's least-squares differences as a post-hoc comparison) was performed on solubility data, comparing the control and hydrolysates with unheated sodium caseinate using SPSS, version 9.0 (*27*). A significant difference in results implies a significant difference at $P < 0.005$.

RESULTS AND DISCUSSION

Preliminary results from SDS-PAGGE patterns of sodium caseinate incubated with TGase at [E:S] values of 1, 2, 5, and 10% (w/w) for 3, 42, 110, 185, and 290 min demonstrated that cross-linking to different extents had occurred (results not shown). Subsequently, two limited levels of cross-linking ([E:S] of 1% (w/w) for 3 and 42 min) and one extensive level of

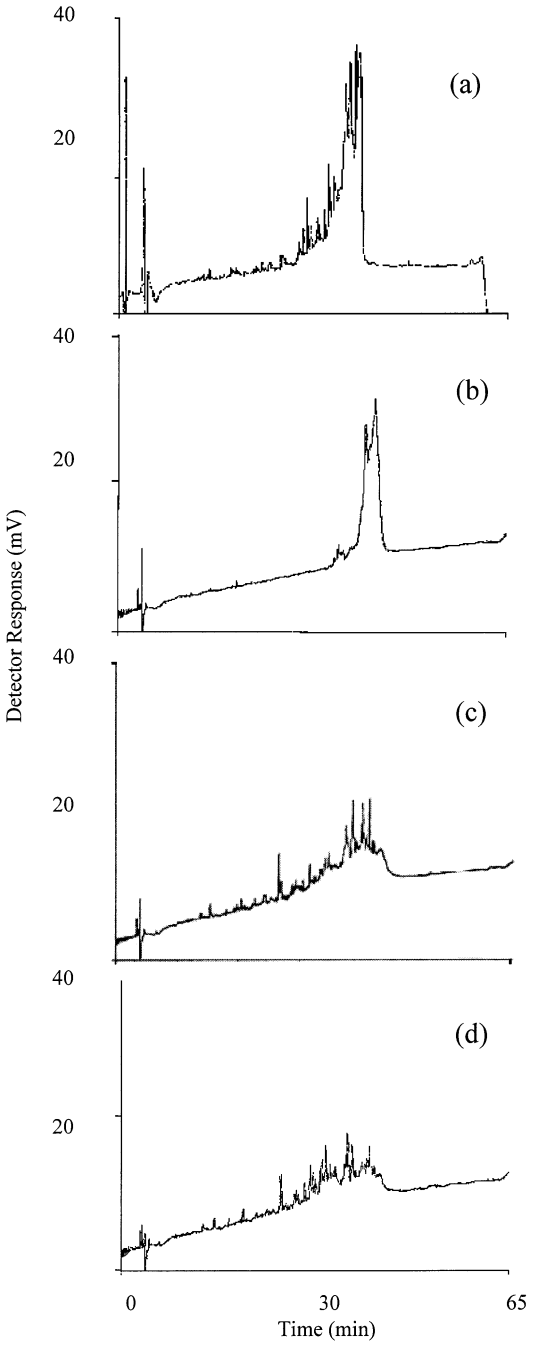

Figure 3. RP-HPLC profiles of NaCN: (a) hydrolyzed with Protamex to 0.5% DH; (b) cross-linked with TGase for 3 min, [E:S] 1% (w/w); (c) cross-linked with TGase for 3 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 0.5% DH; (d) hydrolyzed with Protamex to 0.5% DH after cross-linking with TGase for 3 min, [E:S] 1% (w/w).

