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CHARACTERIZATION OF BYSSOCHLAMYOPEPTIDASE A

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Summary

The enzyme properties of byssochlamyopeptidase A, a chymosin-like enzyme produced by *Byssochlamys fulva* were studied. The enzyme was shown to be electrophoretically and immunochemically pure. Most metallic cations had negligible effect, whereas Hg^{2+} greatly suppressed the enzyme activity. *N*-Bromosuccinimide and I_2 completely inactivated the enzyme. For milk clotting at pH 4.6—6.6, the enzyme was less sensitive to pH than pepsin.

The substrate specificity of the enzyme was studied by incubation of the enzyme at 37°C with whole and individual casein fractions at pH 6.6 and pH 3.0, respectively. The proteolysis by the enzyme was found to be most extensive for α_s-, less for κ-, and least for β-casein. Studies with different synthetic dipeptides and tripeptides revealed that byssochlamyopeptidase A exhibits specificity for Phe-Tyr and Gly-Phe-Phe, but the enzyme did not hydrolyze Ac-Phe-Tyr(I_2).

Introduction

Due to the short supply of animal chymosin (rennin), recent investigations have led to the discovery of a number of microbial enzymes with chymosin-like activity [1—4]. However, most enzymes from bacterial sources cause extensive proteolysis and thus are not acceptable for use in cheese manufacture. Because of high coagulating ability and lesser proteolysis, enzymes from fungi have been considered as a potential source for chymosin substitutes. For example, extracts or partially purified enzyme preparations from *Endothia parasitica* [5], *Mucor pusillus* [6,7] and *Mucor miehei* [8] have been shown to function with some success in cheese-making [3,4].

In a previous investigation, we isolated a fungal enzyme, byssochlamyo-

peptidase A, produced by *Byssochlamys fulva* [9]. The enzyme had a pH optimum at 2.9 and a temperature optimum around 60° C. Preliminary studies showed that the enzyme had high milk-coagulating activity with restricted proteolytic activity. The enzyme was subsequently purified and the physical chemical properties studied [9,10]. Some properties of the purified enzyme, the factors affecting enzyme activity, and the unique action of the enzyme on different substrates are presented in this paper.

Materials and Methods

Materials. Casein (Hammersten quality) was obtained from Mann Research Laboratories. Bovine serum albumin was obtained from Sigma Chemical Company. Raw skim milk was obtained from the dairy plant of the Department of Food Science (University of Wisconsin-Madison). Veal rennet and protease of *M. miehei* were kindly supplied by Dr. John H. Nelson of Dairyland Food Laboratories (Waukesha, WI). Protease of *E. parasitica* was a gift from Dr. John R. Whitaker (University of California-Davis). Protease of *M. pusillus* was obtained from Marschall Division, Miles Laboratories (Clifton, NJ). Pepsin was obtained from Worthington Biochemical Corporation.

Byssochlamyopeptidase A was purified as described [9], with the following modification. The enzyme preparation obtained from the DEAE-cellulose chromatography was subsequently filtered through a Sephadex G-75 column (2.5 × 99 cm) and rechromatographed on a Sephadex G-200 column, and was eluted with 0.05 M sodium acetate buffer (pH 5.2), 0.1 M NaCl [10].

Synthetic peptides of L-Trp-L-Tyr-CONH, L-Trp-L-Glu · H_2O, L-Trp-L-Val and L-Trp-L-Ala-HCl were obtained from Cyclo Chemical, Division Travenol Laboratory, (Los Angeles, CA). L-Ala-L-Ala, Ac-L-Phe-L-Tyr(I_2) Gly-L-Phe, Gly-L-Phe-L-Phe, and L-Phe-L-Gly were obtained from Mann Research Laboratories, (New York). L-Phe-L-Ile, L-Phe-L-Leu, L-Ala-L-Phe, *N*-CBZ-L-Leu-L-Leu-$CONH_2$, DL-Leu-DL-Phe, L-Ala-L-Tyr, L-Ile-L-Phe, L-Leu-L-Leu, DL-Ala-DL-Phe, L-Leu-L-Val, Gly-Gly-L-Tyr and L-Phe-L-Tyr were obtained from Nutritional Biochemicals (Cleveland, OH). L-Phe-L-Met and *N*-CBZ-Gly-L-Phe were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or chemically pure. Deionized water was used throughout this work.

