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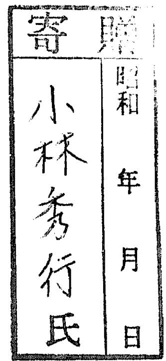

BIOCHEMICAL STUDIES ON THE MILK-CLOTTING ENZYME FROM *IRPEX LACTEUS*

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ABBREVIATIONS

BSA	bovine serum albumin
CM	carboxymethyl
CR	calf rennet
DAN	diazoacetyl-DL-norleucine methyl ester
DEAE	diethylaminoethyl
DFP	diisopropylfluorophosphate
EDTA	ethylenediamine tetraacetic acid
EPNP	1,2-epoxy-3(p-nitrophenoxy) propane
IR	Irpex lacteus milk-clotting enzyme fraction
MIA	monoiodoacetic acid
M.W.	molecular weight
NPN	non-protein nitrogen
PAGE	polyacrylamide gel electrophoresis
p-CMB	p-chloromercurybenzoate
pI	isoelectric point
RP-HPLC	reverse phase- high performance liquid chromatography
SDS	sodium dodecyl sulfate
SN	soluble nitrogen in water at pH 4.6
s.u.	Soxhlet units
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TN	total nitrogen
TPCK	N-p-toluenesulfonyl-L-phenylalanine chloromethyl ketone

CHAPTER I

INTRODUCTION

There is an ancient legend that cheese was accidentally discovered by an Arabian merchant when he stored his milk in a pouch made of sheep´s stomach. During his journey across the desert, the milk was separated into curd and whey. At night he discovered that the whey slaked his thirst and the tasty curd satisfied his hunger.[1)]

With the passage of time, cheeses such as Cheddar(England), Brie, Camembert(France), Emmental(Switzerland) and Grana(Italy) were manufactured after the 10th century in Europe. Cheese is the curd of milk which has been separated from the whey and then processed to yield a savory food. The nutritious merit of this savory food is attributable to the concentrated protein and fat from milk, as well as to attendant some vitamins and minerals. The coagulating enzyme most extensively used to produce cheese is derived from the "fourth" stomach of unweaned calf. The crude enzyme preparation extracted from calf stomachs is referred to as calf rennet while the pure milk-clotting enzyme is designated as calf chymosin.

The exact mechanism of curd formation brought about by calf chymosin is still imperfectly understood. The coagulation of milk is recognized to be a complicated process which can be

separated into two distinct phases, enzymatic and non-enzymatic. The enzymatic step involves the hydrolysis of κ-casein into a soluble glycoprotein and para-κ-casein. In the presence of calcium ions, the resultant para-κ-casein precipitates along with the other casein fractions.[2,3]

Although calf rennet is regarded as an ideal enzyme for cheese manufacture, a great deal of efforts have been made to search for its substitutes. Calf rennet has become less available, because instead of slaughtering calves to obtain rennet, they are preferably utilized to provide meat for human consumption.

In order for any rennet enzyme to substitute successfully for calf rennet, it must measure up to some criteria.[1] These criteria are: (1) the weight yield of the curd produced must be no less than that of the calf rennet, (2) the physical characters of the curd should be unaffected, and (3) there should be no development of off-flavors.

One of the most important specifications that should be satisfied is the organoleptic quality of the cheese. Off-flavors, such as bitterness and rancidity, are considered to be distasteful generally. Many potential calf rennet substitutes are unacceptable because they impart bitterness to the cheese. The reason for development of bitterness is not completely understood, but there is evidence that it may be due to the formation of bitter peptides derived from β-casein as a result of the proteolytic activity of both rennet and starter culture enzyme.[4]

Among rennets of animal sources, pepsin yields cheese of passing quality.[5] In practice, pepsin is not used by itself to make cheese because of certain disadvantages when it is employed. These are (1) a long period of time is required for clot formation, (2) the curd formed is not as firm as calf rennet´s, (3) there is some loss of fat in the whey, and (4) organoleptic quality of the cheese is inferior to that of calf rennet´s. As a consequence, pepsin is used together in equal proportion with calf rennet.[2]

Most of the milk-clotting enzymes produced by bacteria have a poor ratio of milk-clotting activity to proteolytic activity. However, there are a few bacteria whose enzymes have been used to produce cheese. For example, the enzyme of *Bacillus cereus* is fairly thermostable and has been used to produce Cheddar cheese.[6] No bitterness was detected after eight months of ripening, although the texture was considered to be faulty. Enzymes from *B. mesentericus*, *B. polymyxa* and *B. subtilis* have been investigated for production of some types of cheeses,[6] but, these enzymes are not available in commercial scale.

Several investigators have studied the distribution of milk-clotting enzymes in selected fungi. Only three fungal enzymes derived from *Endothia parasitica*, *Mucor miehei*, and *Mucor pusillus Lindt* have proved to be successful in the preparation of cheese on a commercial scale.

E. parasitica enzyme has been crystallized and characterized in 1968.[7,8] It is a thermolabile carboxyl proteinase with a pH

stability rage of 4.0 - 5.5. Its isoelectirc point has been reported to be 4.6 and the molecular weight is estimated to be 37,000. It is less sensitive to calcium ion concentration in milk than calf rennet. The ratio of milk-clotting activity to proteolytic activity of the enzyme is higher among microbial rennets. Many types of cheeses including Camembert, kCheddar, Emmental and Italian varieties have been reported to be successfully prepared with this enzyme.[1)]

M. pusillus var. Lindt enzyme was also crystallized in 1968.[9)] This enzyme is more thermostable than calf rennet and exhibits its pH stability in the range of 4.0 - 6.0. Its isoelectric point renders between pH 3.5 - 3.8, and the molecular weight is estimated to be between 29,000 - 30,600. The enzyme is more sensitive to calcium concentration in milk than calf rennet. This enzyme exhibits one of the highest ratios of milk-clotting activity to proteolytic activity among microbial rennets.[10)] A number of cheeses including Camembert, Cheddar, Edam, Gouda and Italian varieties have been made successfully with this enzyme.

Sternberg crystallized the milk-clotting enzyme of M. miehei NRRL 3420 in 1971.[11)] The enzyme has an isoelectric point at pH 4.2 and its molecular weight is estimated to be 38,000. The enzyme hydrolyzed hemoglobin at pH 3.5 maximally and proved to be stable over the wide range of pH 2.0 to 6.0 at 40°C for 24 hr. The ratio of milk-clotting activity to proteolytic activity of the enzyme was reported to be the highest among microbial rennets. Many types of cheeses including Camembert, Cheddar,

Edam and Gouda have been prepared successfully with this enzyme.[12)]

These enzymes possess distinct advantages, e.g. costs below that of calf rennet; stable supplies, possibilities for accelerated cheese ripening, greater thermostability(M. pusillus and M. miehei enzymes), and less sensitivity to variation in milk pH(E. parasitica enzyme). Milk-clotting enzymes from E. parasitica, M. pusillus, and Mucor miehei were used as admixtures with calf rennet for the manufacturing of more than 50% of the cheese produced in the world in 1980.

Kawai and Mukai[13-15)] undertook a screening test to evaluate 44 strains of basidiomycetes as potential calf rennet substitutes. They found that the most promising substitute among them was Irpex lacteus. The optimum pH of Irpex enzyme for hydrolysis of casein was 2.5 and the optimum temperature was 55 to 60°C. They also found that the enzymatic proteolysis of milk at pH 6.0 continued to increase with time to a greater extent than that of calf rennet. In actual cheese-making trials, Cheddar cheese made with the coagulant was of good quality, however, detailed data were not shown. Although chymosin and milk-clotting enzymes from M. miehei, M. pusillus and E. parasitica were well characterized, milk-clotting enzyme from I. lacteus was not characterized sufficiently.

In this Ph. D. thesis, I described the isolation and characterization of milk-clotting enzymes from Irpex lacteus in chapter II. In chapters III, IV and V, I described the substrate

specificity of the milk-clotting enzyme on insulin B chain, αs1-casein and β-casein, respectively. I also described the evaluation of the milk-clotting enzymes as a calf rennet substitute for cheese-making in chapter VI and the purification and characterization of proteinase other than milk-clotting enzymes existing in the crude preparation in chapter VII.

CHAPTER II

URIFICATION AND CHARACTERIZATION OF TWO MILK-CLOTTING ENZYMES ROM *IRPEX LACTEUS*

SUMMARY

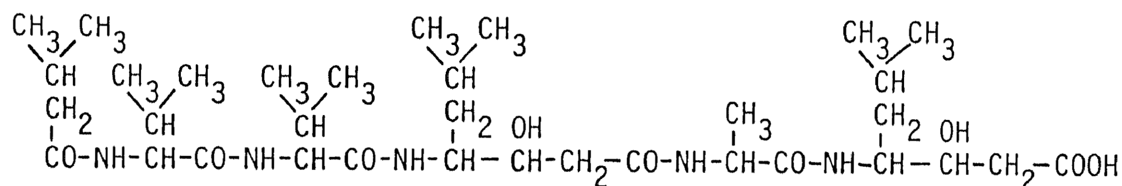
Two carboxyl proteinases with high milk-clotting activity esignated enzymes A and B were isolated by affinity chromatoraphy with dehydroacetylpepstatin as ligand, DEAE-cellulose hromatography and isoelectric focusing. The final preparations ere judged homogeneous by polyacrylamide gel electrophoresis. he molecular weights of enzymes A and B were determined by gel iltration and SDS-PAGE to be 36,000. The isoelectric points of and B were 4.9 and 5.3, respectively.

A and B had similar amino acid compositions lacking sulfurontaining amino acids, such as cysteine and methionine. And oth of the enzymes were inhibited by carboxyl proteinase inhibiors such as pepstatin, DAN and EPNP. These results indicate hat enzymes A and B are similar to calf chymosin and other icrobial milk-clotting enzymes in their active site structure aving two different carboxyl groups, although they showed minor ifference in regard to enzymatic and molecular properties. nzymes A and B exhibited almost the same ratio of milk-clotting ctivity to proteolytic activity as commercial microbial milk-

clotting enzymes obtained from *Mucor pusillus* and *Mucor miehei*.

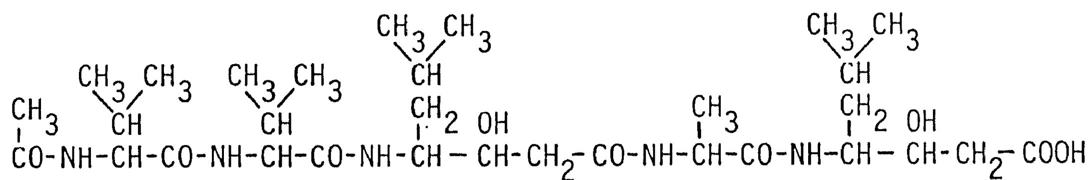
INTRODUCTION

A carboxyl proteinase inhibitor, pepstatin A[16] (*N*-acylated pentapeptide), has been used effectively as a ligand for affinity chromatography in the purification of various carboxyl proteinases from mammals.[17-25] However, the application of this affinity column to the purification of microbial carboxyl proteinase was limited. This is due to the very high affinity of microbial enzymes to pepstatin A.[26]

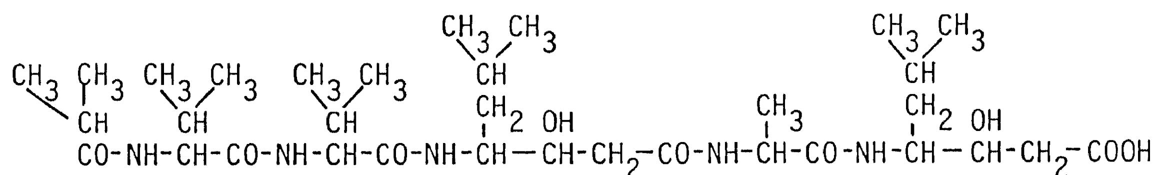


N-isovalerylpepstatin (pepstatin A)

Two modifications of the pepstatin A molecule are feasible for reduction of its affinity to carboxyl proteinase. One modification is the decrease of carbon atoms in *N*-acyl group of pepstatin A(*N*-isovalerylpepstatin). As shown in the previous paper,[26] I prepared two affinity columns including *N*-acetyl-pepstatin or *N*-isobutyrylpepstatin and used them effectively for the purification of microbial milk-clotting enzymes from *M. miehei* and *E. parasitica*.

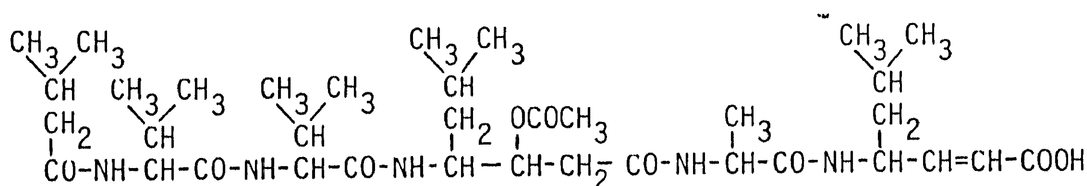


N-acetylpepstatin



N-isobutyrylpepstatin

Another modification is acetylation of hydroxyl group of central static residue of pepstatin A molecule. Marcinizyn[27] and Aoyagi[28] reported individually that the acetylation of this hydroxyl group drastically decreased the inhibitory activity of pepstatin A against carboxyl proteinases. Aoyagi[29] also reported that dehydroacetylpepstatin (O-acetyl derivative of dehydropepstatin) was 100 times less active than pepstatin A against pepsin.



dehydroacetylpepstatin

Thus, I intended to prepare a new affinity column including

dehydroacetylpepstatin for the rapid purification and characterization of *Irpex lacteus* milk-clotting enzyme. Because *I. lacteus* enzyme is adsorbed to a pepstatin A or *N*-acetylpepstatin-aminohexyl-agarose column so tightly that it can not be eluted from the column under stable conditions.

MATERIALS AND METHODS

Irpex lacteus rennet powder(KY2981) was kindly supplied by Kyowa Hakko Co. Ltd. *Mucor miehei* rennet(Marzyme, single strength) was purchased from Miles Laboratories, Inc., *Mucor pusillus* rennet was from Meito Sangyo and *Endothia parasitica* rennet(Liquid surecurd, triple strength) was from Pfizer Inc. Porcine pepsin, bovine serum albumin, ovalbumin, myoglobin, bovine trypsinogen and DAN were from Sigma. EPNP was from Eastman Kodak. Pepstatin, chymostatin, antipain and leupeptin were from the Protein Research Foundation(Japan). Sepharose 4B and Sephadex G-100 were from Pharmacia, DEAE-cellulose(DE-52) was from Whatman, ampholite was from LKB, and all other chemicals were of reagent grade.

Affinity column. Dehydroacetylpepstatin was prepared according to the method of Umezawa *et al*.[16] Dehydroacetyl-pepstatin-aminohexyl-agarose was prepared by the method of Murakami and Inagami.[30] One ml of the affinity gel contained 1.0 to 1.5 μ moles of covalently bound dehydroacetylpepstatin

which was determined by amino acid analysis of the gel.

Enzyme assays.

Milk-clotting activity. Ten grams of skim milk powder (Morigaga-nyugyo Co.) were suspended in 100 ml of 0.01 M calcium chloride solution and used as a substrate. To 5 ml of the substrate, 0.5 ml of enzyme solution which could clot the milk at 35°C between 1 to 3 minutes was added. The moment when the thin film of milk breaks into visible particles was noted as the clotting time. The pH of the reaction mixture was 6.3. Milk-clotting activities are calculated by following equation.

$$\text{Soxhlet Unit (S. U.)} = 2400/T \times 5/0.5 \times D$$

where, T : clotting time (second)

D : dilution factor of enzyme

Proteolytic activity. The assay mixture contained 1 ml of 1.2% casein (or hemoglobin) in 0.1 M glycine-HCl buffer (pH 3.0) and 200 µl of the enzyme solution. The reaction was carried out at 35°C for 30 min, and was stopped by the addition of 1 ml of 0.55M trichloroacetic acid, and the absorbance was measured at 280 nm after filtration. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 1.0 at 280 nm at pH 3.0 and 35°C for 30 min. Protein concentration was measured by absorbance at 280 nm, assuming that absorbance at the concentration of 1 mg/ml is 1.0.

Polyacrylamide gel electrophoresis. Polyacrylamide gel

electrophoresis was run in a 6 cm gel containing 7.5% polyacrylamide with 2.5% cross-linkage at pH 8.9 with a constant current of 3 mA per gel for 3 hr with the stacking gel according to Davis.[31) Protein bands were stained by Coomassie brilliant blue R solution.

Molecular weight. The molecular weight of the enzyme was estimated on a Sephadex G-100 column (1.5 x 90 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl. The column was calibrated with bovine serum albumin (68,000), ovalbumin (45,000) and myoglobin (17,800) as molecular weight markers. SDS-polyacrylamide gel electrophoresis was conducted in 0.1% SDS-0.1 M sodium phosphate buffer, pH 7.2, at 8 mA per gel according to Weber and Osborn.[32)]

Amino acid composition. The pure enzyme preparations were subjected to amino acid analysis in a Durrum D-500 type analyzer after hydrolysis in 6 N HCl at 110°C for 24, 48 and 72 hr. Amino acid values of serine, threonine and tyrosine were corrected by extrapolating to zero time of hydrolysis. The values for valine and isoleucine are average of 48- and 72-hr hydrolyzates. The values for other amino acids are averages of three samples. Tryptophan was determined by the method of Goodwin and Morton.[33)] Cysteine was determined after HCl hydrolysis in 0.2 M dimethyl sulfoxide according to the method of Spencer and Wald.[34)]

Inhibition studies. Inhibition studies with DAN and EPNP were performed by the method of Kageyama and Takahashi.[35)]

Trypsinogen activating ability. A trypsinogen activating ability was carried out as follows: the reaction mixture for activation contained 0.5 ml of 0.1% trypsinogen in 0.05 M glycine-HCl buffer, pH 3.0, and 0.5 ml of enzyme solution (40 s.u./ml). After exactly 10 min reaction at 35°C, 0.5 ml of the reaction mixture was then added to 2.5 ml of 0.6% casein in 0.1 M phosphate buffer, pH 7.8, and maintained at 35°C for 10 min. The reaction was terminated by the addition of 2.5 ml of 0.55 M TCA and the absorbance of resultant filtrate was measured at 280 nm.

Purification of *I. lacteus* milk-clotting enzyme. All operations of enzyme purification were carried out at 4°C. One gram of *I. lacteus* rennet powder was dissolved in 100 ml of 0.01 M sodium acetate buffer (pH 5.5) and applied to a dehydroacetyl-pepstatin-aminohexyl-agarose column equilibrated with the same buffer (Fig. 1). After washing with the same buffer (pH 5.5), active fractions with high proteolytic activity were eluted with 0.5 M NaCl in the starting buffer. Then, active fractions with high milk-clotting activity (brackets, Fig. 1) were eluted from the affinity column by changing the pH to 3.0 with 0.1 N acetic acid, concentrated and applied to a DEAE-cellulose column. Two active fractions were eluted with a NaCl gradient from the DEAE-cellulose column (Fig. 2). The main fraction (brackets, Fig. 2)

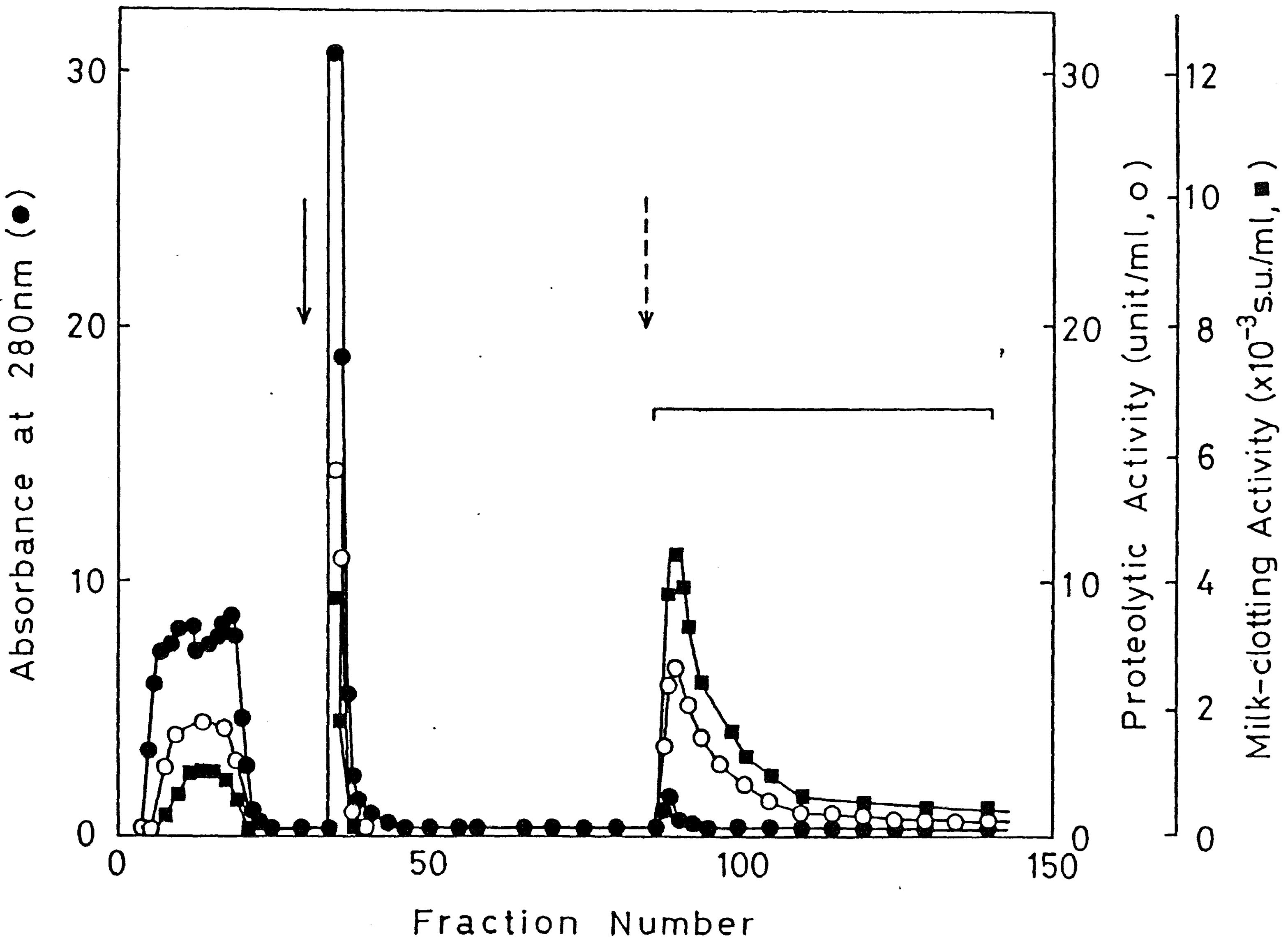


Fig. 1. Affinity Chromatography of Crude Irpex lacteus Rennet on Dehydroacetyl pepstatin-aminohexyl-agarose(1.0 x 5.0cm). The flow rate was 20 ml/hr and 4 ml fractions were collected. ↓ , 0.01 M sodium acetate buffer containing 0.5 M NaCl (pH 5.5); ⇣ , 0.1 N acetic acid.

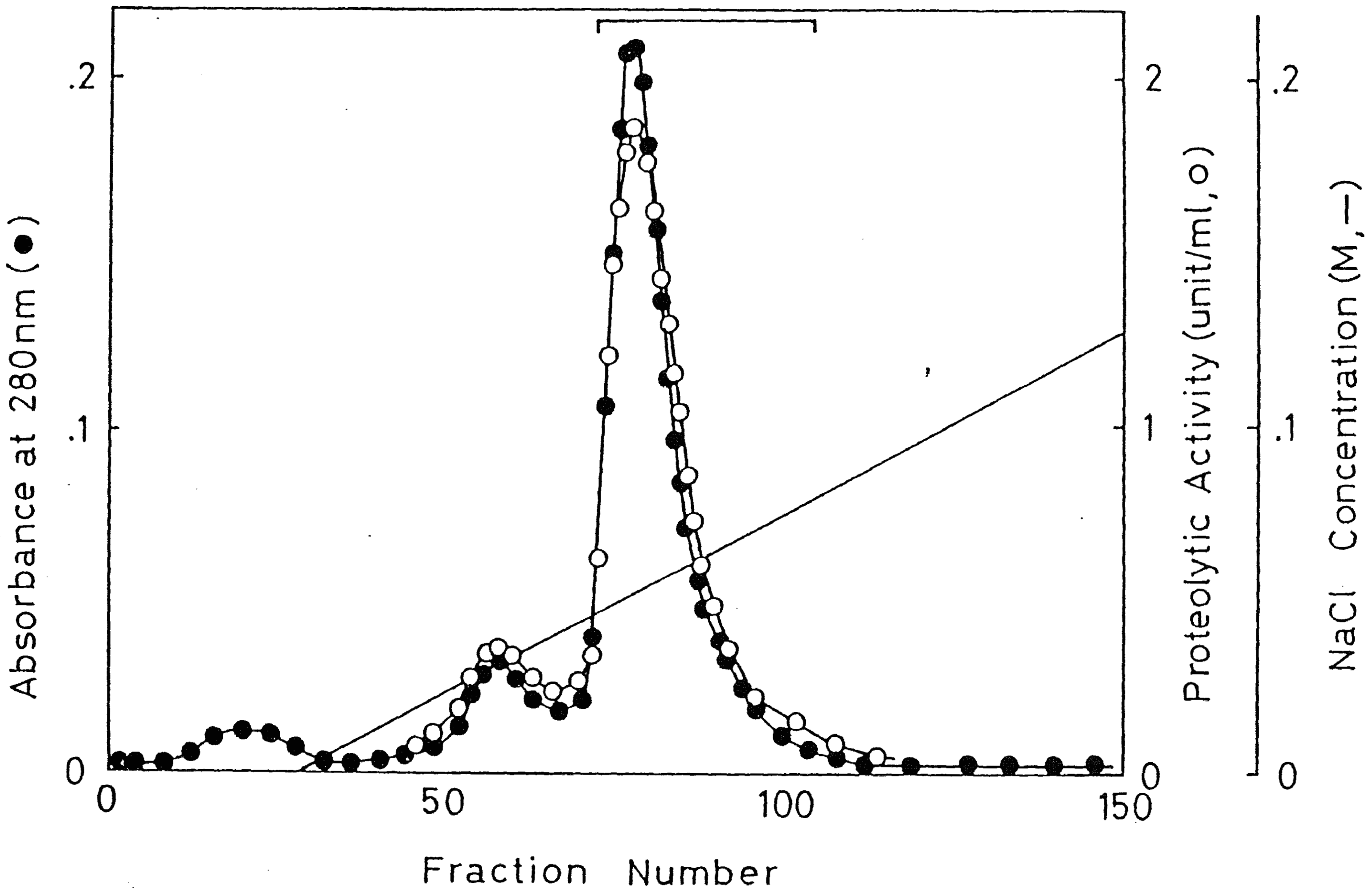


Fig. 2. Chromatography of Fraction with Bracket (Fig. 1) on the DEAE-cellulose Column. 200 ml of the active fraction with bracket (25 mg protein) was dialyzed against 0.05 M phosphate buffer (pH 6.0) and applied to a DEAE-cellulose column (1.5 x 25 cm) previously equilibrated with the same buffer. This column was washed with the same buffer and further eluted using 500 ml linear gradient of 0 - 0.2 M NaCl in the same buffer.

was concentrated to 30 ml, dialyzed against 5 mM sodium acetate buffer, pH 5.5, and further purified by isoelectric focusing in the pH range of 4-6. The active fractions separated into two components and designated as enzymes A and B in order of elution (Fig. 3).

