Studies on the Protease of *Pseudomonas*

Part III. Specific Action of the Protease

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Received September 29, 1956

The specific action of the crystalline protease obtained from *Ps. myxogenes* sp., was examined from its action on various proteins, determination of the N-terminal residues of the enzymatically degraded gelatin—which was most readily digested by the enzyme—and comparison with other proteinases, such as trypsin, papain and pepsin. It was shown that the enzyme might be classified as a collagenase and the mode of digestion of gelatin had a more close resemblance to papain than trypsin or pepsin.

INTRODUCTION

Gelatinolytic enzymes (collagenases) appear to have extremely wide spread distribution in the world of bacteria¹⁾. However, as it was pointed out by Yamamoto²⁾, specificity of the proteinases originating from different bacterial species seems to differ among each other. For example, it has been shown by Yonezawa³⁾ that a conversion of crystalline egg-albumin to plak-albumin is not observed by treatment with the crystalline protease* of *Ps. myxogenes* sp., but it is possible by the enzyme of *B. subtilis*⁴⁾.

In order to examine the specificity of the crystalline protease obtained from *Ps. myxogenes* sp., digestion of some proteins (gelatin, hide-powder, gluten, haemoglobin, casein and egg-albumin) was studied in comparison with other known proteinases such as trypsin, papain and pepsin. Moreover, further determination was made on the N-terminal residues of the enzymatically degraded gelatin using Sanger's fluorodinitrobenzene technique⁵⁾.

EXPERIMENTAL AND METHODS

Materials: The twice recrystallized protease⁶⁾ of *Ps. myxogenes* sp. (abbrev. *Ps.* enzyme), crystalline trypsin (Mochida & Co., Ltd.), purified pepsin (Wako & Co., Ltd., 1:10000), and crude papain (Merck & Co., Ltd., 1:350) were used for comparative study.

Proteolytic Degradation: An appropriate amount of enzyme was added to 60 cc (or 20 cc) of a 5% (or 7.5%) substrate solution adjusted to the pH-optima of each protease—i.e., *Ps.* enzyme at pH 7.0, trypsin at 8.0, papain at 6.0 with Sørensen's phosphate buffer, and pepsin at pH 2.2 with Kolthoff's citrate buffer—placed in a 100-cc Erlenmeyer flask previously kept at 35° for 30 minutes, and the mixture brought to reaction at 35° in addition with 1 cc of toluene. Only in the case of papain, HCN was supplemented in a concentration of 0.3% to the substrate solution for activation.

Determination of Proteolysis: Non protein nitrogen (N.P.N) was determined by adding 3 cc of digest to 6 cc of 30% trichloroacetic acid at room temperature and this was kept for 30 minutes and filtered. A semimicro-Kjeldahl nitrogen determination was performed on the filtrate. Amino nitrogen (NH₂-N) was determined on 5 cc of the digest according to the method of Pope & Stevens⁷⁾.

N-Terminal Determination: The method used

1) Emil L. Smith, "The Enzymes" Vol. 1, p. 862 (1951).

2) T. Yamamoto, "Biseibutsu Kōgyō" edited by Asai (Asakura Shoten) p. 522 (1956).

3) D. Yonezawa, Private communication.

* Supplied by the author.

4) N. Egg-Larsen, K. Linderstrøm-Lang, and M. Ottesen, *Arch. Biochem.*, **19**, 340 (1948).

5) F. Sanger, *Biochem. J.* **39**, 507 (1945).

6) K. Morihara, *This Bulletin*, **21**, 11 (1957).

7) C.G. Pope & M.F. Stevens, *Biochem. J.* **33**, 1070 (1939).

here was the same as that of A. Courts^{8,9,10}.

To 20 cc of degraded gelatin solution (7.5%), 6 N-HCl (or 8% NaHCO₃) was added to adjust the pH to 1.5 (to 7.0 with pepsin) to inactivate proteolytic activity. After more than two hours' storage at 30°, the solution was neutralized with 8% NaHCO₃ and made up to 25 cc with water. A mixture composed of 5 cc of the digest, 5 cc of water, and 10 cc of 8% NaHCO₃ to adjust the pH to 8.5, was shaken mechanically with 0.6 cc of FDNB for 24 hrs. The solution was initially kept at 35°, then brought to room temperature after an elapse of about one hour.

