Substrate Specificity of the Milk-clotting Enzyme from *Irpex lacteus* on κ - and β -Casein

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Irpex lacteus milk-clotting enzyme hydrolyzed the Phe(105)-Met(106) bond of κ -casein, causing the precipitation of para- κ -casein along with other casein fractions in the presence of calcium ions, with a mechanism similar to other milk-clotting enzymes. Furthermore, *Irpex* enzyme hydrolyzed at the positions Leu(79)-Ser(80) and Tyr(30)-Val(31) of para- κ -casein.

Degradation patterns of β -casein by *Irpex* and *Mucor miehei* enzymes were almost the same by polyacrylamide gel electrophoresis, but *Endothia parasitica* enzyme showed a different degradation pattern. Under the conditions employed, β -casein appeared to be scarcely hydrolyzed by chymosin.

Comparing the specificity of *Irpex* enzyme on β -casein with that of chymosin, the common cleaving points were Leu(165)-Ser(166), Ala(189)-Phe(190), and Tyr(192)-Glu(193). The difference in the specificity between the enzymes was exhibited in the cleavage at the Leu(139)-Leu(140) bond by chymosin and of the Ser(142)-Trp(143) bond by *Irpex* enzyme. Although the cleaving points of β -casein by both enzymes resembled each other, each enzyme exhibited different degradation patterns of β -casein because of their different order of cleavage.

Two milk-clotting enzymes excreted by a basidiomycete, *Irpex lacteus*, have been purified and characterized.¹⁾ The main enzyme has maximum proteolytic activity on hemoglobin at pH 3.0 and has almost the same ratio of milk-clotting activity to proteolytic activity as commercial rennet substitutes from *Mucor pusillus* and *Mucor miehei*.

The coagulation of milk is initiated by the hydrolysis of κ -casein into a soluble glycoprotein and para- κ -casein. In the presence of calcium ions, para- κ -casein precipitates along with the other casein fractions. Calf chymosin and other milk-clotting enzymes cleave the bond of Phe(105)-Met(106) of κ -casein.²⁾

As described previously,³⁾ the degradation pattern of α_{s1} -casein by the action of *Irpex* enzyme was similar to that of *Endothia parasitica* enzyme, but was quite different from those of chymosin and *Mucor miehei* enzyme. *Irpex* enzyme hydrolyzed α_{s1} -casein at positions His(8)-Gln(9), Phe(23)-Phe(24), Lys(103)-Tyr(104), and Phe(153)-Tyr(154).

Compared with the specificity of calf chymosin, the only common cleavage point was the Phe(23)-Phe(24) bond of α_{s1} -casein.

It was also previously reported⁴⁾ that there was no significant difference in the PAGE patterns between cheese made with *Irpex* enzyme and that made with calf rennet. β -casein is a source of bitter peptides formed by the action of proteinases. For example, a peptide that constitutes residues Arg(202)-Val(209) of β -casein, is 250 times more bitter than caffeine.⁵⁾ Therefore, it is apparently important to investigate the specificity of milk-clotting enzymes on β -casein.

It has been shown⁶⁾ that chymosin hydrolyzes isolated bovine β -casein at residues 189 ~ 190 and/or 192 ~ 193, 163 ~ 164 and/or 165 ~ 166, and 139 ~ 140 to yield β -I, β -II, and β -III, respectively. On the other hand, the proteolytic specificity of microbial milk-clotting enzymes on β -casein has not yet been established.

This paper deals with the proteolytic speci-

ficiencies of the milk-clotting enzyme from *I. lacteus* on κ -casein and β -casein at pH 6.0 in solution.

MATERIALS AND METHODS

Dansyl chloride, dansyl amino acids, *N*-ethylmorpholine, and micropolyamide sheets (Schleicher and Schuell F1700) were purchased from the Pierce Chemical Co. Phenyl isothiocyanate, *N,N*-dimethylallylamine, trifluoroacetic acid, PTH amino acids, 1,2-dichloroethane, and benzene (sequential grade) were obtained from Wako Pure Chemical Industries, Ltd. Analytical TLC plates (K6F) and DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were from Whatman and carboxypeptidases A-DFP and B-DFP were from Sigma. Molecular weight markers (ranging from 2,500~17,000) for SDS-PAGE were from BDH Biochemicals, and pepstatin was from the Protein Research Foundation of Japan. Other chemicals used were of reagent grade.

