BBA 66027

PURIFICATION AND CHARACTERIZATION OF A PROTEINASE IN THE VENOM OF *TRIMERESURUS FLAVOVIRIDIS*

COMPLETE SEPARATION OF THE ENZYME FROM HEMORRHAGIC ACTIVITY

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(Received July 14th, 1969)

SUMMARY

1. A proteinase was purified from the venom of *Trimeresurus flavoviridis*, by gel filtration on Sephadex G-100 and column chromatography on Amberlite CG-50 (Type 2), with a 27% over-all yield and a 9-fold increase in specific activity. This enzyme, named H_2-proteinase, is the major proteinase in the venom.

2. The purified enzyme was completely free from hemorrhagic activity. It was proven that large amounts of this enzyme had contaminated all preparations of Hemorrhagic Principle 2 so far obtained.

3. The purified enzyme was proved to be homogeneous by ultracentrifugation, sucrose-density-gradient centrifugation, electrophoresis on cellulose acetate membrane and from the absence of any of the other enzyme activities detected in the original venom.

4. H_2-proteinase had an $s_{20,w}$ of 2.43 S and a molecular weight of 24 000, as shown by the sedimentation velocity and the approach-to-equilibrium methods. The enzyme was most active at pH 9.5 with casein as substrate and the enzyme activity was abolished completely by addition of Cd^{2+}, Ni^{2+}, EDTA or cysteine.

5. H_2-proteinase split the oxidized B chain of insulin at the three peptide bonds, $-Asn^3-Gln^4-$, $-His^{10}-Leu^{11}-$ and $-Ala^{14}-Leu^{15}-$. Of the synthetic leucine-containing peptides of varying sizes tested, the longest one, Z-Gly–Pro–Leu–Gly–Pro·OH, alone was split by the enzyme at the peptide bond –Pro–Leu–. A certain dependence of the enzymatic hydrolysis on the chain length of the substrate was suggested.

INTRODUCTION

Most crotalid and viperid venoms manifest severe hemorrhage starting at the site of the bite[1-3]. The hemorrhage was ascribed to an action of proteolytic enzyme(s)

Abbreviation: M.H.D., minimum hemorrhagic dose.

in the venom[4]. This hypothesis does not appear to be very convincing, as pointed out by some workers[3,5,6], although it has widely been accepted[7–9]. Several investigators reported purification of "hemorrhagic proteinases" from crotalid venoms, namely, proteinase *b*[10] from Mamushi *(Agkistrodon halys blomhoffi)* venom, and H_α-[11] and H_β-[12] proteinases from Habu *(Trimeresurus flavoviridis)* venom. The reports from this[13] and other[14,15] laboratories indicated, however, the presence of hemorrhagic principles devoid of proteolytic activity.

Earlier work from this laboratory demonstrated the presence of at least two distinct hemorrhagic principles, Hemorrhagic Principle 1 and Hemorrhagic Principle 2, in Habu venom[16]. They were separable from each other by starch column electrophoresis[16], column chromatography on CM-cellulose[13] or gel filtration on Sephadex G-100 (ref. 17). The Hemorrhagic Principle 2 preparations obtained so far were associated consistently with the potent proteolytic activity originally present in the crude venom[2,13,15–17]. MAENO[15] concluded that Hemorrhagic Principle 2 was identical with a proteolytic enzyme ("H_β-proteinase") from the similarity of the proteolytic and hemorrhagic activities in chromatographic behavior, as well as in the susceptibility to inhibitors. We recently attempted further fractionation of the Hemorrhagic Principle 2 preparations by column chromatography on Amberlite CG-50 and finally succeeded in obtaining the major proteolytic enzyme in a pure state, the hemorrhagic activity having been completely eliminated.

The present paper describes the purification of the proteinase from which the hemorrhagic activity (Hemorrhagic Principle 2) was completely eliminated and some general properties of the enzyme including substrate specificity.

The present success in isolating the major proteinase free from the hemorrhagic activity (Hemorrhagic Principle 2) will answer the fundamental question whether the principle responsible for the hemorrhage and the proteolytic enzyme is the same entity. The unique substrate specificity demonstrated with the purified enzyme will serve as a useful tool for analyzing amino acid sequences of certain proteins.

MATERIALS AND METHODS

Snake venom. The venom used was a pool of dried venom (Batch No. 64A) taken from the specimens of Habu *(Trimeresurus flavoviridis)* collected in the Amami Oshima Islands in 1964. The venom was dissolved at a concentration of 10% in 5 mM Tris–HCl buffer (pH 8.5) containing 0.15 M NaCl and the insoluble material removed by centrifugation. The supernatant fluid was subjected to gel filtration on Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) as reported previously[17]. The Hemorrhagic Principle 2 fraction thus separated from Hemorrhagic Principle 1 served as a starting material for purification of the proteinase.

Oxidized B chain of insulin. Crystalline bovine insulin purchased from Sigma Chemical Company, St. Louis, Mo., U.S.A. was oxidized with performic acid according to the method of SANGER[18]. The oxidized B chain was isolated by chromatography on Dowex 50-X4 (The Dow Chemical Company, Midland, Michigan, U.S.A.) according to the method of MYCEK *et al.*[19].

Synthetic substrates. Benzoyl-L-arginineamide, benzoyl-L-tyrosine ethylester, *N*-benzoyl-DL-arginine-β-naphthylamide and glutaryl-L-phenylalanine-*p*-nitroanilide were purchased from Mann Research Lab., Inc., New York, N.Y., U.S.A. Z–Gly–L-

Val·NH_2, Z–Gly–L-Phe·NH_2, Gly–L-Leu·OH, Z–Gly–L-Leu·OH, Z–Gly–L-Leu·NH_2, Z–Gly–L-Pro–L-Leu·OH, Z–Gly–L-Pro–L-Leu–Gly·OH and Z–Gly–L-Pro–L-Leu–Gly–L-Pro·OH were obtained from the Peptide Center, the Institute for Protein Research, Osaka University, Osaka, Japan.