Supplementation of 5 g of MgSO₄ and acidification with N-HCl caused a yellow gel (DNP-derivatives of the hydrolysate) to be deposited. If the degradation is mild, the mother liquors contain only a negligible quantity of soluble-DNP-peptides (presumably large molecules) which may be neglected, as was shown by A. Courts¹⁰. But the greater the degradation, more of the DNP-peptides (presumably small molecules) may become soluble in the mother liquor by the treatment. Thus, the experimental value on the high degradation may become smaller than the exact value. The yellow gel thus obtained was dried on P₂O₅ and NaOH, crushed, and washed with acetone until no further yellow colour was removed. The powder was dried at 105° and allowed to equilibrate with the atmosphere.

Fifty mg of the powder was heated with 5 cc of 6 N HCl for 16 hrs. in a sealed-tube at 100°. This causes a destruction of free DNP-proline and DNP-hydroxyproline, while the unchanged amounts of DNP-alanine, DNP-glycine, DNP-glutamic acid, DNP-serine and DNP-aspartic acid were 80%, 40%, 75%, 85% and 60% respectively. After cooling, the hydrolysates (mixtures composed of DNP-amino acid) were extracted three times with an equal volume of ether.

The ether soluble-DNP-amino acid mixtures thus obtained were separated chromatographically on buffered Celite* columns. DNP-aspartic acid (R 0.10), DNP-serine (R 0.15) and DNP-glutamic acid (R 0.4) were separated using a column of 4.5 g Celite (0.5 cc stationary phase/1 g Celite) buffered at pH 4 with phosphate-citric buffer (54 cc 0.2 M Na₂HPO₄ + 46 cc 0.2 M citric acid) and chloroform-ether (90:10 by vol.) as the organic phase eluent, and DNP-glycine (R 0.46) using chloroform as the solvent. By a column of 3 g

Celite buffered at pH 6 (77.5 cc Na₂HPO₄ + 22.5 cc citric acid) using chloroform, DNP-alanine (R 0.15) was separated. These elutions may be speeded up by the addition of ether.

Each DNP-amino acid solution collected was taken to dryness, dissolved in 10 cc of 1% NaHCO₃ and estimated colorimetrically with the use of the Beckmann photometer, at 360 mμ. The quantity of N-terminal residues (moles) released was calculated from optical density and the destructive factor from the hypothesis that DNP-derivatives used for acid-hydrolysis was 100,000 g (approximate to the moles of gelatin), and the ash content and moisture of each sample were the same.

RESULTS

1) Optimum pH and Temperature. The course of action of *Ps.* enzyme on gelatin at various pH values and temperatures was measured as shown in Figs. 1 and 2. The optimum pH was found to be in the range 7.0–8.5, the optimum temperature was approximately 45°.

2) Rates of Hydrolysis of Substrates. The action of *Ps.* enzyme on various proteins was studied and the results are shown in Fig. 3. As it can be seen, this enzyme attacks gelatin and hide-powder greatly but the other proteins slightly.

From the course of digestion of the sub-

FIG. 1. Optimum pH of the Action of the Crystalline Protease.

Concentration of substrate was 5% and 1 mg/60 cc of the enzyme was used.

8) A. Courts, *Biochem. J.* **58**, 70 (1954).

9) A. Courts, *Biochem. J.* **58**, 74 (1954).

10) A. Courts, *Biochem. J.* **59**, 382 (1955).

* Celite 545, Johns Manville Co.

FIG. 2. Optimum Temperature of the Action of the Crystalline Protease.

Concentration of substrate was 5%, and 0.5 mg/60 cc of enzyme was used.

FIG. 3. Comparison of the Rates of Hydrolysis of Various Substrates by the Crystalline Enzyme.

Concentration of substrate was 5%, and 1 mg/60 cc of enzyme was used. The reaction was carried out at 35° for 5 hrs.

(■), 24 hrs. (▨) and 48 hrs. (□).

substrates, they were divided into 3 groups, i.e. 1) gelatin and hide powder, 2) gluten and haemoglobin, and 3) casein and egg-albumin.