κ -Casein and β -casein. Crude κ -casein and β -casein were prepared from acid-precipitated whole casein by the methods of Zittle and Custer,⁷⁾ and Aschaffenburg,⁸⁾ respectively. Crude κ -casein was alkylated at 4°C by the method of Rose *et al.*⁹⁾ The alkylated κ -casein and β -casein were purified by ion-exchange chromatography on a DEAE-cellulose column by the method of Davies and Law.¹⁰⁾

Enzymes. Rennets from calf, *Mucor miehei*, and *Endothia parasitica* were purchased from Chr. Hansen Lab., Miles Lab., and the Pfizer Co., respectively. These enzymes were purified by affinity chromatography.^{11,12)} The *I. lacteus* rennet powder was kindly supplied by Kyowa Hakko Co., Ltd. and the milk-clotting enzyme B was purified as described previously.¹⁾

Milk-clotting activity. Milk-clotting activity was determined according to the method described previously¹¹⁾ and expressed as Soxhlet units (s.u.) per ml of enzyme solution.

Action of milk-clotting enzymes on κ -casein and β -casein. Each solution of κ - and β -casein (1.5 ml, 0.1%, w/v) in 0.02 M phosphate buffer, pH 6.0, was mixed with 0.03 ml of enzyme solution (500 s.u./ml) and incubated at 35°C. Aliquots (0.2 ml) were taken at various times, and the reaction was stopped by mixing the samples with 0.2 ml of 9 M urea containing 5×10^{-5} M pepstatin. The samples were then used for PAGE analysis.

Gel electrophoresis. SDS-PAGE was performed with 12.5% acrylamide in the presence of 8 M urea and 0.1% SDS by the method of Swank and Munkres.¹³⁾ PAGE was done with 7.5% gel at pH 8.9 or at pH 4.3 in the presence

of 4.5 M urea as described by Davis.¹⁴⁾ The protein was stained with 0.05% Coomassie brilliant blue R-250 in acetic acid-methanol-water (1:1:5).

Isolation of κ -casein degradation products. Fifty milliliters of a 0.1% κ -casein solution in 0.02 M phosphate buffer, pH 6.0, was incubated with *Irpex* enzyme at 35°C (enzyme-substrate = 1:500 mol/mol). After 5 min incubation, the reaction was ended by heating in a boiling water bath for 5 min. One fifth volume of 60% trichloroacetic acid was added and centrifuged at $1500 \times g$ for 20 min. The resultant precipitates were dissolved in a minimum volume of 0.02 M phosphate buffer, pH 6.0, containing 6 M urea, and were dialyzed against the same buffer. This sample was then chromatographed on a column of CM-cellulose using a linear NaCl gradient (0~0.3 M) in the same buffer. Each fraction was then used for PAGE, to confirm the homogeneity, and after purification was used for determination of amino acid composition and C-terminal and N-terminal amino acids.

The supernatant obtained after centrifugation was adjusted to pH 8.0 with 1 M NaOH and concentrated on an evaporator. The concentrate was then put on a column of Sephadex G-50 equilibrated with 0.1 M acetic acid. Each fraction obtained was then purified by reverse phase high performance liquid chromatography (RP-HPLC, Beckman model 340) and used for identification as stated previously.

Isolation of β -casein degradation products. One hundred milliliters of a 0.1% β -casein solution in 0.02 M phosphate buffer, pH 6.0, was incubated with *Irpex* enzyme at 35°C (enzyme-substrate = 1:400 mol/mol). After 2 hr incubation, the reaction was terminated by heating in a boiling water bath for 5 min. The sample was dried in a rotary evaporator and dissolved in 10 ml of 0.01 M Tris-HCl buffer containing 0.06 M NaCl and 6 M urea, at pH 8.6, and put on a Sephadex G-100 column equilibrated with the same buffer. Each of the separated fractions was put on a DEAE-cellulose column or a CM-cellulose column, then eluted by a linear NaCl gradient or by a pH gradient. Furthermore, all fractions were purified with RP-HPLC [Altex, Ultrosphere-ODS, Ultrosphere-octyl; Yoyo Soda, TMS-250; Beckman model 340] and used for determination of amino acid composition and C-terminal and N-terminal amino acids.