RESULTS

Crude I. lacteus milk-clotting enzyme was inhibited to different degrees by pepstatin A and its derivatives. Concentrations of pepstatin A, N-acetylpepstatin and dehydroacetylpepstatin giving 50% inhibition were 2×10^{-7} M, 2×10^{-7} M and 2×10^{-5} M, respectively (Fig. 4). Dehydroacetylpepstatin is 100 times less active than pepstatin A in the inhibition against crude I. lacteus milk-clotting enzyme. This suggested that affinity between I. lacteus enzyme and dehydroacetylpepstatin is not so high and thus it could be used as a ligand suitable for an affinity column in the purification of I. lacteus milk-clotting enzyme.

Purification of milk-clotting enzyme. The purification steps were summarized in Table I. Purified enzymes A and B showed 16-fold and 19-fold purification over the crude enzyme solution, which were about 5% and 15% recoveries, respectively. Enzymes A and B were dialyzed against 0.01 M sodium acetate buffer, pH 5.5, stored at -20°C and used for subsequent charac-

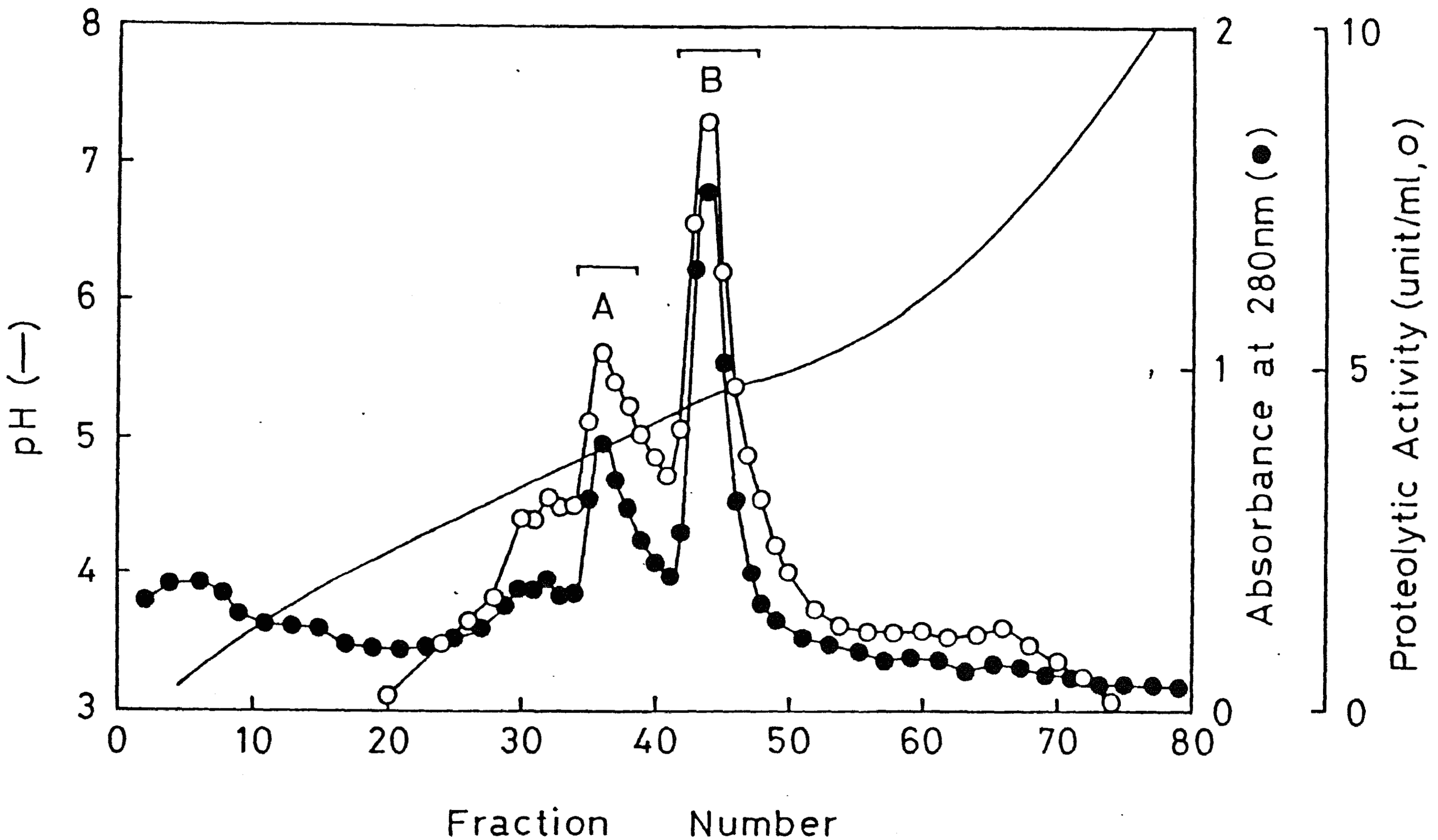


Fig. 3. Isoelectric Focusing of Main Fraction with Bracket in Fig. 2.

Concentrated enzyme preparations (14 mg protein) from DEAE-cellulose were applied onto a 110 ml electrofocusing column in 2% (w/v) ampholines (pH 4-6). The voltage was maintained at 900 V for 24 hr at 4°C (1.4 ml fraction; flow rate 30 ml/hr). Activity and pH determinations were performed immediately.

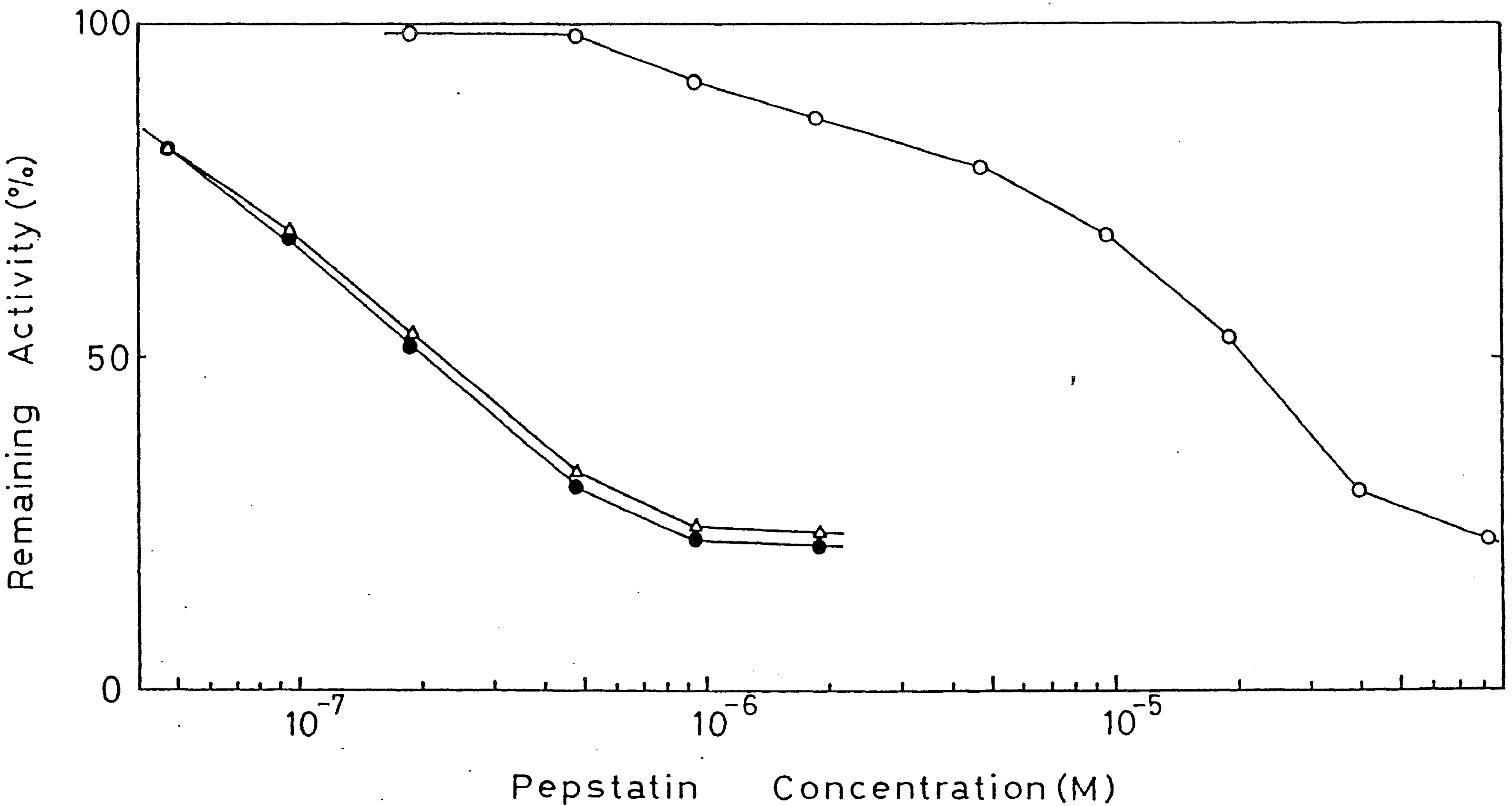


Fig. 4. Inhibition of *Irpex lacteus* Rennet by Pepstatin A, SPI (*N*-acetylpepstatin) and Dehydroacetylpepstatin. To 1 ml of the crude enzyme solution (200 s.u./ml) were added 0.01 ml of pepstatin A, SPI and dehydroacetylpepstatin (10^{-2}-10^{-6} M) in dimethylformamide and residual milk-clotting activity was determined as described in Materials and Methods. ●, pepstatin A; △, SPI; ○, dehydroacetylpepstatin.

Table I. Purification of <u>Irpex lacteus</u> Milk-clotting Enzymes

	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg protein)	Yield (%)	Purification (x fold)
Crude extract	638.9	1016.9	0.63	100	1.0
Dehydroacetyl-pepstatin gel	197.4	25.3	7.80	31.0	12.4
DEAE-cellulose	149.9	14.3	10.47	23.0	16.6
Isoelectric focusing A	31.3	3.2	9.78	4.9	15.5
B	93.3	7.7	12.12	14.6	19.2

terization studies as reported in this investigation.

Evidence of purity of enzymes A and B. When the purified enzymes were examined by polyacrylamide gel electrophoresis at pH 4.3, each gave rise to one discrete band after the staining for protein (Fig. 5)

Molecular weight. The molecular weight determined by the gel filtration on Sephadex G-100 was calculated to be 36,000 for each of enzymes A and B. SDS-PAGE in the presence of 2-mercapto-ethanol produced a single discrete band at a position correspond-ing to a molecular weight of 36,000 (A and B). In estimation of the molecular weight, good agreement was obtained between the gel filtration and SDS-PAGE methods.

Isoelectric point. As shown in Fig. 4, enzymes A and B had isoelectric points of 4.9 and 5.3, respectively.

Extinction coefficient. The ultraviolet adsorption spectra of these purified enzymes were measured in 0.01 M sodium acetate buffer, pH 5.5. The $E^{1\%}_{1cm}$ at 280 nm of A and B were determined to be 11.6 and 12.1, respectively.

Proteolytic and milk-clotting activities. Enzymes A and B were most active at pH 3 toward protein substrates. The highest rates of proteolysis among proteins examined were obtained with

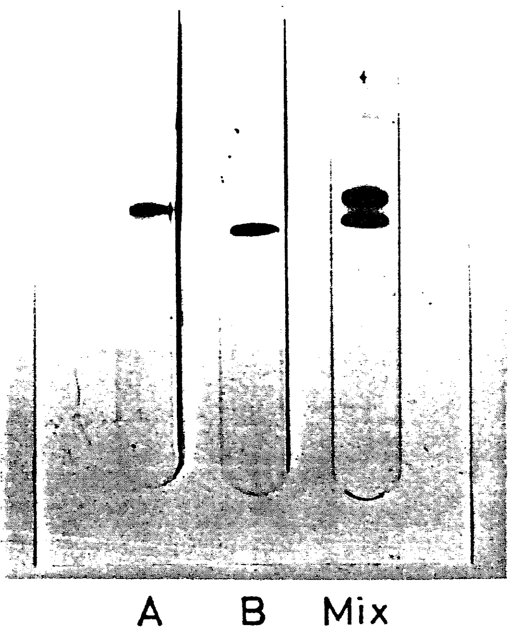


Fig. 5. Polyacrylamide Gel Electrophoresis of Enzymes A and B. The electrophoresis was performed at pH 4.3, 3 mA per tube, 4°C for 3 hr in 0.5 x 7 cm columns of 7.5% polyacrylamide gel according to Davis.[31] Mix contains purified enzymes A and B. Protein bands were stained by Coomassie brilliant blue R.

hemoglobin and myoglobin (Table II). Casein was hydrolyzed by these enzymes at about one-third the rate for hemoglobin. The optimal temperature of A and B for the maximum rate of milk-clotting was 45°C(Table III).

Stability. No loss of proteolytic activity of the purified enzymes A and B was noted on storing at - 20°C for 1 month after freezing of the purified enzyme solution, at pH 5.5. Almost no loss of activity of the enzymes was observed at pH 3 - 6 when they were incubated at 30°C for 2 hr. Both of the enzymes became unstable beyond 6 and were completely inactivated at 50°C and pH 6.0 for 20 min. As compared to other milk-clotting enzymes such as chymosin and enzymes from *M. miehei* and *M. pusillus*, enzymes A and B are relatively unstable at neutral pH. Molecular and enzymatic properties of A and B were summarized in Table III.

Amino acid composition. The amino acid compositions of enzymes A and B were summarized in Table IV and showed striking similarities to each other. Sulfur-containing amino acids, such as cysteine and methionine, could not be detected in each hydrolyzate, 24, 48, and 72 hr. Cystine and cysteine were also not detected as cysteic acid after oxidation of enzymes A and B with dimethylsulfoxide.

Inhibitors. The time courses of inactivation of enzymes A and B, and pepsin by DAN and EPNP are shown in Fig. 6. Pepsin

Table II. Relative Activity toward Various Proteins

Substrate	Relative activity	
	A	B
Casein	100	100
Hemoglobin	264	263
Ovalbumin	17	38
BSA	121	122
Myoglobin	270	235
Elastin	4	0

Each reaction mixture contained 1 ml of 1.2% protein solution (0.1 M glycine-HCl buffer, pH 3.0) and 0.2 ml enzyme solution.

Table III. Properties of *Irpex lacteus* Milk-clotting Enzymes

	A	B
Molecular weight*	36,000	36,000
Isoelectric point	4.9	5.3
Optimal pH for casein	3.0	2.9
for hemoglobin	2.8	2.8
pH stability	3-5	3-6
Optimal temperature (proteolysis)	62°C	62°C
(milk clotting)	45°C	45°C
Thermostability	0-45°C	0-45°C

* Determined by gel filtration on Sephadex G-100 and SDS-polyacrylamide gel electrophoresis.

Table IV. Amino Acid Compositions of Enzymes A and B

	Irpex lacteus		Chymosin[36]	*M. pusillus*[37]	*M. miehei*[38]	*E. parasitica*[39]
	A	B				
Asp	33	32	36	44	42	26
Thr	48	49	23	21	18	50
Ser	43	43	31	22	25	44
Glu	15	15	33	20	15	15
Pro	10	10	15	14	11	13
Gly	34	34	28	34	24	38
Ala	25	25	15	16-17	22	29
Val	22	22	26	24	16	22
Cys	0	0	6	2	4	2
Met	0	0	8	3	5	1
Ile	14	15	19	12	11	17
Leu	29	28	23	15	14	19
Tyr	13	12	19	13	13	19
Phe	11	11	17	19	14	13
Lys	8	8	9	11-12	8	12
His	1	1	5	1-2	2	3
Arg	5	5	6	4	4	2
Trp	2	2	4	2-3	3	3
Total	313	312	323	277-281	251	328

The compositions are expressed as amino acid residues/mole of protein, based on molecular weights for A and B of 36,000.

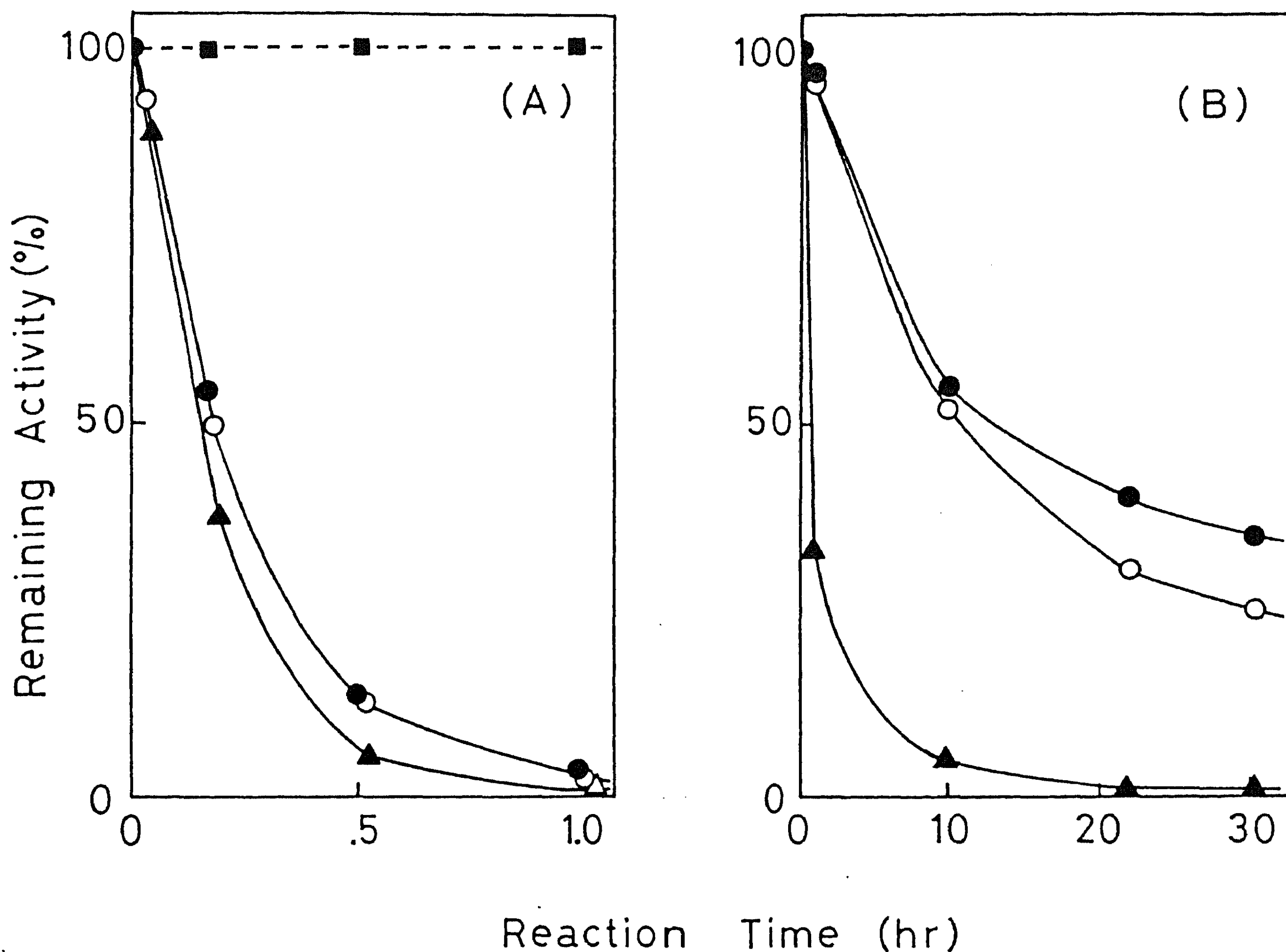


Fig. 6. Time Courses of Inhibitors on Enzymes A and B, and Porcine pepsin. ○, A; ●, B; ▲, porcine pepsin. A) Each (30 μg) of the purified enzymes in 1.0 ml of 0.01 M sodium acetate buffer, pH 5.5, was mixed with 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.0, and 25 μl of 0.01 M cupric sulphate. After incubation of the mixture at 25°C for 10 min, 25 μg of DAN in 50 μl methanol was added to the mixture. The reaction was allowed to proceed at 14°C for 1hr. At the indicated intervals, 200 μl aliquots were withdrawn and used for the assay of enzymatic activity. ■, without Cu^{+2}. B) Enzyme (30 μg) in 1.0 ml of the acetate buffer, pH 5.5, was mixed with 1.0 ml of 0.1 M citrate buffer, pH 4.6, and 500 ug EPNP in 50 μl ethanol. The reaction mixture was maintained at 25°C with gentle stirring. Aliquots of 200 μl were withdrawn and used for the assay of proteolytic activity.

was slightly more sensitive to these inhibitors than the two milk-clotting enzymes. DAN was not inhibitory in the absence of Cu^{+2}. Thiol proteinase inhibitors such as p-CMB and MIA, metal proteinase inhibitors such as EDTA, serine proteinase inhibitors such as PMSF and DFP showed no effect on the activities of enzymes A and B (Table V). Moreover, chymostatin, antipain and leupeptin did not inhibit A and B (Table V).

The ratio of milk-clotting activity to proteolytic activity. Each ratio of the purified enzymes A and B was 3 times higher than that of the crude enzyme. Although these values of A and B were lower than that of calf chymosin, they were almost the same as those of *Mucor* enzymes and four times higher than that of *E. parasitica* enzyme (Table VI).

Trypsinogen activating ability. Table VII shows trypsinogen activating ability of some milk-clotting enzymes at pH 3.0. Although the enzyme from *E. parasitica* showed the ability considerably, enzymes from *Mucor* and *Irpex*, and chymosin hardly activate trypsinogen.

Effect of calcium concentration on milk-clotting activity. As shown in Fig. 7, enzymes A and B were less sensitive than calf chymosin to calcium ion concentration in milk. This is an importance, since calcium concentration in milk vary with season and geography.

Table V. Effect of Various Inhibitors

Reagent	Concentration (mM)	Remaining activity (%) A	B
None		100	100
Pepstatin	0.01	0	0
Chymostatin	0.01	100	100
Antipain	0.01	100	100
Leupeptin	0.01	100	100
DFP	10	100	99
MIA	10	99	100
_p_-CMB	1	100	99
EDTA	10	99	100

Each of the purified enzymes was preincubated with each reagent in 0.01 M sodium acetate buffer, pH 5.5, at 30°C for 15 min and residual activity was assayed. Enzyme activities are expressed as percentages of the activity in the absence of reagents.

Table VI. Ratio between Milk-clotting Activity and Proteolytic Activity

Enzyme	Milk-clotting activity / Proteolytic activity
Crude *Irpex lacteus* rennet	493
Enzyme A	1,424
Enzyme B	1,657
Calf rennet	4,778
Mucor pusillus rennet	1,742
Mucor miehei rennet	1,819
Endothia parasitica rennet	394

Milk-clotting activity was determined by the method as described in MATERIALS AND METHODS and proteolytic activity was measured at 35°C for 30 min at pH 6.0 with 1.2% casein solution.

Table VII. Trypsinogen Activating Ability of Various Milk-clotting Enzymes

Enzyme	Trypsin activity generated (A_{280})	Relative activity (%)
Crude *Irpex lacteus* rennet	0.092	17
Enzyme A	0.020	4
Enzyme B	0.016	3
Calf chymosin	0.000	0
Porcine pepsin	0.008	1
Mucor miehei rennet	0.020	4
Mucor pusillus rennet	0.015	3
Endothia parasitica rennet	0.556	100

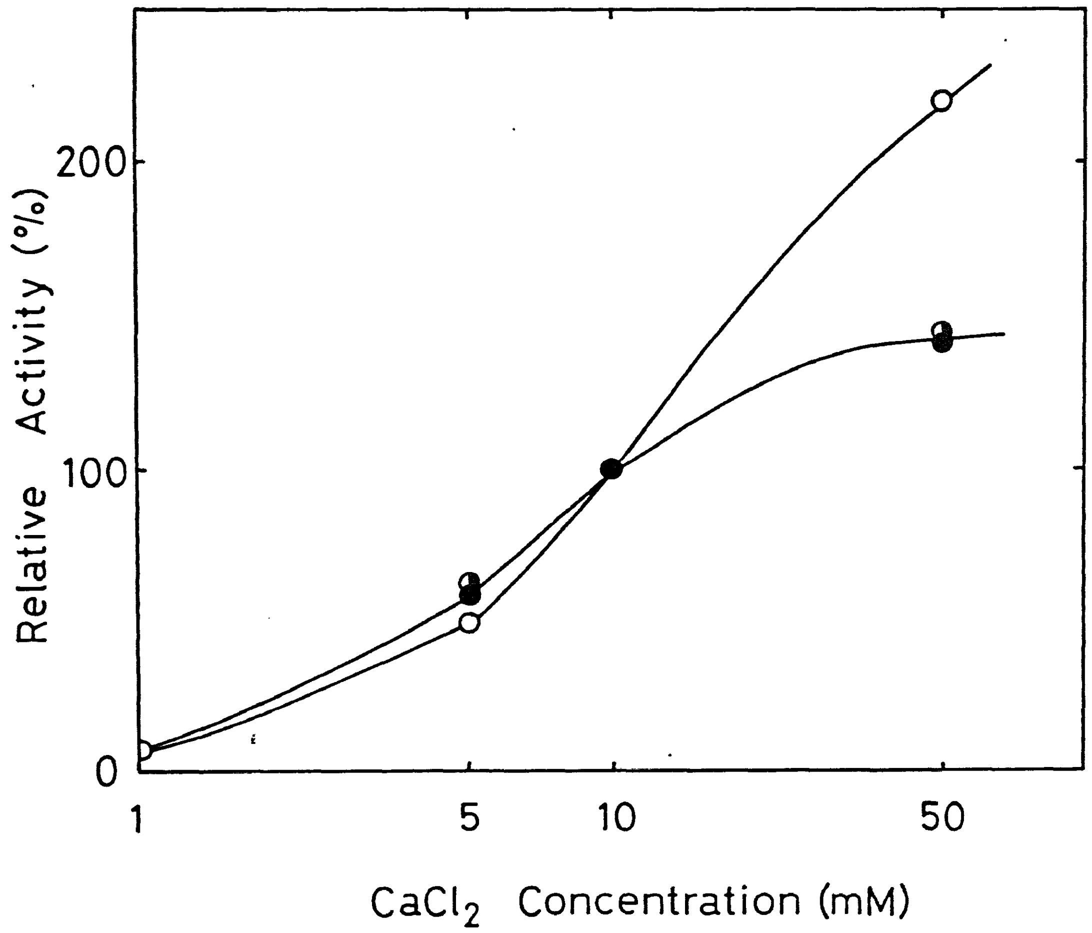


Fig. 7. Effect of Calcium Concentration on Milk-clotting activities of Enzymes A and B, and Chymosin. ○, chymosin; ◐, A; ●, B.

Digestion of casein. Electrophoretic patterns of whole casein digestion by A and B were shown in Fig. 8. A protein band between αs- and β-casein appeared after 2 hr incubation of whole casein with either A or B, but no change was observed with chymosin.