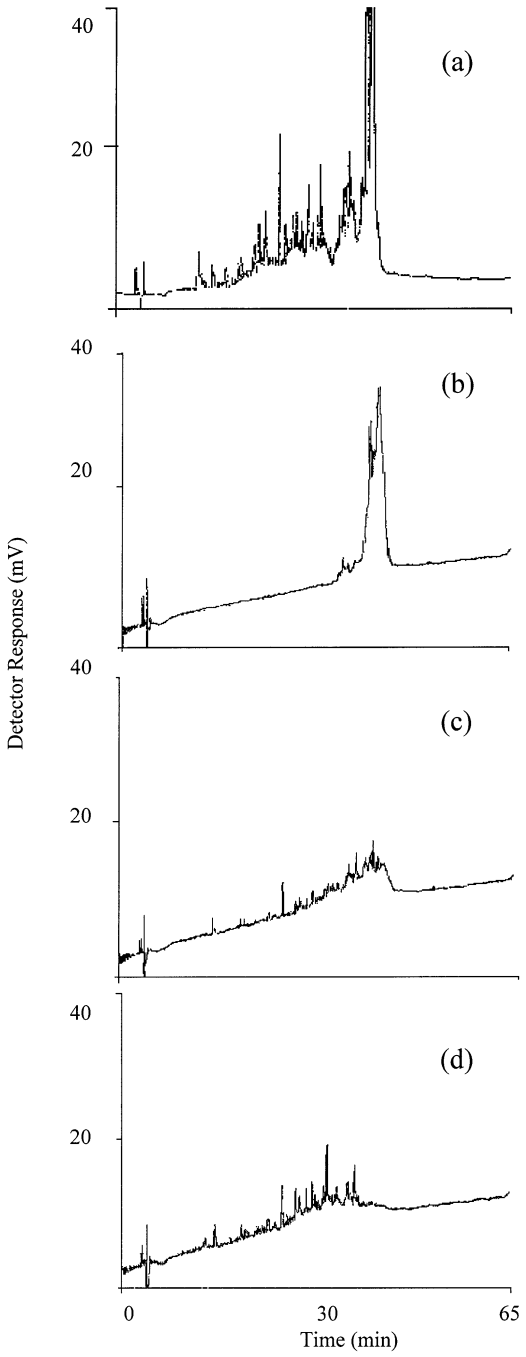

Figure 4. RP-HPLC profiles of NaCN: (a) hydrolyzed with Protamex to 1.3% DH; (b) cross-linked with TGase for 42 min, [E:S] 1% (w/w); (c) cross-linked with TGase for 42 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 1.3% DH; (d) hydrolyzed with Protamex to 1.3% DH after cross-linking with TGase for 42 min, [E:S] 1% (w/w).

cross-linking ([E:S] of 10% (w/w) for 290 min) of NaCN were chosen for further study.

Limited enzymatic hydrolysis has been shown to be more beneficial for improving functional properties of sodium caseinate compared to extensive hydrolysis (*6*). However, NaCN hydrolysates having a high DH value, incubated extensively with TGase pre- and post-hydrolysis, may also result in peptides with distinctively different functional properties. Therefore, the effects of three levels of incubation with TGase pre- and post-hydrolysis (two limited and one extensive) on the solubility and physicochemical properties of 0.5, 1.3, and 17.5% DH Protamex generated hydrolysates of NaCN were determined.

It was found that a small amount of protein precipitated on freezing, notably in the CH (c) sample, where almost 7% of the protein present precipitated on thawing at room temperature. However, after heating to 37 °C and stirring, the precipitate fully re-entered solution. The HC (c) sample did not have the characteristic milky color of the other samples, and no protein precipitation was evident after freezing.

Electrophoretic Profiles. *SDS-PAGGE.* Unheated NaCN and heat-treated NaCN eluted as four major bands around 30 kDa on SDS-PAGGE with some bands also eluting near the dye front (**Figure 1a−c**, lanes 2 and 3). Reasons for the larger than expected apparent molecular masses of the intact caseins have

previously been put forward by Creamer and Richardson (*28*). Upon hydrolysis to 0.5% DH, some breakdown of the casein bands was evident, along with the formation of a range of low molecular weight bands, most notably at 21.3, 20.5, and 16.2 kDa (**Figure 1a**, lane 4). Increasing the DH to 1.3% resulted in the complete disappearance of the casein bands, accompanied by the further breakdown of the 21.3, 20.5, and 16.2 kDa bands evident in the 0.5% DH hydrolysate (**Figure 1b**, lane 4). No bands were visible on the gel after extensive hydrolysis (17.5% DH) (**Figure 1c**, lane 4).

Incubation of NaCN with TGase resulted in the appearance of bands at 74.5 kDa in the CL-1 sample (**Figure 1a**, lane 5). Higher molecular mass cross-linked polymers were visible having molecular masses >94 kDa in the CL-2 sample (**Figure 1b**, lane 5). Extensive TGase cross-linking, as in CL-3, resulted in polymers that were unable to enter the gel (**Figure 1c**, lane 5). Increases in molecular mass upon TGase cross-linking of NaCN (*18*, *23*) and reconstituted skim milk powder (*29*) have been reported previously.

