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LEUCOSTOMA PEPTIDASE A: A METALLOPROTEASE FROM SNAKE VENOM

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SUMMARY

The substrate specificity and metal ion content of *leucostoma* peptidase A from the venom of *Agkistrodon piscivorus leucostoma* were investigated. The enzyme was found to contain calcium and zinc in a 2:1 ratio, and on the basis of a mol. wt of 22 500, it appeared that these values represented 2 gatoms of calcium and 1 gatom of zinc per mole. Activity was inhibited by exposure of the enzyme to *o*-phenanthroline in the presence of excess calcium, but the activity of enzyme treated in this manner was restored by the addition of zinc ions. Exposure to EDTA, however, removed both zinc and calcium and resulted in irreversible inactivation.

Leucostoma peptidase A hydrolyzed several N-substituted or doubly substituded dipeptides in which an aromatic or a basic residue contributed the amino group. It hydrolyzed four of nine polyamino acids tested, and cleaved the B-chain of oxidized insulin at positions Phe^{1}–Val^{2}, His^{5}–Leu^{6}, His^{10}–Leu^{11}, Ala^{14}–Leu^{15}, Gly^{20}–Glu^{21}, Gly^{23}–Phe^{24}, and Phe^{24}–Phe^{25}.

These data indicate that the properties of *leucostoma* peptidase A are those of the general class known as neutral proteases, examples of which have previously been reported only in microorganisms.

INTRODUCTION

Neutral proteases constitute one of the best known classes of microbial proteolytic enzymes, but enzymes of this type have not been reported to occur in other sources. These metallo-endopeptidases possess pH optima near neutrality and are inhibited by metal chelating reagents, but are insensitive to DFP[1]. They are not known to possess amidase activity and their substrate specificities are governed by

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the identity of the residue contributing the amino group to the peptide bond; substrates having bulky, hydrophobic residues in this position are the most readily hydrolyzed. These enzymes are produced by various members of the bacterial genera *Bacillus*, *Pseudomonas*, *Aeromonas* and *Serratia*, and by various species of *Streptomyces* and *Aspergillus*[1]. Insofar as we are aware, there is no previous report of such metalloendopeptidases in animals or higher plants, and they have been generally regarded as being restricted to microorganisms.

Earlier experiments in this laboratory[2] resulted in the purification of a protease from the venom of the western cottonmouth moccasin *(Agkistrodon piscivorus leucostoma)*. This enzyme, for which we suggested the trivial name *leucostoma* peptidase A, was shown to be homogeneous by several criteria, and a number of its physical characteristics were described[2]. Our earlier experiments had also shown that *leucostoma* peptidase A possessed none of the esterase activity toward benzoyl-L-arginine ethyl ester or the arylamidase activity toward L-leucyl-β-naphthylamide found in the unfractionated venom. Preliminary efforts to find a susceptible synthetic substrate for the protease were negative and we were therefore prompted to undertake a more thorough investigation of its substrate requirements by examining the action of the enzyme on synthetic substrates, on a large peptide of known sequence, and on homopolymers of amino acids. Also, our previous report of inhibition by EDTA suggested that *leucostoma* peptidase A might be a metalloenzyme, and it appeared appropriate more fully to investigate that possibility. We present in this paper evidence to show that *leucostoma* peptidase A is a metalloenzyme containing zinc and calcium, and that it possesses a substrate specificity and pH optimum that place it in the class of endopeptidases known as neutral proteases.

MATERIALS AND METHODS

Enzyme

In our earlier work[2] on the isolation of *leucostoma* peptidase A, most of the isolation experiments were carried through Step 4, then individual aliquots as needed were either treated with DFP to inactivate the small amount of residual esterase (Step 5A) or were rechromatographed on DEAE-Sephadex at pH 9.6 to accomplish removal of the esterase physically (Step 5B). To demonstrate the effectiveness of the latter procedure in the present work, two complete isolation experiments were conducted and processed through Step 5B, omitting Step 5A. The results of one of these experiments are shown in Table I, which reveals a recovery of 31% of the original proteolytic activity in a product having a specific activity of 7.7 units per mg. Both of these values represent an improvement over those reported earlier[2], and electrophoresis in polyacrylamide gel indicated a product of high homogeneity, comparable in all respects to the enzyme prepared previously[2]. Enzyme from the two preparations made as summarized in Table I was used in all of the experiments reported in this paper except for one of the analyses for metal ion content and an experiment on EDTA inhibition, both of which were done with a preparation from an earlier isolation experiment taken through Step 5A of the original procedure[2]. This preparation was made by DFP treatment of material from Step 4 which had a specific activity of 7.55; it was devoid of both esterase and arylamidase activity determined as described below.