Chymosin was purified by the method of Castle and Wheelock [11].

Whole casein was prepared by precipitation at the isoelectric point with 1 N HCl at pH 4.6 from fresh raw skim milk. The precipitate was filtered through a cloth bag, washed with distilled water and redispersed with 1 N NaOH. Finally, it was washed exhaustively with distilled water and lyophilized.

The κ-casein was prepared from whole casein by gel filtration through a 2.5 × 95 cm Sephadex G-200 column, equilibrated with 0.005 M Tris/citrate buffer (pH 8.6), 6 M urea [12].

The α_s- and β-casein fraction were prepared by the method of Nakai et al. [13]. β-casein was obtained from the first half of the elution peak of a Sephadex G-100 column (2.5 × 86 cm) equilibrated with 0.01 M phosphate buffer (pH 11) at 4° C. A trace of α_s-casein was eliminated by precipitating the eluant at pH 4.4 and 4° C. α_s-Casein was purified from the second half of elu-

tion peak by precipitating with 0.1 M $CaCl_2$.

Enzyme assays and protein determination. Enzymic assays for milk-clotting and proteolytic activities were as described previously [9]. Unless otherwise stated, the substrate for milk-clotting assay was 10% (w/v) non-fat dry milk in 0.05 M sodium acetate buffer (pH 5.7); the substrate for proteolytic activity assay was 1% (w/v) casein in 0.05 M glycine-HCl buffer (pH 3.0). In the experiment on the effect of pH on milk-clotting activity, non-fat dry milk was dispersed in deionized water to 10% and the pH adjusted to the desired value. One unit of milk-clotting activity is defined as that amount of enzyme in 0.1 ml which will clot 1 ml substrate in 1 min at 37°C and pH 5.7. One unit of proteolytic activity is that amount of enzyme which liberates 1 μg tyrosine from 1 ml 1% casein in 1 min at 37°C. The protein concentration was determined by the method of Lowry et al. [14] using bovine serum albumin as standard.

Preparation of rabbit antiserum against byssochlamyopeptidase A. Antibody against byssochlamyopeptidase A was prepared by injection of purified enzyme in rabbits. The immunization consisted of an initial injection of 2 mg purified enzyme into each rabbit via foot pads and a booster of 1 mg protein through the thigh two weeks after the first injection (total dose, approx. 5 mg/rabbit). In general, 1 ml enzyme was mixed with 1 ml complete Freund's adjuvant, and 1 ml mixture was injected into each rabbit. The animals were bled through the marginal ear vein 10 days after the last booster.

Immunochemical tests. For the gel double-diffusion test, small plastic petri dishes (Falcon plastic No. 100 G) (50 × 12 mm), plated with 3 ml 1.2% noble agar (0.001% thimersol) were used. 20-μl samples were used and developed for 24—72 h at room temperature.

For neutralization studies, two approaches were used. The first method involved the direct interaction of antibody with different chymosin-like enzymes. Various amounts of anti-byssochlamyopeptidase A serum were added to 0.3 ml enzyme solution containing 1.5 clotting units and incubated at 4°C for 24 h. 0.1 ml incubation mixture was used for enzyme assay. The second method involved the use of the antisera and AH-Sepharose 4B conjugated with goat anti-rabbit-γ-globulin. A constant amount of enzyme was incubated with antisera for 20 min at 37°C, followed by addition of 0.1 ml goat anti-IgG Sepharose gel. After incubation at 4°C for 24 h, the mixture was filtered through a Millipore filter (HAWP type) and washed with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme activity in the filtrate was determined.

Effect of metal ions and organic compounds on enzyme activity. Solutions containing 3.7 clotting units/ml purified byssochlamyopeptidase A were incubated at room temperature (25°C) for 5 min at pH 5.7 with equal volumes of the inhibitors or activators at a concentration of 10 mM. The proteolytic activities were determined.