Increases in the molecular mass of cross-linked hydrolysates were not evident, which may be due to low concentrations of a variety of different cross-linked products being formed on incubation with TGase that were not detectable on SDS-PAGGE (**Figure 1a**−**c**, lane 6). These results concur with Lorenzen et al. (*23*), who reported very small increases in the molecular mass of TGase-cross-linked tryptic hydrolysates of NaCN compared to the non-cross-linked hydrolysate. TGase-catalyzed cross-linking of the hydrolysates leads to a reduction in the intensity of the bands at ~20 kDa and a disappearance of bands at ~30 kDa (**Figure 1a**, lanes 4 and 6).

Furthermore, the order of combined enzymatic treatment resulted in peptides of similar apparent molecular masses as observed on SDS-PAGGE (**Figure 1a**−**c**, lanes 6 and 7). This suggests that the formation of the ϵ-(γ-glutamyl)lysine isopeptide bond in the TGase-cross-linked samples did not subsequently sterically hinder the proteinase activity. However, Lorenzen et al. (*23*) found that gel filtration analysis of tryptic hydrolysates of TGase-cross-linked NaCN revealed the presence of some residual nonhydrolyzed and partly hydrolyzed polymers. Furthermore, Sharma et al. (29) observed a 20−30% decrease in the number of amino acids released by extensive proteolysis with various enzymes of reconstituted skim milk pretreated with 0.1% (w/w) TGase for 30 min, compared to unmodified reconstituted skim milk. This indicated that complete proteolysis of the TGase cross-linked product was not possible.

As the extent of combined enzymatic modification increased, fewer bands were evident on the SDS-PAGGE profile (**Figure 1a**−**c**, lanes 6 and 7). This may be attributed to the formation of peptides of molecular mass <10 kDa in the extensively hydrolyzed samples.

Urea-PAGE. Hydrolysis to 0.5% DH resulted in limited degradation of α-, β-, and κ-casein bands (**Figure 2a**, lane 5). The 1.3% DH hydrolysate sample exhibited extensive degradation of the α-, β-, and κ-casein bands (**Figure 2b**, lane 5). Hydrolysis to 17.5% DH resulted in complete disappearance of all bands on urea-PAGE (**Figure 2c**, lane 5). Incubation of NaCN with TGase to CL-1 resulted in a decrease in the intensity of the casein bands, with the appearance of streaking (**Figure 2a**, lane 6). Incubation of NaCN with TGase to CL-2 resulted in more extensive loss of the α-, β-, and κ-casein bands (**Figure 2b**, lane 6). However, the polymers formed from incubation of NaCN with TGase to CL-3 are again clearly evident, as they do not enter the gel (**Figure 2c**, lane 6). The CH (a) and HC (a) samples showed almost complete loss of the casein bands, with bands eluting in the proteose−peptone and γ-casein region (**Figure 2a**, lanes 7 and 8). After the combined enzymatic treatments corresponding to CH (b) and HC (b), bands were also evident in the proteose−peptone and γ-casein region (**Figure 2b**, lanes 7 and 8). The combined enzymatic treatments CH (c) and HC (c) resulted in complete disappearance of all bands on urea-PAGE (**Figure 2c**, lanes 7 and 8).