TABLE I

SUMMARY OF THE PURIFICATION OF *Leucostoma* PEPTIDASE A, STEPS 1–5B

Step	*Treatment*	*Total protein (mg)*	*Total enzyme units**			*Specific activity*			*% Recovery protease*
			Protease	*Esterase*	*Aryl-amidase*	*Protease*	*Esterase*	*Aryl-amidase*	
1	Dialysis	2568	3186	5100	3870	1.24	1.99	1.51	100
2	Chromatography (DEAE-Sephadex A-50, pH 8.5)	668	2022	528	429	3.03	0.79	0.64	64
3	Rechromatography (DEAE-Sephadex A-50, pH 9.2)	389	1702	229	132	4.38	0.59	0.34	53
4	Gel filtration (Sephadex G-75)	211	1260	23	0	5.97	0.11	0.0	40
5B	Rechromatography (DEAE-Sephadex A-50, pH 9.6)	128	984	0	0	7.69	0.0	0.0	31

* See text for definition of units.
** Represents the cumulative recovery in the isolation procedure.

Substrates

Substrates were obtained as follows: poly-L-leucine, poly-L-alanine, polyglycine, poly-L-tryptophan, poly-L-glutamic acid, poly-L-lysine, and poly-L-phenylalanine were purchased from Yeda Research and Development Company; poly-L-histidine was purchased from Miles Research Laboratories and crystalline bovine insulin from Boots Pure Drugs. All dipeptide and blocked dipeptide substrates were purchased from either Cyclo Chemical Corporation or Mann Research Laboratories and were tested for purity by thin-layer chromatography. This technique was also used to confirm the identities of the constituent amino acids in substrates that proved susceptible to enzymatic action.

Other materials

Tris and Bicine buffers, purchased from Calbiochem, were used without further purification. Adsorbosil 1, with 5% binder, was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. All other chemicals were reagent or analytical grade obtained from commercial sources.

Enzymatic assays

Assays were run as previously described[2] during the isolation of the enzyme to quantitate the protease, esterase, and arylamidase activities of each fraction. Protease assays were performed with denatured hemoglobin substrate, pH 8.5, incubated at 37 °C. One unit was defined as that amount of enzyme that caused an increase of 1.0 unit in absorbance at 280 nm in 10 min. The esterase activity of the various fractions was measured at 25 °C by the spectrophotometric procedure of Schwert and Takenaka[3] with 1 mM benzoyl-L-arginine ethyl ester in 5 mM Tris–HCl (pH 7.6); one unit was defined as the amount of enzyme that caused an absorbance

increase of 1.0 per min at 253 nm. Arylamidase was determined by the method of Goldbarg and Rutenburg[4] with L-leucyl-β-naphthylamide substrate (2 mg per ml 0.1 M citrate buffer, pH 6.7). One unit was the amount of enzyme that liberated in 30 min sufficient β-naphthylamine to yield a colored product with an absorbance of 1.0 at 560 nm.