The digestion of Group 1 was rapid in the early stages, up to the formation of 100% N.P.N. by digestion of gelatin and 60% N.P.N. by hide powder. But the extend of digestion of hide powder on over 60% N.P.N. was considerably slow. The initial digestion of Group 2. seemed to be very slow, but kinetics of the reaction almost obeyed the second-order reaction ($K=1/t \cdot x/a(a-x)$). That is, when the substrate concentration was 5% and 1 mg/60 cc enzyme was used in the reaction at 35°, $K \times 10^4$ was calculated as 2.05 in gluten and 1.7 in haemoglobin. Moreover,

FIG. 4. Activity of the Enzyme on Gluten.

Substrate was 1% and contained Sørensen's phosphate buffer of pH 7.0. The reaction was carried out at 35° for 24 hrs.

activity curves¹¹⁾ were found to be represented by the formula: $x = Kt \sqrt{E}$, where x = percentage digestion; K = constant; t = time; E = percentage of enzyme concentration, as is shown in Fig. 4. The digestion of Group 3. in comparison with the former two groups described was very slow in all stages.

The splitting action on peptides or acylated amino acids such as glycylglycine, diglycylglycine, glycyl-L-leucine, glycyl-DL-leucine, glycyl-DL-phenylalanine, glycine-ethyl ester and acetyl-DL-methionine was not observed by examination of amino acid liberated with the use of a paper-partition chromatography.

Next, experiments were made to determine

11) E. Bidwell and W.E. Heyningen, *Biochem. J.* **42**, 140 (1948)

the difference of action with the other known proteinases on various substrates. The course of action of each enzyme on gelatin with the requisite amount of enzyme (mg/60 cc) is shown in Fig. 5. From the determination of the N.P.N ((A) in Fig. 5), it is found that

FIG. 5. Comparison of the Action of Various Proteinases on Gelatin.

Requisite amounts of enzyme were added as was shown in the figure to 60 cc of 5% substrate solution and the reaction was carried out at 35°.

- crystalline protease of *Pr. myxogenes* sp. (P_s)
- crystalline trypsin (Tr)
- purified pepsin (Pep)
- crude papain (Pa)

P_s enzyme acts remarkably well on gelatin in comparison with the others, the digestion rate per mg of P_s enzyme being about 5 to 10 times stronger¹² than that of trypsin, and about 40–80 times that of crude papain.

* The results measured by the viscosity method (η /mg), as shown in Part II of this series and the results obtained by determination of N.P.N liberated in this experiment are approximately the same as those in the reaction for 30 minutes.

Pepsin hardly digests gelatin under these conditions. Interestingly, the action of trypsin is rapid in the early stages up to the formation of 60% N.P.N after which the extend of digestion slows down in comparison with the P_s enzyme (this reason will be discussed later on).

The relationship between the liberated N.P.N and NH_2-N ((B) in Fig. 5) is shown to be linear except with the P_s enzyme, which shows formation of more NH_2-N per N.P.N with the elapse of time and increase of the enzyme content suggesting that it acts still more on the gelatin-hydrolysate not precipitated with trichloroacetic acid up to the formation of about 17% NH_2-N (per N.P.N). The value of NH_2-N per N.P.N is about 8–17% (varying with time or the enzyme content) in P_s enzyme, 7.5–9.5% in trypsin, and 7.5% in papain. This means that P_s enzyme attacks more bonds with peptide linkage of gelatin in comparison with trypsin or papain. The next experiment supports this view more clearly. After the addition of appropriate amounts of P_s enzyme to the digest of trypsin (90% N.P.N liberated, and $NH_2-N/N.P.N$ was 9.5%) the mixture was kept for one night at 35°. The formation of NH_2-N increased greatly and reached 17% (per N.P.N). On the other hand, even with the addition of trypsin to the digest of P_s enzyme, not any increase of the NH_2-N was observed. Fukumoto, et al.¹²⁾ using preparations of crystalline proteinase from *B. amylo-liquefaciens* Fukumoto have reported that NH_2-N formation by the action on gelatin or casein was less than that of trypsin. Perhaps, the P_s enzyme may be a different type of proteinases.