DISCUSSION

In the present study, a new pepstatin derivative, dehydroacetylpepstatin, was used effectively as a ligand for the affinity gel in the purification of milk-clotting enzyme in *I. lacteus*. The affinity gel including the new ligand enabled us to purify the enzyme more simply with a higher yield compared with the conventional method consisting of 6 steps.[40] With 4 ml of this gel it was possible to obtain 25 mg of the highly purified milk-clotting enzyme from 1 g of crude enzyme preparation. Furthermore, this affinity gel was very stable and could be used over a hundred times by regenerating it with the initial buffer without significant deterioration. The two purified carboxyl proteinases, enzymes A and B, resemble each other in their molecular and enzymatic properties other than their isoelectric points. Enzyme B, a major carboxyl proteinase in *I. lacteus*, is similar to an carboxyl proteinase purified partially from *I. lacteus* by Kawai.[15] But enzyme A seems to be a new type of enzyme in *I. lacteus* judged from its isoelectric point.

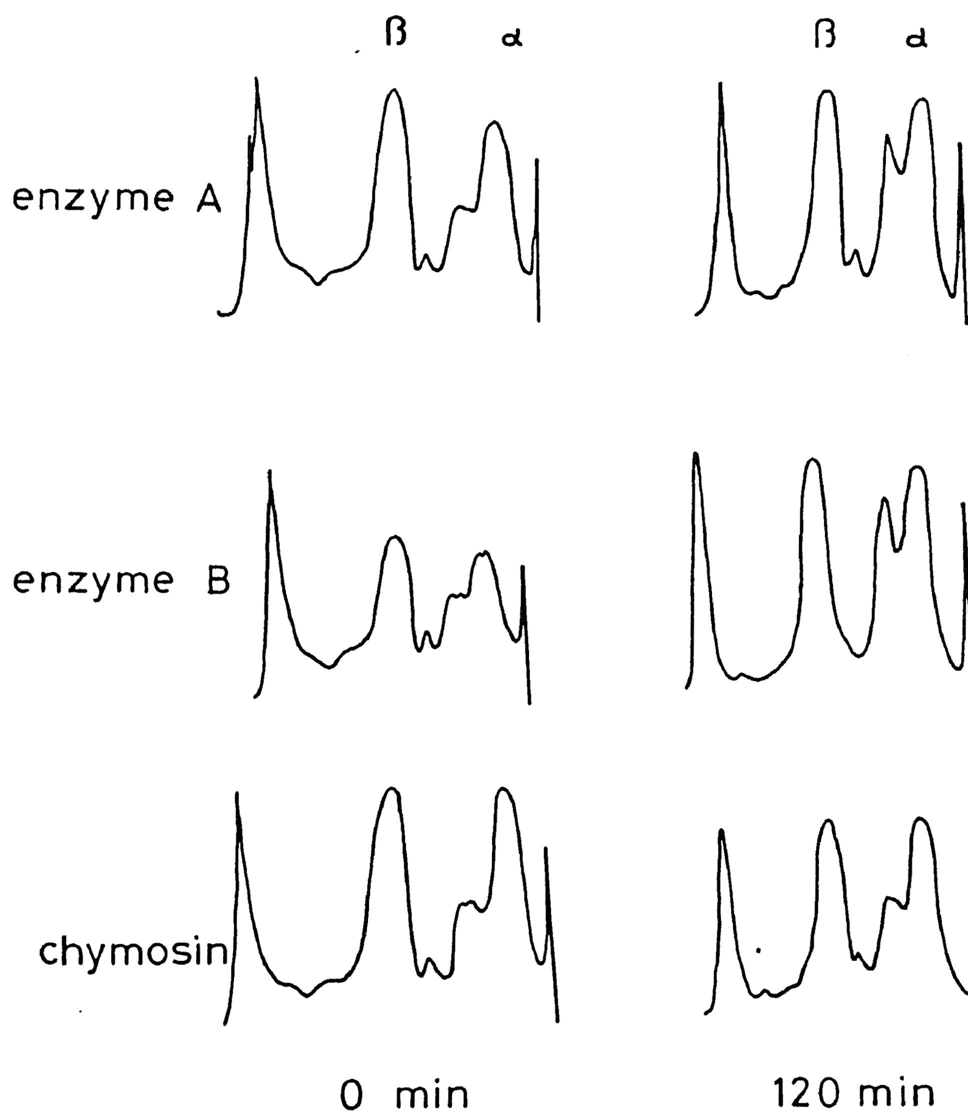


Fig. 8. Polyacrylamide Gel Electrophoresis of Whole Casein Digested by Enzymes A and B, and Calf Chymosin. To 2 ml of substrate (2% whole casein in 0.1 M phosphate buffer, pH 6.3), 0.2 ml of the enzyme solution (300 s.u./ml) was added and the reaction mixture was incubated at 35°C. Samples taken after zero and 120 min were diluted with 9 M urea to final concentration of 6 M and 0.05 ml of the diluted samples was examined by PAGE at pH 8.9.

Enzymes A and B were inhibited by pepstatin, DAN and EPNP as well as calf chymosin,[41] pepsin[41] and M. pusillus[42] enzyme. The inhibition of A and B by EPNP and DAN indicates that these enzymes contain two different carboxyl groups as their active site like all other gastric and microbial carboxyl proteinases.

In general, enzymes A and B were similar to E. parasitica enzyme in amino acid composition except for cysteine and methionine contents. As compared to chymosin and microbial milk-clotting enzymes from M. miehei and M. pusillus, I. lacteus enzymes A and B were rich in threonine, serine and leucine, but deficient in glutamic acid, cysteine, methionine and histidine (Table IV).

The ratios of milk-clotting activity to proteolytic activity of A and B were the same as those of the microbial milk-clotting enzymes which are used commercially, indicating that enzymes A and B have good properties as calf rennet substitute for cheese-making.

CHAPTER III

SUBSTRATE SPECIFICITY OF MILK-CLOTTING ENZYME B FROM *IRPEX LACTEUS* ON OXIDIZED INSULIN B CHAIN AND ANGIOTENSIN I

SUMMARY

Substrate specificity of enzyme B from *Irpex lacteus* on oxidized insulin B chain and angiotensin I was investigated. In the case of insulin B chain, the peptide bonds mainly susceptible to the enzyme were Leu(11)-Val(12), Ala(14)-Leu(15), Phe(24)-Phe(25) and Thr(27)-Pro(28) bonds, and the Ala(14)-Leu(15) bond was hydrolyzed most preferentially. The specificity of *Irpex lacteus* enzyme B is distinct from other commercial microbial milk-clotting enzymes and it has a more restricted specificity than chymosin and porcine pepsin. Moreover, a notable property of enzyme B is that it hydrolyzed the Thr(27)-Pro(28) bond which is hydrolyzed very little by any of the carboxyl proteinases ever studied.

Irpex lacteus enzyme B hydrolyzed the Tyr(4)-Ile(5) bond much more rapidly than the Val(3)-Tyr(4) bond of angiotensin I. This specificity resembled that of *Pycnoporus coccineus* carboxyl proteinase.

INTRODUCTION

In chapter II, the author described the purification and characterization of two carboxyl proteinases with high milk-clotting activity from *Irpex lacteus*. The main enzyme, carboxyl proteinase B, has a molecular weight of 36,000, an amino acid composition similar to that of *Endothia parasitica* carboxyl proteinase, and has maximum proteolytic activity on hemoglobin at pH 2.8. The enzyme B exhibits almost the same ratio of milk-clotting activity to proteolytic activity as commercial rennet substitutes from *Mucor pusillus* and *Mucor miehei*. Substrate specificities of carboxyl proteinases including chymosin, pepsin and microbial milk-clotting enzymes on oxidized insulin B chain have been investigated[43] and comparisons of specificities of these enzymes were reported.[44,45] On the other hand, Ichishima *et al*. investigated substrate specificities of several carboxyl proteinases on angiotensin I and classified them into four groups. In this chapter, I intended to investigate the site of cleavage towards the oxidized insulin B chain and angiotensin I by the carboxyl proteinase B from *Irpex lacteus* and compare the specificity with those of other carboxyl proteinases reported so far.

MATERIALS AND METHODS

Bovine pancreatic insulin was from Sigma. Dowex 50W-X2 and Sephadex LH-20 were from Dow Chemical Co. and Pharmacia, respectively. Thin layer chromatography plate (silica gel 60) was from Merck and fluorescamine was from Roche. Dansyl chloride, dansyl amino acids and N-ethylmorpholine were from the Pieace. Micro-polyamide sheets(F1700) was from Schleicher and Schuell GmbH. Angiotensin I was from the Protein Research Foundation (Japan). Other chemicals used were of reagent grade.

Purified carboxyl proteinase B from *Irpex lacteus* was prepared according to the methods described in Chapter II. The oxidized insulin B chain used as the substrate was prepared from crystalline bovine pancreatic insulin according to the method of Sanger[46)] and the purity of the preparation was ascertained by amino acid analysis and N-terminal amino acid analysis by the method of Hartley.[47)]

Hydrolysis of oxidized insulin B chain. The enzyme B (0.2 mg) was added to a solution of 40 mg of insulin B chain in 10 ml of 0.1 M acetic acid (pH 3.0, substrate:enzyme=200:1). After 2 hr incubation at 35°C, the reaction was terminated by lyophilization.

Purification of peptides formed by the action of enzyme B. The lyophilized powder was dissolved in 1 ml of 50% formic acid and applied on a column of Dowex 50W-X2 (0.9 x 60cm) equilibrated with 0.2 M pyridine-acetic acid buffer, pH 3.1, at 50°C at a constant rate of 30 ml/hr. After washing with the same buffer, peptides were separated by the linear gradient generated between 0.75 liters of 0.2 M pyridine-acetic acid buffer, pH 3.1, and 0.75 liters of 2 M pyridine-acetic acid buffer, pH 5.0. A 5 ml volume was collected for each tube(Fig. 9). Peptides were detected as follows: borate buffer (1.8 ml, 0.5 M, pH 8.5) and 20 µl of each sample were mixed and 0.15 ml of fluorescamine solution (30 mg/100 ml of acetone) was rapidly added with agitation. Fluorescent intensities were measured using Hitach fluorescence spectrophotometer model 650-10S and expressed in arbitrary units. The wave lengths of exitation and emission were 390 nm and 480 nm, respectively. The fractions of each peptide shown with blackets in Fig. 9 were concentrated and applied on a Sephadex LH-20 column (0.9 x 90cm) equilibrated with 0.1 N acetic acid. Each of the fractions was then subjected to thin layer chromatography to determine the homogeneity of its components, using n-butanol-pyridine-acetic acid-water=30:23:6:24 as a solvent. Peptides were detected with fluorescamine.

Identification of peptides formed by the action of enzyme B The isolated peptides were subjected to the analysis of amino acid compositions as described in MATERIALS AND METHODS of

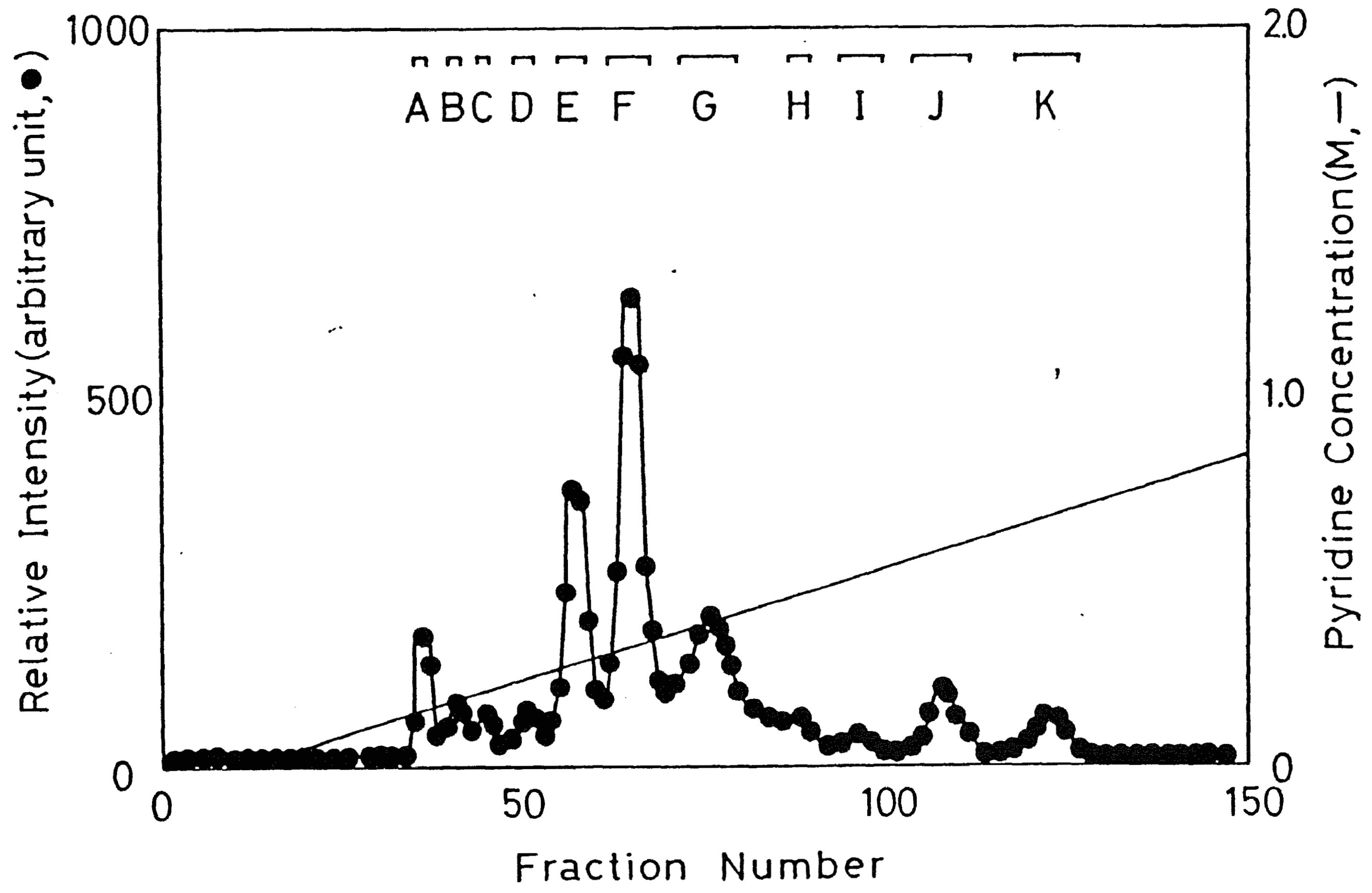


Fig. 9. Column Chromatography of the Digest of the Oxidized Insulin B Chain on Dowex 50W-X2.

Chapter II. N-terminal amino acids of each peptide were determined by the method of Hartley.[47)]

<u>Determination of the cleavage sites of angiotensin I.</u> Angiotensin I (1 mg, 0.77 nmole) was dissolved in 2 ml of 0.1 N acetic acid at pH 3.0, and incubated at 30°C for 3 hr with enzyme B (substrate:enzyme=2000:1). After that, the reaction was terminated by lyophilization. The N-terminal amino acids were determined as described above.

RESULTS

<u>Identification of peptides formed by the action of enzyme B</u> The amino acid compositions and N-terminal amino acids of these peptides were summarized in Table VIII. In the reaction with fluorescamine, the fluorescent intensity of the peptide having proline as its N-terminal amino acid, such as peptide H, is not increased(Fig. 9). Therefore, peptide H appeared as a minor peak, although it is one of the main components, as seen in Table VIII. The peptide bonds mainly susceptible to the enzyme were the Leu(11)-Val(12), Ala(14)-Leu(15), Phe(24)-Phe(25) and Thr(27)-Pro(28) bonds, and the Ala(14)-Leu(15) bond was hydrolyzed most preferentially. Leu(11)-Val(12), Phe(24)-Phe(25) and Thr(27)-Pro(28) were hydrolyzed to a relatively great extent, whereas hydrolyses at Gly(23)-Phe(24) and Phe(25)-Tyr(26) were minor.

Table VIII. Composition of Peptides Formed by the Action of Enzyme B from *Irpex lacteus* on Oxidized Insulin B Chain

Peptide	N-terminal	Amino acid found and its molar ratio	Possible amino acid sequence	Recovery(%)*
A	Val	Glu(1.06),Ala(1.00),Val(1.00)	Val(12)-Ala(14)	3
B**	-	-	-	
C	Leu	Cys(0.96),Glu(1.36),Gly(2.14),Val(1.17),Leu(1.85), Tyr(0.61),Arg(0.94)	Leu(15)-Gly(23)	2
D	Leu	Cys(1.03),Glu(1.30),Gly(2.08),Val(1.16),Leu(1.84), Tyr(1.17),Phe(2.05),Arg(1.03)	Leu(15)-Phe(25)	2
E	Leu	Cys(1.05),Glu(1.75),Gly(2.03),Val(1.14),Leu(1.76), Tyr(0.93),Phe(1.08),Arg(1.02)	Leu(15)-Phe(24)	10
Fa	Phe	Cys(1.08),Asp(1.06),Ser(1.03),Glu(1.89),Gly(1.12), Ala(1.06),Val(2.00),Leu(2.20),Phe(0.99),His(2.07)	Phe(1) -Ala(14)	14
Fb	Phe	Thr(0.96),Tyr(0.89),Phe(1.15)	Phe(25)-Thr(27)	5
Ga	Phe	Cys(1.07),Asp(0.80),Ser(0.97),Glu(1.33),Gly(1.04), Ala(0.66),Val(1.11),Leu(2.08),Phe(0.85),His(1.75)	Phe(1) -Leu(11)	7
Gb	Leu	Cys(1.24),Thr(0.76),Glu(1.24),Gly(2.23),Val(1.08), Leu(1.71),Tyr(1.68),Phe(2.16),Arg(1.11)	Leu(15)-Thr(27)	7
H	Pro	Pro(1.00),Ala(1.00),Lys(1.00)	Pro(28)-Ala(30)	11
I	Tyr	Thr(1.01),Pro(1.00),Ala(1.00),Tyr(0.77),Lys(1.02)	Tyr(26)-Ala(30)	2
J	Phe	Thr(1.13),Pro(1.08),Ala(1.11),Tyr(o.74),Phe(0.77), Lys(1.16)	Phe(25)-Ala(30)	5
K	Phe	Thr(1.05),Pro(0.99),Ala(0.97),Tyr(1.00),Phe(1.93), Lys(1.10)	Phe(24)-Ala(30)	3

Amino acid compositions were analyzed by Durrum amino acid analyzer model D-5 after hydrolysis with 6 N HCl at 110°C for 24hr. The dansyl peptides were hydrolyzed in 6 N HCl at 110°C for 20 hr and the dansyl amino acids were isolated by the method of Woods and Wang[48]) with polyamide sheet.
* The recovery of peptides was determined by amino acid analysis and expressed as the percentage of total peptide fractions eluted from the Dowex 50W-X2 column.
** No attempt was made to assign the amino acid composition because of its heterogeneity.

DISCUSSION

The specificities of several other proteolytic enzymes as determined on the oxidized B chain of insulin are compared in Fig. 10. The specificity of *Irpex lacteus* enzyme B is distinct from other commercial microbial milk-clotting enzymes. It has a more restricted specificity than chymosin and porcine pepsin.

Kawai[7] previously reported that *Trametes sanguinea* (now designated *Pycnoporus coccineus*) produced a proteinase with high milk-clotting activity, to the same degree as *Irpex lacteus*. But the *Irpex lacteus* enzyme was chosen as a rennet substitute because it produced Cheddar cheese of good quality. As shown in Fig. 10, the Ala(14)-Leu(15) and Phe(24)-Phe(25) bonds were commonly cleaved by both enzyme B from *Irpex lacteus* and the carboxyl proteinase from *Pycnoporus coccineus*, but Leu(11)-Val(12) and Tyr(16)-Leu(17) bonds were only cleaved by the enzyme B and *Pycnoporus coccineus* enzyme, respectively. Moreover, a notable property of enzyme B is that it hydrolyzed the Thr(27)-Pro(28) bond which is hydrolyzed very little by any of the carboxyl proteinases ever studied.

On the other hand, Ichishima *et al.*[49] reported that *Aspergillus sojae* carboxyl proteinase I and *Scytalidium lignicolum* carboxyl proteinase B hydrolyzed the peptide bond of His(6)-Pro(7) of angiotensin I. He classified carboxyl proteinases into four groups[50] (A to D in Fig. 11) according to

No.	Residue	Enzyme 1	2	3	4	5	6	7	8
	Phe		←	⇠	**←**				
	Val								
	Asn					←			
	Gln			←		**←**			
(5)	His								
	Leu								
	Cys SO_3H						**←**		
	Gly								
	Ser								
(10)	His								⇠
	Leu	←	⇠	←		←	**←**		
	Val								
	Glu		**←**	⇠				**←**	
	Ala	**←**	←	⇠			**←**	**←**	**←**
(15)	Leu		←	⇠	**←**	⇠	←		
	Tyr		←	←	**←**	**←**	←	←	**←**
	Leu		**←**				**←**		
	Val								
	Cys SO_3H								
(20)	Gly					⇠			
	Glu						←	←	
	Arg								
	Gly	⇠		⇠					
	Phe	←	←	←	**←**	**←**	**←**	**←**	**←**
(25)	Phe	⇠	←	←	**←**	⇠	**←**		
	Tyr				⇠			**←**	
	Thr	←							
	Pro								
	Lys								
(30)	Ala								

Fig. 10. Comparison of Cleavage Sites of Oxidized Insulin B chain by Some Carboxyl Proteinases.

The arrows indicate the bond split, the degree of hydrolysis being as follows:

1, _Irpex lacteus_ enzyme B; 2, calf chymosin[43]; 3, porcine pepsin[43]; 4, _Mucor miehei_ enzyme[44]; 5, _Endothia parasitica_ enzyme[45]; 6, _Scytalidium lignicolum_ carboxyl proteinase A[50]; 7, _Scytalidium lignicolum_ carboxyl proteinase B[51]; 8, _Pycnoporus coccineus_ carboxyl proteinase[52].

their specificities toward angiotensin I. The specificity of enzyme B from Irpex lacteus on angiotensin I was investigated. As shown in Fig. 11, Irpex lacteus enzyme B hydrolyzed the Tyr(4)-Ile(5) bond much more rapidly than Val(3)-Tyr(4) bond. This specificity resembled that of Pycnoporus coccineus carboxyl proteinase. Using the numbering system of Schechter and Berger,[53] enzyme B generally required hydrophobic amino acids for exhibiting of its activity in the P_1 and P_1' positions.

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

group	enzyme	Val-Tyr	Tyr-Ile	His-Pro	Phe-His
A	1		↑	↑	
	1'		⇡	↑	
B	2		↑		↑
C	3		↑		
D	4	↑	⇡		
	5	⇡	↑		

Fig. 11. Comparison of Cleavage Sites of Angiotensin I by Some Carboxyl Proteinases.

The arrows indicate the bond split. Solid arrows, major sites; broken arrows, minor sites. 1, *Aspergillus sojae* carboxyl proteinase I[50]; 1', *Scytalidium lignicolum* carboxyl proteinase B[50]; 2, *Scytalidium lignicolum* carboxyl proteinase A[50]; 3, *Pycnoporus coccineus* carboxyl proteinase[50]; 4, porcine pepsin[50]; 5, *Irpex lacteus* enzyme B.

CHAPTER IV

SUBSTRATE SPECIFICITY OF MILK-CLOTTING ENZYME B FROM *IRPEX LACTEUS* ON αS1-CASEIN

SUMMARY

Milk-clotting enzymes may be classified into two groups according to their degradation pattern of αs1-casein in solution at pH 6.0. On the one hand, calf chymosin and *Mucor miehei* enzyme produced only one degradation product corresponding to αs1-I under the conditions employed. On the other hand, *Irpex lacteus* and *Endothia parasitica* enzymes produced several degradation products accompanied by a product corresponding to αs1-I. The *Irpex* milk-clotting enzyme hydrolyzed αs1-casein at the positions of His(8)-Gln(9), Phe(23)-Phe(24), Lys(103)-Tyr(104), and Phe(153)-Tyr(154). *Irpex* enzyme has only one common cleaving site with calf chymosin, that is, the Phe(23)-Phe(24) bond of αs1-casein.

INTRODUCTION

The specificity of *Irpex* enzyme B on the oxidized insulin B chain, which resembled that of *P. cossineus* carboxyl proteinase,

is distinct from other commercial microbial milk-clotting enzymes and is more restricted than chymosin and porcine pepsin as described in Chapter III.

Calf chymosin is the ideal enzyme for cheese manufacture due to its high milk-clotting activity and its limited proteolysis of caseins. αs1-Casein is reported to be extensively hydrolyzed by calf chymosin during cheese ripening, while β-casein remains almost unchanged.[54]

It has been shown that there are three chymosin-susceptible bonds in αs1-casein and it is hydrolyzed at pH > 5.8 by chymosin to form, in this order, αs1-I, αs1-II, and αs1-III.[55] The proteolytic specificity of microbial milk-clotting enzymes on αs1-casein has not yet been established, therefore it is important to investigate the substrate specificity of enzyme B on αs1-casein.

In this chapter, I described the proteolytic specificity of the milk-clotting enzyme from *Irpex lacteus* on αs1-casein in solution at pH 6.0 and compared it with that of calf chymosin.

MATERIALS AND METHODS

Dansyl chloride was purchased from Pierce, and carboxypeptidase A-DFP and carboxypeptidase B-DFP from Sigma. Molecular weight markers (ranging from 2,500-17,000) for SDS-PAGE were obtained from BDH Biochemicals. Pepstatin was secured from the Protein Research Foundation of Japan. Reagent grade chemicals

were used.

αs1-casein. Crude αs1-casein was prepared from acid precipitated whole casein by the method of Zittle and Custer.[56] It was purified by ion-exchange chromatography on DEAE-cellulose column according to the method of Davies and Law.[57]

Enzymes. Calf chymosin, and milk-clotting enzymes from *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.[24,26] The *Irpex lacteus* milk-clotting enzyme B was also purified as described in Chapter II.

Milk-clotting activity. Milk-clotting activity was determined according to the method described in Chapter II and expressed as Soxhlet units per ml of enzyme solution.

Action of milk-clotting enzymes on αs1-casein. A solution of αs1-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. Samples (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. Then the samples were analyzed by PAGE.

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.[58] PAGE was carried out with 7.5% gel at pH 8.9 in the presence of 4.5 M urea by the method of Davis.[31] The protein was stained with 0.05% Coomassie brilliant blue R-250 in acetic acid-methanol-water (1:1:5) mixture.

Isolation of αs1-casein degradation products. One hundred milliliters of a 0.1% αs1-casein solution in 0.02 M phosphate buffer, pH 6.0, was incubated with enzyme B at 35°C (enzyme/substrate=1/300mole/mole). After 30 min of incubation, the reaction was terminated by heating in a boiling water bath for 5 min. The sample was dried in a rotary evaporator, dissolved in 10 ml of 0.01 M Tris-HCl buffer containing 0.06 M NaCl and 6 M urea, pH 8.6, and applied to a Sephadex G-100 column equilibrated with the same buffer. Each of the separated fractions was chromatographed on a DEAE-cellulose column equilibrated with the same buffer, then eluted by a linear NaCl gradient from 0.06 M to 0.3 M. Furthermore, all fragments from the fractions were purified with RP-HPLC [Altex, Ultrosphere-ODS, Ultrosphere-octyl(4.6 x 250mm); Toyo Soda, TMS-250(7.5 x 75mm); Beckman model 340] and identified.