Tests on the substrate specificity of the purified protease, devoid of activity toward benzoyl-L-arginine ethyl ester and L-leucyl-β-naphthylamide, were performed by several procedures, depending upon the nature of the substrate. The enzyme-catalyzed hydrolysis of polyamino acids was assessed both by determining the increase in ninhydrin-reactive groups and by potentiometric titration at constant pH. In the former method, the substrate solutions of polyamino acids contained 0.75 to 1.5 mg of substrate per ml in 50 mM Bicine buffer (pH 8.5), and enzyme solutions contained 0.2 mg/ml of *leucostoma* peptidase A. The assays were performed by incubating 1 ml of enzyme and 1 ml of substrate in optically matched tubes for the desired time period, at either 37.5 °C or 25 °C; 1 ml of ninhydrin reagent[5] was then added and the mixture was heated at 100 °C for 15 min. Tubes were read at 570 nm in a Coleman model 6A spectrophotometer to determine the increase in ninhydrin-reactive products resulting from enzymatic hydrolysis. The ability of *leucostoma* peptidase A to hydrolyze polyamino acids was also tested by potentiometric titration in a pH-stat (Radiometer TT1c/SBR2c/SBU1a) equipped with a jacketed titration vessel maintained at 37.5 °C by a circulating water bath. Substrate solutions were prepared by dissolving 3.75 mg of the polyamino acid in 5 ml of CO_2-free deionized water (adjusted to pH 8.5); to this was added 0.5 ml of enzyme solution containing 138 μg of purified *leucostoma* peptidase A. Drying tubes filled with Ascarite were attached to all outlets of the titration vessel to prevent absorption of CO_2 from the atmosphere. Any products of uncatalyzed hydrolysis of the substrates were titrated before addition of the enzyme, and activity was determined by measuring the amount of 10.5 mM NaOH required to maintain the reaction mixture at pH 8.5.

Experiments using dipeptide and N-substituted dipeptide substrates were performed by the ninhydrin procedure described above for polyamino acids. Substrate solutions of the synthetic dipeptides were made in 50 mM Tris–HCl (pH 8.5) to a concentration of either 10 mM or 20 mM when their solubilities permitted; some of the substrates were made to 1 mM, 2 mM, or 5 mM concentrations because of their low solubilities. The concentration of enzyme used in these experiments was 125 μg per ml of the final reaction mixture. The conditions of incubation and the detection of amino groups liberated were carried out by the same procedure used with polyamino acids.

Quantitative tests for esterase and amidase activities of purified *leucostoma* peptidase A were accomplished in the pH-stat at 25 °C with 10 mM solutions of L enantiomorphs and 20 mM concentrations of racemic substrates. The assays were performed by adding 0.2 ml of enzyme (60 μg) to 10 ml of substrate dissolved in CO_2-free deionized water adjusted to pH 8.5; the titriant was 24 mM NaOH. The hydrolysis of N-substituted dipeptides and of poly-L-lysine was investigated by a spectrophotometric assay based upon the decrease in absorbance of the peptide bond at 230 nm, a value selected on the basis of difference spectra of each substrate used. The assays were run in a Gilford Model 222 spectrophotometer equipped with an automatic cuvette positioner and offset control. Double thermospacers on the

spectrophotometer were connected to a water bath set to maintain a temperature of 25 °C inside the cuvette compartment. The enzyme sample (0.1 ml) was injected from a Hamilton microsyringe into a cuvette containing 2.9 ml of temperature-equilibrated substrate (pH 8.5) and mixed immediately with a plastic stirring rod. The sample was read against a blank containing the same molar concentrations of products that would have resulted from complete hydrolysis of the substrate.

Digestion of B-chain of oxidized insulin

A 140-mg sample of freeze-dried B-chain of oxidized insulin, prepared by the method of Griffin, Wagner and Prescott[6], was dissolved in 20 ml of 5 mM Tris–HCl buffer, pH 8.5, and to it was added 1.0 mg of *leucostoma* peptidase A in a volume of 1.5 ml. The mixture was incubated at 37 °C under nitrogen for 5.5 h, the pH being periodically readjusted to 8.5 with 10 mM NaOH. The reaction was terminated by the addition of glacial acetic acid and the hydrolytic products were stored at 4 °C. The peptides resulting from the enzymatic action were separated by high-voltage electrophoresis, eluted and analyzed as follows.