Liquefying activity (from N.P.N) of P_s enzyme on the other substrates was compared with those of trypsin, papain and pepsin, and the results are shown in Table I.

Subsequent to the activity of each proteinase

12) J. Fukumoto & H. Negi, *Symposia on Enzyme Chemistry* (Japan), 7, 12 (1952).

TABLE I
COMPARISON OF THE ACTIVITY OF *Ps* ENZYME ON SUBSTRATES WITH THE OTHER PROTEINASES

The values shown in the Table show the activity ratio of each enzyme when compared with *Ps* enzyme, calculated from enzyme amounts necessitated for the same degree of digestion (N.P.N%) as that of *Ps* enzyme. Substrate concentration was 5%, and the reaction was carried out at 35° for 5 hrs.

Substrate	Rate of activity			
	<i>Ps</i> enzyme* ¹	trypsin* ¹	papain* ²	pepsin* ³
Gelatin	1	0.15	0.02	0.00
Hide-powder	"	0.15	0.02	0.00
Gluten	"	0.4	1.5	0.05
Haemoglobin	"	0.5	0.2	0.1
Casein	"	5	0.9	0.04
Egg-albumin	"	10	3	0.7
*1 crystalline material				
" 2 crude " (Merck, 1:350)				
" 3 purified " (Wako, 1:10000)				

on the substrates being calculated as standard with the activity of *Ps* enzyme (as 1), substrate specificity of each enzyme was found to be differentiable with each other. From the comparative study, it is shown that *Ps* enzyme digests gelatin and hide-powder remarkably well, trypsin digests egg-albumin and casein, and papain (even crude material) digests strongly egg-albumin, gluten and casein. Consequently, the author considers that *Ps* enzyme may be classified as a collagenase.

Further determination revealed that the $\text{NH}_2\text{-N}$ formation (per N.P.N) of *Ps* enzyme and the extend of digestion of *Ps* enzyme with the time elapse were generally higher than those of the other proteinases (i.e., *Ps* enzyme > trypsin > papain).

3) **N-Terminal Residue of Enzymatically Degraded Gelatin.** In order to examine the specific action of *Ps* enzyme more clearly, the N-terminal residues of the enzymatically degraded gelatin was determined by Sanger's fluorodinitrobenzene technique. This technique does not permit the bonds broken to be specified, but its application indicates that the amino acid residues carrying the amino groups are released during hydrolysis.

Digesting the gelatin with *Ps* enzyme, the N-terminal residues were measured with the time elapse (Table II), and the enzyme exhibited a marked specificity for peptide

bonds involving the amino group of alanine in the initial stage of reaction (within 30 minutes). However, with reaction exceeding five hours, the specificity was shown to be scattered in all bonds of amino groups without distinction. That is to say, primary specificity is shown to be alanine at the site of amino groups of the peptides, and secondary specificity is observed in rapid success in all the other amino groups.

The change in end groups at various temperatures in the digestion of gelatin with *Ps* enzyme is summarized in Table III. Thermal degradation under these conditions was negligible. The lower the temperature, the less N-terminal residues were released, and the residue liberated primarily (i.e., as shown at the low temperature), is clearly shown to be alanine similarly as in the initial stage of reaction in the former experiment. At high temperatures, the specificity was scattered too widely, and not distinguished.

Effect of the pH on degradation was measured and the results are shown in Table IV. The primary specificity is shown to be alanine by the degradation at pH 4.0 and 9.0-10.5, at which the reaction was mild. At pH 5.5-7.0 the degradation was remarkably increased and so the specificity was not distinguished. At pH 9.0-10.5, only alanine groups increase remarkably, while little increase of the other

TABLE II

LIBERATION OF N-TERMINAL RESIDUES DURING THE DEGRADATION OF GELATIN BY P_5 ENZYME

Into 20 cc of a 7.5% solution of gelatin containing Sørensen's phosphate buffer of pH 7.0, 0.8 mg of P_5 enzyme was added and the mixture was allowed to react at 35° for a suitable time in addition with 0.5cc of toluene.

