Characterization of a Nonproteolytic Arginine Ester-hydrolyzing Enzyme from Snake Venom*

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SUMMARY

An enzyme that catalyzes the hydrolysis of N-benzoyl-l-arginine ethyl ester and p-toluenesulfonyl-l-arginine methyl ester has been isolated from the venom of Agkistrodon contortrix laticinctus (broadbanded copperhead) by means of DEAE-cellulose chromatography. A high degree of homogeneity is suggested by sedimentation velocity, gel filtration, polyacrylamide electrophoresis, and isoelectric focusing. The purified enzyme has a sedimentation coefficient of 2.7 S, a diffusion coefficient of $8.3 \times 10^{-7}$ cm² per sec, and a molecular weight of 30,000. The isoelectric point, as determined by means of isoelectric focusing, was found to be 9.1. Enzymatic assays showed the preparation to be specific for arginine esters. The $K_m$ values determined with N-benzoyl-l-arginine ethyl ester and p-toluenesulfonyl-l-arginine methyl ester are $1.17 \times 10^{-4}$ and $1.49 \times 10^{-4}$, respectively. The enzyme was inhibited by diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and N-bromosuccinimide. Optimal rates of hydrolysis were observed from pH 7.5 to pH 8.5.

The ability of various venoms to hydrolyze amino acid esters has been well documented. Recent evidence suggests that these esterases might play an important role in local tissue destruction (1, 2). Tu, Passey, and Tu (3) and Tu, Chua, and James (4) tested the venoms of all four families of venomous snakes for their ability to hydrolyze p-toluenesulfonyl-l-arginine methyl ester and N-benzoyl-l-arginine methyl ester, and concluded that only venoms of the families Crotalidae and Viperidae possess enzymes capable of this hydrolysis. In these same investigations, the substrates N-acetyl-l-tyrosine ethyl ester and N-benzoyl-l-tyrosine ethyl ester were not hydrolyzed by most of the venoms, suggesting that the substrate specificities were similar to those of trypsin, rather than chymotrypsin. However, venom enzymes were not inhibited by soybean or ovomucoid trypsin inhibitors, showing that venom enzymes were different from trypsin.

To date, a study of the various physical and enzymatic parameters of a purified amino acid esterase has not been reported. This paper will describe a procedure for the isolation of an esterase shown to be homogeneous by sedimentation velocity, polyacrylamide electrophoresis, and isoelectric focusing, and report on some of its enzymatic and physical properties.

EXPERIMENTAL PROCEDURE

Materials—Lyophilized Agkistrodon contortrix laticinctus venom was purchased from Miami Serpentarium, Miami, Florida. Substrates used in the enzymatic assays were obtained from Sigma Chemical Company, Calbiochem, Nutritional Biochemicals Corporation, Mann Research Laboratories, Pierce Chemical Company, and Fisher Scientific Products.

Standards for molecular weight determinations utilizing Sephadex G-75 were purchased from Mann Research Laboratories. Sephadex G-75 and G-10 were obtained from Pharmacia Fine Chemicals, Inc. DEAE-cellulose was the product of Bio-Rad Laboratories. Ampholine carrier ampholytes used in electrophoresis were purchased from LKB Instruments.

Enzyme Assays—Assays for proteolytic activity using casein as substrate were carried out following the modified method of Kunitz (5) as previously described (1). Those proteolytic enzyme assays in which hemoglobin was used as substrate were performed by incubating 0.5 ml of enzyme solution (1 mg per ml) with 1.0 ml of 2% urea-denatured hemoglobin (in 0.01 M NaH₂PO₄, pH 7.0) at 37°C for 30 min (6). The reaction was terminated by the addition of 2.0 ml of 5% trichloracetic acid, and the absorbance of the acid-soluble products was determined at 280 nm. Proteolytic activity, as measured by the hydrolysis of the synthetic substrates Azocoll and Congocoll (Calbiochem) (7), was determined by incubating 5 mg of substrate in 3.0 ml of Tris-HCl buffer (0.1 M, pH 8.5) with 0.5 ml of enzyme solution (1 mg per ml). After 30 min, the solution was filtered and the absorbance of the liberated dye measured at 495 nm (Congocoll) or 580 nm (Azocoll). Esterase activity toward BAEE3 was routinely followed during the course of purification by the spectrophotometric method previously described (1). Enzymatic activities against casein, hemoglobin, Azocoll, Congocoll, and BAEE were expressed as specific activity = (absorbance change per min)/(milligram of venom) × 1000.

