Acta Alimentaria, Vol. 32 (2), pp. 169–179 (2003)

ACTION OF THE CHYMOSIN ON RECONSTITUTED CASEIN SYSTEMS

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(Received: 2 January 2002; accepted: 12 November 2002)

The aim of this work was to study the chymosin-catalysed hydrolysis of reconstituted casein systems containing α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein or β-casein modified via chemical glucosylation and/or enzymatic dephosphorylation. The systems containing modified β-casein instead of κ-casein were destabilised after release of peptides in trace amounts. The coagulation of the systems reconstituted using κ-casein required release of much more peptides than coagulation of those containing modified β-casein. Proteolysis range in both classes of reconstituted systems was much smaller than proteolysis range in milk. The specificity of chymosin against reconstituted systems was typical. The major proteolysis products were para-κ-casein and caseinomacropeptide in the systems reconstituted using κ-casein as well as fragment 1–23 of α_{s1}-casein and fragment 193–209 of β-casein in all the systems used. Only the systems containing κ-casein formed gel with a structure similar to this obtained via casein coagulation in milk.

Keywords: casein, dephosphorylation, glucosylation, proteolysis, reconstituted casein systems

Chemical and enzymatic modifications are considered to be a tool for alteration of physico-chemical and functional properties of milk proteins (LUDWIG et al., 1995; IMAFIDON et al., 1997). One of objects of interest is β-casein modified via chemical glycation and/or enzymatic dephosphorylation (YOSHIKAWA et al., 1974; 1981; LORENZEN & REIMERDES, 1992; HUSBAND et al., 1997; DZIUBA et al., 1998; DAREWICZ et al., 1998; 1999 a; b; 2000; DAREWICZ & DZIUBA, 2001; MINKIEWICZ et al., 2002). Previous works were concentrated mainly on properties of isolated β-casein. It was found that systems containing α_{s1}-casein and glycated and/or dephosphorylated β-casein were stable in the presence of calcium ions (YOSHIKAWA et al., 1981; DAREWICZ et al., 1999 a). It may suggest that the modified β-casein may act as stabiliser of casein systems. This function would be similar to that of κ-casein in casein micelles. The problem of stability of casein systems covers, among others susceptibility to the chymosin action.

The aim of this experiment was to study the chymosin action on the casein systems containing β-casein modified via glycation and/or dephosphorylation and comparison of

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their properties with the systems containing κ-casein. The experiment included measurements of the proteolysis extent, identification of main products of proteolysis and investigations on microstructure of gels formed due to chymosin action.

1. Materials and methods

1.1. Materials

Whole casein was precipitated from skim milk of individual, selected Jersey cows with a specified genotype from the experimental herd of Justus Liebing University, Giessen, Germany. Alkaline phosphate [EC 3.1.3.1] (Cat. No P-0905) and chymosin [EC 3.4.23.4] (Cat. No 7751) were from Sigma. Unless stated otherwise all reagents were of analytical grade.

1.2. Preparation of caseins

The following milk proteins: α_{s1}-casein (α_{s1}-Cn), α_{s2}-casein (α_{s2}-Cn), β-casein (β-Cn) and κ-casein (κ-Cn) were used in the experiment. Each casein fraction resulted from the separation of whole casein by ion-exchange chromatography according to THOMPSON (1966) and was dialysed and lyophilised afterwards. Genetic variant and purity of caseins were checked using isoelectric focusing (SEIBERT et al., 1985). The following genetic variants of caseins were used: α_{s1}-Cn A; α_{s2}-Cn A; β-Cn A^1A^2 and κ-Cn A.

1.3. Modification of β-casein

β-Casein was dephosphorylated according to DAREWICZ and co-workers (2000). The protein was incubated with alkaline phosphatase (2 units per milligram of protein) in 50 mM TRIS-KCl buffer (pH 8.0). After that the 24 h reaction was terminated by heating at 80 °C for 5 min. Next, the protein was dialysed and lyophilised. All phosphate groups were removed as judged using FIL-IDF (1987) method.