Other chemicals. Casein (Hammarsten) was purchased from E. Merck and Co., Inc., Rahway, N.J., U.S.A. Cellulose acetate membrane, 'Separax', was a commercial product (Fuji Photo Film Co., Ltd., Tokyo, Japan) as was DFP (Sigma Chemical Company, St. Louis, Mo., U.S.A.). All other chemicals were of analytical reagent grade.

Determination of protein. Protein content was estimated either by the method of LOWRY *et al.*[20], with bovine serum albumin as standard, or by measurement of absorbance at 280 nm with a 1-cm cell in a Hitachi type EPU-2A spectrophotometer. A factor of 1.20 was used to convert $A_{280\ nm}$ to mg protein per ml.

Determination of proteolytic activity. (a) Casein method: Proteolytic activity on casein was assayed by the method of KUNITZ[21] with a slight modification. The reaction mixture consisted of 1 ml of a 2% casein solution in 0.2 M Tris–HCl buffer (pH 8.5) and 0.5 ml of an enzyme solution. After incubation at 37° for 10 min, 1.5 ml of 0.44 M trichloroacetic acid were added to the mixture and the resulting precipitate removed by filtration. The filtrate was read spectrophotometrically at 280 nm. 1 unit of the activity was defined as the amount of enzyme liberating acid-soluble materials to the same absorbance as that of 1 μg of L-tyrosine per min under the specified conditions.

(b) Ninhydrin method: The enzyme-catalyzed hydrolysis of synthetic peptide was followed by determining the production of ninhydrin-reactive groups. The reaction mixture contained 0.3 ml each of a 30 mM substrate solution and 0.1 M borax–HCl buffer (pH 8.0), together with 50 μl of an enzyme solution. At appropriate intervals, 0.1-ml portions were taken and each was diluted to 0.5 ml with 0.1 M citrate buffer (pH 5.0) in order to determine ninhydrin-reactive groups, according to the method of YEMM AND COCKING[22].

Determination of activities of other enzymes. Phosphodiesterase (EC 3.1.4.1) was determined according to the method of BJÖRK[23], non-specific alkaline phosphomonoesterase (EC 3.1.3.1) to the method of SULKOWSKI *et al.*[24], 5'-nucleotidase (EC 3.1.3.5) to the method of PRIVAT DE GARILHE AND LASKOWSKI[25] and hyaluronidase (EC 4.2.99.1) to the method of DI FERRANTE[26]. L-Amino acid oxidase (EC 1.4.3.2) was determined, with L-methionine[27] as substrate, by the ninhydrin method; phospholipase A (EC 3.1.1.4) was determined indirectly by measuring the hemolyzing activity on sheep red cells[28].

Determination of hemorrhagic activity. Hemorrhagic activity was determined by the method reported previously[29]. This method consists of an intracutaneous injection of 0.1 ml of a test solution into the depilated back skin of a rabbit, measurement of the size of the hemorrhagic spot after 24 h from the visceral side of the removed skin and estimation of the activity by the parallel-line-assay method. One minimum hemorrhagic dose (M.H.D.) was defined as the amount of venom producing a hemorrhagic spot of 10 mm in diameter under the specified conditions. 0.2 μg of the crude venom used contained hemorrhagic activity corresponding to 1 M.H.D.

Amino acid analysis. Hydrolysis of the peptide (about 10 μmoles) was carried out with 0.5–1.0 ml of 6 M HCl in an evacuated tube for 24 h at 110 ± 1°. The hydrolysate was analyzed automatically in a Yanagimoto amino acid analyzer, model

LC-5. No correction was made for destruction of certain labile amino acids during hydrolysis or for incomplete hydrolysis of peptide bonds.

FDNB-end-group analysis. The terminal amino residue was identified by the FDNB procedure according to SANGER[30]. The dinitrophenyl peptides were hydrolyzed with 6 M HCl for 16 h at 105 ± 1°. The dinitrophenyl amino acids were identified by two-dimensional paper chromatography with the solvent system described by KOCH AND WEIDEL[31].

Determination of molecular weight. Molecular weight was determined by the methods of ARCHIBALD[32] and of YPHANTIS[33] in 10 mM Tris–HCl (pH 8.5, $I = 0.1$) at 20°, based on the assumption that the partial specific volume of the enzyme was 0.75.

RESULTS

Purification of proteinase

The crude preparation of Hemorrhagic Principle 2 obtained by gel filtration on Sephadex G-100 was subjected to column chromatography on Amberlite CG-50 (Type 2) with the results shown in Fig. 1. Both the proteolytic and hemorrhagic activities were substantially separated, although the hemorrhagic fraction (Hemorrhagic Principle 2) still contained a small amount of proteolytic activity. The main peak of proteinase, designated as H_2-proteinase, was completely devoid of hemorrhagic activity. Rechromatography of this peak (Fractions 148–165 in Fig. 1) on Amberlite CG-50 (Type 2) under the same conditions yielded a symmetrical protein peak