End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Amino-acid (M)	Time of reaction (min.)	0	10	30	60	120	300	2400
Aspartic acid+Serine		0.09	0.60	1.04	1.82	2.23	3.55	5.21
Glutamic acid		0.12	3.33	4.00	4.42	5.20	5.75	8.03
Glycine		0.39	1.98	2.26	3.58	4.22	5.00	14.91
Alanine		0.08	4.83	6.44	7.08	7.20	6.95	7.79
Approx. increase in alanine			× 60	× 80	× 86			
Approx. increase in glutamic acid			× 28	× 33	× 37			

* The value of the control was almost equal to that of zero hour's reaction, and therefore negligible.

TABLE III

EFFECT OF TEMPERATURE ON THE DEGRADATION OF GELATIN BY P_5 ENZYME

For conditions, see Table II. The reaction was carried out for 1 hr. at various temperatures as shown in the Table. End groups are expressed as moles/100,000 g DNP gelatin (or-hydrolysate).

Amino-acid (M)	Temperature (C°)	15	25	35	45	55
Aspartic acid+Serine		0.70	1.41	1.82	2.73	2.40
Glutamic acid		1.47	3.57	4.42	5.86	5.08
Glycine		2.52	3.02	3.58	4.74	4.58
Alanine		5.40	6.73	7.08	7.16	6.70
Approx. increase in alanine		× 67	× 84	× 86		
Approx. increase in glutamic acid		× 12	× 30	× 37		

TABLE IV

EFFECT OF pH VALUES ON THE DEGRADATION OF GELATIN BY P_5 ENZYME

For conditions, see Table II. Various buffer solutions were prepared. Michaelis acetate buffer at pH 4.0, Sørensen's phosphate buffer at pH 5.5-7.0, Clark & Lub's borate buffer at pH 9.0, and Kolthoff's buffer at pH 10.5-12. The reaction was carried out at 35° for 2 hours. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Amino-acid (M)	pH	4.0	5.5	7.0	9.0	10.5	12
Aspartic acid+Serine		0.74	3.41	2.23	0.87	0.94	0.11
Glutamic acid		1.67	5.81	5.20	1.44	1.07	0.08
Glycine		1.46	4.41	4.22	6.26	4.80	0.52
Alanine		3.21	5.60	7.20	8.37	8.24	0.16
Approx. increase in alanine		× 40	× 70	× 90	× 105	× 103	
Approx. increase in glutamic acid		× 14	× 45	× 43	× 12	× 9	
Approx. increase in glycine		× 3.7	× 11.3	× 10.8	× 16	× 12	

TABLE V
EFFECT OF ENZYME CONCENTRATION ON THE DEGRADATION OF GELATIN BY *P_S* ENZYME

Various amounts of *P_S* enzyme were used for the degradation, other conditions being the same as in Table II. The reaction was carried out at 35° for 5 hrs. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Amounts of enzyme added (mg/20 cc)	0.00	0.01	0.03	0.1	0.3	0.8
Amino- acid (M)						
Aspartic acid+Serine	0.09	0.66	0.74	1.05	3.60	3.55
Glutamic acid	0.12	1.77	1.82	2.40	4.74	5.75
Glycine	0.39	2.15	1.74	2.35	4.24	5.00
Alanine	0.08	4.07	3.42	4.13	5.60	6.95

residues occur which differentiates this from the degradation at pH 5.5-7.0.

Effect of enzyme concentrations on the degradation of gelatin was measured and the results are shown in Table V. When a low concentration of enzyme was used, the primary specificity on alanine appeared as the N-terminal residue released, while when a high concentration of enzyme was used all the other residues also increased without distinction.

Obvious results on the degradation of gelatin under various conditions show that *P_S* enzyme initiates attacks bonds involving alanine groups as N-terminal residues, and in rapid succession splits bonds containing other amino groups as N-residues. The reason for the excellent extent of the degradation of

P_S enzyme may be attributable to the extensive specificity on gelatin.

The digestions of gelatin were determined by examining the difference of specificity between *P_S* enzyme and other known proteinases (Table VI).