Two-dimensional high-voltage electrophoresis was done in an apparatus similar to that described by Michl[7], using a formic acid–acetic acid buffer (pH 1.9) and a pyridine–formic acid–acetic acid buffer (pH 6.5); Varsol was the coolant. The procedures for electrophoretograms were essentially identical to those described earlier[8]. Electrophoretograms were dipped in a solution of trinitrobenzene sulfonic acid in acetone, then were air dried to reveal the location of the peptides. Each yellow spot was cut from the paper, macerated in 30% acetic acid solution and washed on a fritted glass funnel with approximately seven 10-ml portions of 30% acetic acid. After evaporation of the solutions to dryness, the peptides were dissolved in 1 ml of water, the trinitrophenyl group was removed by the addition of 0.5 ml of 0.5 M NH_4OH, and the samples were again evaporated to dryness. Each sample was dissolved in 1 ml of distilled water and streaked across the center of a 23 cm × 56 cm sheet of Whatman 3MM paper. The paper was dried, then wetted with the pH 4.38 electrolyte (pyridine–acetate–water, 10:20:2500, v/v/v) and electrophoresed for 30 min at 4000 V on a Savant flat plate apparatus. The resulting peptide bands were located by cutting 0.5 cm strips and staining with ninhydrin–cadmium reagent[9]. The peptide bands thus identified were cut from the electrophoretogram and eluted from the paper with the pH 4.38 electrolyte. The solutions were evaporated to dryness in a rotary evaporator, redissolved in 0.5 ml of distilled water, and 0.2-ml samples were removed for amino acid analysis. To each was added 0.2 ml of concentrated HCl, and the sample tubes were evacuated and sealed under 50 μm of pressure according to the procedure of Moore and Stein[10]. Amino acid analyses were run in a Technicon amino acid analyzer on a 130-cm column of Chromobeads B, or on a 75-cm column by the procedure of Ellis and Prescott[11].

Experiments on metal ion content of the enzyme

Precautions as outlined by Thiers[12], including cleansing of the glassware, were used to minimize contamination by adventitious metal ions. Glass-distilled water was further purified by passage through a column of mixed ion-exchange resin (Illcoway research model, Illinois Water Treatment Company). Buffer for metal analysis was prepared by extracting 10 mM heavy metal-free Tris–HCl (pH 9.6) with

0.01% dithizone in carbon tetrachloride; the excess dithizone was removed by extraction with carbon tetrachloride, and the extracted buffer was adjusted to pH 8.5. Dialysis tubing was first soaked in hot deionized water for 20 min, then placed in 5 mM EDTA for 4 h and washed with deionized water. Metal ions were analyzed by atomic absorption spectrophotometry (Techtron Model AA3) using the dialysis buffer as a blank.

Other procedures

In all reactions in which hydrolysis of the substrates was detected, the products were qualitatively identified by one-dimensional thin-layer chromatography on Adsorbosil 1. The solvent system used to develop chromatograms of the products of polyamino acid hydrolysis was 95% ethanol–34% NH_4OH (7:3, v/v) as described by Brenner *et al.*[13]; that used in chromatographing the products of dipeptide hydrolysis was 77% ethanol. The color reagent used was 0.2% ninhydrin in water saturated *n*-butanol. In some instances, it proved necessary to concentrate the products of hydrolysis by perevaporation before they could be identified by thin-layer chromatography.

Protein concentrations were determined by the procedure of Lowry *et al.*[14], with crystalline bovine plasma albumin used as the standard. All measurements of pH were made with a Radiometer TTT1c titrator.

RESULTS

Metal ion content

The participation of metal ions in the action of the venom protease was suggested by the fact that activity was completely inhibited by incubation with EDTA in concentrations as low as 2.3 mM, and by dialysis for 24 h against 0.1 mM EDTA. Analysis of purified *leucostoma* peptidase A for zinc, calcium, magnesium and cobalt by atomic absorption spectrophotometry revealed the presence of only calcium and zinc in stoichiometrically significant amounts. As is shown in Table II, these metal

TABLE II

METAL CONTENT OF *Leucostoma* PEPTIDASE A

All values represent differences between atomic absorption analyses of the enzyme samples and the buffers against which they had been dialyzed. Preparation 1 was made from a batch of Step 4 material (specific activity 7.55) by DFP treatment as we have previously described[2]; Preparations 2 and 3 were from different isolation experiments carried through Step 5B. Preparations 1 and 2 were dialyzed against dithizone-extracted Tris buffer (5 mM; pH 8.0 or 8.5) before analysis; Preparation 3 was dialyzed against 5 mM Tricine buffer, pH 8.5, not treated with dithizone but certified by the manufacturer to be of low metal ion content.