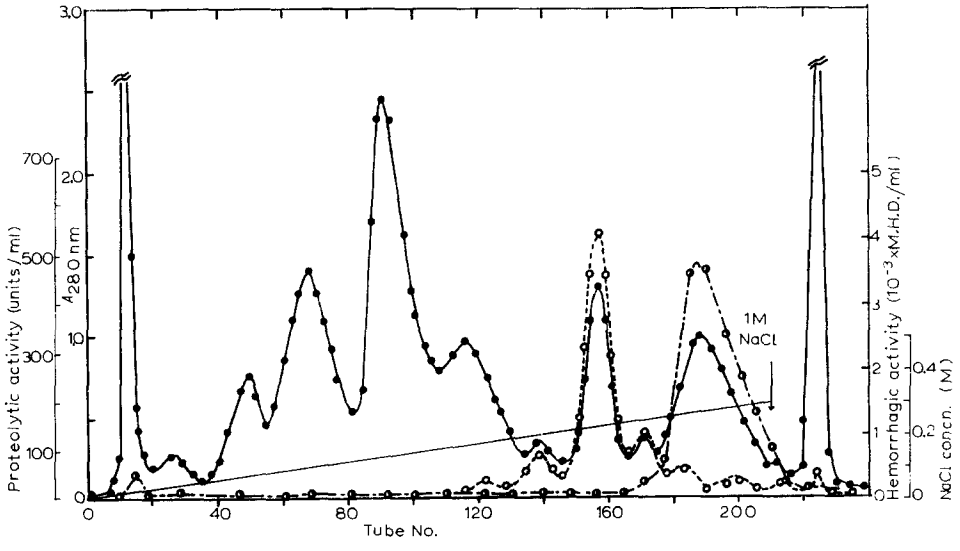


Fig. 1. Chromatography of crude Hemorrhagic Principle 2 on Amberlite CG-50. The crude Hemorrhagic Principle 2 fraction obtained by gel filtration on Sephadex G-100 of 2 g of crude venom was dialyzed against 5 mM borax–HCl buffer (pH 8.3) containing 2 mM Ca^{2+}. After the precipitate had been removed by centrifugation, the dialysis residue (15 ml) containing 1.4 g protein was applied to a column (2.2 cm × 39 cm) of Amberlite CG-50 (Type 2) equilibrated with the same buffer. Linear gradient elution was carried out with 1.1 l of the buffer in the mixing vessel and the same volume of 0.3 M NaCl in the buffer in the reservoir. 10-ml fractions were collected. The flow rate was 20–30 ml/h. ●—●, $A_{280\,nm}$; ○ · · · ○, proteolytic activity; ◑— – —◑, hemorrhagic activity; – – –, NaCl concentration.

TABLE I

SUMMARY OF THE PURIFICATION OF H_2-PROTEINASE

Step	*Protein**		*Activity*		*Specific activity*
	Total (mg)	*Recovery (%)*	*Total (10^{-5} × units)*	*Recovery (%)*	*(10^{-2} × units/mg protein)*
Crude venom	3000	100	1.63	100	0.54
Sephadex G-100	2280	76	1.37	84	0.60
Dialysis	1746	58	1.29	79	0.74
Amberlite CG-50 (1st)	101	3.7	0.49	30	4.80
Amberlite CG-50 (2nd)	89	3.0	0.45	27	5.03

* Estimated by the method of LOWRY *et al.*[20].

throughout which a constant specific activity was obtained. The purification procedure and the yield at each step are summarized in Table I. An approx. 9-fold increase in specific activity was attained, with an over-all yield of 27%.

Relationship between H_2-proteinase and "H_β-proteinase"

MAENO[15] claimed that Hemorrhagic Principle 2 was identical with a proteolytic enzyme ("H_β-proteinase") from the similarity of the proteolytic and hemorrhagic activities in chromatographic behavior as well as in the susceptibility to inhibitors. We examined, therefore, the relationship between H_2-proteinase and "H_β-proteinase". "H_β-proteinase" was prepared according to the method of MAENO *et al.*[12], which consisted of fractionation with $(NH_4)_2SO_4$ (0.55–0.75 saturation) and of chromatography on Amberlite CG-50 (Type 1) with stepwise elution. The resulting preparation

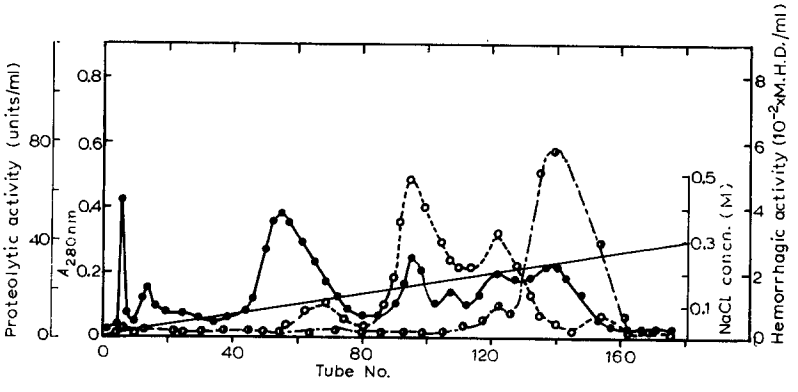


Fig. 2. Chromatography of "H_β-proteinase" on Amberlite CG-50. "H_β-proteinase" was prepared from 0.5 g of Habu venom according to the method of MAENO *et al.*[12]. The preparation was dialyzed against 5 mM borax–HCl buffer (pH 8.3) containing 2 mM Ca^{2+}. The precipitate formed was removed by centrifugation. The dialysis residue (10 ml) was applied to a column (1.3 cm × 35 cm) of Amberlite CG-50 (Type 2) equilibrated with the same buffer. Linear gradient elution was carried out with 400 ml of the buffer in the mixing vessel and an equal volume of 0.3 M NaCl in the buffer in the reservoir. 4.5-ml fractions were collected. ●—●, $A_{280\,nm}$; ○— — —○, proteolytic activity; ◑— - —◑, hemorrhagic acitvity; ———, concentration of NaCl.

was subjected to chromatography on the Amberlite CG-50 (Type 2) of our system and was further resolved into at least three peaks of proteinase (Fig. 2). The main peak alone, being completely devoid of hemorrhagic activity (Hemorrhagic Principle 2), coincided with H_2-proteinase with respect to elution volume. The other minor proteinases emerging subsequently were being contaminated with hemorrhagic activity (Hemorrhagic Principle 2). Thus "H_β-proteinase" was a mixture of H_2-proteinase, Hemorrhagic Principle 2 and some other minor proteinases.