A titrimetric method was employed to determine the hydrolysis of the esters and amides listed in Table III, by the purified en-
enzyme. Added to the substrate dissolved in 3 ml of deionized water were 25 μl of enzyme solution (0.2 mg per ml). The pH was adjusted to 8.35 and the hydrolysis was followed by recording the amount of 3.45 × 10⁻⁸ M NaOH required to maintain the pH at 8.35. If no hydrolysis was detected, 500 μl of enzyme (1 mg per ml) were added and the assay was repeated. All experiments were performed using a Radiometer titrator equipped with an autoburette and recorder.

Inhibition Experiments—The inhibitory effects of EDTA, 1, 10-phenanthroline, diisopropyl fluorophosphate, p-chloromercuribenzoic acid, phenylmethylsulfonyl fluoride, and thioglycolic acid were tested by incubation of varying concentrations of each with venom in Tris buffer (final concentration 10 mM, pH 8.4) for 2 hours at 37°. The phenylmethylsulfonyl fluoride solution was prepared by dissolving phenylmethylsulfonyl fluoride in 2-propanol, then diluting with 40 mM Tris-HCl, pH 8.40, to give a 30% solution in 2-propanol.

The inhibition by N-bromosuccinimide was tested by adding 40 μl of a 25 mM solution of N-bromosuccinimide in sodium acetate buffer (50 mM, pH 4.5) to 1.5 ml of venom dissolved in the same buffer. After a 30-min incubation at 25°, aliquots were removed and assayed for enzymatic activity.

Isoelectric Fractionation—The electrofocusing column was filled with a linear gradient from Solution A to Solution B. The gradient was produced by means of a 115-ml divided box described by Svensson (8). Solution A consisted of 5 mg of purified enzyme and 0.70 g of carrier ampholyte dissolved in 55 ml of 50% sucrose (w/v). Solution B consisted of 0.30 g of carrier ampholyte dissolved in 55 ml of deionized water. The applied voltage, initially 100 volts, was gradually increased to 500 volts by the end of the experiment, maintaining the power output at about 0.8 watts by manual adjustment. After separation for 48 to 60 hours at 4°, the column was drained and 1.0-ml fractions were collected.

Chromatographic Procedures—DEAE-cellulose was suspended in the first buffer to be used in the elution procedure, and allowed to stand for several hours. After the fine particles had been decanted and the procedure repeated several times, 2 mM NaCl dissolved in this buffer was added. The cellulose was then reequilibrated with buffer containing no NaCl, the column was poured and equilibrated for 24 hours with the first buffer to be used in the elution procedure. Sephadex G-75 and G-10 were dispersed in the eluting solvent, and allowed to swell for 4 and 24 hours, respectively. After the fine particles had been decanted several times, the gel was poured into the columns and washed with 24 hours with eluting solvent. Column effluents were monitored with an ISCO model UA-2 ultraviolet analyzer and the elution patterns recorded with an ISCO B-inch chart recorder.

Amino Acid Analysis—A 2.5-μg enzyme sample was dissolved in 3.0 ml of constant boiling HCl and placed in a heavy walled ignition tube. The tube was sealed under vacuum and the protein was hydrolyzed for 24 hours at 110°. Following hydrolysis, the sample tubes were opened and the HCl was removed by drying over NaOH pellets under vacuum. After three washings, the residue was dissolved in 2.5 ml of 0.2 M sodium citrate buffer, pH 2.2. Amino acid analyses were performed on a Technicon amino acid analyzer, equipped with a column (1.6 × 40 cm) of Chromobeads B.