Enzyme preparation	*Calcium*		*Zinc*	
	%	*gatoms per mole*	*%*	*gatoms per mole*
1	0.26	1.44	0.22	0.76
2	0.38	2.13	0.39	1.35
3	0.29	1.60	0.24	0.83
Average	0.31	1.72	0.28	0.98

ions were present in a ratio of approx. 2:1 (calcium:zinc), and calculations based on a molecular weight of 22 500 indicated that the enzyme contains 2 gatoms of calcium and 1 gatom of zinc per mole. The efficacy of EDTA in removing both of these metals was shown by an experiment in which a sample containing 1.44 gatoms of calcium and 0.76 gatoms of zinc per mole was dialyzed for 24 h against 0.1 mM EDTA. This treatment reduced the content of calcium by more than 70%, the zinc content by approx. 90%, and diminished enzymatic activity below the point of quantitative detection.

From analogy with the neutral proteases of microorganisms, it appeared that zinc might be the metal ion involved in the enzymatic activity of the venom protease and that the role of calcium might be to stabilize the protein's structure. In order to test this possibility, a sample of *leucostoma* peptidase A was dialyzed for 24 h against 10 mM Tricine buffer (pH 8.0) containing 5 mM *o*-phenanthroline and 10 mM calcium acetate, conditions which provided for an excess of Ca^{2+} for stabilization of the enzyme and an excess of chelating agent for removal of zinc from the protease[15,16]. When assayed with homoglobin substrate, the sample thus treated showed only 16% of the activity in the original sample, but the addition of 5 mM $ZnCl_2$ restored proteolytic activity to an extent of 87% of the original value.

Action of the protease on small synthetic substrates

The purified enzyme failed to hydrolyze such unsubstituted dipeptides as Gly–Tyr-OH and Leu–Tyr-OH, and preliminary experiments using less highly purified enzyme had previously shown no activity toward such exopeptidase substrates as Leu-NH_2, Lys-NH_2, Arg-OMe, Lys-OMe, Phe-OEt and Tyr-OEt. The venom protease thus appeared to be a true endopeptidase and the evidence suggested that the minimal substrates susceptible to its action were dipeptides, amides or esters having substituted NH_2-terminal residues. Consequently, 33 compounds consisting of N-substituted dipeptides, doubly-blocked dipeptides, and NH_2-substituted amino acid amides and esters were investigated for their ability to serve as substrates for *leucostoma* peptidase A. The results shown in Table III reveal the hydrolysis of only nine compounds, all of which possessed either a basic or an aromatic residue in the COOH-terminal position. These substrates were then subjected to quantitative assessment of their susceptibility, and the rates of hydrolysis are shown in Table IV, which reveals Z-Gly–Arg-OH to be the most rapidly hydrolyzed.

Polyamino acids as substrates

The ability of various polyamino acids to serve as substrates for the venom protease is shown in Table V, which reveals polylysine to be hydrolyzed at the highest rate of those tested. It is evident that only polymers of aromatic amino acids and of basic amino acids other than histidine were cleaved.

Action on B-chain of oxidized insulin

The action of the venom protease on the B-chain of oxidized insulin is shown in Fig. 1, which also affords a comparison of the points of cleavage of this substrate by three other snake venom proteases. It is apparent that with this large substrate, the enzyme primarily hydrolyzed bonds to which leucine and phenylalanine had contributed the amino group, and despite its activity toward small substrates in