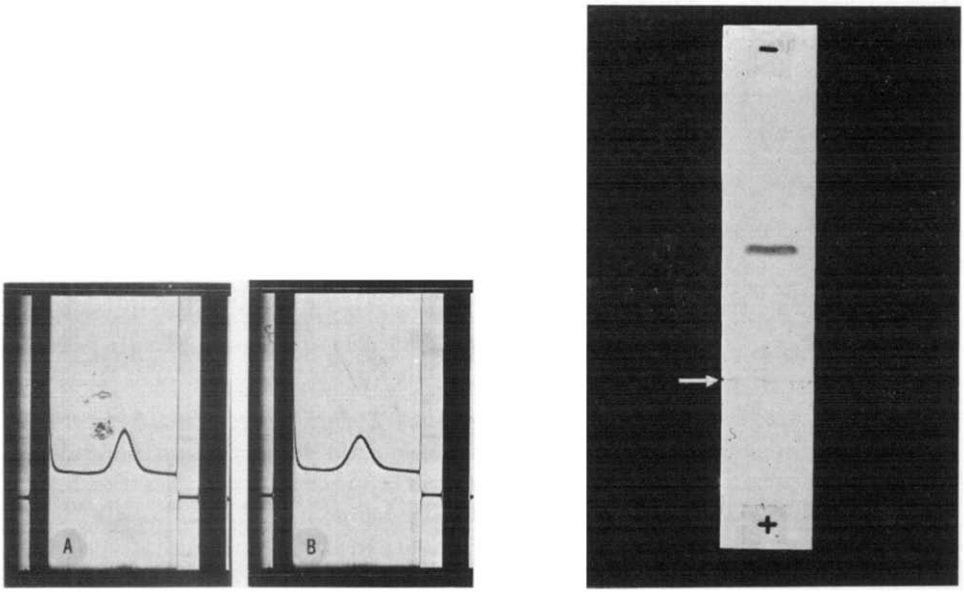


Fig. 3. Ultracentrifugal patterns of the purified H_2-proteinase. A 0.7% solution of H_2-proteinase in 5 mM Tris–HCl buffer (pH 8.5) containing 0.1 M NaCl was centrifuged in a Spinco Model E ultracentrifuge at 59 780 rev./min and 20°. Photographs A and B were taken 16 and 32 min, respectively, after the maximum speed had been attained.

Fig. 4. Electrophoresis of the purified H_2-proteinase on 'Separax'. The electrophoretic run on a piece (11 cm × 3 cm) of 'Separax' was conducted in 5 mM borax–NaOH buffer (pH 9.5, I = 0.1) at 4° for 1 h with a constant current of 3.5 mA and a potential drop of about 150 V. At the end of the run, the protein band was located by staining with a 0.1% alcoholic solution of bromophenol blue saturated with $HgCl_2$. The arrow indicates the place where the sample was applied.

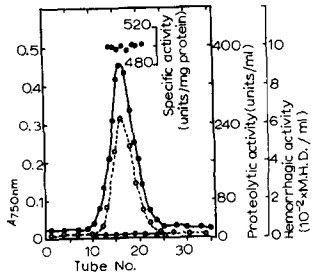
Homogeneity of the purified enzyme

The purified H_2-proteinase in 5 mM Tris–HCl buffer (pH 8.5) containing 0.1 M NaCl was subjected to ultracentrifugation at protein concentrations of 0.7% and 1.0%; only a single symmetrical boundary was observed (Fig. 3).

Upon electrophoresis on 'Separax' (cellulose acetate membrane) at different pH values from 7.0 to 12.0 and at I = 0.1, the enzyme migrated towards the cathode in a single band. A typical electrophoretic pattern at pH 9.5 is given in Fig. 4.

Centrifugation of the proteinase in a density gradient of sucrose yielded a single protein peak; a constant specific activity was observed throughout the peak (Fig. 5).

In the purified preparation of the enzyme were detected none of the other enzymes contained in crude venom, such as non-specific phosphomonoesterase,



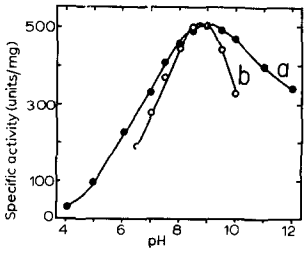


Fig. 5. Sucrose-density-gradient centrifugation of the purified H_2-proteinase. 0.2 ml of a sample solution (4.8 mg/ml) was layered on top of a linear sucrose gradient (3–15%, w/v) in 5 mM Tris–Tris–HCl buffer (pH 8.5) of 4.8 ml in a 5-ml tube. The tube was centrifuged at 39 000 rev./min for 19 h in a Hitachi SW-39 swinging-bucket rotor. The tube was then punctured with a hypodermic needle and 12-drop fractions were collected. ●—●, $A_{750\ nm}$ (Folin); ○- - - -○, proteolytic activity; ◑—◑, hemorrhagic activity.

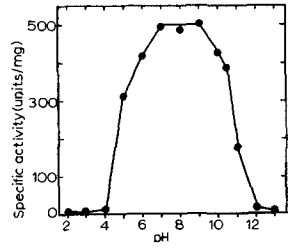
Fig. 6. pH-activity curve of H_2-proteinase. The enzymatic activity was determined with casein and Z–Gly–Pro–Leu–Gly–Pro·OH as substrates. The following buffers were used at a final concentration of 0.01 M; the final ionic strength was adjusted to 0.1 by the addition of NaCl. Denatured casein was dissolved in acetate buffer (pH 4.0–6.5), Tris–HCl buffer (pH 7.0–9.5) or borax–NaOH buffer (pH 10.0–12.0); the synthetic peptide was dissolved in phosphate buffer (pH 6.0–7.0), borax–HCl buffer (pH 7.5–9.0) or borax–NaOH buffer (pH 9.5–11.0). Curve a, with casein as substrate; Curve b, with Z–Gly–Pro–Leu–Gly–Pro·OH as substrate.

phosphodiesterase, 5′-nucleotidase, L-amino acid oxidase, hyaluronidase and phospholipase A.