Amino acid analysis. Each of the κ - and β -casein fractions was hydrolyzed *in vacuo* in 6 N HCl at 110°C for 24 hr. The amino acid compositions of the hydrolyzates were determined on a Durrum amino acid analyzer, model D-5.

C-Terminal amino acid analysis. Purified κ - and β -casein fractions dissolved in 0.2 M *N*-ethylmorpholine-acetate buffer, pH 8.5 were incubated with DFP-treated carboxypeptidases A and B for 0, 0.5, 1 and 2 hr. The released

amino acids were determined by the amino acid analyzer with norleucine as an internal standard.

N-Terminal amino acid analysis. Dansylation of the N-terminal amino acid of each fragment was done by the method of Hartley¹⁵⁾ and the resultant dansyl amino acid was identified with micropolyamide sheet by the method of Woods and Wang.¹⁶⁾ Edman degradation was also done by the method of Sauer¹⁷⁾ and the PTH amino acid obtained was identified by TLC as described by Jeppsson.¹⁸⁾

RESULTS

Degradation of κ -casein by several microbial milk-clotting enzymes and calf chymosin

In all cases, κ -casein (MW 19,000) almost completely disappeared within 5 min and a new product corresponding to MW 12,000 was formed (Fig. 1). In addition, microbial milk-clotting enzymes, especially *I. lacteus* enzyme, produced a degradation product having a MW of about 10,000.

Isolation and identification of degradation products formed by the action of *Irpex* milk-clotting enzyme on κ -casein

The fraction insoluble in 12.5% trichloroacetic acid was purified by ion exchange chromatography on a CM-cellulose column. Figure 2 shows that three fractions were eluted by a NaCl linear gradient and these were designated I, II, and III in the order of elution. Each fraction appeared to be homogeneous by pH 4.3 PAGE. The elimination of contaminat-

ing free amino acids and peptides was done by RP-HPLC (Ultrosphere-octyl) and samples were identified. Table I shows the N-terminal amino acids, C-terminal amino acid sequences, molecular weights and amino acid compositions of purified I, II, and III. Therefore, I, II, and III were identified as PyrGlu(1)-Leu(79), PyrGlu(1)-Phe(105) and Val(31)-Phe(105) of κ -casein, respectively, by taking into consideration the facts shown in Table I. Yields of these fragments are also presented in Table I.

On the other hand, when the fraction soluble in 12.5% trichloroacetic acid was chromatographed on Sephadex G-50, two fractions were obtained and designated IV and V. Each fraction was checked for homogeneity by RP-HPLC (Ultrosphere-octyl). Fraction IV appeared to be homogeneous but V was separated into many peaks. The two major peaks shown in Fig. 3 were designated V and VI. Table II shows the amino acid compositions, N-terminal amino acids, and C-terminal amino acid sequences of these fractions. Taking into consideration the results shown in Table II, fragments IV, V and VI were identified as Met(106)-Val(169), Ser(80)-Phe(105), and PyrGlu(1)-Tyr(30) of κ -casein, respectively. Yields of these fragments are also shown in Table II.

Degradation of β -casein by some microbial milk-clotting enzymes and calf chymosin

Figure 4 shows that although *Irpex* enzyme

FIG. 1. SDS-PAGE Patterns of κ -Casein Incubated with Calf Chymosin (A), *I. lacteus* Milk-clotting Enzyme (B), *M. miehei* Milk-clotting Enzyme (C) and *E. parasitica* Milk-clotting Enzyme (D).

FIG. 2. Ion Exchange Chromatography on CM-cellulose of Insoluble κ -Casein Degradation Products Formed by the Action of *I. lacteus* Enzyme.

The fraction insoluble in 12.5% trichloroacetic acid was put on a column of CM-cellulose (0.9 $\times$ 25 cm, Whatman CM-52) equilibrated with 0.02 M phosphate buffer containing 6 M urea, pH 6.0. Degradation products were eluted by 500 ml each of an NaCl concentration gradient (0 $\sim$ 0.3 M). The flow rate was 20 ml/hr and 4.0 ml fractions were collected. ●, absorbance at 280 nm; —, NaCl concentration.