Glucosylation of β-Cn was performed in the 0.05 M sodium phosphate buffer (pH 7.4). The concentrations of protein and glucose were 0.5 mg ml^{-1} and 0.5 m, respectively. Sodium azide (0.003 M) was added to prevent bacterial growth. The reaction was performed at 37 °C for 24 h (NESSAR & FURTH, 1991; DAREWICZ & DZIUBA, 2001). Approximately six glucose residues were attached to one β-Cn chain as judged according to NESSAR and FURTH (1991).

1.4. Reconstitution of casein systems

Casein micellar systems were reconstituted in the simulated milk ultrafiltrate (SMUF) prepared according to JENNESS and KOOPS (1962), containing 50 mM $CaCl_2$. The final solutions contained 2.6% (w/v) protein. The molar ration α_{s1}-Cn : α_{s2}-Cn : β-Cn : κ-Cn or glucosylated β-Cn (Glc-β-Cn) or dephosphorylated β-Cn (DP-β-Cn) or glucosylated and dephosphorylated β-Cn (Glc-DP-β-Cn) was 3:1:3:1, respectively. Appropriate

amounts of individual caseins were weighted taking into consideration their known molecular masses (SWAISGOOD, 1992) and protein content in particular lyophilisates were determined using the Kjeldahl method. The content of individual caseins in reconstituted systems was also determined using gel electrophoresis (see section 1.7). Whole casein dissolved under the same conditions was used as a reference system.

1.5. Hydrolysis of reconstituted casein systems by chymosin

The hydrolysis was performed using chymosin in the concentration of 1.25 μg of enzyme ml^{-1} of casein suspension at 32 °C. Coagulation time of whole casein was 30 min. Samples designed to electrophoretic and chromatographic analysis were centrifuged (3000×*g*, 20 min). Pellet and supernatant were lyophilised.

1.6. Measurement of the extent of proteolysis

The extent of proteolysis was measured as percentage of nitrogen soluble in 2 and 12% trichloroacetic acid (TCA), released by chymosin (WILSON & WHEELOCK, 1972).

1.7. Gel electrophoresis

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of pellet obtained after hydrolysis by chymosin (see section 1.5) was performed according to references (LAEMMLI, 1970; DZIUBA & MIODUSZEWSKA, 1997) in 12.5% gel. The protein bands were stained with Coomassie Brilliant Blue K. Individual casein fractions were identified by comparing their R_f values with those of standards (SHAPIRO et al., 1967; SWANK & MUNKERS, 1971). Relative content of individual caseins in reconstituted systems was determined according to DZIUBA and MIODUSZEWSKA (1997).

1.8. Reversed-phase high-performance liquid chromatography (RP-HPLC) on-line with UV spectroscopy

Lyophilised supernatants after hydrolysis of micellar systems by chymosin were dissolved in a reducing buffer (VISSER et al., 1991). The final concentration was ca 1.5 mg of lyophilisate ml^{-1}. The water deionised using MilliQsystem (Millipore), acetonitrile and trifluoroacetic acid for HPLC (Baker) were used. The RP-HPLC separations were carried out using Shimadzu HPLC equipment consisting of two LC-10AD pumps, CTO10-AS column oven, SCL-10AD controller and SPD-10AW photodiode-array detector. The chromatographic column Hi-Pore RP-318 of 250×4.6 mm size (Bio-Rad Laboratories Gmbh) was applied. The Class-VP 5.03 software was used for data acquisition and processing. Peptide separations were performed in acetonitrile gradient. Solvent A was a mixture of acetonitrile-water-trifluoroacetic acid (TFA) in the ratio of 100:900:1 (v/v/v). Solvent B contained the same components in the ration of 900:100:0.7 (v/v/v) (VISSER et al., 1991). The following gradient was used: 15% B at the start; 29% B after 22 min; 45% B after 40

min. After gradient completion the concentration of solvent B was increased to 80% during 3 min, then kept constant over 5 min and returned to the initial values during 2 min. Next, the column was equilibrated in the initial conditions during 10 min. The separations were carried out at 30 °C and the flow rate was 0.8 ml min^{-1}. UV spectra were taken from the chromatograms within the wavelength range 190–300 nm. The wavelength step was 1 nm. Data acquisition time was 45 min. The reference peptides were obtained as described previously (MINKIEWICZ et al., 2000).