Hydrolysis of whole casein at pH 6.6. A 2% solution was prepared by dissolving 2 g whole casein in 0.05 M phosphate buffer (pH 6.6). 10 ml solution were heated to 80°C for 10 min to destroy any microorganisms or enzymes, then cooled and supplemented with a few drops of toluene. 1 ml rennin or byssochlamyopeptidase A solution containing 4 clotting units was added and the reaction mixture was held at 37°C. 1.5-ml samples were removed at appropriate

intervals and pipetted into 1.5 ml 4% trichloroacetic acid. The resulting suspension was mixed and held for 60 min at room temperature, then centrifuged at 5000 rev./min for 10 min. Protein content of the supernatant was determined by the Lowry-Folin method [14] using tyrosine as standard. The 10 h incubation sample was also subjected to thin-layer chromatography. The trichloroacetic acid precipitates were solubilized in 4 ml Tris-HCl buffer (pH 8.6) for polyacrylamide gel electrophoresis.

In the experiment to determine the effect of NaCl on whole casein digestion, a 4% whole casein in 0.05 M phosphate buffer (pH 6.6) was used as substrate. Enzyme concentration was adjusted to 5 clotting units/ml. NaCl was added to the casein solution to give final concentration of 5 and 10% NaCl.

Hydrolysis of casein fractions at pH 3.0. 1 ml chymosin, pepsin or byssochlamyopeptidase A solution (containing 1.5 clotting units) was added to 9 ml purified α_s-, β-, or κ-casein in 0.01 M glycine-HCl buffer (pH 3.0), and the reaction mixture was kept at 37° C. At appropriate intervals, 1.5-ml aliquots were withdrawn from each solution and mixed with 1.5 ml 4% trichloroacetic acid. After standing at room temperature for 1 h, the solutions were centrifuged at 5000 rev./min for 10 min. The non-protein nitrogen was estimated by measuring the absorbance of the supernatant at 280 nm. The trichloroacetic acid precipitates were solubilized in 1 ml Tris-HCl buffer (pH 8.6) for polyacrylamide gel electrophoresis. The electrophoretic patterns were scanned at 560 nm. Percentage of digestion on the individual casein fractions was determined by weighing the peak areas on an analytical balance.

Hydrolysis of synthetic peptides. The reaction mixture (5 ml) containing 0.3—0.7 mM substrate, 2 enzyme clotting units, 0.05 M acetic acid and 10% dimethylformamide. The reaction mixture was at equilibrium at 40° C. 1 ml samples were withdrawn at 0 and 24 h and put into a test tube containing 1 ml 0.5 M buffer (pH 5.0), 0.6 ml 5% ninhydrin in methylcellosolve and 2.5 ml KCN (250 ml methylcellulosolve 5 ml 10 mM aqueous KCN). The tubes were then heated in a boiling water-bath for 15 min to inactivate the enzyme and to develop the color. 10 ml 60% ethanol were added to each solution and the absorbance at 570 nm was recorded. Controls containing enzyme without substrates and substrates without enzyme were run concurrently.

The sites of action of the enzyme on the tripeptides were determined by thin-layer chromatography of the hydrolyzates with reference to the synthetic compounds. The degree of hydrolysis was calculated based on L-leucine as standard.

Thin-layer chromatography and polyacrylamide gel electrophoretic analyses. Silica gel plates (5 × 20 cm and 20 × 20 cm) were prepared using silica gel G (Brinkmann Instruments, Westbury, NY). The plates were spotted with 1.0—5.0 μl solution and were developed with the organic layer of butanol/acetic acid/water (4 : 1 : 5, v/v). The plates were dried at 110° C for 10 min, cooled to room temperature, and then kept in an atmosphere of ammonia fumes for about 5 min. They were then sprayed with 0.05% fluorescamine in acetone [15], air-dried for several seconds, and examined for fluorescence under long-wave ultraviolet light.

The purity of enzyme preparations was determined by polyacrylamide gel electrophoresis as described by Davis [16]. The electrophoresis of the trichloro-

acetic acid-insoluble fraction of rennin and byssochlamyopeptidase A action on whole casein was determined in 4 M urea gels at pH 9.8 by the same technique of Davis [16], except that 2% mercaptoethanol was added to the buffer.