Properties of the purified proteinase

The ultracentrifugal analysis of the enzyme (Fig. 3) yielded a sedimentation coefficient ($s_{20,w}$) of 2.43 S. Molecular weight determinations according to the methods of ARCHIBALD[32] and YPHANTIS[33] gave values of 23 600 and 24 400, respectively, a mean value being 24 000.

The casein-digesting activity of H_2-proteinase was determined at 37° as a function of pH. As illustrated in Fig. 6, digestion occurred in the alkaline region with an optimum pH of around 9.0 (see Curve a). The proteinase was stable between



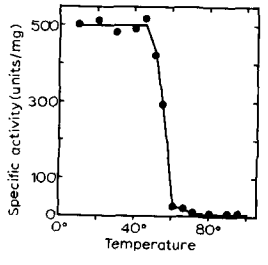
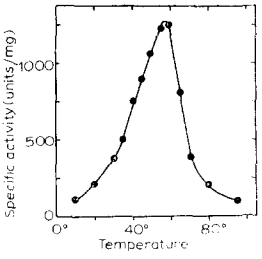


Fig. 7. pH-stability of H_2-proteinase. Proteinase solution (0.3 mg/ml) was incubated at varying pH values and at I = 0.1 and 37° for 1 h, and then the enzymatic activity on casein was determined as described in the text. The buffers used were the same as those used for obtaining the Curve a in Fig. 6. The enzyme solution was adjusted to pH 2 or 13 with a dilute solution of either HCl or NaOH.

Fig. 8. Heat stability of H_2-proteinase. The enzyme at 0.3 mg protein/ml of 5 mM Tris–HCl buffer (pH 8.5, I = 0.1) was incubated for 5 min at varying temperatures and then quickly cooled to room temperature. The enzymatic activity on casein was determined as described in the text.



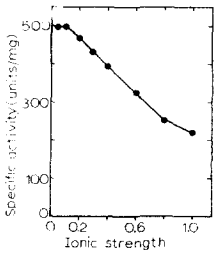


Fig. 9. The enzymatic activity as a function of temperature. The enzymatic activity on casein was determined as described in the text, with a slight modification; 1.5 ml of the substrate solution, after pre-incubation for 15 min, were mixed with 30–100 μl of an enzyme solution (0.6 mg/ml) in order to allow the reaction to proceed at an indicated temperature for 5 min. In enzyme-less controls performed at 35, 60 and 95°, no appreciable degradation of the substrate was observed.

Fig. 10. The enzymatic activity as a function of ionic strength. The substrate solution used was 2% casein in 0.01 M Tris–HCl buffer (pH 8.5) added with an appropriate amount of NaCl required to attain the indicated ionic strength. The enzymatic activity was determined as described in the text.

pH 7.0 and 9.0, as illustrated in Fig. 7. Incubation of the enzyme at pH values below 6.0 or above 10.0 resulted in an abrupt decrease in the enzymatic activity. Heat treatment of the enzyme in 5 mM Tris–HCl buffer (pH 8.5, $I = 0.1$) for 5 min at varying temperatures indicated that the enzyme retained its full activity up to 45°, but lost the activity almost completely above 60° (Fig. 8). Figs. 9 and 10 show that the enzyme is most active at a temperature around 60° and at an ionic strength between 0.05 and 0.10.

TABLE II

EFFECTS OF SOME BIVALENT CATIONS AND GROUP-SPECIFIC REAGENTS ON THE ENZYMATIC ACTIVITY

H_2-proteinase was dialyzed thoroughly against 0.1 M *N*-methylmorpholine–HCl buffer (pH 7.9) at 4°. Each addition at a final concentration of 0.2 mM or 2 mM was mixed with the enzyme solution (finally 0.1 mg protein/ml). The mixture, after kept standing at 37° for 10 min, was assayed for the enzymatic activity on casein. All the bivalent cations tested were of chloride form.

Addition	*Relative activity (%)*
None	100
Ca^{2+}	102
Ba^{2+}	100
Mg^{2+}	90
Mn^{2+}	96
Co^{2+}	90
Zn^{2+}*	62
Cu^{2+}*	29
Hg^{2+}*	24
Cd^{2+}*	0
Ni^{2+}*	0
EDTA*	0
Cysteine*	0
p-Chloromercuribenzoate	76
Monoiodoacetic acid	95
DFP	102

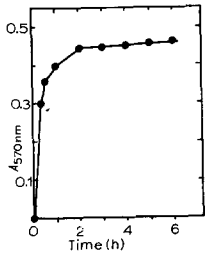
* These additions were tested at 0.2 mM; the others at 2 mM.

The effects of some bivalent cations and group-specific reagents on the enzymatic activity are summarized in Table II. The presence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, monoiodoacetic acid and DFP, each at a concentration up to 2 mM, had no effect on the activity. A significant degree of inhibition (24%) was observed with *p*-chloromercuribenzoate at a concentration of 2 mM. Hg^{2+}, Cu^{2+} and Zn^{2+}, each at a concentration of 0.2 mM, impaired the activity to an extent of 76%, 71% and 38%, respectively. The enzymatic activity of H_2-proteinase was lost completely in the presence of 0.2 mM of Cd^{2+}, Ni^{2+}, EDTA or cysteine; this inactivation was restored neither by dialysis against 5 mM Tris–HCl buffer (pH 8.5) containing 2 mM Ca^{2+} nor by addition of any other metallic ions.