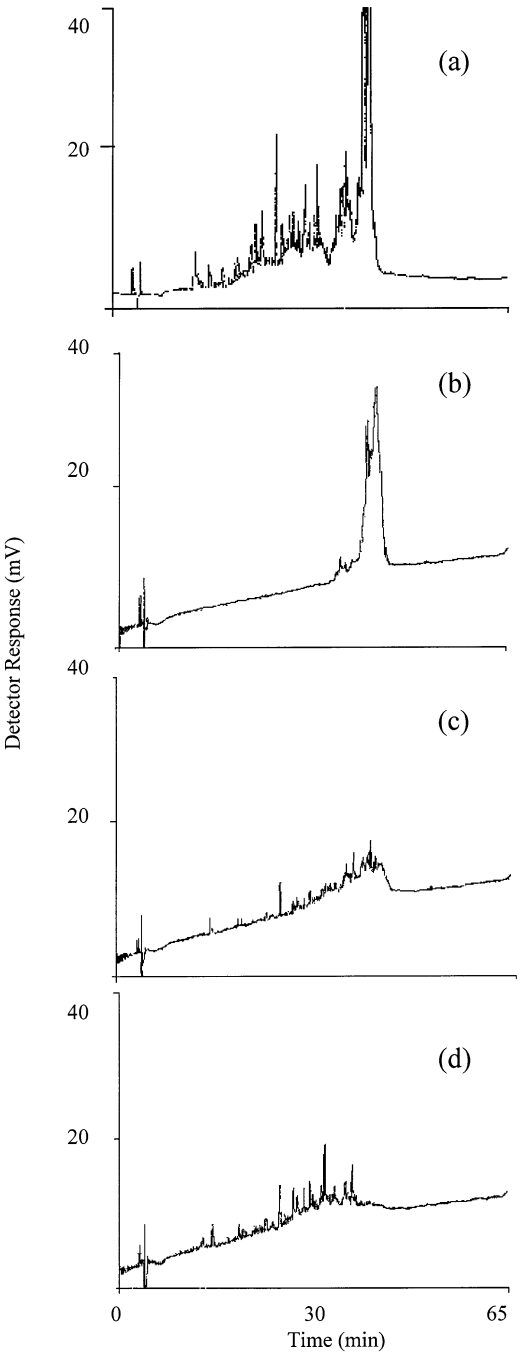

Figure 5. RP-HPLC profiles of NaCN: (a) hydrolyzed with Protamex to 17.5% DH; (b) cross-linked with TGase for 290 min, [E:S] 10% (w/w); (c) cross-linked with TGase for 290 min, [E:S] 10% (w/w) after hydrolysis with Protamex to 17.5% DH; (d) hydrolyzed with Protamex to 17.5% DH after cross-linking with TGase for 290 min, [E:S] 10% (w/w).

The similarities between the CH and HC samples on SDS-PAGGE profiles were also observed after urea-PAGE (**Figure 2a**−**c**, lanes 7 and 8). Furthermore, it appears that the ϵ-(γ-glutamyl)lysine isopeptide bond did not sterically hinder the