Results

Polyacrylamide gel electrophoresis and immunochemical tests

Enzyme preparation obtained from Sephadex G-200 gel filtration shows only one protein band which coincides with enzyme activity in the gel electrophoresis. In the Ouchterlony double-diffusion test, only a single precipitin line was shown when the anti-byssochlamyopeptidase A-serum reacted with the purified enzyme.

The specific reaction between byssochlamyopeptidase A and the anti-byssochlamyopeptidase A-serum was also evident from a neutralization experment. Fig. 1 shows that the enzymatic activity was progressively decreased as the amount of anti-serum increased in the incubation mixture. Inhibition of enzymatic activity was also observed with serum obtained from non-immunized rabbit (normal rabbit serum), but the degree of inhibition was considerably less (top line, Fig. 1). Similar results were obtained when the experiment was carried out with a double-antibody technique using an AH-Sepharose 4B conjugated with goat anti-rabbit IgG.

Reaction of various chymosin-like enzymes with anti-byssochlamyopeptidase A-serum

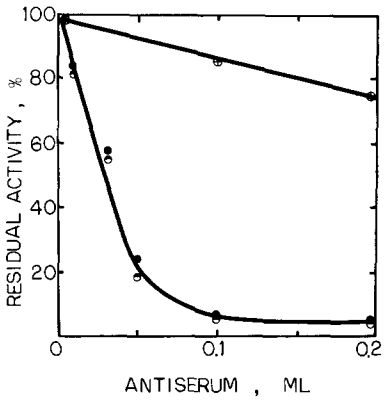
The effect of anti-byssochlamyopeptidase A-serum on the milk-clotting activity of rennin and various chymosin-like enzymes is shown in Table I. Except for pepsin, which was greatly inhibited by normal control serum, rennin and other chymosin-like enzymes were inhibited by the anti-byssochlamyopeptidase A-serum to some degree. The *M. pusillus* enzymes were found to be less sensitive to anti-byssochlamylopeptidase A-serum.

TABLE I

EFFECT OF ANTI-BYSSOCHLAMYOPEPTIDASE A RABBIT SERUM AND NORMAL RABBIT SERUM ON THE MILK-CLOTTING ACTIVITY OF DIFFERENT CHYMOSIN-LIKE ENZYMES

The reaction was carried out by incubation of different enzymes with anti-byssochlamyopeptidase A-serum or control serum at 4°C overnight and then the clotting activity determined.

Enzyme used	Enzyme activity (clotting time, min)		
	No serum added	Control serum	Anti-byssochlamyopeptidase A-serum
Byssochlamyopeptidase A	2.8	3.0	12
E. parasitica protease	4.3	8.6	>4 h
M. pusillus protease	3.4	3.9	6.9
M. miehei protease	3.1	4.0	135
Pepsin	4.0	>4 h	>4 h
Chymosin	3.4	4.5	>4 h



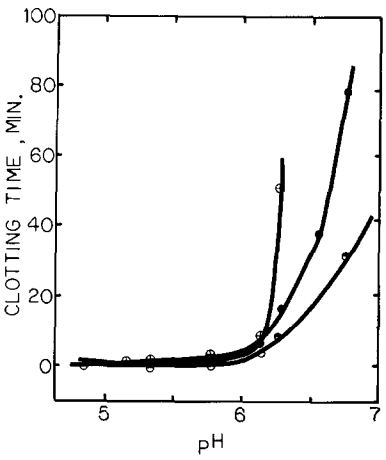


Fig. 1. Inhibition of byssochlamyopeptidase A enzyme activity by rabbit anti-enzyme-serum. Top line (⊖) represents a control experiment by incubating enzyme with normal rabbit serum. The symbols (●) and (◓) represent a data obtained from direct incubation method and by double-antibody method, respectively. In the double-antibody method, a constant amount of enzyme was incubated with various amounts of antisera and 0.1 ml of AH-Sepharose 4B gel conjugated with goat anti-rabbit-γ-globulin. After incubation and filtration, the gel was washed from the Millipore filter and the enzyme activity was determined.