The substrate specificity

The following synthetic compounds were examined for susceptibility to H_2-proteinase: benzoyl-L-tyrosine ethylester and glutaryl-L-phenylalanine-*p*-nitroanilide, both known as typical substrates for chymotrypsin (EC 3.4.4.5), *N*-benzoyl-DL-arginine-β-naphthylamide and benzoyl-L-arginineamide, known as typical substrates for trypsin (EC 3.4.4.4), and two dipeptides, Z–Gly–Val·NH_2* and Z–Gly–Phe·NH_2. None of the compounds tested was hydrolyzed by H_2-proteinase. As a consequence of these negative results, the effect of H_2-proteinase on long chain peptides of known sequence was then investigated.

(a) Digestion of the oxidized B chain of insulin. The oxidized B chain of bovine insulin was susceptible to the enzyme (Fig. 11); the ninhydrin color value of the incubation mixture reached a plateau after 2 h, where the value was about 2.5 times



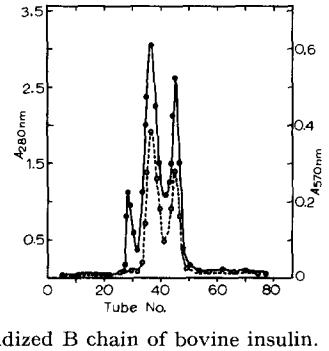


Fig. 11. Time-course of hydrolysis with H_2-proteinase of the oxidized B chain of bovine insulin. The reaction mixture contained 5 mg of the oxidized B chain of bovine insulin and 0.25 mg of the enzyme in 5 ml of 0.1 M borax–HCl buffer (pH 8.0). The reaction was allowed to proceed at 37°. At appropriate intervals, 0.1-ml portions were withdrawn for analysis for the ninhydrin value; the value of the enzyme-less control, being approx. 0.20, was subtracted.

Fig. 12. Preliminary fractionation on Sephadex G-25 of the oxidized B chain of bovine insulin digested with H_2-proteinase. 100 mg of the oxidized B chain were incubated with 5 mg of the enzyme in 5 ml of 0.1 M borax–HCl buffer (pH 8.0) at 37°. After 2 h, 3 ml of acetic acid were added in order to terminate the reaction. The digest was applied to a column (0.9 cm × 90 cm) of Sephadex G-25 equilibrated with a 50% acetic acid solution; elution was carried out with the same solution at 4°, 2-ml fractions being collected. The flow rate was 7 ml/h. The ninhydrin determination was made with 0.1-ml portions of every other fraction according to the method of YEMM AND COCKING[22]. ●—●, $A_{280\ nm}$; ○— — —○, $A_{570\ nm}$.

* It was reported[34] that among the three peptide bonds in the B chain of insulin cleaved by "H_β-proteinase" from Habu venom, two contained the amino group donated by valine.

TABLE III

AMINO ACID COMPOSITIONS OF THE PEPTIDES ISOLATED FROM H_2-PROTEINASE-DIGEST OF THE OXIDIZED B CHAIN OF BOVINE INSULIN

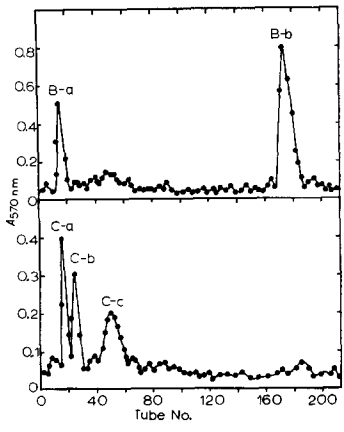
The oxidized B chain of insulin was digested with the enzyme as described in the legend to Fig. 12. The isolated peptides were hydrolyzed with 6 M HCl at 110° for 24 h. The hydrolysates were analyzed in a Yanagimoto model LC-5 amino acid analyzer. The figure in parentheses represents the theoretical number of amino acid residues in a given peptide. The names of the peptides correspond to the peak numbers in Fig. 13.

Amino acid	*Peptide*				
	B-a	*B-b*	*C-a*	*C-b*	*C-c*
Lys		0.71 (1)			
His	2.31 (2)		2.10 (2)		
Arg		1.08 (1)			
Cysteic acid	1.42 (1)	1.00 (1)	1.03 (1)		
Asp					1.32 (1)
Thr		0.94 (1)			
Ser	1.18 (1)		0.66 (1)		
Glu	1.93 (2)	1.04 (1)	1.13 (1)	1.25 (1)	
Pro		1.24 (1)			
Gly	1.41 (1)	2.09 (2)	1.0 (1)		
Ala	0.89 (1)	0.99 (1)		0.93 (1)	
Val	0.77 (1)	0.95 (1)		1.0 (1)	1.29 (1)
Ile					
Leu	2.00 (2)	2.13 (2)	1.04 (1)	1.22 (1)	
Tyr		1.76 (2)			
Phe		2.02 (2)			1.13 (1)
Relative yield*	58	88	18	22	100
Residues	4–14	15–30	4–10	11–14	1–3

* The yield of Peptide C-c was taken as 100.

as high as that at time zero. For identification of the peptide bonds to be hydrolyzed by the enzyme, 100 mg of the oxidized B chain was treated with 5 mg of the enzyme in 5 ml of 0.1 M borax–HCl buffer (pH 8.0) at 37° for 2 h. Fractionation of the digest through a column of Sephadex G-25 yielded three peaks which were in turn designated Peaks A, B and C (Fig. 12). Peak A contained the enzyme. The peptides in Peaks B and C were further fractionated on a column of Dowex 50-X4 according to the method of GERWIN *et al.*[35]. The eluate, after alkaline hydrolysis[36], was analyzed for ninhydrin reactivity with the results shown in Fig. 13. The fractions included in Peaks B-a, B-b, C-a, C-b and C-c were pooled separately. Upon paper chromatography with the solvent system, *n*-butanol–pyridine–acetic acid–water (30:20:6:24, by vol.)[37], each of the pooled fractions yielded a single ninhydrin-positive spot. The amino acid compositions of the isolated peptides are summarized in Table III. The relative yields of the individual peptides were: Phe^{1}–Asn^{3} (100%), Gln^{4}–His^{10} (18%), Leu^{11}–Ala^{14} (22%), Gln^{4}–Ala^{14} (58%) and Leu^{15}–Ala^{30} (88%). The extent of hydrolysis of the bond –His^{10}–Leu^{11}– was, therefore, estimated to be about one-fourth that of the bond, –Asn^{3}–Gln^{4}– or –Ala^{14}–Leu^{15}–.