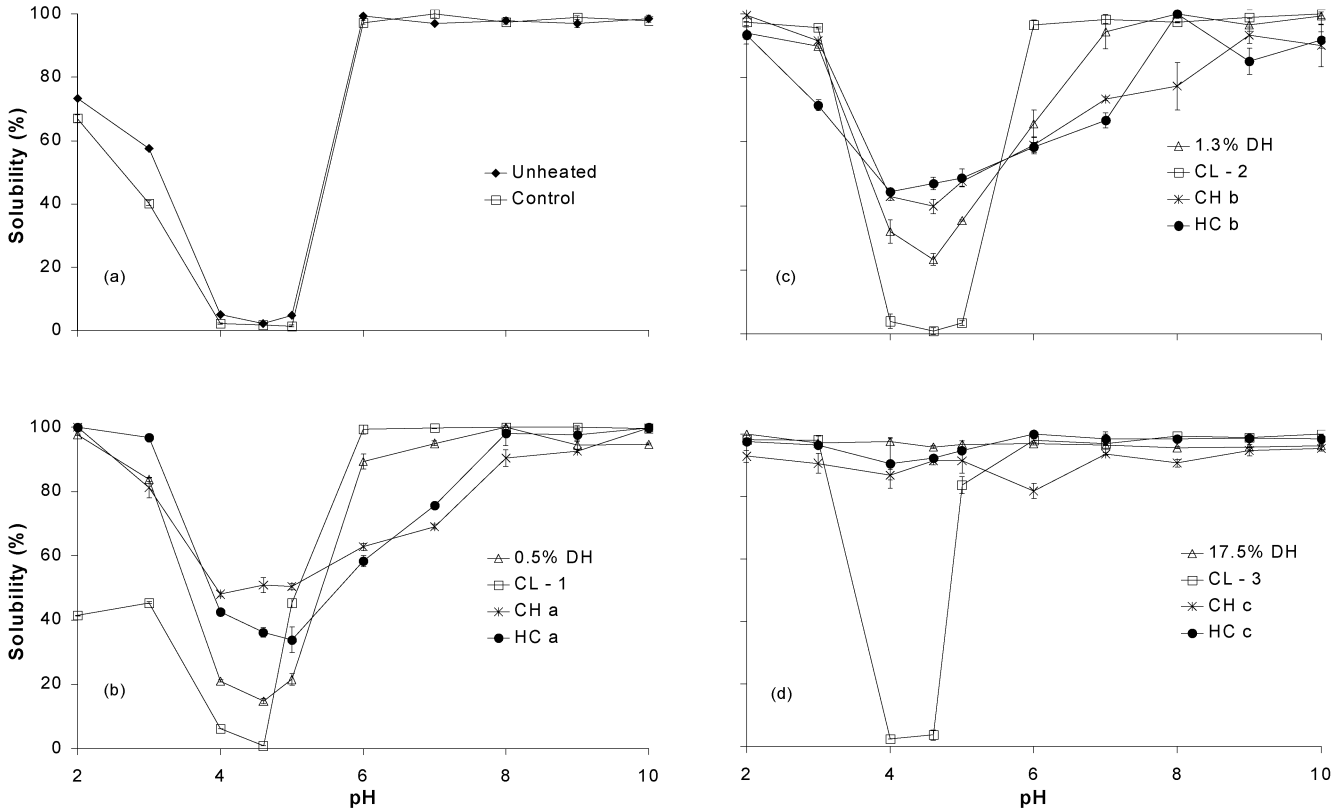

Figure 6. Effect of pH on the NSI % of NaCN: (a) heat-treated (80 °C for 20 min, □) compared to unheated NaCN (●); (b) hydrolyzed with Protamex to 0.5% DH (△), cross-linked with TGase for 3 min, [E:S] 1% (w/w) (CL-1, □), cross-linked with TGase for 3 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 0.5% DH (CH a, ∗), and hydrolyzed with Protamex to 0.5% DH after cross-linking with TGase for 3 min, [E:S] 1% (w/w) (HC a, ●); (c) hydrolyzed with Protamex to 1.3% (1.3% DH, △), cross-linked with TGase for 42 min, [E:S] 1% (w/w) (CL-2, □), cross-linked with TGase for 42 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 1.3% DH (CH b, ∗), and hydrolyzed with Protamex to 1.3% DH after cross-linking with TGase for 42 min, [E:S] 1% (w/w) (HC b, ●); (d) hydrolyzed with Protamex to 17.5% (17.5% DH, △), cross-linked with TGase for 290 min, [E:S] 10% (w/w) (CL-3, □), cross-linked with TGase for 290 min, [E:S] 10% (w/w) after hydrolysis with Protamex to 17.5% DH (CH c, ∗), and hydrolyzed with Protamex to 17.5% DH after cross-linking with TGase for 290 min, [E:S] 10% (w/w) (HC c, ●). Data points plotted are means ± SD.

proteinase activity, as complete breakdown of the TGase-polymerized protein was evident from urea-PAGE profiles (**Figure 2c**, lanes 7 and 8).

RP-HPLC. Hydrolysis of NaCN with Protamex resulted in distinctly different RP profiles for the 0.5, 1.3, and 17.5% DH samples (**Figures 3a**, **4a**, **5a**), respectively. Gallagher et al. (*30*) also observed clearly defined peak patterns for the RP-HPLC profile of whole casein following hydrolysis with a *Bacillus* proteinase. Furthermore, as the extent of hydrolysis increased from 0.5 to 17.5% DH, the peptide material eluted earlier, indicating a decrease in hydrophobicity. This agrees with previous findings where extensive hydrolysis of NaCN with Protamex resulted in an apparent decrease in hydrophobicity compared to limited hydrolysis (*7*).

The RP-HPLC profiles of CL-1 and CL-2 appeared to be very similar, with most of the material eluting between 40 and 45 min (**Figures 3b** and **4b**). The CL-3 sample also eluted in the same time interval; however, the area of the peak was much reduced. This could be due to loss of high molecular mass polymers on filtration through 0.2 μm membranes prior to HPLC analyses (**Figure 5b**).

Distinctly different peaks were obtained (**Figure 3c**,**d**) after limited combined enzymatic modification (CH a and HC a), compared to hydrolysis and incubation with TGase per se (**Figure 3a**,**b**). Furthermore, increasing the extent of hydrolysis, either pre- or postincubation with TGase, resulted in earlier eluting peaks, indicative of a decrease in hydrophobicity (**Figures 3**, **4**, and **5c**,**d**). Highly hydrophobic peptides are generally regarded as being bitter (*31*), suggesting that increasing the extent of hydrolysis either before or after incubation with TGase potentially results in the formation of less bitter peptides. In addition, the peptides in the samples that were hydrolyzed after cross-linking (HC) generally eluted earlier than the CH samples (**Figures 3**, **4**, and **5c**,**d**). Sensory evaluation of the samples in the present study was not performed due to the presence of sodium azide.