TABLE III

ACTION OF *Leucostoma* PEPTIDASE A ON SUBSTITUTED DIPEPTIDES, AMIDES AND ESTERS

Substrate	*Reaction observed*	*Substrate*	*Reaction observed*
Z-Gly–Gly-OH	–	Z-α-Glu–Tyr-OH	–
Z-Gly–Ala-OH	–	Z-Tyr–Leu-OH	–
Z-Gly–Leu-OH	–	Z-Trp–Ala-OH	–
Z-Gly–Ser-OH	–	Z-Trp–Phe-OH	–
Z-Gly–Met-OH	–	Z-Trp–Tyr-OH	+
Z-Gly–α-Asp-OH	–	Ac-Gly–Lys-OH	+
Z-Gly–α-Glu-OH	–	Ac-Phe–NH_2	–
Z-Gly–Arg-OH	+	Ac-Tyr–NH_2	–
Z-Gly–His-OH	–	Z-Gly–Phe-NH_2	+
Z-Gly–Phe-OH	–	Z-Leu–Leu-NH_2	–
Z-Gly–Tyr-OH	+	Ac-DL-Phe-OEt	–
Z-Gly–Trp-OH	+	Ac-His-OMe	–
Z-Ala–Phe-OH	–	Ac-Gly–Gly-OMe	–
Z-Leu–Phe-OH	–	Z-Gly–Phe-OMe	+
Z-Leu–Tyr-OH	+	Z-Leu–Leu-OMe	–
Z-Leu–Leu-OH	–	Ac-Gly–Lys-OMe	+
Z-α-Glu–Phe-OH	–		

TABLE IV

RELATIVE RATES OF HYDROLYSIS OF SOME SUBSTITUTED DIPEPTIDES BY *Leucostoma* PEPTIDASE A

Substrate	*Concentration (mM)*	*Rate of hydrolysis* ($\mu moles \cdot min^{-1} \cdot mg^{-1}$)*
Z-Gly–Arg-OH	10	2.83
Ac-Gly–Lys-OMe	10	2.59
Ac-Gly–Lys-OH	10	2.41
Z-Gly–Phe-OMe	10	2.39
Z-Gly–Phe-NH_2	1	2.36
Z-Gly–Tyr-OH	5	1.59
Z-Leu–Tyr-OH	10	1.19
Z-Trp–Tyr-OH	5	0.79

* Activities were determined from kinetic plots under initial velocity conditions. The assays were run spectrophotometrically by determination of the rate of decrease in absorbance at 230 nm as described in the text.

TABLE V

ACTION OF *Leucostoma* PEPTIDASE A ON SOME POLYAMINO ACIDS

Substrate: homopolymer of	*Relative activity* (%)*
Lysine	100.0
Tyrosine**	50.5
Phenylalanine**	35.9
Tryptophan**	33.2
Alanine	0.0
Glycine	0.0
Glutamic acid	0.0
Histidine	0.0
Leucine**	0.0

* Activities were determined from the kinetic slope under initial velocity conditions at 37.5 °C and at a substrate concentration of 0.75 mg per ml. Relative activities shown above are expressed as a percentage of activity toward poly-L-lysine which was hydrolyzed at a rate of 0.88 μmole per min per mg of enzyme.

** Tested as a suspension.

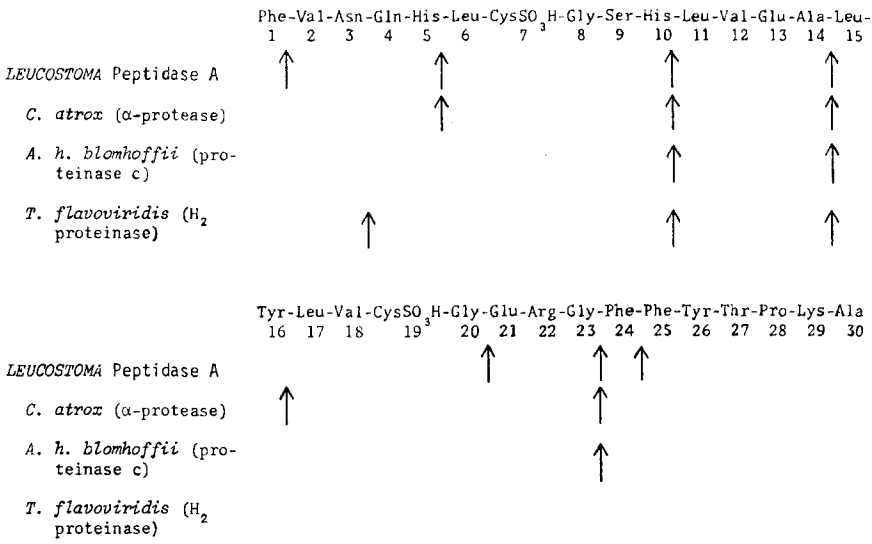

Fig. 1. Points of cleavage of oxidized B-chain of insulin by *leucostoma* peptidase A, compared with those of some other venom proteases. The data for α-protease of *Crotalus atrox* is that of Pfleiderer and Krauss[24], the data for proteinase c of *Agkistrodon halys blomhoffii* is from Satake *et al.*[26], and the bonds hydrolyzed by H_2 proteinase from *Trimeresurus flavoviridis* is from Takahashi and Ohsaka[20].