Amino acid analysis. Each of the αs1-casein fragments 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. α s1-Casein fragments 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.

N-terminal amino acid analysis. The N-terminal amino acid of each fragment was isolated by the method of Hartley.[47)]

RESULTS

Degradation of α s1-casein by some microbial milk-clotting enzymes and calf chymosin As shown in Fig. 12, although the *Mucor* enzyme had slightly higher proteolytic activity than calf chymosin, their proteolytic patterns on α s1-casein showed no significant difference between the two. The proteolytic pattern of *Irpex* enzyme B resembled that of *Endothia* enzyme but both enzymes' pattens were different from those of calf chymosin and *Mucor* enzyme.

Isolation of degradation products by *Irpex* milk-clotting enzyme from α s1-casein As shown in Fig. 13, five fractions designated as A, B, C, D, and E in the order of elution were obtained by Sephadex G-100 column chromatography. Judging from the PAGE (Fig. 13), fraction A was composed of two components

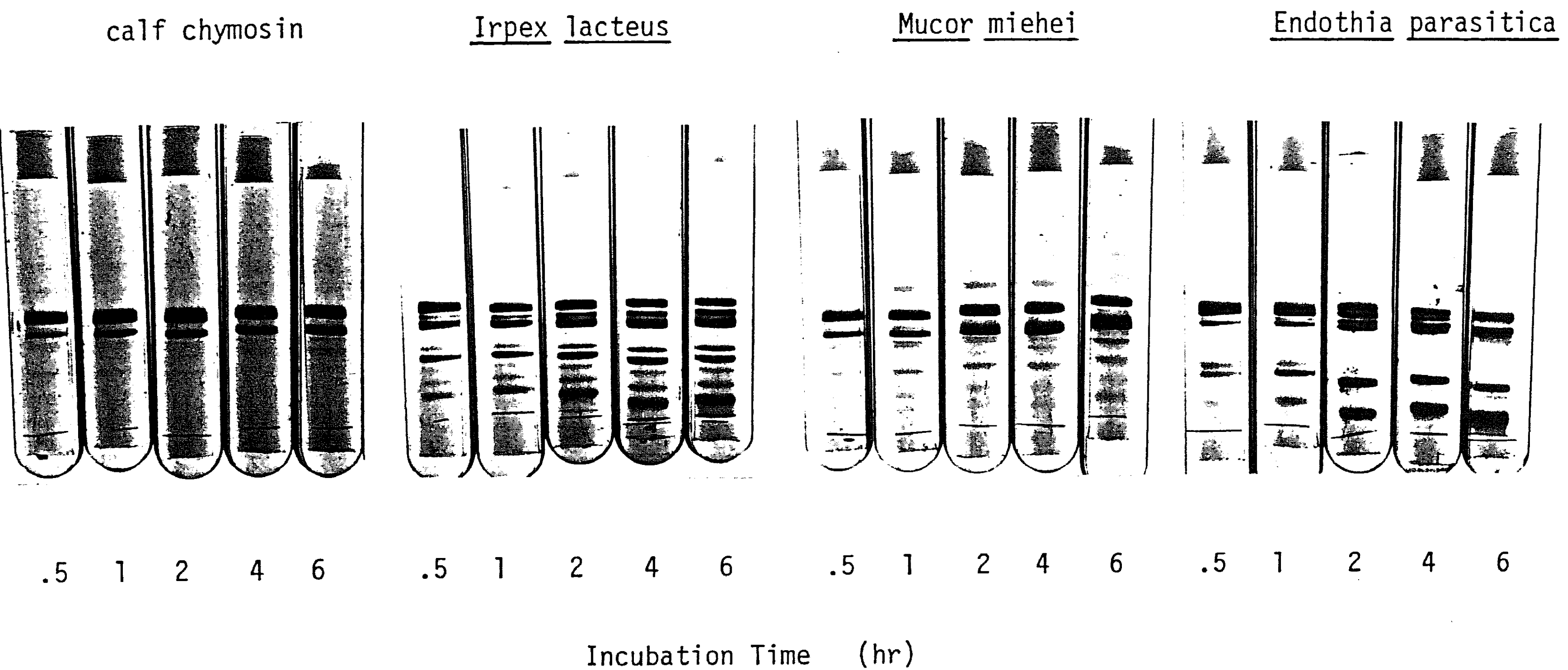


Fig. 12. Disc Gel Electrophoretic Pattern of α_{s1}-Casein Incubated with Calf Chymosin, <u>Irpex lacteus</u> Enzyme B, <u>Mucor miehei</u> Enzyme, and <u>Endothia parasitica</u> Enzyme.

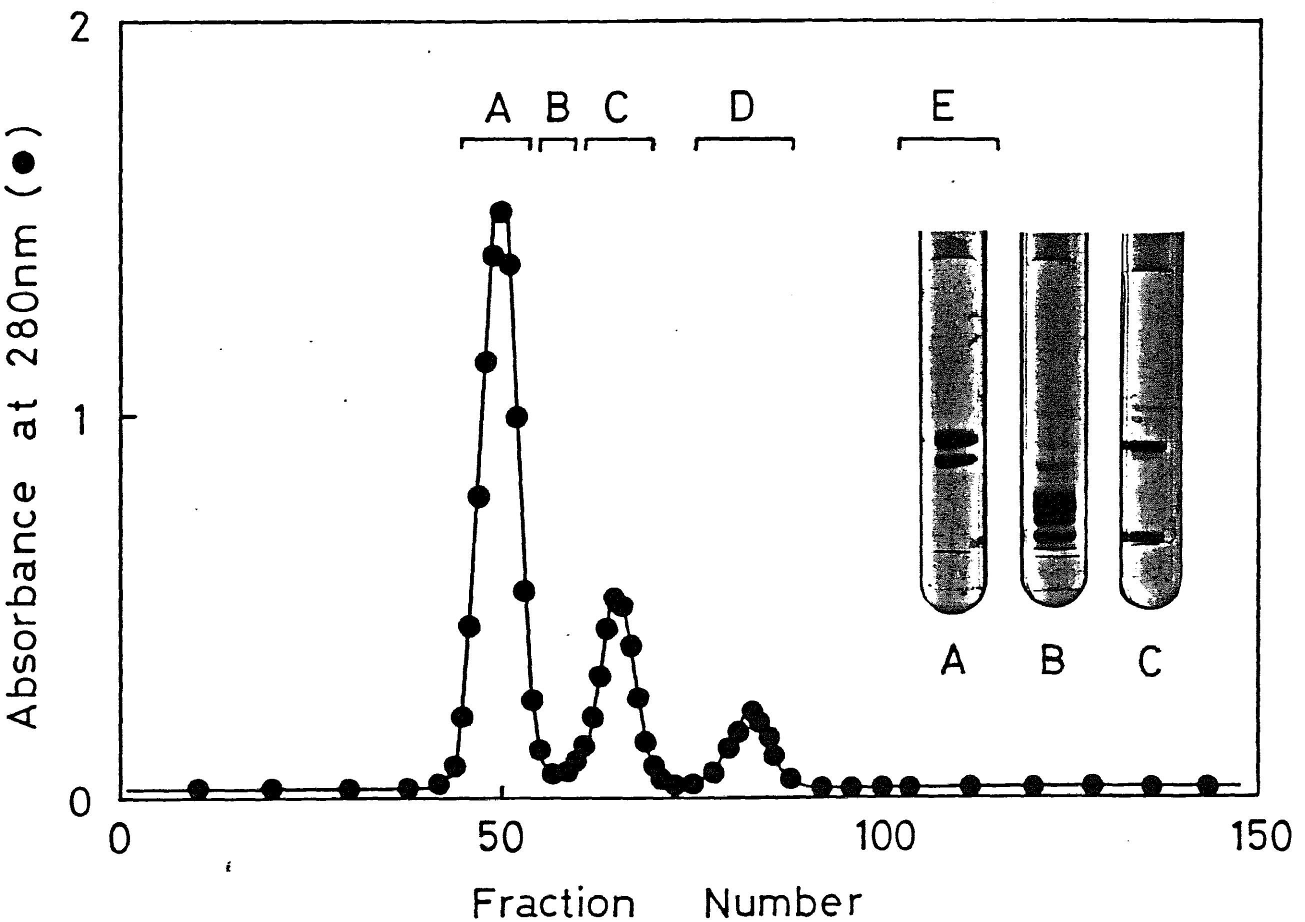


Fig. 13. Gel Filtration of the Reaction Mixture on a Sephadex G-100 Column (4 x 100cm) in 0.01 M Tris-HCl Buffer Containing 0.06 M NaCl and 6 M Urea, pH 8.6. The flow rate was 40 ml/hr and 11 ml fractions were collected.

which were eluted from a DEAE-cellulose column with a pH gradient from 8.6 to 4, as shown in Fig. 14. The first fraction was found to be unchanged αs1-casein itself by PAGE and amino acid analysis. The second peak, I, was judged homogeneous by PAGE (Fig. 14) and by reverse phase high performance liquid chromatography (RP-HPLC) with a TMS-250 column.

Fraction B in Fig. 13 was separated into two fractions using ion-exchange chromatography on a DEAE-cellulose column with a linear NaCl gradient. A main peak appeared homogeneous in PAGE but was separated into two peaks by RP-HPLC (TMS-250) as shown in Fig. 15. The main peak, II, indicated with a bracket, was dried and identified.

Fraction C in Fig. 13 was also separated into two fracions by DEAE-cellulose column chromatography with NaCl gradient elution. As shown in Fig. 16, a main peak, III, and a minor one were obtained and the homogeneity of III was confirmed by PAGE and RP-HPLC with TMS-250 column.

Fraction D in Fig. 13 was chromatographed on RP-HPLC (Ultrosphere-octyl) and two main peaks, IV and V, were obtained as shown in Fig. 17.

Similarly, fraction E in Fig. 13 was separated into many peaks by RP-HPLC (Ultrosphere-ODS), giving two main peaks, VI and VII(Fig. 18). Each fraction indicated with a bracket was collected, dried, and identified.

<u>Identification of degradation products from αs1-casein</u>. As

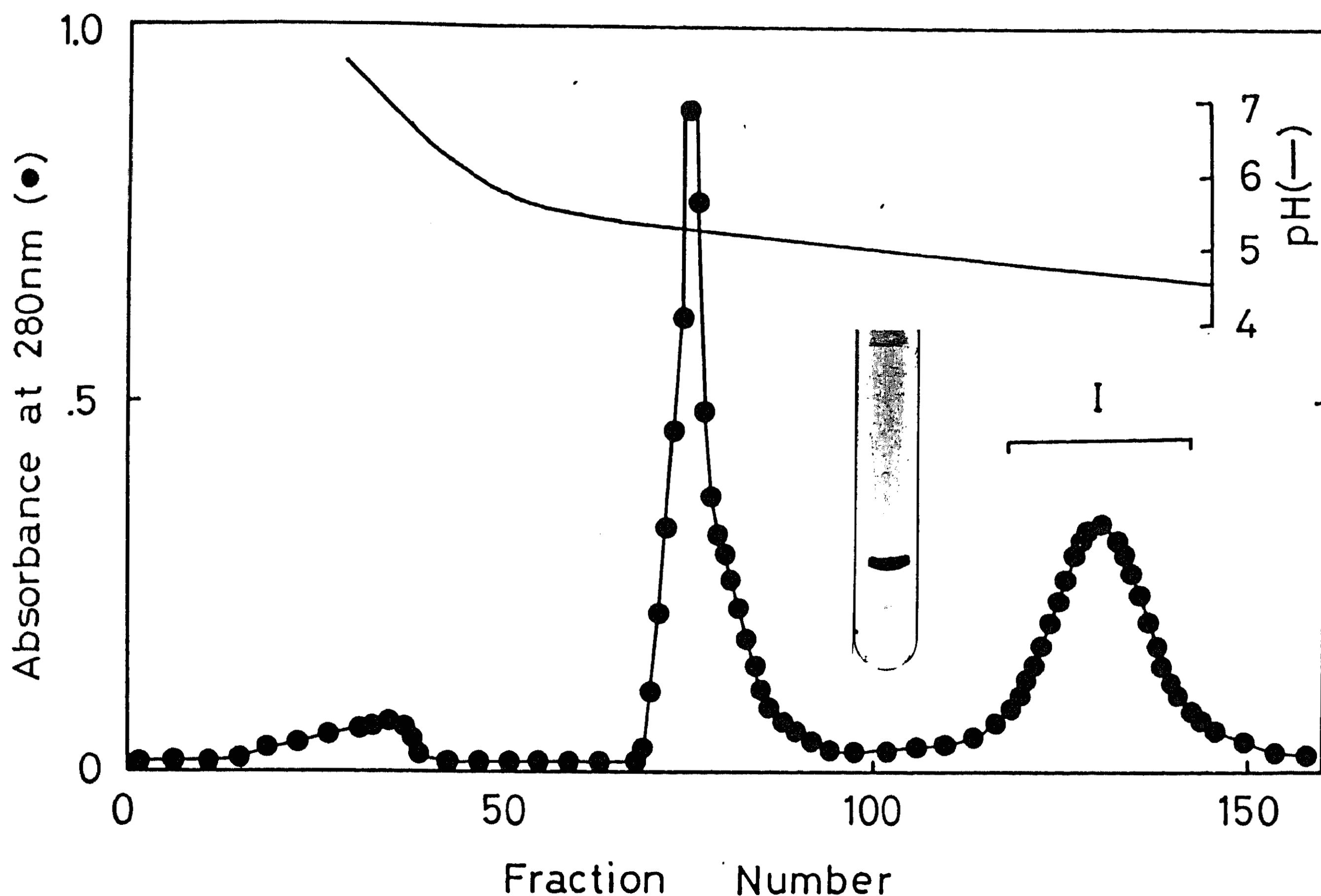


Fig. 14. Ion Exchange Chromatography of Fraction A after Gel Filtration (Fig. 13) on DEAE-cellulose.

Fraction A from the gel filtration on Sephadex G-100, indicated with a blacket in Fig. 13, was applied to a DEAE-cellulose column (0.9 x 20cm, Whatman DE-52) equilibrated with 0.01 M Tris-HCl buffer containing 0.06 M NaCl, 6 M urea, pH 8.6. Degradation products were eluted by a pH gradient decreased from pH 8.6 to 4 generated between 400 ml each of the initial buffer and of 0.3 M acetic acid containing 6 M urea. The flow rate was 30 ml/hr and 4.5 ml fractions were collected.

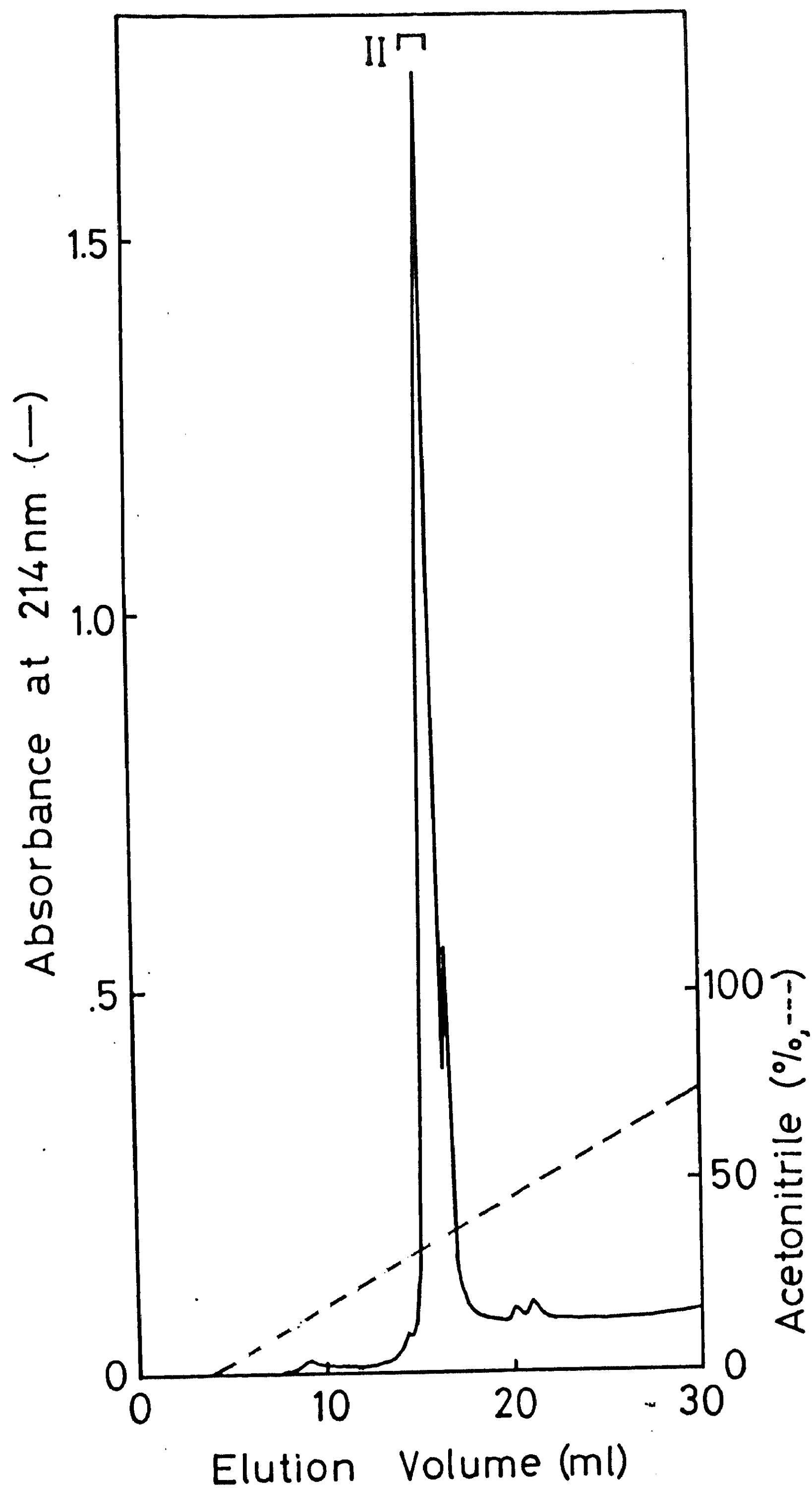


Fig. 15. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) of Fraction B Partially Purified by DEAE-cellulose after Gel Filtration. Partially purified fraction B was adsorbed on a column of TMS-250 (7.5 x75mm) and eluted at a flow rate of 1 ml/min by a linear gradient from 0.01 M trifluoroacetic acid to 90% acetonitrile containing 0.01 M trifluoroacetic acid. The column was operated at 28°C.

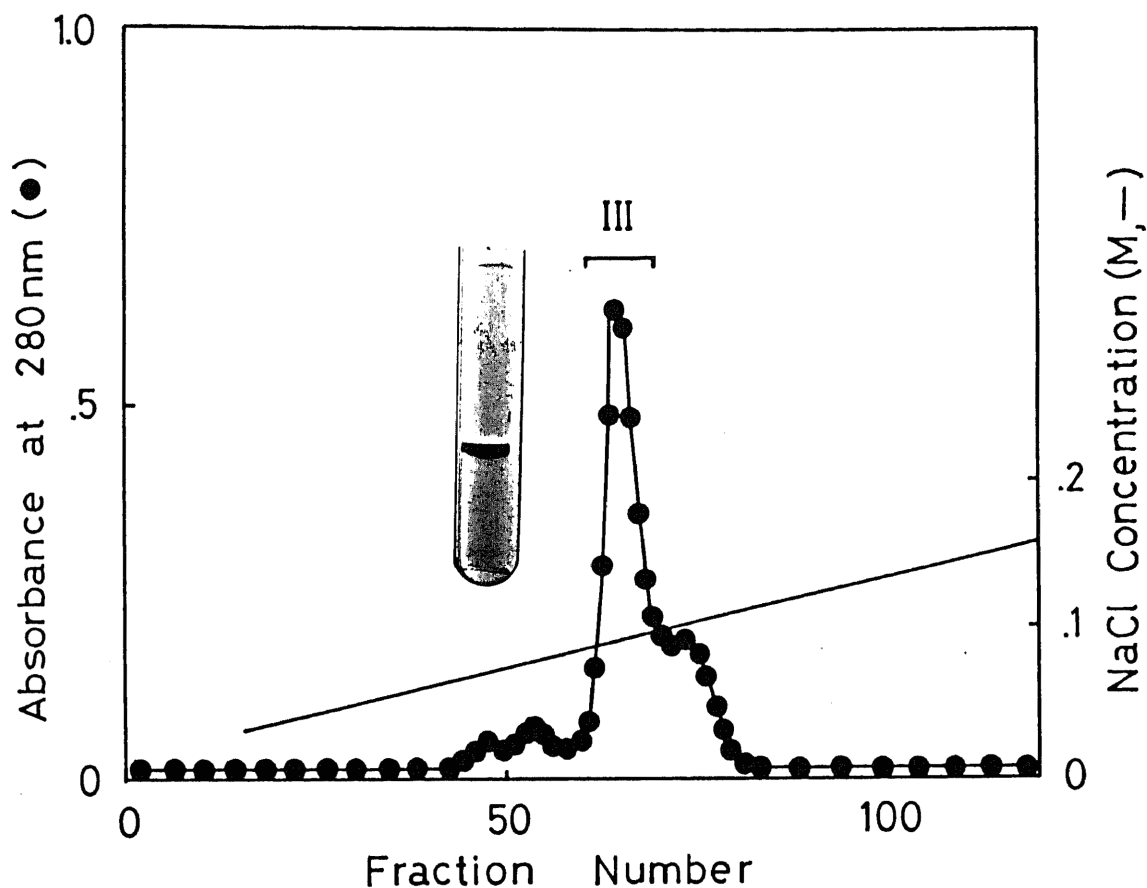


Fig. 16. Ion Exchange Chromatography of Fraction C after Gel Filtration (Fig. 13) on DEAE-cellulose.

Fraction C, indicated with a bracket in Fig. 13, was dialyzed against 0.005 M Tris-HCl buffer containing 0.03 M NaCl and 6 M urea, pH 8.2, and applied to a DEAE-cellulose column (0.9 x 25cm, Whatman DE-52) equilibrated with the same buffer. Degradation products were eluted by a linear NaCl gradient generated between 500 ml each of the initial buffer and of 0.33 M NaCl in the buffer. The flow rate was 30 ml/hr and 4.5 ml fractions were collected.

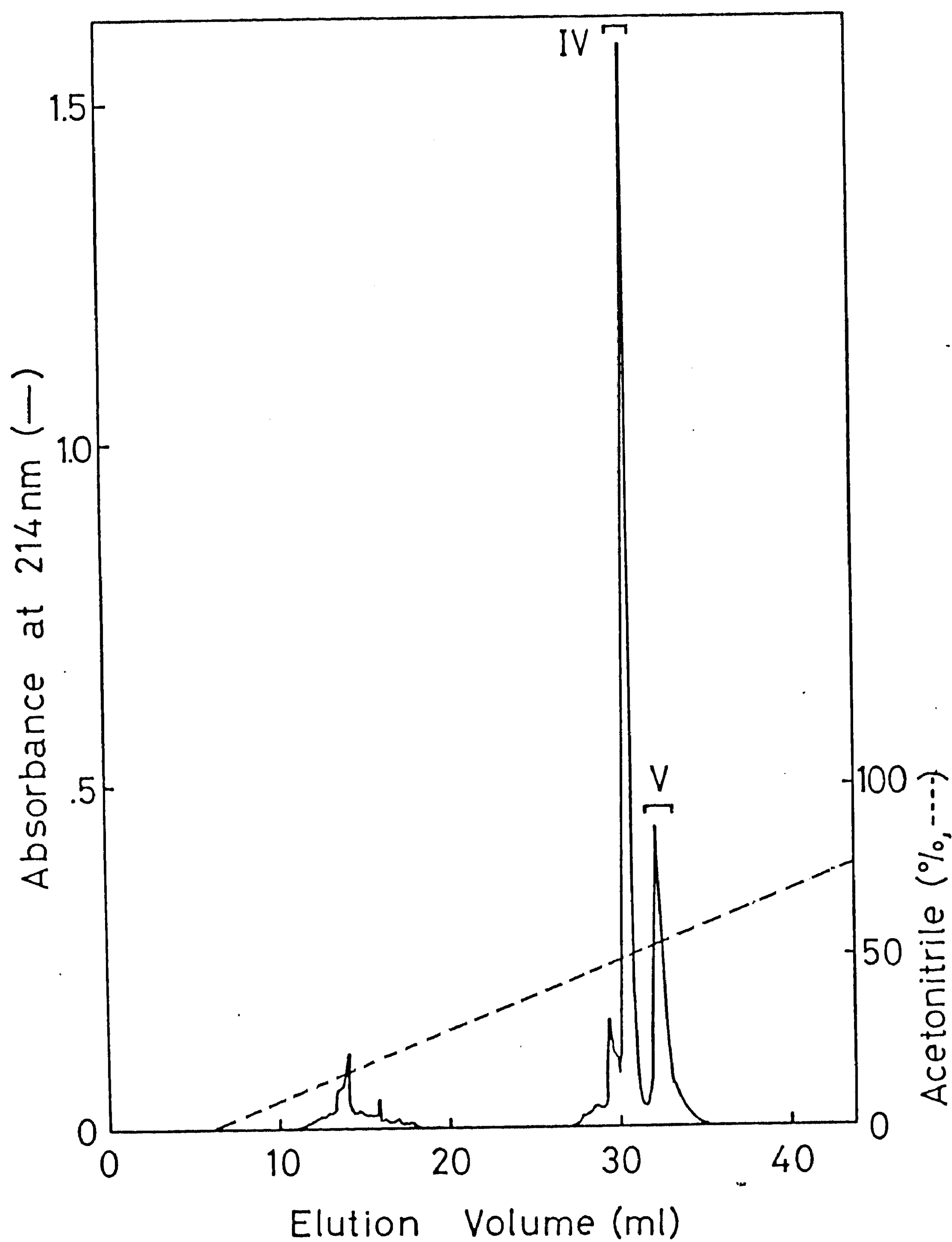


Fig. 17. RP-HPLC of Fraction D after Gel Filtration (Fig. 18). Fraction D was applied to a column of Ultrosphere-octyl (4.6 x250mm) and eluted under the same conditions described in Fig.15.