Watanabe et al. (*13*) observed decreased bitterness in papain, trypsin, and Protease P digests of skim milk after incubation with TGase. Babiker et al. (*20*) reported reduced bitterness in TGase cross-linked chymotrypsin, papain, pepsin, Pronase, and acid hydrolysate digests of soy protein, compared to the non-cross-linked hydrolysate. However, the RP-HPLC profiles of NaCN hydrolysates incubated with TGase pre- and post-hydrolysis do not appear to have been reported previously.

Nitrogen Solubility Index (NSI). The NSI values of unheated, heat-treated, and enzymatically modified NaCN are presented in **Figure 6**. The heat-treated control was found to be significantly less soluble compared to unheated NaCN at pH 2.0 and 3.0 (**Figure 6a**).

Effects of Hydrolysis. Hydrolysis with Protamex to 0.5, 1.3, and 17.5% DH resulted in significantly improved nitrogen solubility between pH 2.0 and 5.0 (**Figure 6**) compared to unheated NaCN. However, a significant decrease in solubility was observed at pH 6.0 for the 0.5 and 1.3% DH hydrolysates

compared to unheated NaCN (**Figure 6a−c**). The significantly improved solubility at the p*I* and the decrease in solubility at pH 6.0 at low DH agree with previous reports on casein hydrolysis with trypsin (*2*), *Staphylococcus aureus* V8 protease (*3*), and Protamex (*6*, *7*). Hydrolysis to 17.5% DH resulted in almost 100% nitrogen solubility over the entire pH range tested.

Effect of Incubation with TGase. The CL-1 sample exhibited significantly lower solubility at pH 2.0 and significantly higher solubility at pH 5.0 compared to unheated NaCN (**Figure 6a**,**b**). The CL-2 sample was found to exhibit significantly greater solubility than unheated NaCN at pH 2.0 and 3.0 (**Figure 6a**,**c**). The CL-3 sample exhibited significantly improved solubility at pH 2.0, 3.0, and 5.0 compared to unheated NaCN (**Figure 6a**,**d**). In general, increasing the extent of incubation with TGase resulted in improved nitrogen solubility at pH 2.0 and 3.0. Furthermore, the CL-3 sample exhibited 84% nitrogen solubility at pH 5.0, compared to 45.3 and 3.3% for the CL-1 and CL-2 samples, respectively. The reason for the relative loss of solubility of the CL-2 sample at pH 5.0 is unclear.

Improvements in nitrogen solubility at pH 5.0, 5.5, and 6.0 of α_{s1}-casein incubated with guinea pig liver TGase (*11*) and deamidated with guinea pig liver TGase (*9*) and immobilized microbial TGase (*12*) were previously reported. Lorenzen (*18*) did not observe any change in the nitrogen solubility at pH 7 upon incubation of NaCN with microbial TGase; however, unmodified NaCN exhibits almost 100% nitrogen solubility at this pH.

Effect of Combined Enzymatic Treatments. Both the CH (a) and HC (a) samples exhibited significantly higher solubility between pH 2.0 and 5.0 and significantly lower solubility at pH 6.0 and 7.0 compared to unheated NaCN. The solubility of the CH (b) sample was found to be significantly higher than that of unheated NaCN between pH 2.0 and 5.0 and significantly lower than that of unheated NaCN between pH 6.0 and 8.0. The HC (b) sample displayed significantly improved solubility at pH 2.0 and between pH 4.0 and 5.0 and significantly lower solubility at pH 6.0 and 7.0, compared to unheated NaCN. Both the CH and HC (c) samples exhibited significantly improved solubility between pH 2.0 and 5.0, compared to unheated NaCN (**Figure 6d**). However, at pH 6.0, the CH (c) sample was found to exhibit significantly lower solubility than unheated NaCN. Extensive combined enzymatic treatments (CH c and HC c) appear to mimic the effect of the fully hydrolyzed samples with solubility $>\sim$85% over the entire pH range tested (**Figure 6d**). Protamex hydrolysates of NaCN incubated with TGase pre- and post-hydrolysis generally exhibited significantly greater nitrogen solubility than NaCN incubated with Protamex or TGase, per se, between pH 4.0 and 5.0.