Ultracentrifugation—A Spinco model E analytical ultracentrifuge equipped with a temperature control unit (RTIC) and a schlieren optical system was used to determine sedimentation and diffusion coefficients. The schlieren patterns were recorded photographically on Eastman Kodak Metallographic plates. The plates were read using a microcomparator (Nikon Model 6C) equipped with a rotational stage. The sedimentation coefficient was calculated from the rate of movement of the maximum ordinate of the refractive index gradient. No attempt was made to use the theoretically more correct second moment procedure of Goldberg (9) because of various experimental difficulties, particularly the low solubility of the enzyme near its isoelectric point. The sedimentation velocity experiments were performed at 4° and 59,780 rpm using a double sector cell with an aluminum-filled Epon center piece. The diffusion coefficient measurements were performed at 4° and 10,589 rpm using a capillary-type synthetic boundary cell according to the procedure described by Schachman (10). The samples were prepared for ultracentrifugation.
Step and treatment | Total protein | Total enzyme units | Specific activity | Recovery of esterase
---|---|---|---|---
I. Chromatography (DEAE-cellulose, pH 8.5) | 382 | $7.8 \times 10^4$ | 2,080 | 71
II. Chromatography (DEAE-cellulose, pH 9.5) | 91 | $5.6 \times 10^5$ | 6,150 | 51
III. Chromatography (DEAE-cellulose, pH 9.5 to pH 8.5) | 11 | $1.6 \times 10^5$ | 16,200 | 29

* Specific activity as defined in text.

**Fig. 2.** Electrophoretic pattern of the purified enzyme on polyacrylamide strip. Sodium acetate (0.03 M), pH 5.5; conditions, 400 volts for 90 min. Arrow indicates point of application of sample. Cathode is on right.

**Fig. 3.** Isoelectric focusing profile of purified esterase. Details of experiment given in text.

**RESULTS**

**Purification of Enzyme**

Lyophilized *A. contortrix laticeps* venom (1.0 g) was dissolved in 5.0 ml of 0.01 M Tris-HCl, pH 8.5, and dialyzed for 24 hours against this buffer. No loss of either proteolytic or estererase activity was observed.

The dialyzed venom sample was added to a column of DEAE-cellulose previously equilibrated with 0.01 M Tris-HCl, pH 8.5. After all unadsorbed material had been completely eluted with the starting buffer, either a NaCl or a Tris-HCl salt gradient, or both, was applied to the column (0 to 0.2 M, followed by 0.2 to 1.0 M).

A representative chromatogram is shown in Fig. 1A. The highest esterase activity toward BAEE was found in Fraction I, with Fraction III also displaying some activity. These two fractions also exhibited the highest proteolytic activity. Our primary interest was in the enzyme represented by Fraction I, and thus, no attempt was made to further purify the other fractions.

Fraction I from the preceding step was lyophilized to dryness, then redissolved in 20 ml of 0.01 M Tris-HCl, pH 9.5. After dialyzing for 24 hours against this buffer, the solution was placed on a column previously equilibrated with 0.01 M Tris, pH 9.5. The sample was eluted by means of a 500-ml gradient of either NaCl or Tris-HCl, or both, of increasing concentration (0 to 0.2 M, followed by 0.2 to 1.0 M). The elution pattern obtained is presented in Fig. 1B. Esterase activity was highest in Fraction B, with Fractions A and C also displaying slight activity toward BAEE. Proteolytic activity was highest in Fraction C, but was also present in Fractions A and B.

Fraction B was concentrated by lyophilization to about 10 ml and dialyzed against 0.01 M sodium glycinate buffer, pH 9.5, for 24 hours. The sample was then placed on a column of DEAE-
cellulose previously equilibrated with the same buffer. As can be seen in Fig. 1C, all material was adsorbed on the column. A pH gradient from pH 9.5 to 8.8 (0.01 M glycine) was then applied to the column, and a single, symmetrical peak was obtained. When 0.1 M glycine, pH 8.5, was passed through the column, a second peak emerged. Activity against BAEK was found in the first peak, while proteolytic activity toward casein was found in the second. A small amount of esterase was also found in the second peak.

The purified esterase was then lyophilized to dryness, dissolved in 2.0 ml of deionized water, and passed through a Sephadex G-10 column using deionized water as the eluting solvent. A summary of the purification steps is presented in Table I, where it can be seen that the specific esterase activity was increased about 20-fold by the isolation procedure. The recovery of protein (11 mg) represents a 1% recovery from unfractionated venom.

**Physicochemical Properties**

*Electrophoresis*—A high degree of purity was indicated by electrophoretic experiments on polyacetate strips. A number of experiments were performed at pH values ranging from 5.0 to 9.0. In each instance, only a single band could be detected. An example of such an experiment is presented in Fig. 2, in which the electrophoretic pattern obtained at pH 5.5 is presented.