TABLE I. IDENTIFICATION OF PEPTIDES FORMED FROM κ -CASEIN BY *Irpex lacteus* MILK-CLOTTING ENZYME

Amino acid	I	PyrGlu(1)-Leu(79)	II	PyrGlu(1)-Phe(105)	III	Val(31)-Phe(105)
Asp	6	6	7	7	4	4
Thr	0	0	3	3	4	3
Ser	4	4	7	7	6	6
Glu	15	15	15	17	10	8
Pro	8	8	12	12	9	10
Gly	1	1	1	1	1	1
Ala	6	6	10	9	8	8
Cys	1	1	2	2	1	1
Val	5	4	5	5	5	5
Met	0	0	1	1	1	1
Ile	5	6	6	6	2	2
Leu	6	6	8	7	6	7
Tyr	8	9	8	9	5	7
Phe	3	3	4	4	2	2
His	0	0	3	3	4	3
Lys	6	5	7	6	3	3
Arg	4	4	5	5	3	3
Trp	— ^a	1	— ^a	1	— ^a	1
Total residues	78	79	104	105	74	75
MW	10,000	9,500	12,000	12,000	9,000	8,600
N-Terminal	— ^a	PyrGlu	— ^a	PyrGlu	Val	Val
C-Terminal	-Val-Leu	-Val-Leu	-Ser-Phe	-Ser-Phe	-Ser-Phe	-Ser-Phe
Recovery (%) ^b	8		79		10	

^a Not detected.

^b The recovery of each fragment was determined by amino acid analysis and expressed as the percentage of total amount of κ -casein used as the substrate.

FIG. 3. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of Fraction V.

Fraction V was concentrated and put on a column of Ultrasphere-octyl (Altex, 4.6×250 mm) and eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0~90%) in 0.01 M trifluoroacetic acid. The column was operated at 28°C.

exhibited more proteolytic activity than *Mucor* enzyme, these enzymes showed almost the same degradation patterns. In the case of *Irpex* enzyme, the main degradation product was observed even at the early stage of proteolysis, and it remained even after a long period of incubation.

Endothia enzyme clearly had a different degradation pattern from the others, producing several products that migrated slower than the substrate and a main product that migrated faster than the degradation product of *Irpex* enzyme. Under the condition employed, β -casein appeared to be hardly hydrolyzed by chymosin.

Isolation and identification of degradation products formed by the action of Irpex milk-clotting enzyme on β -casein

When the reaction mixture was chromatographed on Sephadex G-100, three fractions were eluted and designated A, B, and C in the

TABLE II. IDENTIFICATION OF PEPTIDES FORMED FROM κ -CASEIN BY *Irpex lacteus* MILK-CLOTTING ENZYME

Amino acid	IV	Met(106)-Val(169)	V	Ser(80)-Phe(105)	VI	PyrGlu(1)-Tyr(30)
Asp	5	5	1	1	2	3
Thr	12	12	2	3	1	0
Ser	6	6	3	3	2	1
Glu	11	10	2	2	8	9
Pro	8	8	4	4	2	2
Gly	1	1	0	0	0	0
Ala	5	5	3	3	1	1
Cys	0	0	0	1	0	1
Val	5	6	2	1	0	0
Met	1	1	1	1	0	0
Ile	6	6	0	0	3	3
Leu	1	1	1	1	0	0
Tyr	0	0	0	0	2	2
Phe	0	0	2	1	2	2
His	0	0	2	3	1	0
Lys	3	3	1	1	3	3
Arg	0	0	2	1	2	2
Trp	— ^a	0	— ^a	0	— ^a	0
Total residues	64	64	26	26	29	29
N-Terminal	Met	Met	Ser	Ser	— ^a	PyrGlu
C-Terminal	-Ala-Val	-Ala-Val	-Ser-Phe	-Ser-Phe	-Gln-Tyr	-Gln-Tyr
Recovery (%) ^b	60		8		5	

^a Not detected.

^b The recovery of each fragment was determined by amino acid analysis and expressed as the percentage of total amount of κ -casein used as the substrate.

FIG. 4. Disc Gel Electrophoretic Patterns of β -casein Incubated with Calf Chymosin (A), *I. lacteus* Milk-clotting Enzyme (B), *M. miehei* Milk-clotting Enzyme (C) and *E. parasitica* Milk-clotting Enzyme (D). PAGE was done at pH 8.9.