The increased solubility of the samples that had been subjected to combined enzymatic treatment may be attributed to the effects of hydrolysis, either pre- or postincubation with TGase, as the solubility at pH 4.0 and 4.6 was unaffected by incubation with TGase per se. However, incubation of Protamex-generated hydrolysates with TGase resulted in improved solubility around the p*I*, compared to the hydrolyzed only sample at low levels of enzymatic modification (**Figure 6b**,**c**). For example, nitrogen solubility values of 1, 15, 36, and 51% were observed at pH 4.6 for the CL-1, 0.5% DH hydrolysate, HC (a), and CH (a) samples, respectively. Although small differences were observed between the electrophoretic profiles of the HC (a) and CH (a) samples on SDS-PAGGE and urea-PAGE, highly significant differences were observed in their solubilities. Furthermore, the decrease in solubility of the CH and HC samples at pH 6.0 and 7.0 after limited modification (**Figure 6b**,**c**) is similar to the decrease in solubility of the 1.3% DH sample at pH 6.0.

Improved solubility of plant proteins following combined proteolysis and TGase treatments has been reported. Reduced turbidity of TGase-treated chymotrypsin, pepsin, and Pronase digests of soy protein isolate (*20*) and gluten (*22*) has been observed.

Overall, limited TGase treatment of low-DH Protamex hydrolysates of NaCN pre- and post-hydrolysis significantly improved solubility around the p*I* and generated products that eluted earlier on RP-HPLC than the products resulting from hydrolysis or incubation with TGase per se. These modified products may display differences in other functional properties in addition to the observed differences in solubility reported herein.

ABBREVIATIONS USED

[E:S], enzyme/substrate ratio; CH (a), cross-linked with TGase for 3 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 0.5% DH; CH (b), cross-linked with TGase for 42 min, [E:S] 1% (w/w) after hydrolysis with Protamex to 1.3% DH; CH (c), cross-linked with TGase for 290 min, [E:S] 10% (w/w) after hydrolysis with Protamex to 17.5% DH; CL-1, cross-linked with TGase for 3 min, [E:S] 1% (w/w); CL-2, cross-linked with TGase for 42 min, [E:S] 1% (w/w); CL-3, cross-linked with TGase for 290 min, [E:S] 10% (w/w); DH, degree of hydrolysis; HC (a), hydrolyzed with Protamex to 0.5% DH after cross-linking with TGase for 3 min, [E:S] 1% (w/w); HC (b), hydrolyzed with Protamex to 1.3% DH after cross-linking with TGase for 42 min, [E:S] 1% (w/w); HC (c), hydrolyzed with Protamex to 17.5% DH after cross-linking with TGase for 290 min, [E:S] 10% (w/w); NaCN, sodium caseinate; NSI, nitrogen solubility index; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGGE, sodium dodecyl sulfate−polyacrylamide gradient gel electrophoresis; TGase, transglutaminase; urea-PAGE, urea polyacrylamide gel electrophoresis.

LITERATURE CITED

(1) Panyam, D.; Kilara, A. Enhancing the functionality of food proteins by enzymatic modification. *Trends Food Sci. Technol.* **1996**, *7*, 120−125.

(2) Chobert, J.-M.; Bertrand-Harb, C.; Nicolas, M.-G. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *J. Agric. Food Chem.* **1988**, *36*, 883−892.

(3) Chobert, J.-M.; Sitohy, M. Z.; Whitaker, J. R. Solubility and emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus* V8 protease. *J. Agric. Food Chem.* **1988**, *36*, 220−224.

(4) Agboola, S. O.; Dalgleish, D. G. Enzymatic hydrolysis of milk proteins used for emulsion formation. 2. Effects of calcium, pH, and ethanol on the stability of emulsions. *J. Agric. Food Chem.* **1996**, *44*, 3637−3642.

(5) Smyth, M.; FitzGerald, R. J. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.* **1998**, *8*, 819−827.

(6) Slattery, H.; FitzGerald, R. J. Functional properties and bitterness of sodium caseinate hydrolysates prepared with a *Bacillus* proteinase. *J. Food Sci.* **1998**, *63*, 418−422.

(7) Flanagan, J.; FitzGerald, R. J. Functionality of *Bacillus* proteinase hydrolysates of sodium caseinate. *Int. Dairy J.* **2002**, in press.

(8) Novo Nordisk A/S, Bagsvaerd, Denmark. Product sheet for Protamex, March 1994.