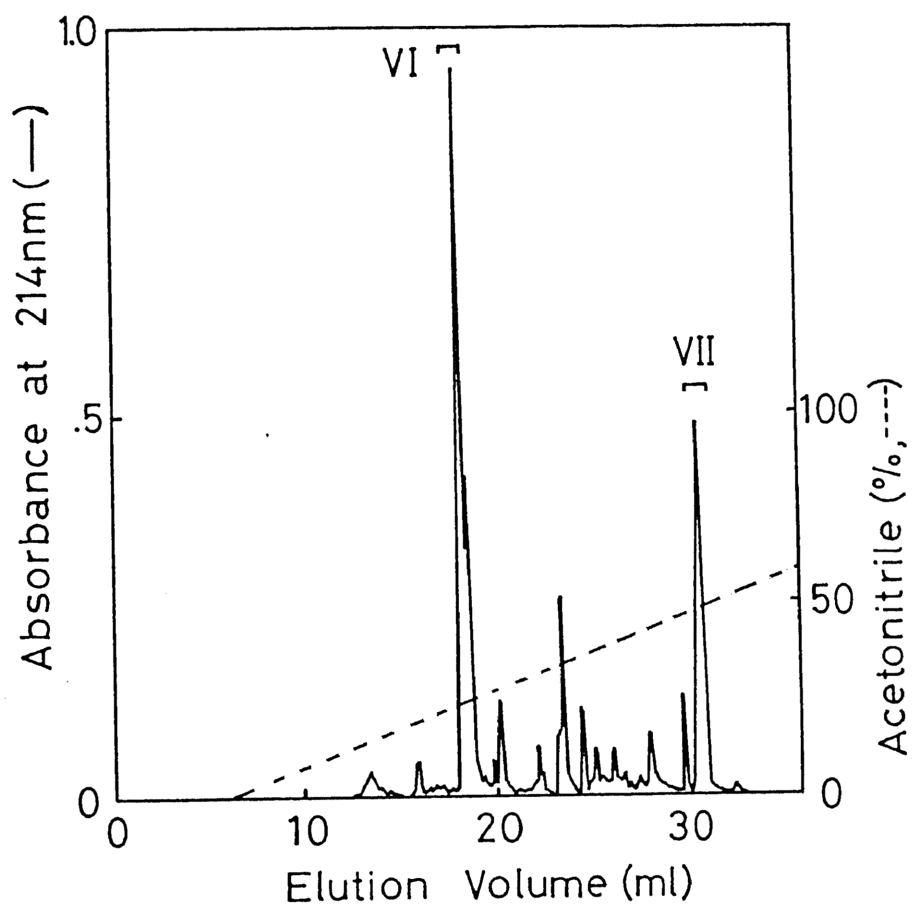


Fig. 18. RP-HPLC of Fraction E after Gel Filtration (Fig. 13). Fraction E was loaded on a column of Ultrosphere-ODS (4.6 x 250mm) and was eluted under the same conditions described in Fig.15.

shown in Table IX, the N-terminal amino acids of I, II, and III were phenylalanine, arginine, and tyrosine, respectively, by dansylation and their corresponding C-terminal amino acid sequences were -Leu-Trp, -Leu-Lys-Lys, and -Leu-Trp, respectively. The molecular weights were estimated to be 20,000, 12,000, and 11,000 by SDS-PAGE for I, II, and III, respectively, and their amino acid compositions are shown in Table IX, together with the compositions of known αs1-casein B segments. Therefore, these fragments, I, II, and III, were identified as Phe(24)-Trp(199), Arg(1)-Lys(103), and Tyr(104)-Trp(199) of αs1-casein, respectively.

As shown in Table X, the N-terminal amino acid of IV was tyrosine by dansylation, and the C-terminal sequence to be -Leu-Trp. Taking the amino acid composition into consideration, fragment IV was identified as Tyr(154)-Trp(199) of αs1-casein. In the same manner, fragments V, VI, and VII were identified as Tyr(104)-Phe(153), Arg(1)-His(8), and Gln(9)-Phe(23) of αs1-casein, respectively.

DISCUSSION

Milk-clotting enzymes may be classified into two groups according to their degradation pattern of αs1-casein as shown in Fig. 12. Calf chymosin and Mucor enzyme belong to the first group which produces almost entirely one degradation product corresponding to αs1-I from αs1-casein. The second group

Table IX. Identification of Peptides Formed from αs1-Casein by *Irpex lacteus* Milk-clotting Enzyme B

Amino acid	I	24 199 Phe-Trp	II	1 103 Arg-Lys	III	104 199 Tyr-Trp
Asp	14	13	7	8	7	7
Thr	5	5	1	1	4	4
Ser	17	16	7	9	7	7
Glu	37	35	27	22	17	16
Pro	14	14	7	7	10	10
Gly	9	8	5	5	5	5
Ala	9	9	3	3	6	6
Cys	0	0	0	0	0	0
Val	9	10	6	7	5	4
Met	4	5	2	2	3	3
Ile	8	10	5	6	4	5
Leu	14	13	9	10	8	7
Tyr	9	10	2	2	8	8
Phe	6	7	4	4	4	4
His	3	3	3	3	2	2
Lys	12	12	11	10	4	4
Arg	4	4	4	4	2	2
Trp	-	2	-	0	-	2
Total residues	174	176	103	103	94	96
M. W.	20,000	20,000	12,000	12,000	11,000	11,000
N-terminal	Phe	Phe	Arg	Arg	Tyr	Tyr
C-terminal	-Leu-Trp	-Leu-Trp	-Lys-Lys	-Lys-Lys	-Leu-Trp	-Leu-Trp
Recovery(%)	26		3		17	

The recovery of fragments was determined by amino acid analysis and expressed as the percentage of total amounts of αs1-casein used as the substrate.

Table X. Identification of Peptides Formed from αs1-Casein by _Irpex lacteus_ Milk-clotting Enzyme B

Amino acid	IV	154 199 Tyr-Trp	V	104 153 Tyr-Phe	VI	1 8 Arg-His	VII	9 23 Gln-Phe
Asp	5	5	3	2	0	0	1	1
Thr	4	4	0	0	0	0	0	0
Ser	5	5	2	2	0	0	0	0
Glu	4	4	0	12	0	0	4	4
Pro	6	6	4	4	2	2	1	1
Gly	3	3	3	2	0	0	2	2
Ala	3	3	3	3	0	0	0	0
Cys	0	0	0	0	0	0	0	0
Val	1	1	3	3	0	0	1	1
Met	1	1	2	2	0	0	0	0
Ile	2	2	3	3	1	1	0	0
Leu	3	3	4	4	0	0	4	4
Tyr	4	4	3	3	0	0	0	0
Phe	1	1	3	3	0	0	1	1
His	0	0	2	2	2	2	0	0
Lys	1	1	3	3	2	2	0	0
Arg	0	0	2	2	1	1	1	1
Trp	-	2	-	0	-	0	-	0
Total residues	43	45	50	50	8	8	15	15
N-terminal	Tyr	Tyr	Tyr	Tyr	Arg	Arg	Glu	Glu
C-terminal	-Leu-Trp	-Leu-Trp	-Gln-Phe	-Gln-Phe	-Lys-His	-Lys-His	-Arg-Phe	-Arg-Phe
Recovery(%)	6		4		10		8	

The recovery of fragments was determined by amino acid analysis and expressed as the percentage of total amounts of αs1-casein used as the substrate.

consists of *Endothia* and *Irpex* enzymes, which produce several degradation products accompanied by a product corresponding to αs1-I. Therefore,the first group is less proteolytic and shows more restricted specificity on αs1-casein than the second under the conditions employed. Although the similarities of their specificity on αs1-casein by both enzymes in either group had been pointed out, the four enzymes differed significantly from each other in their specificity on oxidized insulin B-chain, as shown in Chapter III. This indicates that the specificity on protein substrates is affected not only by the amino acids adjacent to the cleaving point but also by the conformation of the substrate surrounding the cleaving point.

The specificity of *Irpex* enzyme on αs1-casein is summarized in Fig. 19 and is compared to chymosin. *Irpex* enzyme and calf chymosin have only one common cleaving point, that is, at Phe(23)-Phe(24), and differ on subsequent cleavage sites. It is well known that chymosin produces αs1-I, αs1-II, and αs1-III by a sequential manner at pH 6.0. At the beginning, chymosin cleaves the Phe(23)-Phe(24) bond of αs1-casein to form αs1-I[Phe(24)-Trp(199)], and then it acts on the Leu(169)-Gly(170) bond of αs1-I to form αs1-II[Phe(24)-Leu(169)], and finally it hydrolyzes the Leu(149)-Phe(150) bond of αs1-II to produce αs1-III[Phe(24)-Leu(149)]. *Irpex* enzyme cleaves the Phe(23)-Phe(24) bond of αs1-casein in the same manner as chymosin to form αs1-I, however, the next cleaving point is the Lys(103)-Tyr(104) bond of αs1-I to form III, Tyr(104)-Trp(199). III is hydrolyzed at Phe(153)-

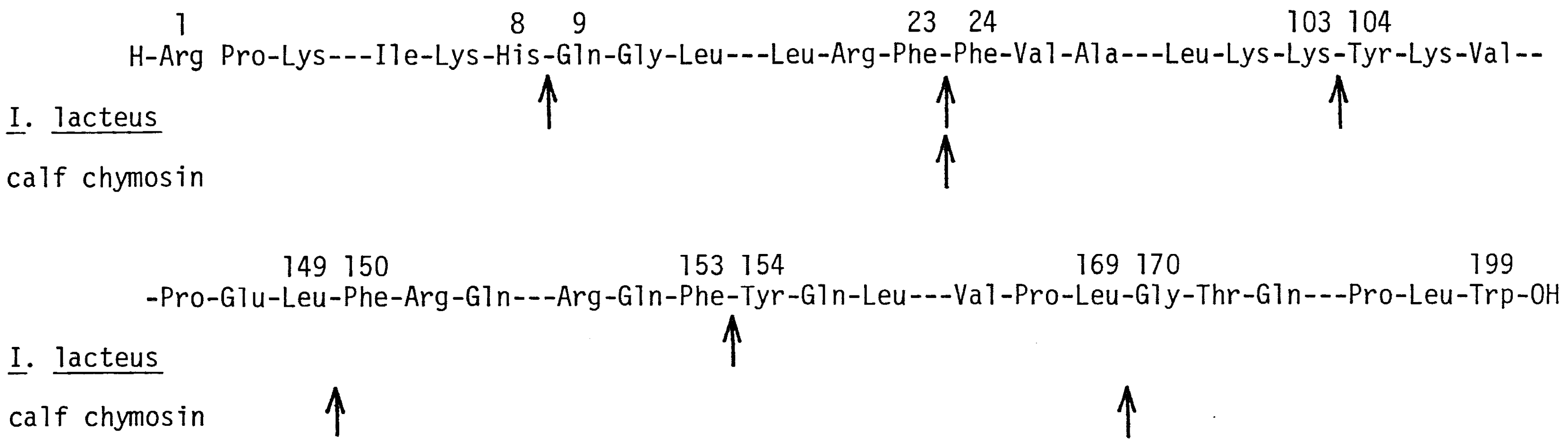


Fig. 19. Comparison of Cleavage Sites of αs1-Casein by Milk-clotting Enzyme B from *Irpex lacteus* and Calf Chymosin

Tyr(154) forming IV, Tyr(104)-Phe(153) and V, Tyr(154)-Trp(199). Yields of fragments I, II that is Arg(1)-Lys(103), and III are estimated to be 26%, 3% and 17%, respectively. This may indicate that a certain fragment [Phe(24)-Lys(103)], which is not identified, is formed during proteolysis or is hydrolyzed further. The two fragments VI, Arg(1)-His(8), and VII, Gln(9)-Phe(23) were produced from the hydrolysis of Arg(1)-Phe(23) by *Irpex* enzyme.

As shown in Table XI, chymosin always requires a hydrophobic amino acid such as phenylalanine or leucine in the P_1 site as mentioned by Schechter and Berger.[53] *Irpex* enzyme is specific for the peptide bonds formed by two amino acids with large hydrophobic side chains such as Phe(23)-Phe(24) and Phe(153)-Tyr(154). A noticeable exception is the hydrolysis of the His(8)-Gln(9) bond by *Irpex* enzyme.

It is established that microbial enzymes possessing trypsinogen activating ability, such as the acid proteinases of *Aspergillus niger*, *A. saitoi*, and *Rhizopus chinensis* are able to hydrolyze Lys-X bond preferentially.[59] Although the *Irpex* enzyme, as well as the *Mucor miehei* enzyme, *M. pusillus* enzyme, and pepsin, did not activate trypsinogen, the *Irpex* enzyme hydrolyzed the Lys(103)-Tyr(104) bond to form III, Tyr(104)-Trp(199) with high yield. This might be attributed to the large hydrophobic region formed by the neighboring six amino acids from the P_3 to P_3' sites. In addition, Tang[60] reported that even low frequencies of hydrolysis at the Lys-X bond by pepsin were

Table XI. Comparison of Cleavage Sites of αs1-casein by <u>Irpex lacteus</u> Milk-clotting Enzyme B and Calf Chymosin on the Basis of the Hydrophobicities of Side Chain of Amino Acid Residues Adjacent to the Bonds to be Split

	P_4	P_3	P_2	P_1 ↑	P_1'	P_2'	P_3'
Enzyme B	Pro	Ile	Lys	His	Gln	Gly	Leu
	*2.6	3.0	1.5	0.5	-0.1	0	1.8
	Leu	Leu	Arg	Phe	Phe	Val	Ala
	*1.8	1.8	0.7	2.5	2.5	1.5	0.5
	Arg	Leu	Lys	Lys	Tyr	Lys	Val
	*0.7	1.8	1.5	1.5	2.3	1.5	1.5
	Phe	Arg	Gln	Phe	Tyr	Gln	Leu
	*2.5	0.7	-0.1	2.5	2.3	-0.1	1.8
Chymosin	Leu	Leu	Arg	Phe	Phe	Val	Ala
	*1.8	1.8	0.7	2.5	2.5	1.5	0.5
	Tyr	Val	Pro	Leu	Gly	Thr	Gln
	*2.3	1.5	2.6	1.8	0	0.4	-0.1
	Tyr	Pro	Glu	Leu	Phe	Arg	Gln
	*2.3	2.6	0.5	1.8	2.5	0.7	-0.1

*: Δgt, hydrophobicities of side chain of amino acid residues (kcal/mol) cited from the references (61,62). The arrow indicates the splitting point. The positions (P_4 - P_3') on the substrate are designated according to Schechter and Berger.[53)]

observed in protein substrates. Furthermore, amino acids at the P_1 site were classified into three groups in the order of susceptibility as follows: Phe, Leu and Tyr belonged to the first group of highly susceptible ones; Glu, Asp, His and Lys, to the second group of susceptible ones; and Gly, Ile, Pro and Arg, to the third group of non-susceptible ones. Therefore, the susceptibility of Lys(103)-Tyr(104) and His(8)-Gln(9) bonds, and the non-susceptibility of Ile(6)-Lys(7), Pro(5)-Ile(6) and Pro(12)-Gln(13) bonds by Irpex enzyme coincided with the specificity of pepsin.

Morihara et al.[63)] reported that the elongation of the peptide chain length from P_1 to P_3 resulted in a marked increase of hydrolysis with acid proteinases of microbial origin and with pepsin. This indicates that the P_3 site of the substrate is important to the secondary interaction between the enzyme and the substrate. As shown in Table XI, hydrophobic amino acids located in the P_4 and/or P_3 site(s) might be important in the secondary interaction between the enzyme and the substrate in both cases of chymosin and Irpex enzyme B.

CHAPTER V

SUBSTRATE SPECIFICITY OF MILK-CLOTTING ENZYME B FROM *IRPEX LACTEUS* ON κ- AND β-CASEINS

SUMMARY

Irpex lacteus milk-clotting enzyme B 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 B 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 B 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 B. Although the cleaving

points of β-casein by both enzymes resembled each other, each enzyme exhibited different degradation patterns of β-casein in PAGE because of their different order of cleavage.

INTRODUCTION

It is well known that 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.[64]

As described in Chapter IV, the degradation pattern of αs1-casein by the action of Irpex enzyme B was similar to that of Endothia parasitica enzyme, but was quite different from those of chymosin and Mucor miehei enzyme. Irpex enzyme B hydrolyzed αs1-csein 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 cleaving point was the Phe(23)-Phe(24) bond of αs1-casein while others were different from that of chymosin.

β-Casein is a source of bitter peptides formed by the action of proteinases. For example, a peptide, which constitutes residues Arg(202)-Val(209) of β-casein, posseses bitterness 250 times stronger than that of caffeine.[65] Therefore, it is apparently important to investigate the specificity of milk-

clotting enzymes on β-casein.

It has been shown[66] 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.

In this chapter, I described the proteolytic specificities of the milk-clotting enzyme B 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 (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,[56] and Aschaffenburg,[67] respectively. Crude κ-casein was alkylated at 4°C by the method of Rose *et al.*[68] 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.[57]

Enzymes. Calf chymosin, and milk-clotting enzymes from *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 as described in the previous papers.[24,26] The milk-clotting enzyme B from *Irpex lacteus* was purified as described in Chapter II.

Milk-clotting activity. Milk-clotting activity was determined according to the method described previously 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-caseins (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 time intervals, 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 subjected to 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.[58] PAGE was carried out with 7.5% gel at pH 8.9 or at pH 4.3 in the presence of 4.5 M urea described by Davis.[31] The protein was stained with 0.05% Commassie 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=1/500mole/mole). After 5 min incubation, the reaction was terminated by heating in a boiling water bath for 5 min. One fifth volume of 60% trichloroacetic acid was added and centrifuged at 1500 x g for 20 min. The resultant precipitates were dissoved 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 comfirm 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 in an evaporator. The concentrate was then put on a column of Sephadex G-50 equilibrated with 0.1 N 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=1/400mole/mole). 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; Toyo 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 performed by the method of Hartley[47] and the resultant dansyl amino acid was identified with micropolyamide sheet by the method of Woods and Wang.[48] Edman degradation was also carried out by the method of Sauer[69] and the PTH amino acid obtained was identified by TLC as described by Jeppsson.[70]

RESULTS

Degradation of κ-casein by several microbial milk-clotting enzymes and calf chymosin In all cases, κ-casein (m.w. 19,000) almost completely disappeared within 5 min and a new product corresponding to m.w. 12,000 was formed (Fig. 20). In addition, microbial milk-clotting enzymes, especially *I. lacteus* enzyme B, produced a degradation product having a m.w. 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% TCA was purified by ion exchange chromatography on a CM-cellulose column. Figure 21 shows that

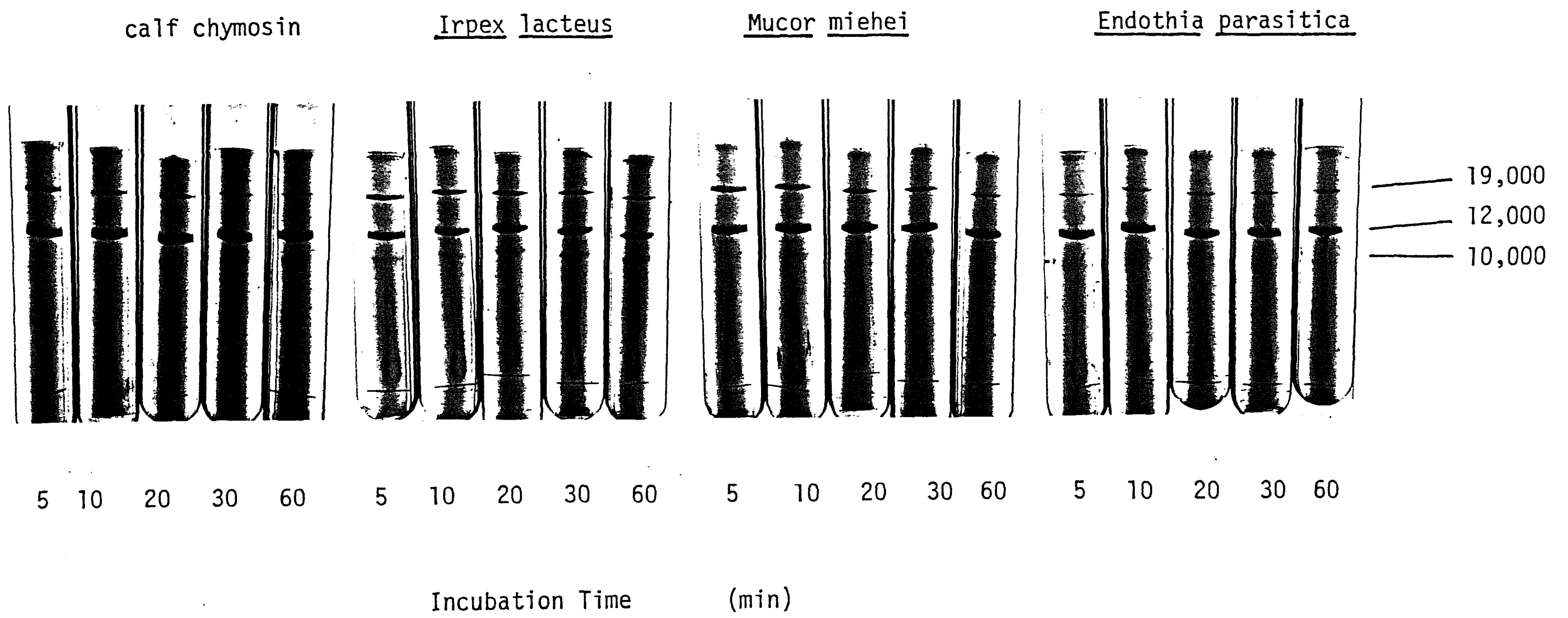


Fig. 20. SDS-PAGE Patterns of κ-Casein Incubated with Calf Chymosin, _Irpex lacteus_ Enzyme B, _Mucor miehei_ Enzyme, and _Endothia parasitica_ Enzyme.

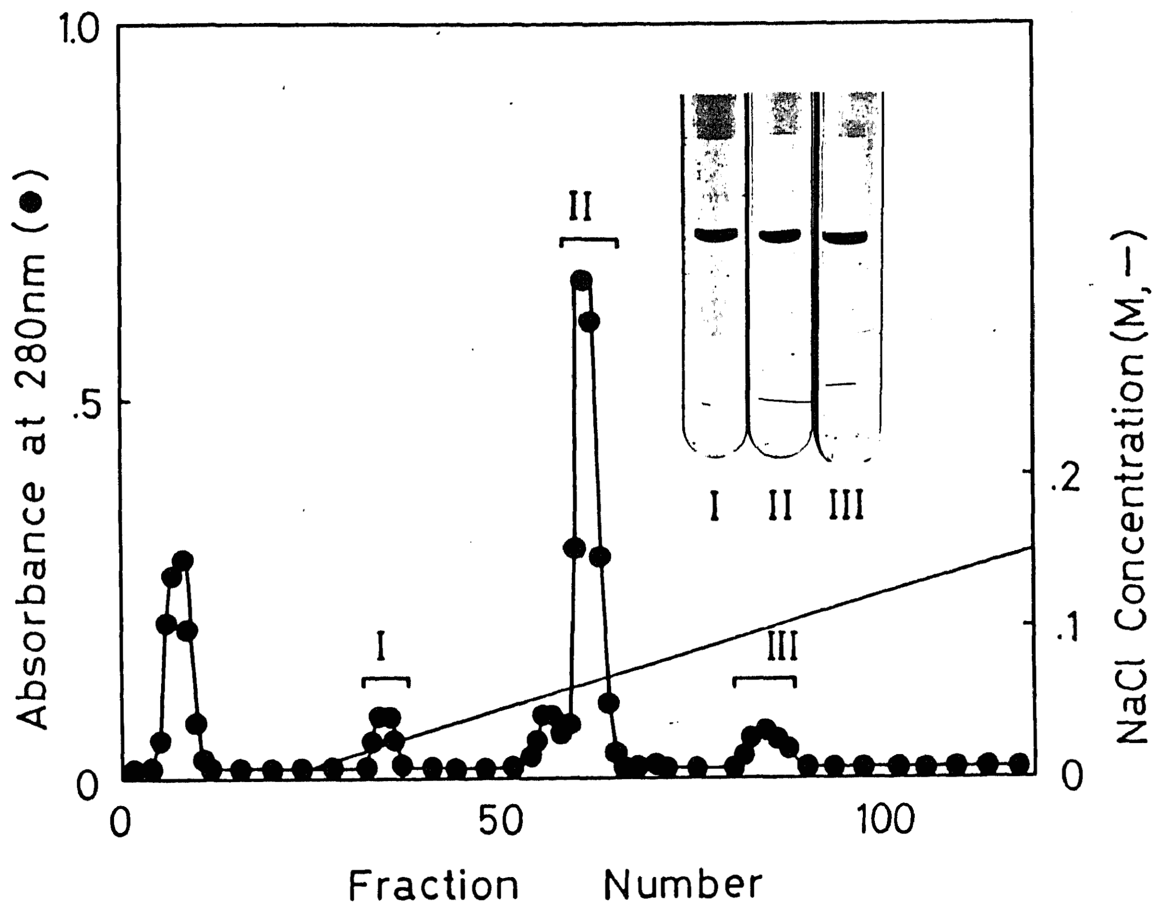


Fig. 21. Ion Exchange Chromatography on CM-cellulose of Insoluble κ-Casein Degradation Products Formed by the Action of I. lacteus Enzyme B.

The fraction insoluble in 12.5% trichloroacetic acid was applied on a column of CM-cellulose (0.9 x 25cm, 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 a NaCl concentration gradient (0 - 0.3 M). The flow rate was 20 ml/hr and 4.0 ml fractions were collected.

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 in pH 4.3 PAGE. The elimination of contaminating free amino acids and peptides was carried out by RP-HPLC (Ultrospere-octyl) and samples were identified. Table XII 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 XII. Yields of these fragments are also presented in Table XII.

On the other hand, when the fraction soluble in 12.5% TCA 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. 22 were designated V and VI. Table XIII 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 XIII, 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 XIII.

Degradation of β-casein by some microbial milk-clotting

Table XII. Identification of Peptides Formed from κ-Casein by <u>Irpex lacteus</u> Milk-clotting Enzyme B

Amino acid	I	1 79 PyrGlu-Leu	II	1 105 PyrGlu-Phe	III	31 105 Val-Phe
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	-*	1	-*	1	-*	1
Total residues	78	79	104	105	74	75
M. W.	10,000	9,500	12,000	12,000	9,000	86,00
N-terminal	-*	PyrGlu	-*	PyrGlu	Val	Val
C-terminal	-Val-Leu	-Val-Leu	-Ser-Phe	-Ser-Phe	-Ser-Phe	-Ser-Phe
Recovery(%)**	8		70		10	

* not detected.
**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.

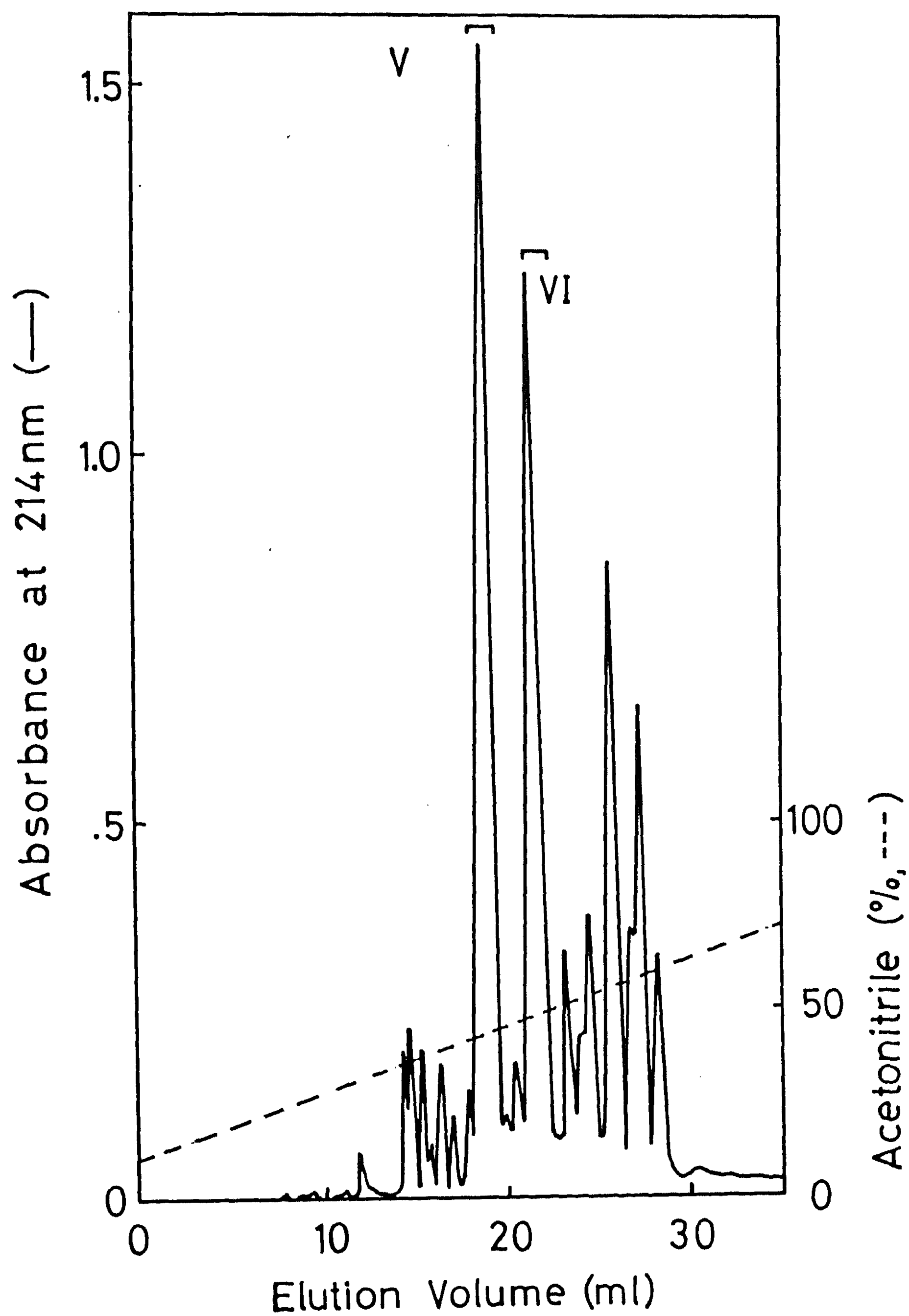


Fig. 22. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) of Fraction V after Gel Filtration on Sehadex G-50.