FIG. 5. Ion Exchange Chromatography of Fraction A after Gel Filtration, on DEAE-cellulose.

Fraction A eluted from the gel filtration on Sephadex G-100 was put on a DEAE-cellulose column (DE-52, Whatman, 0.9×20 cm) equilibrated with 0.01 M Tris-HCl containing 0.06 M NaCl and 6 M urea, pH 8.6. Degradation products were eluted by a decreasing pH gradient from pH 8.6 to pH 4 generated from 500 ml each of the initial buffer and 0.3 M acetic acid containing 6 M urea. The flow rate was 30 ml/hr and 4.5 ml fractions were collected. ●, absorbance at 280 nm; —, pH.

order of elution. Fraction A was adsorbed on a DEAE-cellulose column and eluted by a pH gradient.

As shown in Fig. 5, two peaks designated VII and VIII were obtained. Both VII and VIII were judged homogeneous by PAGE and their mobilities in PAGE coincided with that of the main degradation product. Each of these fractions, VII and VIII, was further purified by RP-HPLC with a column of TMS-250 and was identified.

Fraction B was separated into several peaks by chromatography on CM-cellulose, and IX appeared to be homogeneous in pH 4.3 PAGE (Fig. 6). IX was further purified by RP-HPLC

with a TMS-250 column and identified.

Fraction C was separated into six fractions by RP-HPLC with Ultrosphere-octyl column, but four peaks designated X, XI, XII, and XIII were further investigated (Fig. 7).

As shown in Table III, the N-terminal amino acids of VII, VIII and IX were Arg, Arg, and Trp, respectively, and their corresponding C-terminal amino acid sequences were -Leu-Leu-Gln-Ser, -Leu-Leu-Gln-Ser, and -Ala-Phe-Leu-Leu, respectively. Their molecular weights are shown in Table III, and by considering their amino acid compositions, VII and VIII were identified as Arg(1)-Ser(142), and IX was identified as Trp(143)-

FIG. 6. Ion Exchange Chromatography of Fraction B after Gel Filtration, on CM-cellulose.

Fraction B eluted from the gel filtration on Sephadex G-100 was put on a CM-cellulose column (1.5×25 cm, Whatman DE-52) equilibrated with 0.02 M acetate buffer containing 6 M urea, pH 5.5. Degradation products were eluted by a linear NaCl gradient generated from 500 ml each of the initial buffer and the same buffer containing 0.4 M NaCl. The flow rate was 25 ml/hr and 4.0 ml fractions were collected. ●, absorbance at 280 nm; —, NaCl concentration.

FIG. 7. RP-HPLC of Fraction C after Gel Filtration.

Fraction C eluted from the gel filtration on Sephadex G-100 was loaded on a column of Ultrasphere-octyl (4.6×250 mm) and eluted under the same conditions described in Fig. 3.

Leu(192) of β -casein.

Table IV shows the N-terminal amino acids, C-terminal amino acid sequences, and amino acid compositions of purified X, XI, XII, and

XIII. On account of the results shown in Table IV, X, XI, XII, and XIII were identified as Ser(166)-Ala(189), Tyr(193)-Val(209), Trp(143)-Leu(165), and Phe(190)-Val(209), respectively. Other fractions in Figs. 5, 6, and 7 were not identified because of their heterogeneity.

DISCUSSION

The coagulation of milk is initiated by the specific cleavage of the peptide bond between Phe(105)-Met(106) of κ -casein by calf chymosin and other milk-clotting enzymes.²⁾ When the *Irpex* enzyme acted on κ -casein, fractions II, PyrGlu(1)-Phe(105), and IV, Met(106)-Val(169), were formed with high yields (Tables I and II). This indicates that *Irpex* enzyme hydrolyzes the Phe(105)-Met(106) bond of κ -casein and subsequent milk coagulation occurs in the same manner as with other milk-clotting enzymes. Other degradation products identified here were derived from *para*- κ -casein by the enzyme action but it was not clear how *Irpex* enzyme acted on glycomacropeptides. It suggests that microbial milk-clotting enzymes hydrolyze *para*- κ -casein further because all