Fig. 2. Effect of pH on milk-clotting activity of byssochlamyopeptidase A (●), pepsin (⊕), and chymosin (◓). The enzyme concentrations were adjusted to 14 clotting units/ml at pH 5.7 and 37°C.

Factors affecting enzyme activity

(a) Effect of enzyme concentration and pH of milk. Studies on the effect of enzyme concentration on milk-clotting activity showed that the clotting time was directly proportional to the reciprocal of the enzyme concentration tested ($2.2 \cdot 10^{-4}$—$6.7 \cdot 10^{-4}$ mg/assay). However, enzyme activity was found to be quite dependent on the pH of the milk. Between pH 4.6 and 5.8, the milk-clotting activity of byssochlamyopeptidase A was comparable with chymosin and pepsin (Fig. 2). Above pH 6.0, byssochlamyopeptidase A exhibited greater dependence on milk pH than did chymosin. Pepsin activity was even more affected by high pH.

(b) Effect of metallic ions on enzyme activity. Table II shows the effect of

TABLE II

EFFECT OF METALLIC IONS ON BYSSOCHLAMYOPEPTIDASE A ACTIVITY

Na^+, K^+, Fe^{2+}, Fe^{3+}, Cd^{2+}, Mg^{2+} tested at concentrations of 10 mM did not show any effect on the enzyme activity.

Metallic ions	Residual proteolytic activity (%)
Control	100
Cu^{2+}	98
Zn^{2+}	96
Ca^{2+}	104
Hg^{2+}	59
Al^{2+}	114

metallic ions on enzyme activity of byssochlamyopeptidase A. The clotting activity of byssochlamyopeptidase A was considerably inhibited by Hg^{2+}, and to a lesser degree by Zn^{2+} and Cu^{2+}. Al^{3+} had a slightly enhancing effect for the proteolysis. Other metallic ions had little effect on the enzyme activity.

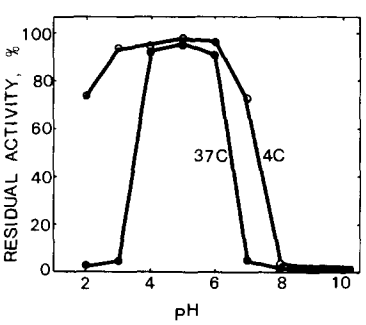
(c) Effect of some organic substances on enzyme activity. Organic substances including hydrazine sulfate, iodoacetic acid, L-cysteine, glutathione, lima bean trypsin inhibitor, *p*-nitrophenylacetate and EDTA did not affect the proteolytic activity of the enzyme. However, *N*-bromosuccinimide and I_2 completely inactivated the enzyme activity and sodium thiosulfate and 2-mercaptoethanol exhibited 13% and 22% inhibition on the enzyme activity, respectively.

Enzyme stability

Byssochlamyopeptidase A, in the form of a partially purified solution, was found stable between pH 3 and 6 at 4°C and between pH 4 and 5.5 at 37°C for 24 h incubation (Fig. 3). Solutions of the purified enzyme were not very stable, especially at low concentration. Both freeze-drying and storage affected enzyme activity. However enzyme activity was found to be stable when it was stored in buffer containing 0.4 M NaCl (pH 5.2). The inhibitory effect of NaCl on enzyme activity was reversible; ie. enzyme activity could be restored after a brief dialysis.

Hydrolysis of whole casein at pH 6.6 by byssochlamyopeptidase A and chymosin

The proteolytic action of byssochlamyopeptidase A and chymosin on whole casein at pH 6.6 and 37°C was examined by comparing the protein content in the trichloroacetic acid-soluble fractions, the fragments in the soluble fractions, as well as the electrophoretic pattern in the trichloroacetic acid-insoluble fracfractions. The results show that there is more extensive hydrolysis of casein by byssochlamyopeptidase A at pH 6.6 than by chymosin (Fig. 4). Thin-layer