Fraction V was concentrated and loaded on a column of Ultrosphere-octyl (Altex, 4.6 x 250mm) 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.

Table XIII. Identification of Peptides Formed from κ-Casein by <u>Irpex</u> <u>lacteus</u> Milk-clotting Enzyme B

Amino acid	IV	106 169 Met-Val	V	80 105 Ser-Phe	VI	1 30 PyrGlu-Tyr
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	-*	0	-*	0	-*	0
Total residues	64	64	26	26	29	29
N-terminal	Met	Met	Ser	Ser	-*	PyrGlu
C-terminal	-Ala-Val	-Ala-Val	-Ser-Phe	-Ser-Phe	-Gln-Tyr	-Gln-Tyr
Recovery(%)**	60		8		5	

* not detected.
** 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.

enzymes and calf chymosin Fig. 23 shows that although *Irpex* enzyme 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 order of elution. Fraction A was adsorbed on a DEAE-cellulose column and eluted by a pH gradient.

As shown in Fig. 24, 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. 25). IX was further purified by RP-HPLC with a

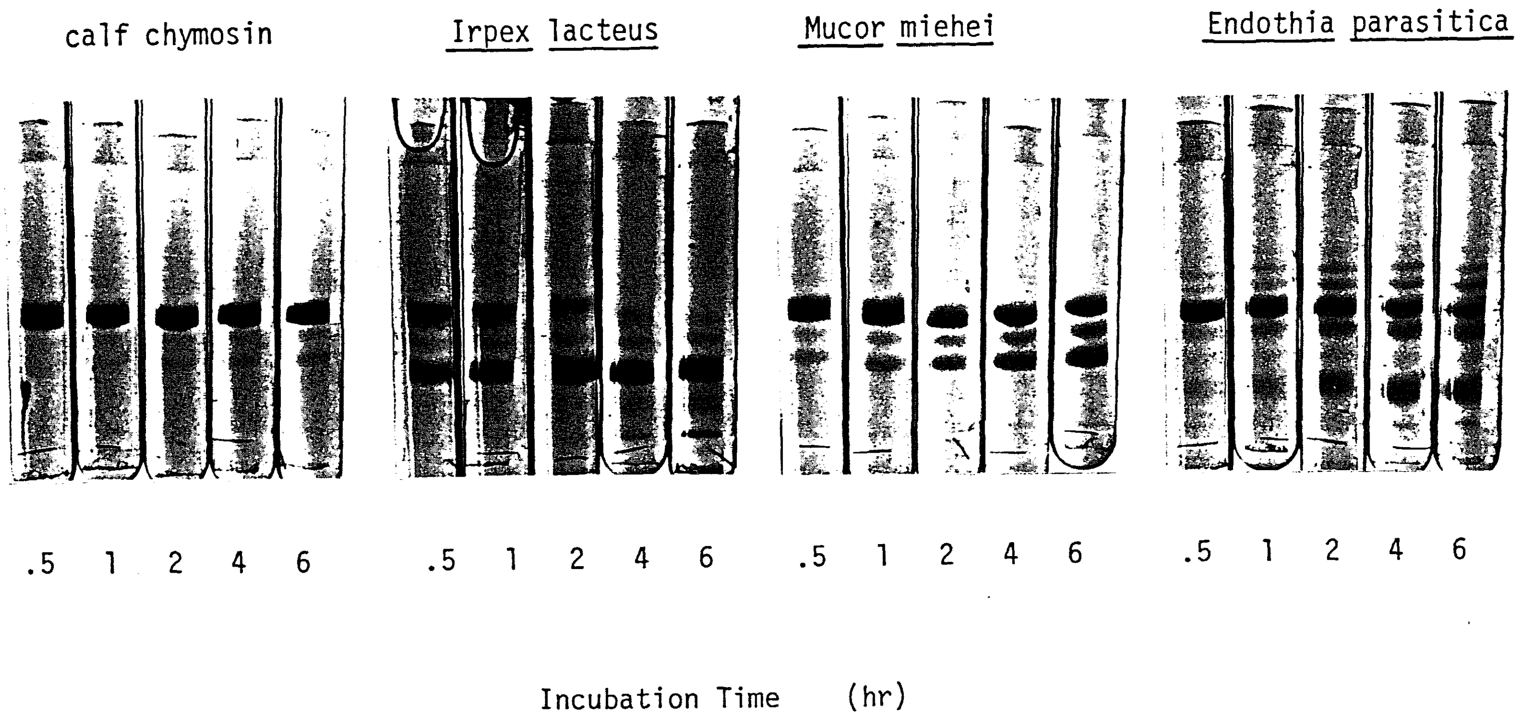


Fig. 23. Disc Gel Electrophoretic Patterns of β-Casein Incubated with Calf Chymosin, Irpex lacteus Enzyme B, Mucor miehei Enzyme, and Endothia parasitica Enzyme.

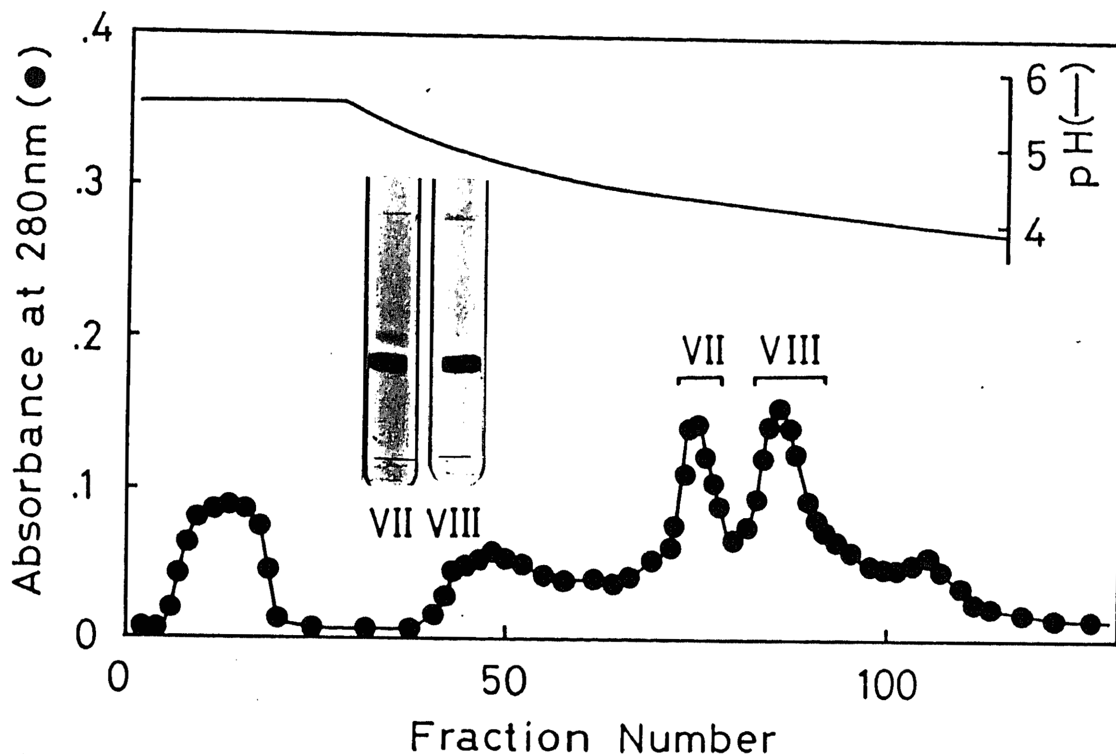


Fig. 24. Ion Exchange Chromatography of Fraction A after Gel Filtration, on DEAE-cellulose.

Fraction A eluted from the gel filtration on Sephadex G-100 was applied to a DEAE-cellulose column (DE-52, Whatman, 0.9 x 20cm) equilibrated with 0.01 M Tris-HCl buffer 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 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.

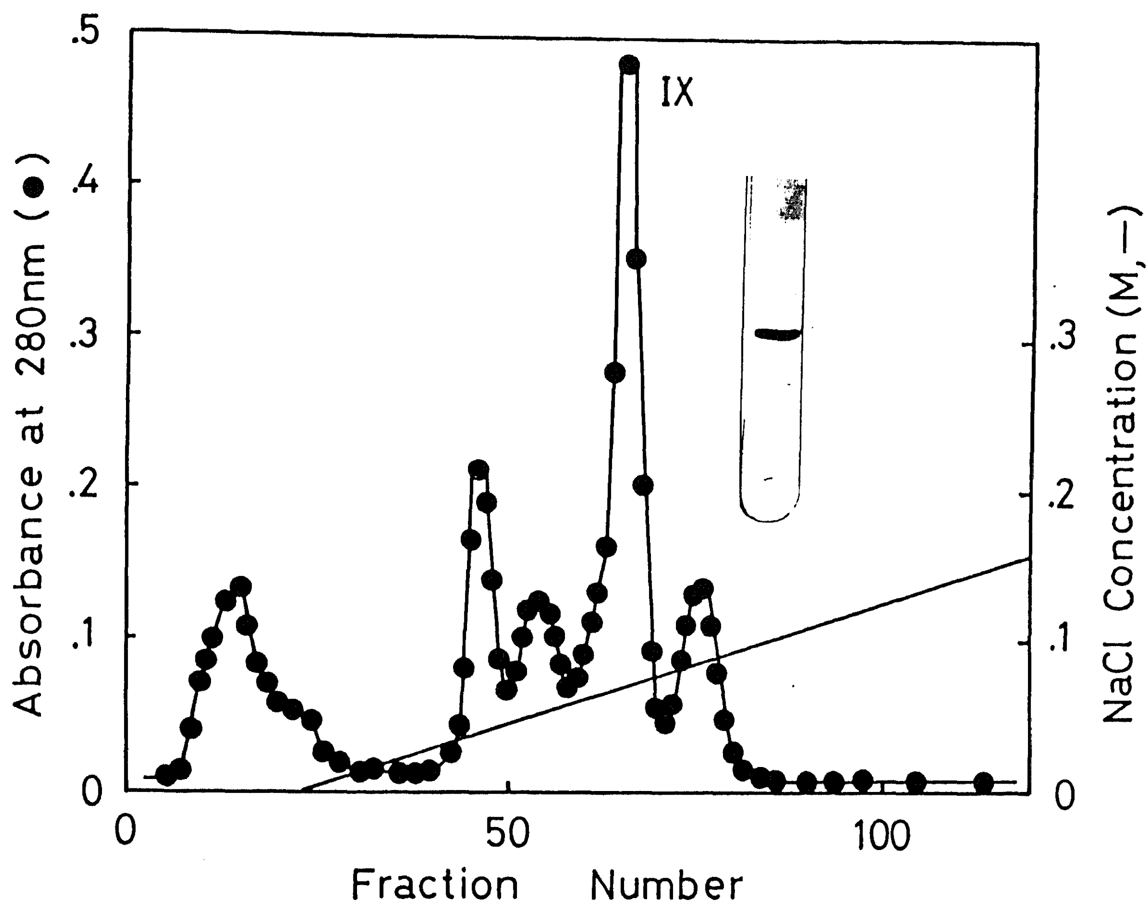


Fig. 25. Ion Exchange Chromatography of Fraction B after Gel Filtration, on CM-cellulose.

Fraction B eluted from the gel filtration on Sephadex G-100 was applied to a CM-cellulose column (1.5 x 25cm, 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 0.3 M NaCl. The flow rate was 25 ml/hr and 4.0 ml fractions were collected.

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. 26).

As shown in Table XIV, 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 XIV, and by considering their amino acid compositions, VII and VIII were identified as Arg(1)-Ser(142), and IX was identified as Trp(143)-Leu(192) of β-casein.

Table XV 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 XV, fractions 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 Fig. 24, 25, and 26 were not identified because of their heterogeneity.

DISCUSSION

It has been established that 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. When the *Irpex* enzyme B acted on κ-casein,

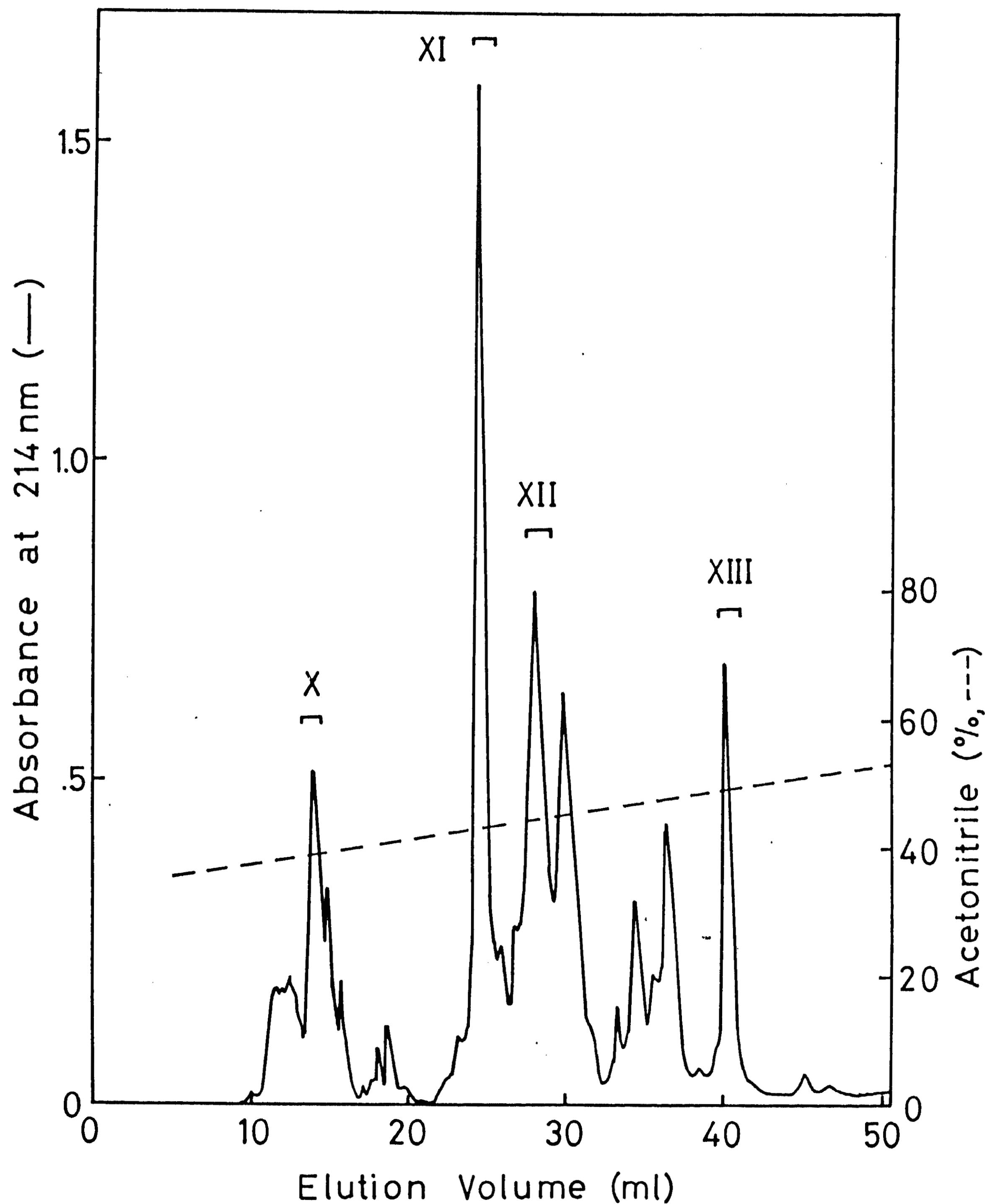


Fig. 26. RP-HPLC of Fraction C after Gel Filtration.

Fraction C eluted from the gel filtration on Sephadex G-100 was loaded on a column of Ultrosphere-octyl (4.6 x 250mm) and eluted under the same conditions described in Fig. 22.

Table XIV. Identification of Peptides Formed from β-Casein by <u>Irpex</u> <u>lacteus</u> Milk-clotting Enzyme B

Amino acid	VII	VIII	1 142 Arg-Ser variant A_1,	A_2,	A_3,	B,	C	IX	143 192 Trp-Leu
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	-*	-*	0					1	1
Total residues	142	142	142	142	142	142	142	50	50
M. W.	16,000	16,000	16,000					5200	5200
N-terminal	Arg	Arg	Arg					Trp	Trp
C-terminal	-Gln-Ser	-Gln-Ser	-Gln-Ser					-Leu-Leu	-Leu-Leu
Recovery(%)**	15	30						20	

* not detected.

** 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.

Table XV. Identification of Peptides Formed from β-Casein by I. lacteus Enzyme B

Amino acid	X	166 189 Ser-Ala	XI	193 209 Tyr-Val	XII	143 165 Trp-Leu	XIII	190 209 Phe-Val
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	-*	0	-*	0	1	1	-*	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(%)**	11		21		9		10	

* not detected.
** 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.

fraction II, PyrGlu(1)-Phe(105), and fraction IV, Met(106)-Val(169), were formed with high yields (Tables XII and XIII). 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 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 microbial enzymes produced a degradation product that migrated just a little faster than para-κ-casein in SDS-PAGE. Figure 27 shows comparative cleaving points of κ-casein by chymosin and _Irpex_ enzyme.

It is well known that β-casein exists in five genetically determined forms which are disignated as A_1, A_2, A_3, B, and C, in the milk of western breeds of cattle.[71] Amino acid compositions of these variants are summarized in Table XIV. 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 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 A_1

and VIII is from variant A_3 of β-casein. This might be due to the fact that β-casein used in this work was prepared from bulk herd milk instead of that from an indiviual. The β-casein preparation contained variants A_1 and A_3 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 apparently the major degradation products of the action of Irpex enzyme B from β-casein. Although IX constitutes a large percentage as determined by the absorbance at 280 nm, as shown in Fig. 25, 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. 27, 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 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.[66] On the other hand, it is postulated that Irpex enzyme B hydro-

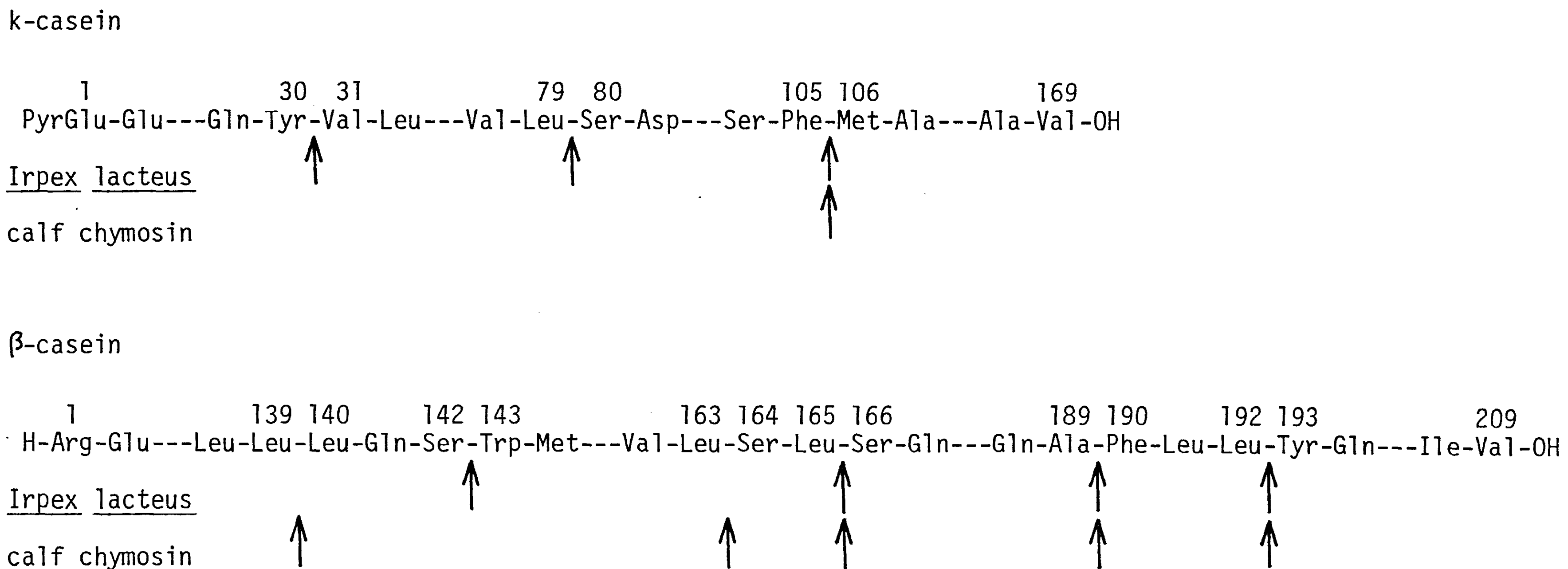


Fig. 27. Comparisons of Cleavage Sites of k-Casein and β-Casein by *Irpex lacteus* Milk-clotting Enzyme B and Calf Chymosin.

lyzes the Ser(142)-Trp(143) bond of β-casein to form VII or VIII and a certain fragment Trp(143)-Val(209) of β-casein first, and then the enzyme attacks Leu(192)-Tyr(193) of the fragment of Trp(143)-Val(209) to produce IX and XI. Irpex enzyme B produced the major degradation products VII and VIII without the band corresponding 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 αs1-casein, chymosin and Irpex enzyme showed different degradation patterns in PAGE and different cleaving sites except for Phe(23)-Phe(24). 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 from each other, resulting in degradation products that showed different PAGE patterns.

Table XVI shows the comparative specificity of Irpex enzyme and chymosin on k-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

Table XVI. Comparison of Cleavage Sites of κ- and β-Casein by _Irpex_ Milk-clotting Enzyme B and Calf Chymosin on the Basis of the Hydrophobicities of the Side Chain of Amino Acid Residues Adjacent to the Bonds to be Split.

	κ-casein P_4	P_3	P_2	P_1 ↑	P_1'	P_2'	P_3'	β-casein P_4	P_3	P_2	P_1 ↑	P_1'	P_2'	P_3'
Enzyme B	Pro *2.6	Ile 3.0	Gln -0.1	Tyr 2.3	Val 1.5	Leu 1.8	Ser -0.3	Leu *1.8	Leu 1.8	Gln -0.1	Ser -0.3	Trp 3.0	Met 1.3	His 0.5
	Trp *3.4	Gln -0.1	Val 1.5	Leu 1.8	Ser -0.3	Asp 0.5	Thr 0.4	Val *1.5	Leu 1.8	Ser -0.3	Leu 1.8	Ser -0.3	Gln -0.1	Ser -0.3
	His *0.5	Leu 1.8	Ser -0.3	Phe 2.5	Met 1.3	Ala 0.5	Ile 3.0	Pro *2.6	Ile 3.0	Gln -0.1	Ala 0.5	Phe 2.5	Leu 1.8	Leu 1.8
								Ala *0.5	Phe 2.5	Leu 1.8	Leu 1.8	Tyr 2.3	Gln -0.1	Gln -0.1
Chymosin	His *0.5	Leu 1.8	Ser -0.3	Phe 2.5	Met 1.3	Ala 0.5	Ile 3.0	Pro *2.6	Pro 2.6	Leu 1.8	Leu 1.8	Leu 1.8	Gln -0.1	Ser -0.3
								Gln *-0.1	Ser -0.3	Val 1.5	Leu 1.8	Ser -0.3	Leu 1.8	Ser -0.3
								Val *1.5	Leu 1.8	Ser -0.3	Leu 1.8	Ser -0.3	Gln -0.1	Ser -0.3
								Pro *2.6	Ile 3.0	Gln -0.1	Ala 0.5	Phe 2.5	Leu 1.8	Leu 1.8
								Ala *0.5	Phe 2.5	Leu 1.8	Leu 1.8	Tyr 2.3	Gln -0.1	Gln -0.1

* Δgt, hydrophobicities of side chain of amino acid residues (kcal/mol). Data from references (61,62). The arrow indicates the splitting point. Positions (P_4-P_3') on the substrate are designated according to Schechter and Berger[53].

site(s) as defined by Schechter and Berger,[53] if k-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).

Chapter IV stated that *Irpex* enzyme has the specificity for the peptide bonds formed by two amino acids with large hydrophobic side chains on αs1-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 located in the P_4 and/or P_3 site(s) might play an important role in the secondary interaction between the enzyme and substrate.

CHAPTER VI

EVALUATION OF MILK-CLOTTING ENZYME FROM *IRPEX LACTEUS* AS A CALF RENNET SUBSTITUTE FOR CHEDDAR CHEESE MANUFACTURE

SUMMARY

The milk-clotting enzyme fraction from *Irpex lacteus* (IR) was obtained by affinity chromatography. To evaluate IR as a calf rennet substitute, Cheddar cheese-making trials were carried out. There was no difference in cheese yield, protein recovery, or fat recovery between cheese made with calf rennet (CR) and that made with IR. Although IR cheese showed a slightly higher extent of proteolysis in comparison to the control during ripening, IR cheese did not develop a bitter taste even after 6 months of ripening. These facts indicate that IR is a promising rennet substitute for cheese making.

INTRODUCTION

Two milk-clotting enzymes secreted by *Irpex lacteus* were purified by affinity chromatography with dehydroacetylpepstatin as ligand. By this step, proteinases with high proteolytic activity, secreted together with milk-clotting enzymes by the microorganism, were eliminated and the ratio of milk-clotting

activity to proteolytic activity of the eluted fraction improved 2-3 times higher than that of the crude preparation. This ratio was shown to be almost the same as the commercial microbial rennet substitutes from *Mucor miehei* and *Mucor pusillus*. In this chapter, I tried to evaluate the milk-clotting enzyme fraction from *Irpex lacteus* as a calf rennet substitute for cheese making by comparing with calf rennet in respects to cheese yield, proteolysis during ripening, and quality of cheese.