1.9. Scanning electron microscopy (SEM)

The microstructure of gels obtained from the systems containing κ-casein was investigated by SEM according to DZIUBA and co-workers (1997).

2. Results and discussion

2.1. Range of proteolysis

The range of proteolysis of reconstituted casein systems is presented in Table 1. All the reconstituted systems except for that reconstituted using κ-Cn were hydrolysed only to a small extent. The amount of nitrogen released was drastically lower, than that released by chymosin during hydrolysis of casein in milk. Chymosin released only trace amount of peptides from the systems containing modified β-Cn instead of κ-Cn. Although our previous results (MINKIEWICZ et al., 2002) indicate that systems stabilised by modified β-casein may show considerable resistance to the action of high temperature or ethanol, they were unstable in the presence of chymosin. Coagulation of systems containing κ-Cn occurred after the release of markedly larger amount of peptides than coagulation of systems containing modified β-Cn, but the amount was still smaller, than in milk. These results show that disruption of micelles and further reconstitution from whole casein or separated casein fraction do not lead to formation micellar system identical to that existing in milk. Differences in stability between systems containing modified β-Cn and systems containing κ-Cn are in agreement with results of YOSHIKAWA and co-workers (1981) and our results (MINKIEWICZ et al., 2002). These authors reported that κ-Cn is a better stabiliser for artificial micellar systems, than glucosylated and dephosphorylated β-casein (Glc-DP-β-Cn).

2.2. High-molecular weight proteolysis products

The example of SDS-PAGE electropherogram of insoluble products of chymosin action on reconstituted casein systems is presented in Fig. 1. The electropherograms of hydrolysis products of systems containing DP-β-Cn and Glc-DP-β-Cn (data not shown) were identical to that of Glc-β-Cn (Fig. 1c'). Hydrolysis of the reconstituted systems containing κ-Cn leads to formation of detectable amount of para-κ-Cn (Fig. 1a' and b').

Table 1. Degree of hydrolysis of reconstituted casein systems by chymosin

Casein system	Peptides (% of casein nitrogen)	
	Soluble in 2% TCA (Mean±SE)	Soluble in 12% TCA (Mean±SE)
Whole casein	0.25 ± 0.01	0.06 ± 0.01
System reconstituted using κ-Cna[a]	0.30 ± 0.01	0.13 ± 0.02
System reconstituted using Glc-β-Cna[a]	0.03 ± 0.01	0.02 ± 0.00
System reconstituted using DP-β-Cna[a]	0.04 ± 0.01	0.02 ± 0.01
System reconstituted using Glc-DP-β-Cna[a]	0.08 ± 0.01	0.03 ± 0.01

[a] For explanation of symbols see section 1.3
SE: standard error

Electropherograms of systems containing modified β-Cn both before and after chymosin hydrolysis contain only bands corresponding to caseins. There were no detectable amounts of any proteolysis products. This result is consistent with the data presented in Table 1, indicating that the degree of proteolysis of systems reconstituted using modified β-Cn was very low. We have not found any additional bands corresponding to the modified β-Cn, which confirms previous findings (DAREWICZ et al., 1999a) that glycation does not lead to formation of additional β-Cn band. Bands of DP-β-Cn and Glc-DP-β-Cn also overlapped with this β-Cn. We did not find additional bands attributed to dephosphorylation of β-Cn.