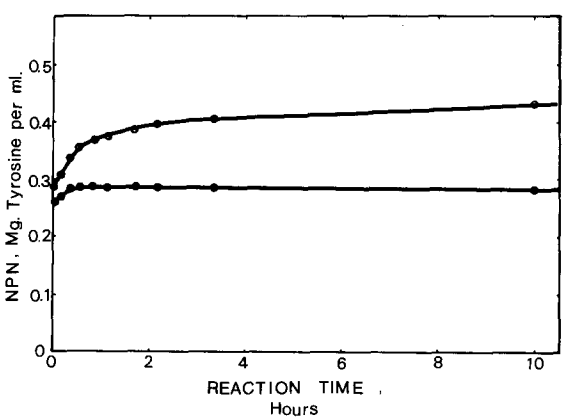


Fig. 3. Effect on pH on the stability of byssochlamyopeptidase A at 4°C (○) and 37°C (●) after 24 h of incubation.

Fig. 4. Release of non-protein nitrogen (NPN) from whole casein after digestion with chymosin (●) and byssochlamyopeptidase A (○) at pH 6.6 and 37°C.

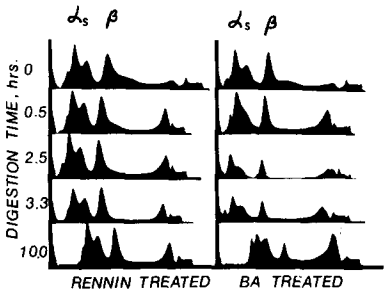


Fig. 5. Electrophoretic patterns of the trichloroacetic acid-insoluble fraction of whole casein after digestion with chymosin and byssochlamyopeptidase A at pH 6.6 and 37°C.

chromatography of the trichloroacetic acid-soluble fraction of the 10-h digestion samples revealed that the hydrolyzed products of the enzymes were different. As many as nine distinct bands were obtained from the byssochlamyopeptidase A-treated sample, whereas only seven bands with different R_F values were found in the chymosin-treated one. Polyacrylamide gel electrophoretic patterns of the trichloroacetic acid-insoluble fractions after 10 h digestion by chymosin and byssochlamyopeptidase A showed little resemblance (Fig. 5) with respect to the degradation of the casein by byssochlamyopeptidase A and by chymosin. In the byssochlamyopeptidase A-treated samples the peaks corresponding to α_s-casein and β-casein changed in shape and diminished in size progressively with time of digestion, whereas the chymosin-treated sample changed only slightly. Also, some new products with mobilities between α_s-casein and β-casein, were formed from the byssochlamyopeptidase A-treated sample. The results suggest the specificities of byssochlamyopeptidase A and rennin are different.

Prevention of extensive hydrolytic action of byssochlamyopeptidase A on casein by NaCl

Proteolysis of whole casein by byssochlamyopeptidase A was inhibited considerably by 5—10% NaCl (pH 6.6) at 37°C. The rate of proteolysis as determined by recording the absorbance (A) at 280 nm of the soluble fraction during the action of byssochlamyopeptidase A on casein with 0, 5 and 10% of added NaCl was found to be $8.8 \cdot 10^{-3}$, $8.4 \cdot 10^{-3}$ and $8.0 \cdot 10^{-3}$ absorbance units/min, respectively.

Hydrolysis of different casein fractions by byssochlamyopeptidase A, chymosin, and pepsin at pH 3

The proteolytic activity of byssochlamyopeptidase A at pH 3.0 on purified casein fractions was compared with chymosin and pepsin. As shown in Table III, the digestibilities of β-casein by byssochlamyopeptidase A and pepsin were comparable, but chymosin practically had no effect on β-casein. All three enzymes showed significant proteolysis on κ-casein fraction, with byssochlamyopeptidase A exhibiting the most.

TABLE III

DIGESTIBILITY OF α_s-, β-, AND κ-CASEINS BY CHYMOSIN, PEPSIN AND BYSSOCHLAMYOPEPTIDASE A AT pH 3.0 AND 37°C

The degree of digestion was calculated based on the zero time sample as zero percentage of digestion.