MATERIALS AND METHODS

Starter. A mixed culture of *Streptococcus cremoris* and *Leuconostoc citrovorum* was used.

Milk. The milk used for cheese making was obtained from the university farm and was pasteurized at 63°C for 30 min.

Coagulants. The milk-clotting enzyme fraction from *Irpex lacteus* (IR) was prepared by affinity chromatography with dehydroacetylpepstatin, a specific inhibitor of carboxyl proteinases, as a ligand. The milk-clotting enzyme fraction was eluted with 0.1 N acetic acid from the affinity gel and the eluate was adjusted to pH 5.5 with NaOH. Calf rennet powder [(CR), Chr. Hansen Lab., Denmark] was used as a control. Milk-clotting activity was determined by the method described in Chapter II.

Proteolysis of casein fractions by CR, IR and crude enzyme from *Irpex lacteus*. To 5 ml of 0.6% casein solution (pH 6.0, 0.05 M phosphate), 0.05 ml of enzyme solution (500 Soxhlet units/ml) was added, and the reaction mixture was incubated at 35°C. Samples taken after 0, 15, 30, 45, and 60 min were mixed with equal volumes of 0.4 M TCA to terminate reaction. After standing for 20 min at 35°C, the resulting precipitate was removed by centrifugation. The supernatant was taken to determine the amount of TCA soluble products using Folin-Ciocalteu reagent.

Cheese-making procedure. Two trials were carried out using a cheese-making procedure described by Yamamoto *et al*.[72] The pasteurized milk (20 liters) was held at 30°C and the starter was added in the proportion of 1.5% of the total volume of milk. The milk was divided into two vats and each coagulant (CR and IR) was added to each vat when the acidity reached 0.2%. After cutting and cooking, the whey was drained. The obtained curd block was milled, salted, hooped, and pressed. The blocks were sealed with paraffin and ripened at 11°C. In each trial, cheese samples were analyzed at 0, 2, 4, and 6 months of ripening.

Preparations of αs1-, β- and κ-casein. αs1-casein and κ-casein were prepared by the method of Zittle and Custer[56] and β-casein was prepared by the method of Aschaffenburg.[67] Each casein was further chromatographed on DEAE-cellulose by the

method of Davies and Law[57] to remove impurities.

Chemical analysis of cheese samples. This included determinations of moisture by heating at 105°C to constant weight, pH, NaCl according to the IDF standard,[73] and fat by ether extraction. The nitrogen soluble at pH 4.6 (SN), the nitrogen soluble in 12.5% TCA (NPN, non-protein nitrogen) and total nitrogen (TN) were determined by the Kjeldahl method.

Free amino acids. The amount of free amino acids in the NPN fraction after the removal of TCA by ether extraction was determined with a Dionex amino acid analyzer model D-5.

Electrophoresis. Disc gel electrophoresis was performed with 7.5% gel at pH 8.9 in the presence of 4.5 M urea by the method of Davis.[31] Insoluble fraction of cheese samples at pH 4.6 was collected, dissolved in 9 M urea, and analyzed.

Organoleptic tests. Cheese sambles were evaluated for flavor quality as well as body and texture.

RESULTS

Proteolysis of casein fractions by CR, IR, and crude enzyme from Irpex lacteus. The extent of hydrolysis found after treatment of whole casein, αs1-casein, β-casein, and κ-casein with

milk-clotting enzymes are shown in Fig. 28. Crude enzyme was considerably more proteolytic on casein fractions than CR and IR. Especially CR hardly degraded any casein fraction under the conditions used.

Cheese-making trial To evaluate IR as a calf rennet substitute, a cheese-making trial was carried out. Each coagulant (IR or CR) added to 10 liters of milk was 30,000 Soxhlet units and clotting times of CR and IR were 29 min and 28 min, respectively. The curd particles made with IR were elastic and did not break during curd making like the control. There were no significant differences in shrinking of curds between IR and CR during cooking. Losses of solid and fat were estimated to be 6.4% and 0.2% for both cheeses in the whey, respectively. The curd made with IR adhered firmly as well as that of CR when it hooped. As shown in Table XVII, there were no differences in cheese yield, protein recovery or fat recovery between control (CR) cheese and that made with microbial coagulant (IR).

Changes during ripening. In both (CR and IR) cheeses, the moisture was observed to decrease about 2% and pH increased slightly during ripening (Table XVII). The rate and extent of proteolysis was estimated by the level of soluble nitrogen formed during ripening. As shown in Fig. 29, both CR and IR cheeses showed regular increases in the NPN and SN with increasing ripening period, but IR cheese showed a slightly higher extent of

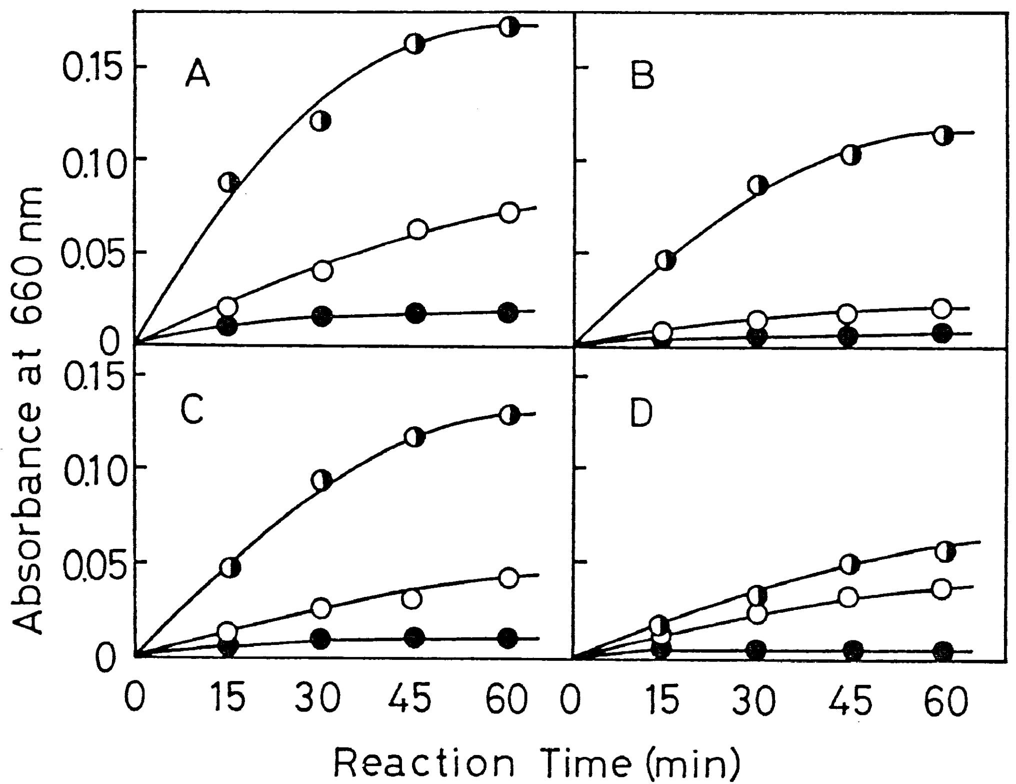


Fig. 28. Time Courses of Proteolysis against Casein Fractions by CR, IR, and Crude Enzyme from *Irpex lacteus*. Casein fractions used were whole casein (A), αs1-casein (B), β-casein (C), and k-casein (D). ◑ , crude enzyme from *I. lacteus*; ○ , milk-clotting enzyme fraction from *I. lacteus* (IR); ● , calf rennet (CR).

Table XVII. Composition of Cheddar Cheese Produced Using Calf Rennet (CR) or Milk-clotting Enzyme Fraction from *Irpex lacteus* (IR) as Milk Coagulant.

Ripening time (months)	Coagulant	Moisture (%)	Fat (%)	Protein (%)	pH	NaCl (%)	Cheese yield (%)wt/milk wt
0	CR	39.5	27.9	25.5	5.06	2.3	8.8
0	IR	39.2	28.1	25.7	5.09	2.1	8.9
6	CR	37.5	27.8	25.7	5.26	2.5	-
6	IR	37.7	27.9	25.8	5.29	2.2	-

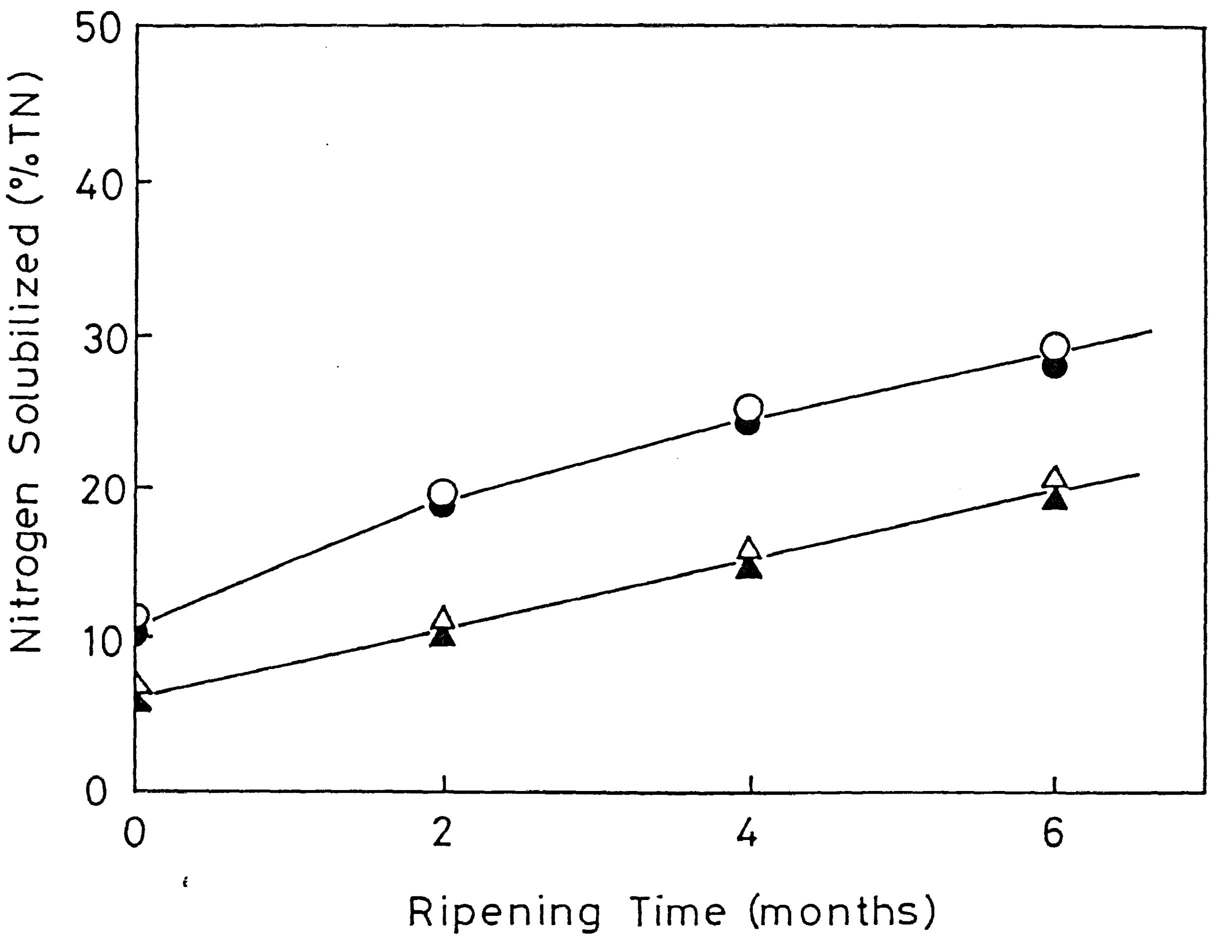


Fig. 29. Development of Water Soluble Nitrogen at pH 4.6 (SN) and Non-protein Nitrogen (NPN) during Ripening.

Open and closed symbols are IR and CR respectively. ○ , ● , SN; △ , ▲ , NPN.

proteolysis in comparison to the control.

In order to estimate the proteolysis of casein during ripening, the insoluble fraction of cheese at pH 4.6 was dissolved in 9 M urea and was analyzed electrophoretically in the presence of urea. As shown in Fig. 30, there was no significant difference in electrophoretic patterns between control cheeses and those made with IR, except two bands located near the top of the gels in IR cheese. As to the degradation rate, CR hydrolyzed αs1-casein faster than IR did, whereas β-casein appeared almost unaffected in both cheeses.

The extent of proteolysis was also acertained by measuring the concentration of the total free amino acids produced. Table XVIII shows a continuous, significant increase in the total free amino acids during ripening for both cheeses. IR cheese, however, showed a higher rate of increase in comparison to the control. Of free amino acids in cheeses, Asp, Thr, Ala, Ile, and Lys were abundant in IR cheese, while Gly, Val, Leu, and Arg are rich in the control. No other amino acids showed significant differences between the two cheeses.

Organoleptic evaluation. There was no difference in the quality of Cheddar flavor between the cheeses, but IR cheese was slightly inferior in the flavor intensity compared to the control. Neither cheese developed bitterness nor rancidity even after 6 months of ripening. As to the texture, there was not significant differences, however, IR cheese was slightly more

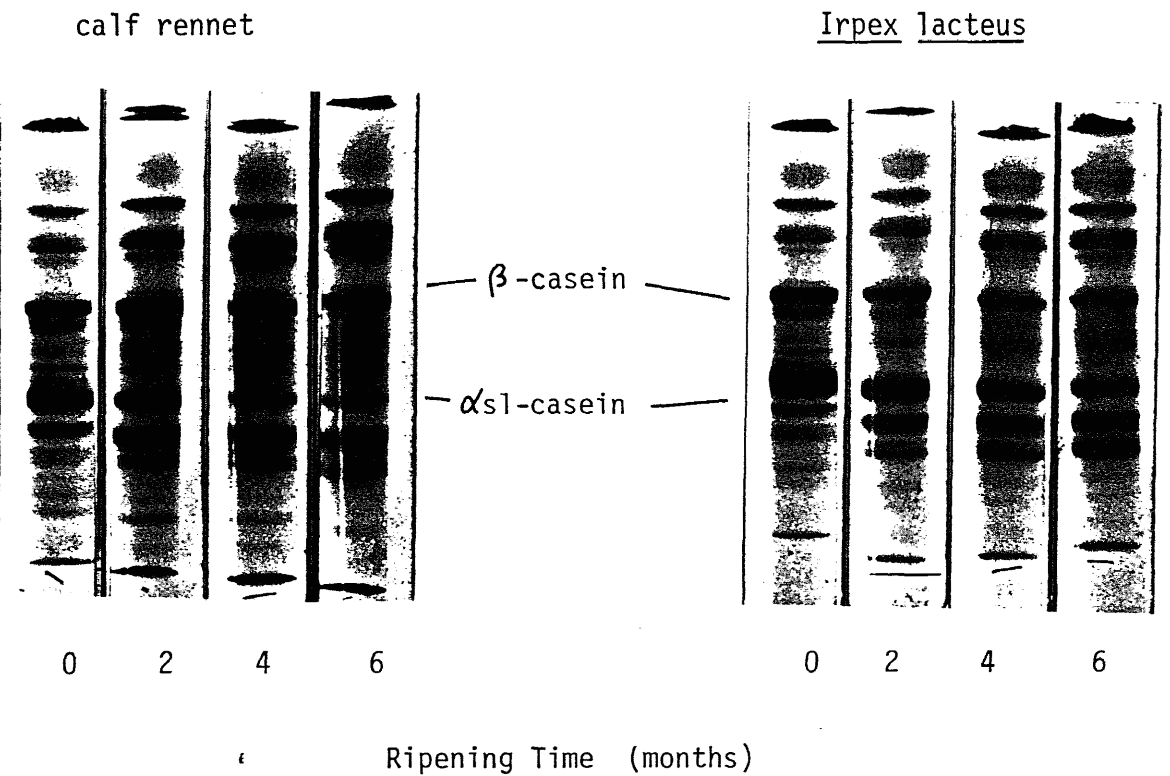


Fig. 30. Polyacrylamide Gel Electrophoresis of Cheddar Cheese Made with Calf Rennet and Milk-clotting Enzyme Fraction from *Irpex lacteus* during Ripening.

Table XVIII. Free Amino Acid Content of Cheddar Cheese Produced Using Calf Rennet (CR) or Milk-clotting Enzyme Fraction from *Irpex lacteus* (IR) as Milk Coagulant.

	Age of cheese (months)							
	0		2		4		6	
Amino acid	CR	IR	CR	IR	CR	IR	CR	IR
Asp	0.8	1.0	1.6	2.4	2.4	3.3	3.4	4.9
Thr	0.7	2.3	1.8	3.3	2.2	3.7	2.4	4.0
Ser	2.7	2.1	2.7	2.1	4.4	4.0	4.3	4.2
Glu	23.3	20.9	20.8	21.4	18.3	21.1	19.7	19.2
Pro	9.6	8.6	5.4	5.0	4.0	3.0	2.5	3.0
Gly	2.2	1.6	2.7	2.2	3.0	2.2	3.3	2.7
Ala	4.8	6.4	4.5	5.5	3.6	4.3	3.4	4.0
Val	8.9	7.3	10.4	9.1	12.5	10.0	12.7	11.0
Met	1.2	1.5	1.5	1.6	1.0	1.0	1.6	1.2
Ile	1.3	2.4	2.5	3.1	2.5	3.0	3.4	4.1
Leu	18.0	16.8	18.1	16.4	18.9	16.5	15.7	13.3
Tyr	3.3	5.3	4.6	4.3	5.3	5.7	4.8	4.3
Phe	9.6	9.3	7.8	7.8	7.7	7.8	6.4	6.6
His	1.8	1.9	2.1	1.9	1.3	1.4	1.4	1.6
Lys	7.6	9.8	9.1	10.1	6.8	9.1	8.5	12.4
Arg	4.1	2.9	4.6	3.1	6.0	4.0	5.5	3.5
Total free amino acid (μmole/g cheese)	14.99	15.76	26.99	28.21	47.52	51.03	67.56	72.48

Values are expressed as percentage of total free amino acid.

mealy than the control.

DISCUSSION

In order to evaluate any enzyme as a calf rennet substitute, it is important to note that the weight yield of the cheese produced must be no less than that made with calf rennet, that is, the enzyme used must not be too proteolytic or lipolytic. It is well known that porcine pepsin is widely used as a calf rennet substitute, especially as an admixture with calf rennet, because it has a high ratio of milk-clotting activity to proteolytic activity, but there is some loss of fat in the whey during cheese making.[74] Many of the proteolytic enzymes from plants, animals, bacteria, and fungi have been investigated because of milk-clotting activity but most of them can not be accepted as calf rennet substitutes because of the great loss of protein during cheese making. Only three fungal rennets derived from _Endothia parasitica_, _Mucor miehei_, and _Mucor pusillus_, have proved to be succesful in the preparation of cheese on a commercial scale.[1] There were no significant differences in cheese yield, protein recovery, or fat recovery between IR cheese and the control cheese (Table XVII). This indicates that IR is a promising calf rennet susbstitute for cheese making.

Although IR is more proteolytic than CR in solution (Fig. 28), IR is only slightly proteolytic than CR in cheeses during ripening. The ratios of SN/TN and NPN/TN in IR cheese are not so

much higher than those in the control cheese. This seems to depend on the presence of NaCl in cheese,[75] the amount of coagulant that remains in the curd,[76] and the instability of IR at neutral pH. Some signigicant differences in the release of free amino acids between CR and IR cheese during ripening were observed, because of a difference in the substrate specificity of IR and CR on caseins.

The most significant flavor defect is the development of bitterness, particularly in long-hold cheeses.[1] In practice, many potential rennet substitutes are rejected because they render cheese bitter. The reasons for the development of bitterness are not completely understood, but there is evidence that it may be due to the formation of bitter peptides from β-casein. Therefore, a coagulant which does not hydrolyze β-casein excessively is suitable for the production of cheeses of good quality without bitterness. IR does not develop bitterness even after 6 months of ripening as well as CR does, and the proteolysis of β-casein by each coagulant is reduced under the conditions of cheese making. In view of the high ratio of milk-clotting activity to proteolytic activity and the production of Cheddar cheese of good quality as described above, if undesirable proteinases which exist in the crude preparation can be eliminated by a simple treatment, the milk-clotting enzyme from _Irpex lacteus_ would be a promising calf rennet substitute for cheese making.

CHAPTER VII

PURIFICATION AND CHARACTERIZATION OF PEPSTATIN-INSENSITIVE CARBOXYL PROTEINASE FROM IRPEX LACTEUS

SUMMARY

A pepstatin-insensitive carboxyl proteinase of Irpex lacteus was purified by the method including affinity chromatography with chymostatin as a ligand.

Although the enzyme exhibited its maximum proteolytic activity on hemoglobin at pH 2.8, it was not affected by carboxyl proteinase inhibitors such as DAN, EPNP and pepstatin. On the other hand, the enzyme was inhibited by chymostatin competitively and its Ki value was estimated to be 1.6×10^{-5} M. The enzyme was very heat labile and was inactivated completely under the following conditions: pH 4.6, heating at 45°C for 15 min. Irpex enzyme and Scytalidium lignicolum enzyme A_1 hydrolyzed the same peptide bond of Z-tetrapeptides, but their primary specificities were slightly different from each other. Irpex enzyme as well as Ganoderma lucidum enzyme contains histidine in the molecule, and amino acid compositions of Irpex enzyme and other pepstatin-insensitive carboxyl proteinases resembled each other.

INTRODUCTION

In Chapter II, two carboxyl proteinases with high milk-clotting activity secreted by a basidiomycete, Irpex lacteus, have been purified by affinity chromatography with dehydroacetyl pepstatin as a ligand, and characterized. The main enzyme B exhibits almost the same ratio of milk-clotting activity to proteolytic activity as commercial calf rennet substitutes from Mucor miehei and Mucor pusillus, which is 2-3 times higher than that of the crude enzyme.

I found that the proteinase other than milk-clotting enzyme existing in the crude enzyme preparation had maximum proteolytic activity on hemoglobin at acidic region but was not inhibited by pepstatin even at the concentration of 10^{-4} M.

It has already been reported[77] that pepstatin-insensitive carboxyl proteinases are widely distributed in basidiomycetes, especially in edible mushrooms, such as Lentinus edodes(Shii-take), Ganoderma lucidum(Mannen-take), Pleurotus ostreatus(Hira-take) and Flammulina velutipes(Enoki-take). Among them, the carboxyl proteinases of Lentinus edodes TMI-567[78,79] and Ganoderma lucidum[80] have been purified and characterized.

In this chapter, the purification and characterization of the pepstatin-insensitive carboxyl proteinase from Irpex lacteus were described.

MATERIALS AND METHODS

Z-tetrapeptides were generously supplied by Dr. K. Morihara of Shionogi Research Lab. Molecular weight markers (ranging from 14,300 to 71,500) for SDS-PAGE were obtained from BDH Biochemicals. Sephadex G-100 and Sepharose 4B were from Pharmacia. Other chemicals used were of reagent grade.

Affinity gel. Dehydroacetylpepstatin-aminohexyl-agarose and chymostatin-aminohexyl-agarose were prepared by the method of Murakami and Inagami.[30]

Enzyme assay. Milk-clotting and proteolytic (hemoglobin) activities were determined as described in Chapter II.

Purification of pepstatin-insensitive carboxyl proteinase. One gram of *Irpex lacteus* rennet powder was dissolved in 100 ml of 0.01 M sodium acetate buffer (pH 5.5) and applied to a dehydroacetylpepstatin-aminohexyl-agarose column(1 x 3cm) equilibrated with the same buffer. After washing with the same buffer, two active fractions were eluted by 0.5 M NaCl and 0.1 N acetic acid from the affinity column as described in Chapter II. The sodium chloride fraction was concentrated and chromatographed on a Sephadex G-100 column (4 x 90cm) equilibrated with 0.01 M sodium acetate buffer, pH 5.5. The active fraction was then

applied to a chymostatin-aminohexyl-agarose column equilibrated with the same buffer. After washing with the same buffer containing 0.5 M NaCl, the active fraction was eluted from the affinity column by changing the pH to 3.0 with 0.1 N acetic acid (Fig. 31). The active fraction was concentrated, dialyzed against 0.02 M sodium acetate buffer (pH 4.6), and applied to a DEAE-cellulose column (1.5 x 25cm) equilibrated with the same buffer. Two active fractions were eluted by a decreasing pH gradient from pH 4.6 to 3.0 (Fig. 32).

Isoelectric focusing. Isoelectric focusing was run in 7.5 cm gel containing 5% polyacrylamide with 4.8% cross linkage and 2% ampholine with a constant voltage of 200V for 5 hr. After run, the gel was sliced into small fragments with 2.5 mm width. Each fragment was extracted with 0.5 ml deionized water and its proteolytic activity was determined as described in Chapter II.

Hydrolysis of various Z-tetrapeptides. The pH of the reaction mixture was adjusted to 4.0 and the hydrolysis was followed by the method according to Oka and Morihara.[81] The initial velocity was measured at 35°C with the enzyme concentration suitably adjusted.

Other methods were the same as described in Chapter II.

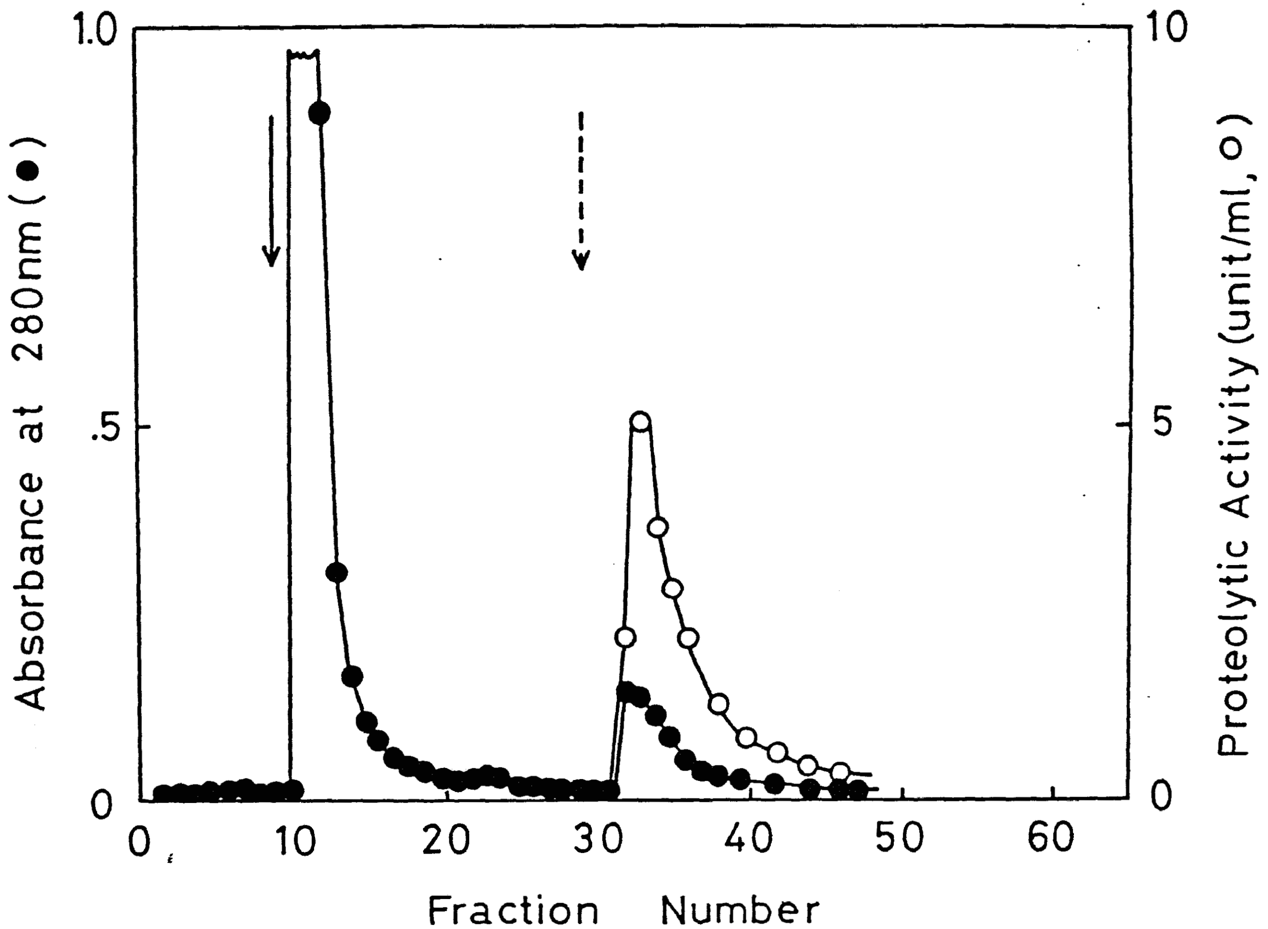


Fig. 31. Affinity Chromatography of Pepstatin-insensitive Carboxyl Proteinase from _Irpex lacteus_ on Chymostatin-aminohexyl-agarose (1 x 3cm).
The flow rate was 20 ml/hr and 4 ml fractions were collected. ↓, 0.01 M sodium acetate buffer containing 0.5 M NaCl; ⇣, 0.1 N acetic acid.