*Isoelectric Focusing*—As can be seen from Fig. 3, the preparation also appeared to be homogeneous by means of isoelectric focusing. This technique also established the isoelectric point to be 9.1.

*Gel Filtration*—Fig. 4 presents the results obtained when the purified enzyme was passed through a column of Sephadex G-75. This technique also indicated homogeneity in the enzyme preparation. The method of Andrews (11) was used to calculate the molecular weight of the purified enzyme. Fig. 5 shows the calibration curve obtained from the G-75 column with a number of proteins of known weight. The purified enzyme gave an elution volume corresponding to a molecular weight of 31,000.

*Amino Acid Composition*—The amino acid composition of the enzyme is shown in Table II. The values for all residues except tryptophan were determined from data obtained using enzyme samples hydrolyzed for 24 hours with constant boiling HCl. The tryptophan content of the protein was determined spectrophotometrically by the method of Benezech and Schmid (12). Cystine was determined from hydrolysates treated with performic acid prior to acid hydrolysis. Based on 1 residue of methionine, the minimum molecular weight was calculated to be 10,800. Assuming that methionine occurs 3 times in the protein, the molecular weight would be 31,000.

*Diffusion Coefficient*—An apparent diffusion coefficient was calculated by the statistical method described by Schachman (10). The diffusion coefficient was calculated to be $D_{0.0} = 2.7 \times 10^{-7}$ cm² sec⁻¹ after correction to a value corresponding to water at 20°C.

*Partial Specific Volume*—The partial specific volume of the enzyme was estimated from the amino acid composition according to the procedure described by Schachman (10). The partial specific volume was calculated to be 0.71 ml per g using the relation $\bar{v} = \sum w_i v_i/\sum w_i$, where $w_i$ and $v_i$ are weight percent and specific volume of Residue $i$.

*Molecular Weight*—The molecular weight, $M$, of the enzyme as determined by sedimentation and diffusion measurements,
TABLE II
Amino acid composition of venom esterase

| Amino acid   | Amino acid residues |   |   |   |
|--------------|---------------------|---|---|---|
|              | A       | B       | C       | Average | Residues | Nearest integer |
| Aspartic acid| 8.89    | 8.69    | 8.85    | 8.81    | 23.0     | 23            |
| Threonine    | 4.32    | 4.61    | 4.58    | 4.50    | 13.3     | 13            |
| Serine       | 8.76    | 8.80    | 9.01    | 8.88    | 30.6     | 31            |
| Glutamic acid| 15.58   | 14.77   | 14.41   | 14.92   | 34.7     | 35            |
| Proline      | 7.48    | 6.23    | 7.15    | 6.95    | 21.4     | 21            |
| Glycine      | 17.00   | 16.12   | 16.83   | 16.67   | 87.4     | 87            |
| Alanine      | 5.96    | 6.40    | 6.21    | 6.19    | 26.1     | 26            |
| Valine       | 3.76    | 3.43    | 3.24    | 3.48    | 10.6     | 11            |
| Half-cystine | 3.00    | 3.29    | 3.16    | 3.15    | 9.1      | 9             |
| Methionine   | 1.18    | 1.35    | 1.27    | 1.27    | 3.9      | 3             |
| Isoleucine   | 3.62    | 3.09    | 3.92    | 3.74    | 9.9      | 10            |
| Leucine      | 6.60    | 7.21    | 6.92    | 6.91    | 18.3     | 18            |
| Tyrosine     | 1.80    | 1.85    | 1.86    | 1.81    | 3.4      | 3             |
| Phenylalanine| 1.45    | 1.09    | 1.42    | 1.52    | 3.1      | 3             |
| Lysine       | 3.68    | 3.98    | 3.43    | 3.70    | 8.7      | 9             |
| Histidine    | 2.33    | 2.52    | 2.38    | 2.41    | 5.3      | 5             |
| Arginine     | 2.02    | 2.55    | 2.42    | 2.33    | 4.4      | 4             |
| Tryptophan   | 2.57    | 2.65    | 2.66    | 2.63    | 4.2      | 4             |

* Determined by performic acid oxidation of separate aliquot prior to hydrolysis.
* Determined spectrophotometrically on intact protein sample by method of Benze and Schmid (12).