Casein fraction	Time (h)	Degree of digestion (%)		
		Pepsin	Chymosin	Byssochlamopeptidase A
α_s	1	6	4	15
	20	21	14	81
β	1	18	0	14
	20	28	6	32
κ	1	28	24	33
	20	60	40	72

Action of enzyme on synthetic peptides

Results on the hydrolytic activity of byssochlamyopeptidase A towards various synthetic dipeptides and some tripeptides are presented in Table IV. The enzyme attacked peptide bonds involving at least one aromatic amino acid residue, or involving two highly hydrophobic amino acids such as L-Leu-L-Leu. The enzyme was found to be inactive against N-acetylated dipeptides including Ac-Phe-Tyr(I_2) which has been reported to be a very good substrate for pepsin and a chymosin-like enzyme from *M. pusillus* [17]. Also, the dipeptide Phe-Met, the sensitive bond of κ-casein in the milk-clotting process [18], was not hydrolyzed by byssochlamyopeptidase A.

In the case of tripeptide hydrolysis, byssochlamyopeptidase A was found highly active towards Gly-Phe-Phe. After a 20 h incubation at 40°C, almost

TABLE IV

HYDROLYSIS OF SYNTHETIC PEPTIDES BY BYSSOCHLAMOPEPTIDASE A

No hydrolysis was observed when *N*-CBZ-Gly-L-Phe, *N*-CBZ-L-Leu-L-Leu, L-Try-L-Tyr amide, Ac-Phe-L-Tyr(I_2), L-Ala-L-Ala, L-Leu-L-Val, L-Phe-L-Met, and L-Try-L-Glu were used as the substrates.

Peptides	Hydrolysis (%)
L-Leu-L-Leu	67
L-Phe-L-Leu	3
DL-Gly-DL-Phe	2
L-Phe-L-Ile	14
L-Try-L-Val	25
L-Ala-L-Tyr	17
L-Leu-L-Phe	22
L-Ile-L-Phe	11
L-Phe-L-Tyr	98
L-Phe-L-Gly	22
L-Try-L-Ala	16
L-Ala-L-Phe	11
Gly-Gly-Tyr	23
Gly-Phe-Phe	98
Phe-Phe-Phe	34

100% hydrolysis was achieved. The digestion products from the tripeptide Gly-Gly-Phe-Phe were shown to be Gly-Phe and Phe by thin-layer chromatographic analyses.

Discussion

Physicochemical and immunological analyses of the purified byssochlamyopeptidase A indicate that the preparation is essentially homogeneous, thus it can be used to study different enzyme properties. The enzyme was originally suggested to be an acidic proteolytic enzyme showing chymosin-like enzyme activity [9]; the present study confirms previous observations by Reps et al. [19] that the enzyme does indeed have both proteolytic and milk-clotting activities. Like other acidic proteinases, the enzyme activity of byssochlamyopeptidase A was not affected by most metallic ions or sulfhydryl reagents [17,20]. Therefore, the enzyme is neither a sulfhydryl enzyme nor has a very sensitive disulfide bond around its active center. However, both iodine and *N*-bromosuccinimide inhibited the enzymic activity completely. Since iodine can react with tyrosine, tryptophan and histidine residues in the enzyme, and since *N*-bromosuccinimide can destroy tryptophan resudues [21], the results suggest that tyrosine, tryptophan or histidine may be present at or near the active site of the enzyme. This suggestion is consistent with observations with other chymosin-like enzymes. While most fungal rennins are not sulfhydryl or serine enzymes [7,20], the role of histidine residues in the active center of *M. pusillus* enzyme has been demonstrated [7]. The inhibitory effect of iodine on pepsin [22] was found to be due to its reaction with tyrosine residues in the enzyme molecule. Both histidine and lysine have been suggested to be present in the active center of calf chymosin [23]. Although the inhibitory effect of Hg^{2+}, Cu^{2+}, and Zn^{2+} is less defined, it is possible that these metallic ions may interact with imidazole, phenolic, or ϵ-amino groups in the enzyme active center. Such interactions could alter the enzyme conformation and modify the enzyme activity.