(9) Motoki, M.; Seguro, K.; Nio, N.; Takinami, K. Glutamine-specific deamidation of α_{s1}-casein by transglutaminase. *Agric. Biol. Chem.* **1986**, *50*, 3025−3030.
(10) Ando, H.; Adachi, M.; Umeda, K.; Matsuura, A.; Nonaka, M.; Uchio, R.; Tanaka, H.; Motoki, M. Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agric. Biol. Chem.* **1989**, *53*, 2613−2617.
(11) Motoki, M.; Nio, N.; Takinami, K. Functional properties of food proteins polymerized by transglutaminase. *Agric. Biol. Chem.* **1984**, *48*, 1257−1261.
(12) Nonaka, M.; Sawa, A.; Matsuura, Y.; Motoki, M.; Nio, N. Deamidation of several food proteins using free and immobilized Ca^{2+}-independent microbial transglutaminase. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 532−533.
(13) Watanebe, M.; Arai, S.; Tanimoto, S.; Seguro, K. Functional peptide obtained by partially hydrolyzing foodstuff protein with protease and/or acid to give hydrolyzed peptide which is treated with transglutaminase. Jpn. Patent JP1992-126039.
(14) Faergemand, M.; Murray, B. S.; Dickinson, E. Cross-linking of milk proteins with transglutaminase at the oil−water interface. *J. Agric. Food Chem.* **1997**, *45*, 2514−2519.
(15) Faergemand, M.; Otte, J.; Qvist, K. B. Emulsifying properties of milk proteins cross-linked with microbial transglutaminase. *Int. Dairy J.* **1998**, *8*, 715−723.
(16) Dickinson, E.; Ritzoulis, C.; Yamamoto, Y.; Logan, H. Ostwald ripening of protein-stabilized emulsions: effect of transglutaminase cross-linking. *Colloids Surf. B: Biointerfaces* **1999**, *12*, 139−146.
(17) Dickinson, E.; Yamamoto, Y. Rheology of milk protein gels and protein-stabilized emulsion gels cross-linked with transglutaminase. *J. Agric. Food Chem.* **1996**, *44*, 1371−1377.
(18) Lorenzen, P. C. Techno-functional properties of transglutaminase-treated milk proteins. *Milchwissenschaft* **2000**, *55*, 667−670.
(19) O'Sullivan, M. M.; Lorenzen, P. C.; O'Connell, J. E.; Kelly, A. L.; Schlimme, E.; Fox, P. F. Influence of transglutaminase on the heat stability of milk. *J. Dairy Sci.* **2001**, *84*, 1331−1334.
(20) Babiker, E. E.; Khan, M. A. S.; Matsudomi, N.; Kato, A. Polymerization of soy protein digests by microbial transglutaminase for improvement of the functional properties. *Food Res. Int.* **1996**, *29*, 627−634.
(21) Babiker, E. E. Effect of transglutaminase treatment on the functional properties of native and chymotrypsin-digested soy protein. *Food Chem.* **2000**, *70*, 139−145.
(22) Babiker, E. E.; Fujisawa, N.; Matsudomi, N.; Kato, A. Improvement in the functional properties of gluten by protease digestion or acid hydrolysis followed by microbial transglutaminase treatment. *J. Agric. Food Chem.* **1996**, *44*, 3746−3750.
(23) Lorenzen, P. C.; Schlimme, E.; Roos, N. Cross-linking of sodium caseinate by a microbial transglutaminase. *Nahrung* **1998**, *42*, 151−154.
(24) Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: London, U.K., 1986; p 122.
(25) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of a bacteriophage T4. *Nature* **1970**, *227*, 680−686.
(26) Andrews, A. T. Proteinase in normal bovine milk and their action on caseins. *J. Dairy Res.* **1983**, *50*, 45−55.
(27) Coakes, S. J. *SPSS—Analysis without Anguish*, versions 7.0, 7.5, and 8.0 for Windows; Wiley: Melbourne, Australia, 1999.
(28) Creamer, L. K.; Richardson, T. Anomalous behaviour of bovine α_{s1}- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers. *Arch. Biochem. Biophys.* **1984**, *234*, 476−486.
(29) Sharma, R.; Lorenzen, P. Chr.; Qvist, K. B. Influence of transglutaminase treatment of skim milk on the formation of ϵ-(γ-glutamyl)lysine and the susceptibility of individual proteins towards cross-linking. *Int. Dairy J.* **2001**, *11*, 785−793.
(30) Gallagher, J.; Kanekanian, A. D.; Evans, E. P. Hydrolysis of casein: a comparative study of two proteases and their peptide maps. *Int. J. Food Sci. Technol.* **1994**, *29*, 279−285.
(31) Matoba, T.; Hata, T. Relationship between bitterness of peptides and their chemical structure. *Agric. Biol. Chem.* **1972**, *36* (8), 1423−1431.

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