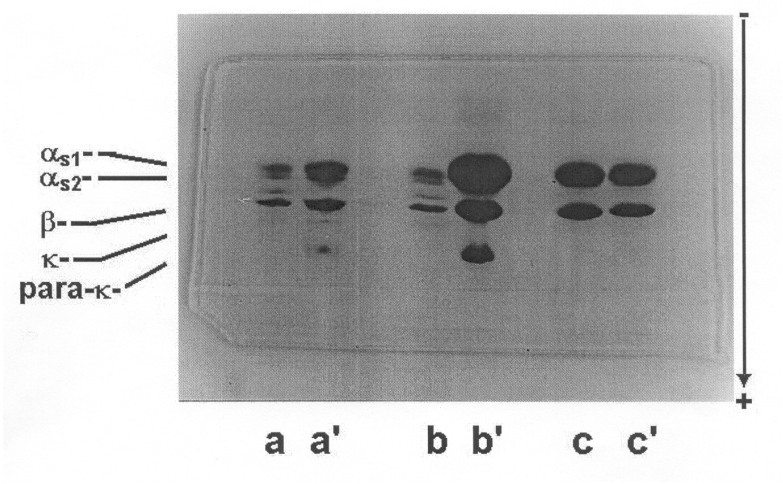

Fig. 1. SDS-PAGE electropherograms of casein systems. a: Whole casein; a': whole casein after chymosin action; b: system reconstituted using κ-Cn; b': system reconstituted using κ-Cn and hydrolysed by chymosin; c: system reconstituted using Glc-β-Cn; c': system reconstituted using Glc-β-Cn and hydrolysed by chymosin. Location of bands corresponding to particular casein fractions according to their R_f values measured using isolated fractions, is indicated

2.3. Peptides released by chymosin

The RP-HPLC chromatogram of products obtained during hydrolysis of the system reconstituted using Glc-β-Cn, is presented in Fig. 2. Peptides from the reconstituted systems were identified on the basis of comparison of retention times and UV spectra with the standard peptides. The major proteolysis products in the systems reconstituted using modified β-Cn were peptides α_{s1}-Cn 1–23 and β-Cn 193–209 (Fig. 2). The first one was identified according to the retention time identical to the reference peptide (Fig. 2). The second one could be identified on the basis of the UV spectrum (Spectrum b in Fig. 3). The second derivative UV spectrum reveals minimum at 283 nm, indicating presence of tyrosine. The β-Cn 193–209 fragment contains one tyrosine residue (SWAISGOOD, 1992). The absorbance at 220 nm, measured at the peak apex, was smaller than 0.06, which is the minimal value recommended to identify peptides or proteins using the second derivatives of UV spectra (DZIUBA et al., 2001). This peak could be characterised using the ratio A_{280nm}/A_{220nm}, which remained reproducible even when absorbance at 220 nm was lower, than 0.01. This ratio was 0.0499±0.0008 (standard deviation for $n = 6$) for the reference peptide β-Cn 193–209 and 0.0503±0.0011 (standard deviation for $n = 10$) for the peptide with corresponding retention time in the chromatograms of peptides obtained from the reconstituted systems. Chromatograms of peptides obtained from systems containing Glc-β-Cn and Glc-DP-β-Cn contained a peak indicated as a in Fig. 2. This peak cannot be attributed to the hydrolysis by chymosin, since it was present also in the chromatograms of reconstituted systems not subjected to the chymosin action (data not shown). The second derivative of its UV spectrum did not contain minima characteristic for aromatic amino acids (PERRIN et al., 1995), there was only one minimum at 265 nm (Spectrum a in Fig. 3). This unidentified substance could be a by-product of the glycation reaction. Chromatograms of the peptides obtained via hydrolysis of systems reconstituted using κ-Cn contained also a peak with the retention time corresponding to sugar-free, monophosphorylated caseinomacropeptide A. The peaks with retention times longer than this 193–209 fragment of β-Cn may be attributed to the casein fractions. On the basis of RP-HPLC experiment, we can conclude that major products of chymosin action on the reconstituted casein systems were the same as the products obtained from isoelectric casein (MINKIEWICZ et al., 2000) or sodium caseinate (MADSEN & ARDÖ, 2001).