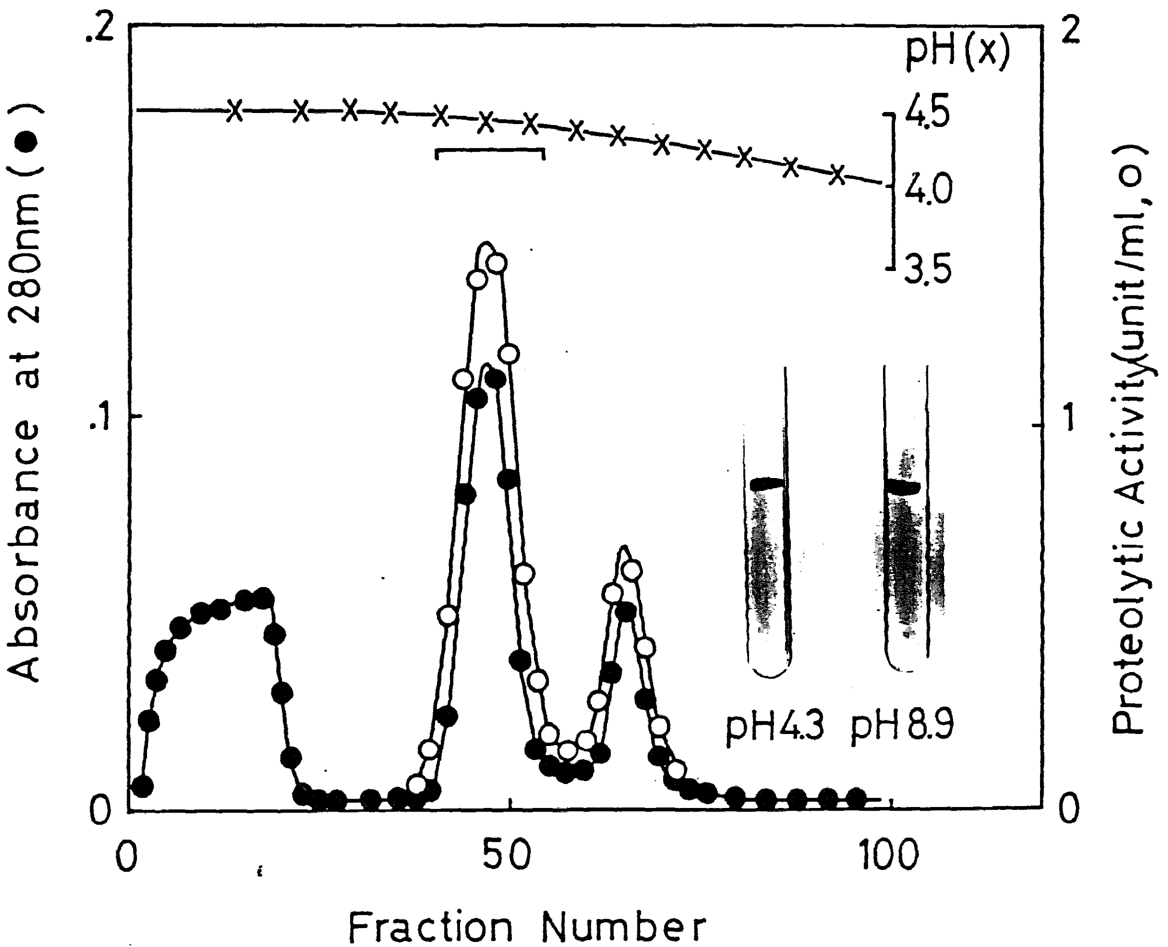


Fig. 32. Ion Exchange Chromatography of Pepstatin-insensitive Carboxyl Proteinase after the Affinity Column (Fig. 31) on a DEAE-cellulose (1.5 x 25cm).
The flow rate was 15 ml/hr and 3 ml fractions were collected. Electrophoresis was performed at pH 4.3 and pH 8.9 according to Davis[31].

RESULTS AND DISCUSSION

Purification The purification steps are summarized in Table XIX. This process purified the enzyme 55-fold, with about 12.5% recovery. The major fraction, indicated with a bracket in Fig. 32, appeared homogeneous at pH 8.9 and pH 4.3 on PAGE. Among the purification steps, chymostatin gel is the most effective for the purification of the pepstatin-insensitive enzyme.

Properties of the enzyme. Table XX shows some properties of *Irpex* enzyme. The enzyme was most active at pH 3.0 on casein and at pH 2.8 on hemoglobin, and the ratio of caseinolytic activity to hemoglobinolytic activity was estimated to be 0.3. The ratio for ordinary carboxyl proteinases (pepstatin-sensitive) was reported to be ranging from 0.5 to 1.0, but *Irpex* enzyme hydrolyzed hemoglobin preferantially similar to other pepstatin-insensitive carboxyl proteinases reported so far.[80)]

The isolated pepstatin-insensitive enzyme revealed that the ratio of milk-clotting activity to proteolytic activity on casein at pH 6.0 was one-fourth that of the milk-clotting enzyme from *Irpex lacteus*(data not shown). Crude enzyme from *Irpex lacteus* was easily separated into two fractions (the pepstatin-insensitive enzyme fraction and pepstatin-sensitive milk-clotting enzyme fraction) by affinity chromatography with dehydroacetyl-pepstatin as ligand. Therefore, as stated in Chapter VI, it

Table XIX. Purification of Pepstatin-insensitive Carboxyl Proteinase from *Irpex lacteus*

Step	Protein (mg)	Activity (units)	Yield (%)	Specific activity (units/mg protein)	Purification (x fold)
Crude extract	1033	1085	100	1.1	1.0
Dehydroacetyl-pepstatin gel	357	312	28.7	1.0	0.9
Sephadex G-100	116	262	24.2	2.3	2.1
Chymostatin gel	6.6	194	17.9	29.3	27.9
DEAE-cellulose	2.4	136	12.5	57.3	54.5

Table XX. Properties of Pepstatin-insensitive Carboxyl Proteinase from _Irpex lacteus_

Optimum pH (hemoglobin)	2.8
(casein)	3.0
pH stability	3 - 5
Optimum temperature	45°C
Heat stability	0 - 30°C
Molecular weight (SDS-PAGE)*	34,000
Isoelectric point**	5.0

*SDS-polyacrylamide gel electrophoresis

**estimated by polyacrylamide gel isoelectric focusing

might be possible to produce good quality cheese by effective elimination of the pepstatin-insensitive carboxyl proteinase from the crude enzyme by affinity chromatography.

The enzyme was stable up to 30°C at pH 4.6 for 15 min of incubation. However, it was completely inactivated at 45°C, indicating that the enzyme was very heat labile compared to other pepstatin-insensitive enzymes reported previously.

Effects of various inhibitors. As shown in Table XXI, specific carboxyl proteinase inhibitors such as DAN, EPNP, and pepstatin did not inactivate the enzyme. This is the common feature which can discriminate these enzymes from ordinary carboxyl proteinase including pepsin. Although leupeptin, antipain, DFP, and EDTA also had no effect on the enzymatic activity, the enzyme was inactivated by chymostatin and TPCK which are known as inhibitors of chymotrypsin. Determination of the inhibition constant (Ki) of chymostatin was carried out with Z-Phe-Leu-Ala-Ala as a substrate at pH 4.0 according to the method of Dixon.[82] The Ki value was calculated to be 1.6×10^{-5} M and chymostatin acted as a competitive inhibitor of *Irpex* enzyme. It is suggested that the inactivation by chymostatin and TPCK, in spite of the maximum enzymatic activity at the acidic region, might be caused by the similarity of substrate specificity to that of chymotrypsin. However, there has been no information about such properties of other pepstatin-insensitive carboxyl proteinases reported so far.

Table XXI. Effect of Inhibitors on the Pepstatin-insensitive Carboxyl Proteinase from _Irpex lacteus_

Inhibitor	Concentration(M)	Remaining activity(%)
DAN	-*	100
EPNP	-**	100
Pepstatin	10^{-3}	100
Chymostatin	10^{-4}	5
Leupeptin	2×10^{-4}	100
Antipain	2×10^{-4}	100
TPCK	10^{-3}	15
DFP	10^{-2}	100
EDTA	10^{-2}	100

* The reaction was carried out at 14°C and pH 5.0 for 1 hr according to the method of Rajagopalan.[83]
Enzyme:DAN:Cu(II)=1:40:30(molar ratio).
** The reaction was carried out with 0.5 ml of 0.01% enzyme in 0.05 M sodium citrate buffer, pH 4.5, and 0.5 mg of solid powder of EPNP at 25°C for 72 hr, according to the method of Tang.[84]

Substrate specificity of the enzyme. Substrate specificity of *Irpex* enzyme was investigated with Z-tetrapeptides as substrates according to the methods of Morihara *et al*.[81,85] The optimum pH for hydrolysis of the synthetic substrate was shown to be pH 4.0 and the enzyme hydrolyzed the Leu-Ala bond of Z-Phe-Leu-Ala-Ala just like *Scytalidium lignicolum* enzyme A_1. Table XXII shows the primary specificity of *Irpex* enzyme as compared to that of the enzyme from *Scytalidium lignicolum*. Although both enzymes require hydrophobic amino acids in the P_1 and P_1' sites designated by Schechter and Berger,[53] *Irpex* enzyme exhibits lower specificity against lysine in the P_1 site of the substrate than *Scytalidium* enzyme does. In addition, the existence of phenylalanine in the P_1 site and alanine in the P_2 site is more effective for the hydrolysis of the substrate by *Irpex* enzyme than in the case of *Scytalidium* enzyme.

Amino acid composition. Table XXIII compares the amino acid compositions of *Irpex* enzyme and other pepstatin-insensitive carboxyl proteinases. In contrast to *Scytalidium* and *Lentinus* enzymes, which contain no histidine in their molecule, both *Irpex* and *Ganoderma* enzymes contain histidine, and amino acid compositions of all these enzymes resemble each other.

Table XXII. Primary Specificity of Pepstatin-insensitive Carboxyl Proteinase from *Irpex lacteus*

Peptide	*Irpex lacteus* enzyme		*Scytalidium lignicolum* enzyme A_1**	
	Km (mM)	Kcat/Km (sec M)	Km (mM)	Kcat/Km (sec M)
Z PheGly-AlaAla	-	10*	-	10*
Z PheAla-AlaAla	-	300*	-	420*
Z PheGlu-AlaAla	3.7	1220	3.7	2540
Z PheLeu-AlaAla	1.0	14300	1.0	10700
Z PheDLeu-AlaAla	-	0	-	0
Z PheTyr-AlaAla	1.3	2220	1.6	1540
Z PheLys-AlaAla	1.3	310	0.8	1770
Z AlaPhe-GlyAla	1.0	1410	1.5	310
Z AlaPhe-LeuAla	0.8	13400	0.6	4600
Z GlyPhe-LeuAla	2.0	1340	1.3	1900

* Calculated on the assumption that the Km is 1.0 mM.

** Data from Reference (86)

The arrow indicates the splitting point.

Table XXIII. Amino Acid Composition of Pepstatin-insensitive Carboxyl Proteinase from *Irpex lacteus*

	*Irpex lacteus**	*Lentinus edodes*[79]	*Scytalidium lignicolum*[87]		*Ganoderma lucidum*[80]	Pepsin[88]
			A_1	B		
Asp	38	31	44	25	30	40
Thr	27	31	31	19	31	25
Ser	47	32	41	31	30	43
Glu	24	17	23	18	19	26
Pro	15	26	22	4	18	16
Gly	39	31	37	18	37	34
Ala	30	31	25	15	36	16
Val	17	18	20	15	16	20
Cys	6	8	9	6	9	6
Met	1	1	1	1	0	4
Ile	11	14	13	11	12	23
Leu	28	22	20	6	25	28
Tyr	8	7	16	5	4	16
Phe	19	19	16	9	20	14
Lys	9	5	4	2	6	1
His	3	0	0	0	2	2
Arg	6	6	2	1	6	2
Trp	ND**	1	1	1	1	6
Total residues	328	300	325	192	302	321

* Based on a molecular weight of 34,000.
** Not determined.

CHAPTER VIII

CONCLUSION

As described in Chapter I, a great deal of efforts have been made to search for calf rennet substitutes, because calf rennet has become less available. Only three fungal enzymes derived from *E. parasitica*, *M. miehei*, and *M. pusillus* have been used successfully in the preparation of cheese on a commercial scale as calf rennet substitutes. *Irpex lacteus* enzyme used in this investigation was found and purified partially by Kawai and Mukai,[15)] but its enzymatic properties were not clarified sufficiently. Although they reported that *Irpex lacteus* crude enzyme could be used as coagulant for cheese making, detailed data were not shown. In the present experiment, I tried to isolate and characterize the milk-clotting enzyme from *Irpex lacteus*.

Affinity gel with pepstatin A as a ligand has been used effectively for purifications of various carboxyl proteinases from mammalian tissues. However, some microbial milk-clotting enzymes including *Irpex lacteus* enzyme were adsorbed so strongly by the affinity column that it could not be eluted from the column in a stable preparation. This is probably due to a very high affinity of microbial enzymes to pepstatin A.

I found that *Irpex lacteus* produced two kinds of carboxyl proteinases. One was pepstatin-sensitive carboxyl proteinases

with high milk-clotting activity and the other was pepstatin-insensitive carboxyl proteinases with high proteolytic activity. It was difficult to separate milk-clotting enzyme from pepstatin-insensitive carboxyl proteinase by conventional steps, because the molecular weights and isoelectric points of these enzymes resembled each other (Chapters II and VII).

It was shown that dehydroacetylpepstatin was an effective ligand for the purification of *I. lacteus* milk-clotting enzyme, because the affinity of dehydroacetylpepstatin for *I. lacteus* milk-clotting enzyme was 100 times weaker than that of pepstatin A.

As described in Chapter II, two carboxyl proteinases with high milk-clotting activity designated as enzymes A and B were purified by the methods including affinity chromatography with dehydroacetylpepstatin as a ligand. The purified enzymes were judged homogeneous by polyacrylamide gel electrophoresis at pH 4.3. The molecular weights of both enzymes A and B were determined by gel filtration and SDS-PAGE to be 36,000, and the isoelectric points were 4.9 and 5.3, respectively. Enzymes A and B had similar amino acid compositions, both lacking sulfur-containing amino acids such as cysteine and methionine. As a whole, enzymes A and B were similar to *E. parasitica* enzyme in amino acid composition.

Both of the enzymes A and B were inhibited by carboxyl proteinase inhibitors such as pepstatin, DAN, and EPNP. These results indicate that enzymes A and B are similar to calf

chymosin and other microbial milk-clotting enzymes in their active site structure, having two different caboxyl groups, although they showed minor differences with regard to enzymatic and molecular properties.

The enzyme was stable up to 45°C at pH 4.6 for 15 min, but was completely inactivated at 50°C and pH 6.0 for 20 min. As compared to other milk-clotting enzymes such as chymosin, M. miehei enzyme, and M. pusillus enzyme, enzymes A and B were relatively unstable at neutral pH. This is important as calf rennet substitute because excess proteolytic activity remaining in the curd affects physical properties of cheese and makes it bitter during ripening.

Enzymes A and B hardly activated trypsinogen at pH 3.0 just like other milk-clotting enzymes such as M. miehei enzyme, M. pusillus enzyme, pepsin, and chymosin. This is recognized to be a common feature of milk-clotting enzymes, which can differentiate them from other microbial carboxyl proteinase[104].

Enzymes A and B exhibited almost the same ratio of milk-clotting activity to proteolytic activity as commercial microbial milk-clotting enzymes obtained from M. pusillus and M. miehei. Enzymes A and B were less sensitive than chymosin to calcium concentration in milk. This is tan important, since calcium concentration in milk can vary with season and locality. These facts would indicate that milk-clotting enzyme from I. lacteus is a promising calf rennet substitute for cheese manufacture.

In Chapter III, the author described the substrate

specificity of Irpex lacteus enzyme B on oxidized insulin B chain and angiotensin I. In the case of insulin B chain, the peptide bonds mainly susceptible to the enzyme were Leu(11)-Val(12), Ala(14)-Leu(15), Phe(24)-Phe(25), and Thr(27)-Pro(28) bonds, with the Ala(14)-Leu(15) bond as the most preferentially hydrolyzed. The specificity of enzyme B was distinct from other commercial microbial milk-clotting enzymes and it had a more restricted specificity than chymosin and porcine pepsin. Moreover, a notable property of enzyme B was that it hydrolyzed the Thr(27)-Pro(28) bond which was barely hydrolyzed by any of the carboxyl proteinases ever studied. Using the numbering system of Schechter and Berger,[53)] enzyme B generally required hydrophobic amino acids for exhibiting its activity in the P_1 and P_1' sites.

Irpex lacteus enzyme B hydrolyzed the Tyr(4)-Ile(5) bond much more rapidly than the Val(3)-Tyr(4) bond of angiotensin I. This specificity resembled that of carboxyl proteinase from Pycnoporus coccineus, a kind of basidiomycetes.

αs1-Casein was reported to be extensively hydrolyzed by calf chymosin during cheese ripening, and the substrate specificity of chymosin on αs1-casein was established. It is important to investigate the substrate specificity of enzyme B on αs1-casein, because substrate specificities of microbial milk-clotting enzymes have not yet been reported.

In Chapter IV, the author described the substrate specificity of enzyme B from Irpex lacteus on αs1-casein. The milk-clotting enzymes may be classified into two groups according

to their degradation pattern of αs1-casein in solution at pH 6.0. On the one hand, calf chymosin and *Mucor miehei* enzyme produced only one degradation product corresponding to αs1-I under the conditions employed. On the other hand, *Irpex lacteus* and *Endothia parasitica* enzymes produced several degradation products accompanied by a product corresponding to αs1-I.

Irpex enzyme B hydrolyzed at the positions of His(8)-Gln(9), Phe(23)-Phe(24), Lys(103)-Tyr(104), and Phe(153)-Tyr(154). *Irpex* enzyme B had only one common cleaving site with calf chymosin, that is, on Phe(23)-Phe(24) bond of αs1-casein. However, other sites were different from those of chymosin. Chymosin cleaved the Phe(23)-Phe24) bond of αs1-casein to form αs1-I[Phe(24)-Trp(199)] at first, and then it acted on the Leu(169)-Gly(170) bond of αs1-I to form αs1-II[Phe(24)-Leu(169)], and finally it hydrolyzed the Leu(149)-Phe(150) bond of αs1-II to produce αs1-III[Phe(24)-Leu(149)]. On the contrary, enzyme B cleaved the Phe(23)-Phe(24) bond of αs1-casein in the same manner as chymosin to form αs1-I, however, the enzyme attacked Lys(103)-Tyr(104) bond of αs1-I to form Tyr(104)-Trp(199), and finally it hydrolyzed at Phe(153)-Tyr(154).

Chymosin always required hydrophobic amino acid such as phenylalanine or leucine in the P_1 site, and *Irpex* enzyme B had the specificity for the peptide bonds formed by two amino acids with large hydrophobic side chains such as Phe-Phe and Phe-Tyr. Although the *Irpex* enzyme B, as well as *Mucor miehei* enzyme, *M. pusillus* enzyme, pepsin, and chymosin, did not activate

trypsinogen, enzyme B hydrolyzed the Lys(103)-Tyr(104) bond to form Tyr(104)-Trp(199) with high yield. This might be attributed to the large hydrophobic region formed by the neighboring six amino acids from the P_3 to P_3' sites.

In addition, Morihara _et al_.[63] reported that the elongation of the peptide chain length from P_1 to P_3 resulted in a marked increase of hydrolysis with microbial acid proteinases and with pepsin. Thus, hydrophobic amino acids located in the P_4 and/or P_3 site(s) might play an important role in the secondary interaction between the enzyme and the substrate in both cases of chymosin and _Irpex_ enzyme B.

The coagulation of milk is initiated by the hydrolysis of κ-casein into glycomacropeptide and para-κ-casein. In the presence of calcium ions, para-κ-casein precipitates along with the other casein fractions. It is well known that β-casein is a source of bitter peptide formed by the action of proteinases. Especially, a peptide, which constitutes residues Arg(202)-Val(209) of β-casein, possesses bitterness 250 times stronger than that of caffeine. Although the substrate specificity of chymosin on β-casein is established, those of microbial milk-clotting enzymes on β-casein have not yet been established. Therefore, it is important to investigate the specific action of milk-clotting enzyme B on β-casein.

In Chapter V, I described the substrate specificity of the milk-clotting enzyme B from _Irpex lacteus_ on κ- and β-casein. Enzyme B hydrolyzed Phe(105)-Met(106) bond of κ-casein, resulting

to the precipitation of para-κ-casein along with other casein fractions in the presence of calcium ions, in the mechanism similar to other milk-clotting enzymes. Furthermore, Irpex enzyme B hydrolyzed at 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 in PAGE, 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 and chymosin on β-casein, 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 Leu(139)-Leu(140) bond by chymosin and of Ser(142)-Trp(143) bond by enzyme B. Chymosin cleave Ala(189)-Phe(190) and/or Leu(192)-Tyr(193) of β-casein to form β-I initially, and then it acted on the Leu(163)-Ser(164) and/or Leu(165)-Ser(166) bond of β-I to produce β-II, and finally it hydrolyzed Leu(139)-Leu(140) to form β-III, sequentially.

On the other hand, enzyme B hydrolyzed the Ser(142)-Trp(143) bond of β-casein to form Arg(1)-Ser(142) and Trp(143)-Val(209) first, and then the enzyme attacked Leu(192)- Tyr(193) to produce Trp(143)-Leu(192) and Tyr(193)-Val(209). Chymosin hydrolyzed β-casein in a sequential manner from the C-terminal to the central point, whereas enzyme B hydrolyzed the central region of β-casein

first and then, it acted on the resultant C-terminal fragment. Although the cleaving points of β-casein by both enzymes resembled each other, each enzyme exhibited different degradation pattern of β-casein in PAGE because of their different order of cleavage. If the substrate used is β-casein, *Irpex* enzyme B 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). It is postulated that in the case of chymosin and *Irpex lacteus* enzyme B, hydrophobic amino acids located in the P_4 and/or P_3 site(s) might be important in the secondary interaction between the enzyme and the substrate.

In view of high ratio of milk-clotting activity to proeolytic activity and almost the same substrate specificity on β-casein with chymosin, it is concluded that milk-clotting enzyme from *Irpex lacteus* is promising as a rennet subtitute for making cheese. In order to evaluate milk-clotting enzyme from *I. lacteus* as a calf rennet substitute, milk-clotting enzyme fraction (IR) obtained by affinity chromatography was used for Cheddar cheese-making trials and the results were described in Chapter VI.

There was no difference in cheese yield, protein recovery, and fat recovery between cheese made with calf rennet (CR) and that made with IR. Although IR cheese showed a slightly higher extent of proteolysis in comparison to the control during ripening, IR cheese did not develop bitter taste even after 6 months of ripening. There were some significant differences in

the release of free amino acids between CR and IR cheese during ripening. This seemed to depend on the difference in the substrate specificity of IR and CR on caseins. In view of the production of Cheddar cheese of good quality, if undesirable proteinases existing in the crude preparation could be eliminated by a simple treatment, milk-clotting enzyme fraction from *Irpex lacteus* would be a promising calf rennet substitute for cheese making.

The proteinase other than milk-clotting enzyme existing in the crude enzyme preparation had maximum proteolytic activity on hemoglobin at acidic region but was not inactivated by pepstatin even at the concentration of 10^{-4}M. These pepstatin-insensitive carboxyl proteinases were widely distributed in basidiomycetes, especially in edible mushrooms, and the carboxyl proteinases of *Lentinus edodes*(Shii-take) and *Ganoderma lucidum*(Mannen-take) have been purified and characterized.

In Chapter VII, I described the purification and characterization of pepstatin-insensitive carboxyl proteinase from *Irpex lacteus*. Pepstatin-insensitive carboxyl proteinase was purified by the methods including affinity chromatographies with dehydroacetylpepstatin and chymostatin as ligands. Although the enzyme's maximum proteolytic activity on hemoglobin was at pH 2.8, it was not affected by carboxyl proteinase inhibitors such as DAN, EPNP, and pepstatin. On the other hand, the enzyme was inhibited by chymostatin competitively and its Ki value was estimated to be 1.6 x 10^{-5}M. It was suggested that the inactiva-

tion by chymostatin might be due to the similarity of substrate specificity with that of chymotrypsin. Among the purification steps, affinity chromatography with chymostatin as a ligand was the most effective for the purification of the enzyme.

The molecular weight of the enzyme was estimated to be 34,000 by SDS-PAGE, and the isoelectric point was determined to be 5.0. The enzyme showed that the ratio of milk-clotting activity to proteolytic activity on casein at pH 6.0 was one-fourth that of the milk-clotting enzyme. The milk-clotting enzyme was easily separated from pepstatin-insensitive carboxyl proteinase by affinity chromatography with dehydroacetylpepstatin as a ligand. Therefore, it was possible to produce good quality cheese by effective elimination of contaminating carboxyl proteinase from the crude enzyme as described in Chapter VI. The enzyme was very heat labile and was inactivated completely under the following conditions: pH 4.6, heating at 45°C for 15 min. _Irpex_ enzyme and _Scytalidium lignicolum_ enzyme A_1 hydrolyzed the same peptide bond of Z-tetrapeptides, but their primary specificities were slightly different from each other. _Irpex_ enzyme as well as _Ganoderma lucidum_ enzyme contained histidine in the molecule, and amino acid compositions of _Irpex_ enzyme and other pepstatin-insensitive carboxyl proteinases resembled each other.

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