Whereas many of the enzymic properties of byssochlamyopeptidase A and the acidic protease produced by *Paecilomyces varioti*, the imperfect stage of *B. fulva*, are alike, there are some distinct differences between these two enzymes. Sawada [24] found that the *P. varioti* protease is sensitive to *p*-chloromercuribenzoate and iodoacetate, and suggested that the enzyme is a SH protease. In addition, the *P. varioti* protease was found to be insensitive to Hg^{2+}, but sensitive to Hg^{+}. Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Sr^{2+}, and Zn^{2+} have a protective effect for the heat inactivation of *P. varioti* protease. Therefore, although the enzymes are produced by genetically and morphologically related fungi, they have different enzymic properties.

Immunochemical analyses reveal that there are some similarities in the structure of byssochlamyopeptidase A and some other microbial chymosins. Since the antisera obtained from rabbit after immunizing with byssochlamyopeptidase A inhibited the clotting activity of *M. miehei* protease, *E. parasitica* protease and animal chymosin, but did not inhibit *M. pusillus* protease, it is likely that the new enzyme and the former three enzymes have a common site(s) for immunological reactions. The inability of anti-byssochlamyopep-

tidase A-serum to inhibit *M. pusillus* protease is consistent with an earlier observation by Iwasaki et al. [25] who found that antibody against *M. pusillus* protease did not react with chymosin, pepsin, or other fungal acidic proteases. Likewise, no cross-reactivities were observed by Martiny and Harboe [26].

Studies on the substrate specificity of byssochlamyopeptidase A reveal that the enzyme is different from chymosin, pepsin and other microbial rennets. The thin-layer chromatographic patterns of the trichloroacetic acid-soluble fraction of whole casein after digestion are different from those reported for other enzymes [27]. The electrophoretic pattern of the trichloroacetic insolubles is different from that obtained with other enzymes, and the digestion pattern of casein fractions is also different. The activity of byssochlamyopeptidase toward a number of di- and tripeptides suggests a specificity for aromatic, or hydrophobic amino acid residues at both sites of the splitting point, a common characteristic of most microbial proteases [28]. The degree of hydrolysis depends upon the nature of the amino acid residues. When both amino acid residues were aromatic, e.g. Phe-Tyr, the hydrolysis was found to be the greatest. In this regard, the enzyme could be useful in the elucidation of protein structure.

The inability of byssochlamyopeptidase A to hydrolyze the Phe-Met dipeptide cannot rule out its ability to attack the Phe-Met bond in κ-casein. Earlier studies have shown that the Phe-Met dipeptide was not susceptible to hydrolysis by the *M. miehei* protease [30] and chymosin [29]. However, when the dipeptide incorporated into a longer peptide chain, it was readily attacked by the enzymes [29,30].

Although byssochlamyopeptidase A exhibits a specificity similar to other acidic proteases for splitting aromatic and hydrophobic bonds, it has a broader specificity than chymosin and pepsin as determined on the hydrolysis of different casein fractions. Gizella and Sanner [31] reported that there were some similarities in enzymic properties between animal chymosin and chymosin from *M. pusillus* and chymosin from *E. parasitica*, yet, the substrate specificity of these three enzymes was quite different. Thus, each individual enzyme, no matter how similar it is to some other enzymes, might still have its unique specificity and kinetic properties.

Whereas byssochlamyopeptidase A has different substrate specificity when compared with animal chymosin, this property does not hinder its use as the chymosin substitute. For example, the amount of non-protein nitrogen liberated from milk by both enzymes in the earlier phase of action was similar. Since aging processes for cheese are carried out at low temperatures. the extent of proteolysis on casein by byssochlamyopeptidase A would be much lower than has been demonstrated in the present study. Proteolysis can be controlled by addition of NaCl also. It has been reported that the degree of hydrolysis of casein by chymosin and by pepsin was maximal for α_s-casein but minimal for the β-casein fraction in the presence of 5—10% NaCl [32]. The inhibitory effect of NaCl to byssochlamyopeptidase A might be also due to the minimized hydrolysis of β-casein. Present study also shows there was an apparent decrease in milk-clotting activity of byssochlamyopeptidase A when the pH of milk is greater than 6.0 (Fig. 2). The effect may be due to the instability of the enzyme as in the case of pepsin.

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