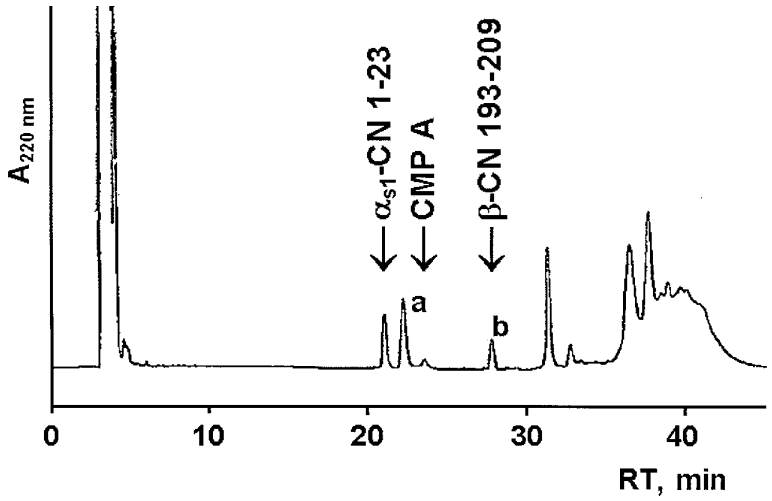

Fig. 2. RP-HPLC chromatogram of peptides obtained via hydrolysis of system reconstituted using Glc-β-Cn by Chymosin. a, b: Fractions, whose spectra are presented in Fig. 3. Arrows indicate retention times of reference peptides: fragment 1–23 of α_{s1}-Cn; sugar-free, monophosphorylated caseinomacropeptide A and fragment 193–209 of β-Cn

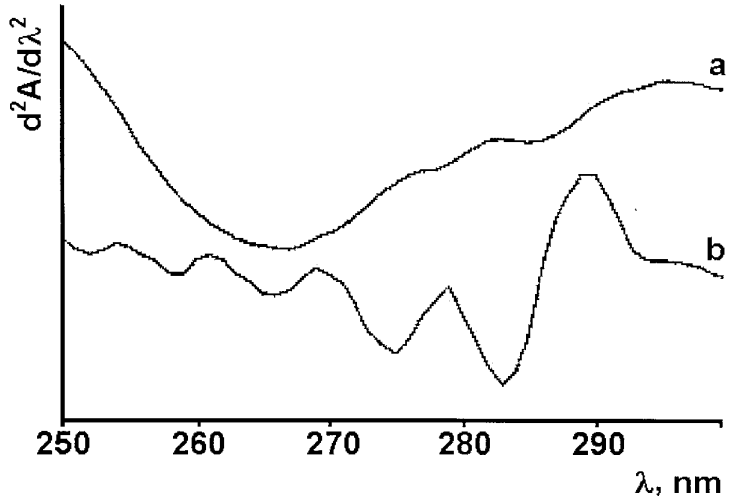

Fig. 3. Second derivatives of UV spectra of fractions a and b separated with RP-HPLC (see Fig. 2)