These results indicate that H_2-proteinase split the oxidized B chain of bovine insulin at the peptide bonds –Asn^{3}–Gln^{4}–, –His^{10}–Leu^{11}– and –Ala^{14}–Leu^{15}– (Fig. 14) and that the peptide bonds –Asn^{3}–Gln^{4}– and –Ala^{14}–Leu^{15}– were cleaved faster than the bond –His^{10}–Leu^{11}–.



Phe-Val-Asn-Gln-His-Leu-CysO_3H-Gly-Ser-His-Leu-Val-
Glu-Ala-Leu-Tyr-Leu-Val-CysO_3H-Gly-Glu-Arg-Gly-Phe-
Phe-Tyr-Thr-Pro-Lys-Ala

Fig. 13. Chromatography on Dowex 50 of the materials from Peaks B and C in Fig. 12. (a) The material recovered from Peak B in Fig. 12 was applied to a column (0.9 cm × 30 cm) of Dowex 50-X4 equilibrated with pyridinium formate buffer (pH 3.25). Elution was initiated with the same buffer. After 20-ml effluent had been collected, gradient elution was started by allowing 4.8 M pyridinium acetate buffer (pH 5.3) to flow into the mixing chamber containing 500 ml of the starting buffer. 2-ml fractions were collected. The ninhydrin determination was made with 0.2-ml portions of every other fraction after alkaline hydrolysis. (b) The material recovered from Peak C in Fig. 12 was subjected to chromatography on Dowex 50-X4 under the same conditions as in (a).

Fig. 14. The peptide bonds in the oxidized B chain of bovine insulin cleaved by H_2-proteinase. Arrows indicate the sites of cleavage.

(b) Hydrolysis of leucine-containing synthetic peptides. In view of the fact that H_2-proteinase hydrolyzed the oxidized B chain of insulin at three peptide bonds, of which two contained the terminal amino function contributed by leucine, the following leucine-containing peptides of varying sizes were examined for their susceptibility to the enzyme: Gly–Leu·OH, Z–Gly–Leu·OH, Z–Gly–Leu·NH_2, Z–Gly–Pro–Leu·OH, Z–Gly–Pro–Leu–Gly·OH and Z–Gly–Pro–Leu–Gly–Pro·OH. The reaction mixture contained 0.4 ml of each peptide solution, containing about 5 mmoles in 0.1 M borax–HCl buffer (pH 8.0) and 30 μl of an enzyme solution (7.5 mg/ml). After incubation at 37° for 0, 24, 48 and 72 h, 50-μl portions were withdrawn for analysis by one-dimensional paper chromatography with *n*-butanol–pyridine–acetic acid–water (30:20:6:24, by vol.) as solvent[37]. Up to the incubation period of 72 h, none of

TABLE IV

KINETIC PARAMETERS OF H_2-PROTEINASE

The values for K_m, v_{max} and K_i at pH 8.0 and 37° were estimated from Fig. 15. The value of v_{max} is expressed in μmoles leucine equivalent/l per h per mg of enzyme.

Leucine-containing peptide	v_{max}	K_m $(10^{-1} \times mM)$	K_i $(10^{-1} \times mM)$
Z–Gly–Leu·OH	—	—	0.42
Z–Gly–Leu·NH_2	—	—	5.50
Z–Gly–Pro–Leu·OH	—	—	1.80
Z–Gly–Pro–Leu–Gly·OH	—	—	0.66
Z–Gly–Pro–Leu–Gly–Pro·OH	0.96	2.20	—

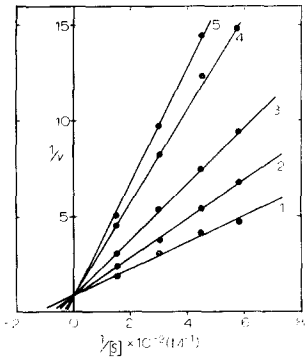


Fig. 15. LINEWEAVER–BURK[38] plot of data on hydrolysis of Z–Gly–Pro–Leu–Gly–Pro·OH with H_2-proteinase, with or without leucine-containing peptide inhibitors. The enzymatic activity on Z–Gly–Pro–Leu–Gly–Pro·OH in 0.1 M borax–HCl buffer (pH 8.0) was determined with or without the inhibitors at 37° by the ninhydrin method. Substrate concentration was changed from 1.7 to 6.8 mM. Enzyme concentration was 0.2 mg/ml. Curve 1, with no inhibitor; Curve 2, with Z–Gly–Leu·NH_2 (15 mM); Curve 3, with Z–Gly–Pro–Leu·OH (15 mM); Curve 4, with Z–Gly–Leu·OH (7.6 mM); and Curve 5, with Z–Gly–Pro–Leu–Gly·OH (15 mM).

the peptides Gly–Leu·OH, Z–Gly–Leu·OH, Z–Gly–Leu·NH_2, Z–Gly–Pro–Leu·OH or Z–Gly–Pro–Leu–Gly·OH yielded new ninhydrin-positive spots. The peptide of the longest size, Z–Gly–Pro–Leu–Gly–Pro·OH, alone was hydrolyzed with H_2-proteinase. FDNB-end-group analysis revealed that cleavage occurred at the peptide bond –Pro–Leu–. As shown in Fig. 6, the pH-activity curve of the enzyme was steeper with Z–Gly–Pro–Leu–Gly–Pro·OH than with casein; the pH optimum with the peptide as substrate was found to be between 8.5 and 9.0 (see Curve b).