TABLE III. IDENTIFICATION OF PEPTIDES FORMED FROM β -CASEIN BY *Irpex lacteus* MILK-CLOTTING ENZYME

Amino acid	VII	VIII	Arg(1)-Ser(142)					IX	Trp(143)- Leu(192)
			Variant A ₁	A ₂	A ₃	B	C		
Asp	8	8	8					1	1
Thr	8	8	8					1	1
Ser	11	11	11			10		3	4
Glu	31	33	30		31		29	7	7
Pro	20	20	19	20		19	19	11	11
Gly	3	4	4					1	0
Ala	4	5	3					2	2
Val	8	8	11					6	5
Cys	0	0	0					0	0
Met	4	3	3					3	3
Ile	6	6	7					1	1
Leu	14	14	15					5	6
Tyr	2	2	2					1	1
Phe	7	6	6					2	2
His	5	3	5	4	3	5	5	2	2
Lys	9	9	9				10	2	2
Arg	2	2	2			3		1	1
Trp	— ^a	— ^a	0					1	1
Total residues	142	142	142	142	142	142	142	50	50
MW	16,000	16,000	16,000					5,200	5,200
N-Terminal	Arg	Arg	Arg					Trp	Trp
C-Terminal	-Gln-Ser	-Gln-Ser	-Gln-Ser					-Leu-Leu	-Leu-Leu
Recovery (%) ^b	15	30						20	

^a Not detected.^b The recovery of each fragment was determined by amino acid analysis and expressed as the percentage of total amount of β -casein used as the substrate.

microbial enzymes produced a degradation product that migrated just a little faster than *para*- κ -casein in SDS-PAGE. Figure 8 shows comparative cleavage points of κ -casein by chymosin and *Irpex* enzyme.

A previous report of ours stated⁴⁾ that Cheddar cheese made with *Irpex* enzyme and calf rennet did not show any difference in the degradation pattern and that β -casein was not degraded. However, it was observed that the degradation pattern of these enzymes on β -casein in solution at pH 6.0 was quite different from each other, and *Irpex* enzyme was shown to be considerably more proteolytic than chymosin under the conditions employed. This depends on (a) the amount of enzyme used in solution at pH 6.0, which is 100 times higher than that in cheese manufacture, (b) the high

level of NaCl in cheese and (c) the variety of protein in cheese as the substrate. Since bitter tastes were not formed in the cheese made with *Irpex* enzyme, it was postulated that the β -casein fragment, which is 250 times more bitter than caffeine, was not produced during ripening.

β -Casein exists in five genetically determined forms which are designated as A₁, A₂, A₃, B, and C, in the milk of western breeds of cattle.¹⁹⁾ Amino acid compositions of these variants are summarized in Table III. The major degradation products VII and VIII were identified as the identical fragment Arg(1)-Ser(142) of β -casein. Although the N-terminal amino acid and C-terminal amino acid sequence of each fragment were identical, they contained different numbers of histidine and glutamic

TABLE IV. IDENTIFICATION OF PEPTIDES FORMED FROM β -CASEIN BY *I. lacteus* ENZYME

Amino acid	X	Ser(166)- Ala(189)	XI	Tyr(193)- Val(209)	XII	Trp(143)- Leu(165)	XIII	Phe(190)- Val(209)
Asp	1	1	0	0	0	0	0	0
Thr	0	0	0	0	0	1	0	0
Ser	1	2	0	0	2	2	1	0
Glu	4	4	2	2	3	3	2	2
Pro	5	5	4	4	6	6	4	4
Gly	0	0	2	2	0	0	2	2
Ala	2	2	0	0	0	0	0	0
Cys	0	0	0	0	0	0	0	0
Val	4	3	4	3	3	2	3	3
Met	1	1	1	0	1	2	0	0
Ile	1	1	1	2	0	0	1	2
Leu	1	1	1	1	3	3	3	3
Tyr	1	1	1	1	0	0	1	1
Phe	0	0	1	1	1	1	2	2
His	0	0	0	0	1	2	0	0
Lys	2	2	0	0	0	0	0	0
Arg	1	1	1	1	0	0	1	1
Trp	— ^a	0	— ^a	0	1	1	— ^a	0
Total residues	24	24	17	17	23	23	19	19
N-terminal	Ser	Ser	Tyr	Tyr	Trp	Trp	Phe	Phe
C-Terminal	-Gln-Ala	-Gln-Ala	-Ile-Val	-Ile-Val	-Ser-Leu	-Ser-Leu	-Ile-Val	-Ile-Val
Recovery (%) ^b	11		21		9		10	