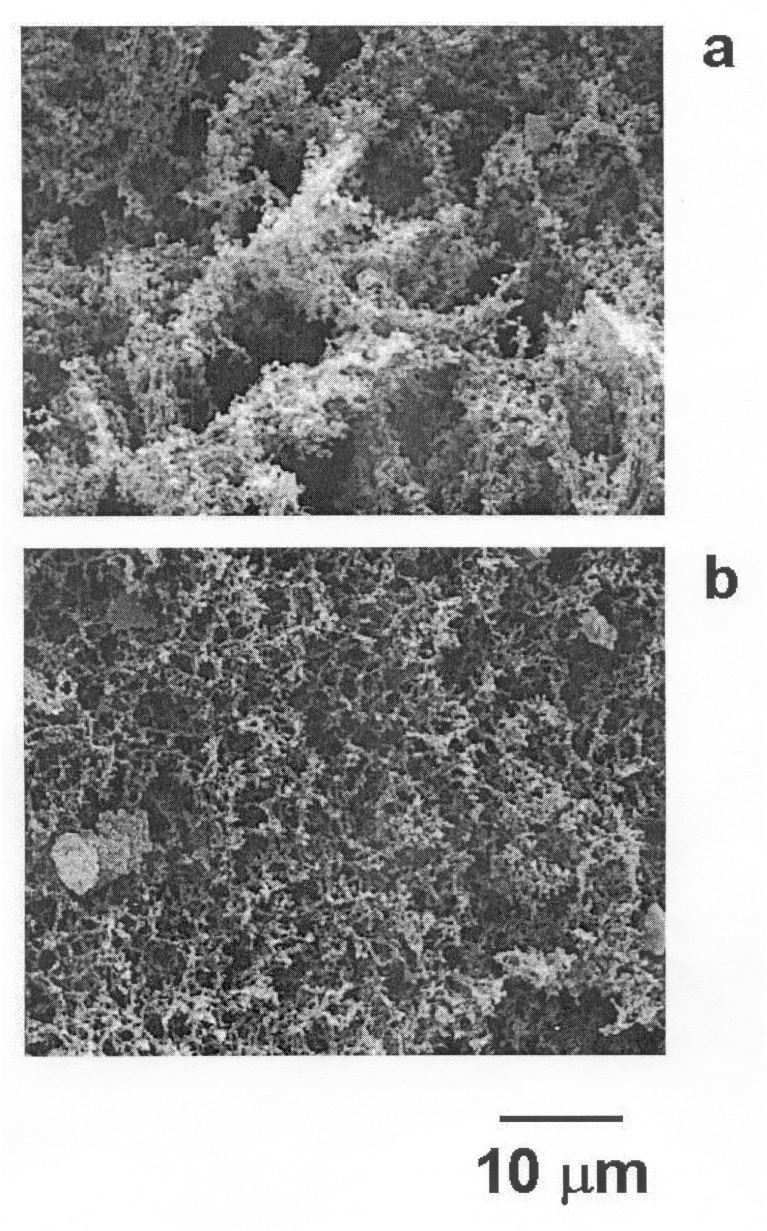

Fig. 4. Electron micrographs of gels obtained after hydrolysis of casein system by chymosin. a: Gel obtained from whole casein; b: gel obtained from system reconstituted using κ-Cn

2.4. Microstructure of gels

Only whole casein and reconstituted system stabilised by κ-Cn formed typical curds after coagulation by chymosin. The reconstituted system formed curd in 50 mM $CaCl_2$ solution, but not in 30 mM solution prepared directly according to JENNESS and KOOPS (1962). The systems stabilised by the modified β-Cn formed only transparent gels similar to those obtained from dephosphorylated casein (VAN HEKKEN & STRANGE, 1994) or from human milk (DZIUBA et al., 1997). The structure of gels obtained from whole casein and system reconstituted using κ-Cn is presented in Fig. 4. Structure of bot gels is typical for gels obtained from casein in milk (OHASHI et al., 1983; KALÁB, 1990). The gels obtained form the whole casein (Fig. 4a) contain larger pores, than those obtained from the reconstituted system (Fig. 4b). This difference may indicate differences in the structure of micelles caused due to casein separation and reconstitution. The fact that systems containing modified β-Cn do not form curd suggests that they do not form micellar structure similar to this occurring in bovine milk.

3. Conclusions

The properties of casein systems containing glucosylated β-casein, dephosphorylated β-casein or glucosylated and dephosphorylated β-casein differ from the properties of those containing κ-casein. Destabilization of systems stabilised by κ-casein requires the release of markedly larger amount of peptides as compared with systems containing modified β-casein. Reconstituted systems containing modified β-casein may be destabilised at very low chymosin-induced hydrolysis degree. Reconstituted systems stabilised by modified β-casein are unable to form chymosin-induced gels similar to those formed in milk. The observed results show that modified β-casein does not interact with other casein fractions via the same mode as κ-casein and structure of aggregates stabilised by the first one differs significantly from the structure of typical casein micelles. Specificity of chymosin against individual casein fractions does not depend on composition of reconstituted systems.

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This study was supported by the State Committee for Scientific Investigations (KBN) within the project 5 P06G 010 16.

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