which arginine, lysine, tyrosine or tryptophan contributed the amino group of the susceptible bond, similar bonds in the B-chain of oxidized insulin were not hydrolyzed. The hydrolysis of the bond Phe^1–Val^2 is not unique among neutral proteases, as this reaction is also catalyzed by several microbial neutral proteases (*cf.* Fig. 4 in ref. 1).

Optimum pH

The activity of *leucostoma* peptidase A toward hemoglobin substrate made in composite buffer and adjusted to various pH values is shown in Fig. 2, which reveals that the enzyme has maximal activity at pH 8.5, a value well within the range of pH optima reported for neutral proteases from other sources[1].

DISCUSSION

To the best of our knowledge, *leucostoma* peptidase A is the first enzyme of animal origin to be shown to possess the properties of a neutral protease (endopeptidase); *i.e.*, a zinc-metalloenzyme with a pH optimum near neutrality and a substrate specificity dictated by the identity of residues whose amino groups are involved in the peptide bond. It is particularly interesting that an enzyme isolated from a snake venom, which is secreted by a highly specialized digestive organ, should have a number of properties similar to those of enzymes known heretofore to be produced only by certain microorganisms. These similarities have been overlooked in the past, however, because relatively few venom proteases have been purified and of these, investigations of metal content have generally not been made, although

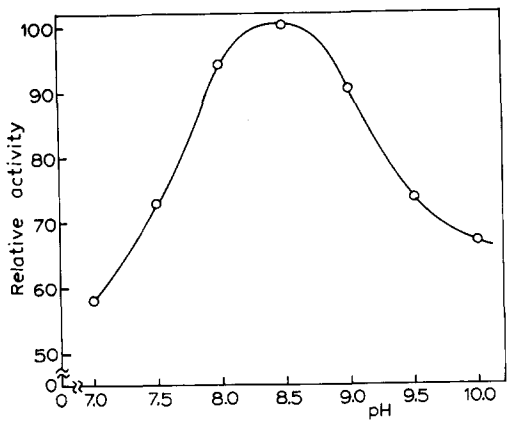

Fig. 2. Activity of *leucostoma* peptidase A as a function of pH. A composite buffer consisting of acetate–phosphate–borate (10 mM with respect to each anion) was used to make up denatured hemoglobin substrate (2%) at each of the pH values shown. Assays were performed at 37 °C as described in the text.

inhibition of proteolytic activity by EDTA has been reported in several crude unfractionated venoms[17–19] and in some purified[20,21] venom proteases.

It has been shown by Latt *et al.*[15] and by Feder and Garrett[16] that exposure of thermolysin to *o*-phenanthroline in the presence of Ca^{2+} permits the removal of zinc, and that this treatment inactivates the enzyme without affecting its stability, which is mediated by calcium. *Leucostoma* peptidase A behaved similarly under such conditions, being inactivated but not destabilized, and it was reactivated by the addition of Zn^{2+}. Thus zinc appears to be the metal ion involved in enzymic activity, as it is in thermolysin and some other microbial neutral proteases[1], and it seems likely that the role of calcium in the venom protease is to stabilize it, as is also the case for various neutral proteases of microbial origin[1]. Indirect evidence for this is furnished by the fact that we have been unable to reactivate *leucostoma* peptidase A after treatment with EDTA, which we found to remove both calcium and zinc (see above), in contrast to the reactivation achieved with Zn^{2+} after treatment with *o*-phenanthroline in the presence of calcium. Similar irreversible losses of activity have been observed when other venom proteases were treated with EDTA[20,21]. While the work reported herein was in progress, Ohshima *et al.*[22] reported the presence of 2 gatoms of calcium per mole of proteinase *b*, an enzyme they obtained in highly purified form from the venom of *Agkistrodon halys blomhoffii*. Removal of the calcium by either electrodialysis or EDTA led to a conformational change and loss of activity, both of which were irreversible. These workers proposed that calcium was involved in the stabilization of the enzyme, and reported that zinc appeared to be present in proteinase *b* only in trace quantities.