The values for K_m and v_{max} at pH 8.0 (0.1 M borax–HCl buffer) and 37° were estimated from the LINEWEAVER–BURK[38] plot shown in Fig. 15. Under the specified conditions, the value of K_m was 0.022 M and that of v_{max} 0.96 μM of leucine equivalent per h per mg of enzyme. Hydrolysis of Z–Gly–Pro–Leu–Gly–Pro·OH with H_2-proteinase was significantly inhibited by the presence of any of the other leucine-containing peptides, with the exception of Gly–Leu·OH. The latter peptide was not tested, since it had given a high ninhydrin value by itself which made evaluation of the inhibitory effect, if any, impossible by the ninhydrin method. The inhibition was competitive between Z–Gly–Pro–Leu–Gly–Pro·OH and any other peptide tested; the extent of inhibition was increasing in the following order: Z–Gly–Leu·NH_2 < Z–Gly–Pro–Leu·OH < Z–Gly–Pro–Leu–Gly·OH < Z–Gly–Leu·OH. The values for K_i, K_m and v_{max} are summarized in Table V.

DISCUSSION

Different lines of evidence have accumulated to show that the venom from a variety of snakes contains more than one proteolytic enzyme[39–45]. Attempts have been made to purify proteinases from snake venoms[11,12,46–50] but only a few have succeeded in obtaining proteinase in a physico-chemically homogeneous state[51–53].

In the present work, the main proteinase of Habu venom was purified by chromatography on Amberlite CG-50 to a physico-chemically homogeneous state.

This enzyme, named H_2-proteinase, was completely free from hemorrhagic activity and it was proven that large amounts of this enzyme had always contaminated all the Hemorrhagic Principle 2 preparations so far obtained[2,13,15–17].

We demonstrated that the "H_β-proteinase" of MAENO[12] was a mixture of H_2-proteinase and Hemorrhagic Principle 2 (Fig. 2), although this author had claimed that "H_β-proteinase" and Hemorrhagic Principle 2 were an identical entity[15].

The Hemorrhagic Principle 2 fraction separated from H_2-proteinase by chromatography on Amberlite CG-50 (Figs. 1 and 2) still contained a little proteolytic activity. This was completely eliminated by chromatography on Bio-Rex 70 (unpublished work). Thus Hemorrhagic Principle 2 and proteinases (caseinolytic) are distinct entities.

The purified H_2-proteinase was demonstrated to be homogeneous by ultracentrifugation, sucrose-density-gradient centrifugation, electrophoresis on cellulose acetate membrane and also by the absence of any of the other enzyme activities detected in the original venom. The enzyme had a sedimentation coefficient ($s_{20,w}$) of 2.43 S and a molecular weight of 24 000 as determined by the methods of ARCHIBALD[32] and YPHANTIS[33]. It was most active at pH 9.0 with casein as substrate and was stable at temperatures below 45° and at pH values between 7.0 and 9.0. The enzyme activity was lost completely by addition of Cd^{2+}, Ni^{2+}, EDTA or cysteine.

The enzyme split the oxidized B chain of insulin at three peptide bonds, namely, –Asn^3–Gln^4–, –His^{10}–Leu^{11}– and –Ala^{14}–Leu^{15}–. Among synthetic leucine-containing peptides of varying sizes such as Gly–Leu·OH, Z-Gly–Leu·OH, Z-Gly–Leu·NH_2, Z–Gly–Pro–Leu·OH, Z–Gly–Pro–Leu–Gly·OH and Z–Gly–Pro–Leu–Gly–Pro·OH, the longest one alone was split by the enzyme at the peptide bond –Pro–Leu–. Thus H_2-proteinase cleaves at least such peptide bonds in which amino groups are donated by leucine. The inability of the enzyme to split the shorter synthetic peptides, namely, those shorter than hexamer*, however, puts forward evidence for a certain dependence of the enzymatic hydrolysis on the chain length of the substrate. The failure of the enzyme to cleave the two leucine bonds, –His^5–Leu^6– and –Tyr^{16}–Leu^{17}–, in the oxidized B chain may be interpreted as indicating the inability of the enzyme to hydrolyze the peptide unless the fragments produced are of the size of a trimer or larger peptides.

A similar dependence of enzymatic hydrolysis on chain length of peptide was postulated with *Crotalus atrox* α-protease[54,55] which, possessing the specificity toward the peptide bond in which the amino group is donated by leucine, valine, isoleucine or alanine, did not cleave such bonds contained in peptides shorter than a hexamer.

Since no other proteolytic enzymes having such dependence on chain length similar to H_2-proteinase or α-protease have been reported, the purified H_2-proteinase will be a useful tool for analyzing the amino acid sequences of certain proteins.

ACKNOWLEDGMENTS

This investigation was supported partly by World Health Organization Grant No. R/00126 and partly by a grant from the Scientific Research Fund of the Ministry of Education of Japan, for which grants the authors wish to express thanks.

* The blocking group (Z) of N-terminal was counted as one amino acid residue with respect to size.

We wish to express our gratitude to the Division of Public Health, Kagoshima Prefecture, Japan, for their generous gifts of Habu venom. We are also grateful to Dr. T. Nakajima, the University of Tokyo for performing the amino acid analyses and to Mr. K. Suzuki of this Institute for carrying out the ultracentrifugal analyses.

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