^a Not detected.^b The recovery of each fragment was determined by amino acid analysis and expressed as the percentage of total amount of β -casein used as the substrate.

acid. The histidine content of VII and the glutamic acid content of VIII were apparently higher than those of VIII and VII, respectively, whereas the contents of serine, lysine, and arginine were identical in both fragments. Therefore, it is postulated that VII is formed from variant A₁ and VIII is from variant A₃ of β -casein. This might be due to the fact that the β -casein used in this work was prepared from bulk herd milk instead of that from an individual. The β -casein preparation contained variants A₁ and A₃ with a ratio of 1:2, because the yields of VII and VIII were estimated to be 15% and 30%, respectively. Thirty percent of the β -casein remained unchanged in the reaction mixture and the total yields of VII and VIII were 45%. This indicates that VII and VIII are the major degradation products of the action of *Irpex* enzyme from β -casein. Although IX constitutes a large percentage as determined by the absorbance at 280 nm, as

shown in Fig. 6, the yields of VII and VIII were higher than that of IX estimated from the amino acid analysis. This fact indicates that the extinction coefficient of IX is much higher than those of VII and VIII, because IX contains the sole residue of tryptophan in β -casein. As presented in Fig. 8, the common cleaving points of *Irpex* enzyme and chymosin on β -casein are Leu(165)-Ser(166), Ala(189)-Phe(190), and Tyr(192)-Glu(193). The difference in the specificity between the enzymes are exhibited by the cleavage at the Leu(139)-Leu(140) bond of β -casein by chymosin and at the Ser(142)-Trp(143) bond by *Irpex* enzyme. In addition, chymosin cleaves Ala(189)-Phe(190) and/or Leu(192)-Tyr(193) of β -casein to form β -I initially, and then it acts on the Leu(163)-Ser(164) and/or Leu(165)-Ser(166) bond of β -I to produce β -II, and finally it hydrolyzes Leu(139)-Leu(140) to form β -III, sequentially.⁶⁾ On the other hand, it is postu-

responding to β -I [Arg(1)-Leu(192) of β -casein] even in the early stage of proteolysis. The yield of XI was estimated to be 21%, which coincided with that of IX, which was 20%. A certain fragment of Trp(143)-Val(209) was hydrolyzed at Leu(165)-Ser(166) and Ala(189)-Phe(190) to yield XII, X, and XIII. The yields of XII, X, and XIII were estimated to be 9%, 11% and 10%, respectively. Chymosin hydrolyzes β -casein in a sequential manner from the C-terminal to the central point, whereas *Irpex* enzyme hydrolyzes the central region of β -casein first and after which, it acted on the C-terminal fragment. With respect to α_{s1} -casein, chymosin and *Irpex* enzyme showed different degradation patterns in PAGE and different cleaving sites except for Phe(23)-Phe(24).³⁾ On the other hand, the enzymes exhibited different degradation patterns on β -casein in PAGE but cleaving points resembled each other closely. This can be deduced from the order of cleavage, which differed, resulting in degradation products that showed different PAGE patterns.

Table V shows the comparative specificity of *Irpex* enzyme and chymosin on κ -casein and β -casein on the basis of hydrophobicity of the side chains of amino acid residues adjacent to the bonds to be cleaved. In the P_1 site and P_4 and/or P_3 site(s) as defined by Schechter and Berger,²²⁾ if κ -casein is used as the substrate, chymosin and *Irpex* enzyme always require hydrophobic amino acids. On the other hand, if the substrate used is β -casein, *Irpex* enzyme and chymosin require hydrophobic amino acids either in the P_1 or P_1 site and in the P_4 and/or P_3 site(s).

A previous report³⁾ stated that *Irpex* enzyme is specific for the peptide bonds formed by two amino acids with large hydrophobic side chains on α_{s1} -casein. Furthermore, chymosin and *Irpex* enzyme always require hydrophobic amino acids in the P_4 and/or P_3 site(s). It is postulated that in the case of chymosin and *Irpex* enzyme, hydrophobic amino acids lo-

cated in the P_4 and/or P_3 site(s) might be important in the secondary interaction between the enzyme and substrate.

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