From Table VI, it is shown that trypsin attacks the peptide-bonds involving the amino group of glycine, and papain on bonds involving alanine. The rate of hydrolysis of gelatin by pepsin was comparatively slow under these conditions, and the residues liberated were not obscure. The above results of the digestion by trypsin and papain are the same as those obtained by A. Courts¹⁰.

The mode of attack of the four proteinases on gelatin are compared in Table VII.

As shown in Table VII, the specificity of

TABLE VI
N-TERMINAL RESIDUES RELEASED IN GELATIN BY TRYPSIN, PAPAIN AND PEPSIN

Appropriate contents of each enzyme were added into 20 cc of 7.5% gelatin solution containing Sørensen's phosphate buffer of pH 8.0 in the case of trypsin, pH 6.0 in the case of papain (containing 0.3% HCN for activation), and Kolthoff's citrate buffer of pH 2.2 in the case of pepsin. The mixed solution was kept at 35°. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Enzyme used	trypsin 3 mg		crude papain 20 mg		purified pepsin 50 mg	
	2.5 hrs.	25 hrs.	2.5 hrs.	25 hrs.	5 hrs.	40 hrs.
Amino- acid (M)						
Aspartic acid+Serine	0.81	1.30	1.51	2.15	0.38	1.11
Glutamic acid	1.08	1.56	2.72	4.84	0.54	1.23
Glycine	8.00	10.63	7.05	9.03	0.63	2.46
Alanine	0.88	1.84	5.21	6.36	0.75	2.17
Approx. increase in glycine	× 20.5		× 18			
Approx. increase in alanine			× 57			

TABLE VII
N-TERMINAL RESIDUES RELEASED DURING
THE PROTEOLYSIS OF GELATIN

Proteinase	Primary specificity	Secondary specificity
<i>Ps</i> enzyme	alanine	glutamic acid, glycine, aspartic acid+serine (very intense)*
trypsin	glycine	glutamic acid, alanine, aspartic acid+serine (mild)*
papain	alanine	glutamic acid, glycine, aspartic acid+serine (intense)*
pepsin	—	alanine, glycine, glutamic acid, aspartic acid+serine (very mild)*

* Degree of liberation in secondary specificity.

Ps enzyme on gelatin observed from N-terminal residues liberated resembles to that of papain, but not trypsin and pepsin. It may be considered that the low extend of digestion of gelatin, over 60% N.P.N by trypsin in comparison with *Ps* enzyme, is attributed to its weak splitting of the secondary specificity.

DISCUSSION

From observations on the character of the crystalline protease of *Ps. myxogenes* sp. in comparison with the well known proteinases such as trypsin, papain, and pepsin from some view points, it can be summarized as follows: (1) *Ps* enzyme is not affected by HCN, and the pH optima is 7.0-8.5. The results show that *Ps* enzyme seems to be similar to trypsin and not to papain and pepsin. (2) From the substrate specificity, *Ps* enzyme seems to be a collagenase clearly differentiable from trypsin, papain and pepsin. (3) The N-terminal residues released from

the enzymatic-hydrolysate of gelatin, show that *Ps* enzyme is similar to papain and not to trypsin and pepsin.

From these three points, it may be concluded that *Ps* enzyme is a collagenase not resembling to trypsin, papain and pepsin, but is closer to papain than trypsin and pepsin from the mode of action.

SUMMARY

1) Experiment were conducted on the enzymatic action of the crystalline protease obtained from cultures of *Ps. myxogenes* sp., and results obtained are as given below (2)-(4).

2) The optimum pH for liquefaction of gelatin was found to be 7.0-8.5, and the optimum temperature about 45°.

3) The enzyme acted remarkably on gelatin and hide-powder, but slightly on the other proteins, i.e., gluten, haemoglobin, casein, egg-albumin. The substrate-specificity is clearly differentiable from those of trypsin, papain and pepsin. Consequently, the enzyme may be assumed to be a collagenase.

4) From the mode of digestion of gelatin when the N-terminal residue technique was applied, it can be said that *Ps* enzyme is more similar to papain, than trypsin and pepsin.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his constant guidance and encouragement in the course of this work, and also to Mr. E. Masuo of this Laboratory.