The experiments involving the action of *leucostoma* peptidase A on synthetic substrates revealed a high degree of selectivity by the enzyme, in that amidase and esterase activities were not found, and the minimum susceptible substrates were NH_2-substituted dipeptides. Of these, moreover, only a limited number were hydrolyzed. *Leucostoma* peptidase A exerts a qualitatively different substrate specificity toward the B-chain of oxidized insulin from that which it displays toward small

synthetic substrates, however, and in this respect, it is similar to some other venom proteases. Takahashi and Ohsaka[20] found purified H_2 proteinase of *Trimeresurus flavoviridis* to cleave the B-chain of oxidized insulin at the bonds His^{10}–Leu^{11} and Ala^{14}–Leu^{15} but not His^5–Leu^6 or Tyr^{16}–Leu^{17}. Of a series of di-, tri-, tetra- and pentapeptides containing leucine, however, only a doubly blocked pentapeptide, Z-Gly–Pro–Leu–Gly–Pro-OH was cleaved. Earlier, Pfleiderer and co-workers[23,25], investigating the specificity of the α-protease of *Crotalus atrox* venom, found that this enzyme did not hydrolyze the di-, tri-, tetra-, or pentapeptide substrates tested, although it cleaved the B-chain of oxidized insulin in five places. Similarly, although *leucostoma* peptidase A cleaved the bonds His^5–Leu^6 and His^{10}–Leu^{11} in the B-chain of oxidized insulin, it did not hydrolyze the dipeptides Z-Gly–Leu-OH, Z-Leu–Leu-OH, Z-Tyr–Leu-OH, Z-Leu–Leu-NH_2 or Z-Leu–Leu-OMe; neither was poly-L-leucine hydrolyzed. It was somewhat surprising, then, that the enzyme was active toward such blocked dipeptides as Z-Gly–Arg-OH, Z-Gly–Lys-OMe, Z-Gly–Trp-OH. These results underscore the complexities involved in the hydrolysis of polypeptides containing a variety of amino acid residues that are capable of affecting both the binding and catalytic steps. It is interesting that all four of the venom proteases whose actions are shown in Fig. 2 cleave the bonds His^{10}–Leu^{11} and Ala^{14}–Leu^{15} of oxidized insulin B-chain, as these bonds are also hydrolyzed by a large number of neutral proteases from microorganisms (Fig. 4 in ref. 1).

It now seems clear that several venom proteases hydrolyze large substrates with a specificity that is directed toward bonds, the amino groups of which are contributed by leucyl and phenylalanyl residues[20,24,26]; no evidence has yet been presented by any investigator to show that a purified venom protease has activity toward such trypsin substrates as arginine esters, which have been used by some investigators to assay for proteolytic activity[17,27,28]. The use of arginine esters as substrates for venom proteases was an outgrowth from experiments in which unfractionated venoms were used as enzyme sources, and was prompted by the observation that many such crude preparations were capable of hydrolyzing not only proteins, but such synthetic substrates for trypsin as benzoyl-L-arginine ethyl ester. We have shown that DFP inhibits amino acid esterase, but not protease, activity, whereas proteolysis, but not esterolysis, was inhibited by EDTA[18]. Moreover, when our earlier experiments yielded *leucostoma* peptidase A in highly homogeneous form, the pure enzyme proved to have no amino acid esterase activity[2]. Delpierre[29] also separated the proteolytic and esterolytic activities of three venoms, and Toom *et al.*[30] later isolated an enzyme that hydrolyzed arginine esters but was inactive toward protein substrates. Thus it is apparent that the earlier view of trypsin-like proteases in snake venoms was occasioned by the then unrecognized fact that proteolytic and esterolytic activities resulted from separate enzymes. It will be interesting to see what range of specificities occurs in venom proteases, but it is necessary that such information be based upon experiments with purified enzymes.

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