Fig. 6. Schlieren patterns of purified esterase. Photographs are shown at times of 12, 44, 108, 156, and 188 min after attaining a rotor speed of 59,780 rpm. The protein concentration was approximately 1% in 0.1 M Tris-HCl and 0.1 M NaCl, pH 7.0. A double sector cell was used, the bar angle was 60°, and the temperature was 4°. Sedimentation was from right to left.

TABLE III
Effect of divalent cations on hydrolysis of BAEE and casein

| Metal      | Esterase activity a | Proteolytic activity b |
|------------|---------------------|------------------------|
| None       | 100                 | 0                      |
| Ca²⁺       | 99                  | 0                      |
| Cd²⁺       | 112                 | 0                      |
| Mg²⁺       | 151                 | 0                      |
| Ni²⁺       | 160                 | 0                      |
| Zn²⁺       | 193                 | 0                      |
| Mn²⁺       | 205                 | 0                      |
| Co²⁺       | 211                 | 0                      |

a The venom solutions were incubated for 1 hour at 20° with 1.0 mM solutions of the respective metals. Assay for esterase was by titration with NaOH at pH 8.35 as described in text.

b Assay for proteolytic activity was with casein as described in text. Venom concentration was 0.2 mg per ml.

was calculated using the equation, \[ M = \frac{RTS}{(1 - \varphi)D} \]
where \( T \) is the absolute temperature, \( R \) the ideal gas constant, and \( \varphi \) the density of the solution. With \( D_{20,w} = 8.3 \times 10^{-2} \) cm² per sec, \( b_{0,w} = 2.7 \times 10^{-13} \) sec, \( \varphi = 0.71 \) cm³ per g, \( p = 0.968 \) g per cm³, and \( T = 20° \), the molecular weight was calculated to be 27,000. This compares with the minimum molecular weight of 27,000.
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### TABLE IV

| Substrate                                                                 | $K_m$ \( \times 10^{-4} \) | Moles per min per mole of enzyme |
|---------------------------------------------------------------------------|-----------------------------|---------------------------------|
| N-Benzoyl-L-arginine ethyl ester                                          | \(1.17\)                     | 3.72                            |
| N-Benzoyl-L-arginine methyl ester                                        | \(4.50\)                     | 3.68                            |
| p-Toluenesulfonyl-L-arginine methyl ester                                 | \(1.49\)                     | 3.55                            |
| p-Nitrophenyl acetate                                                     | \(5.0\)                      | 0.60                            |
| N-Benzoyl-L-alanine ethyl ester                                           | 0                           | 0                               |
| Benzoyloxy carbonyl lysine methyl ester                                   | 0                           | 0                               |
| Benzoyloxy carbonyl lysine benzyl ester                                   | 0                           | 0                               |
| L-Lysine ethyl ester                                                      | 0                           | 0                               |
| L-Lysine-p-nitrophenyl ester                                              | 0                           | 0                               |
| N-Benzoyl-L-arginine-p-nitroanilide                                       | 0                           | 0                               |
| N-Benzoyl-L-arginine-3-naphthylamide                                     | 0                           | 0                               |
| N-Benzoyl-L-arginine amide                                                | 0                           | 0                               |
| \(\alpha\)-N-p-Tosyl-L-arginine amide                                      | 0                           | 0                               |
| N-Benzoyl-L-tyrosine ethyl ester                                          | 0                           | 0                               |
| Acetyl-L-tyrosine ethyl ester                                             | 0                           | 0                               |
| Indophenyl acetate                                                        | 0                           | 0                               |
| Fibrin                                                                    | 0                           | 0                               |
| Casein                                                                    | 0                           | 0                               |
| Hemoglobin                                                                | 0                           | 0                               |
| Congocoll                                                                 | 0                           | 0                               |
| Azocoll                                                                   | 0                           | 0                               |

* Based on molecular weight of 30,000.

### Enzymatic Properties

**Effect of pH**—As shown in Fig. 7, the enzyme exhibited a rather broad pH optimum, being most active at pH values from 7.0 to 9.0. Below pH 7 and above pH 9, the activity dropped off rather rapidly.

**Effect of Divalent Cations**—As can be seen from Table III, the cations Mn\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) approximately doubled the rate of hydrolysis of BAEE by the purified enzyme. Ni\(^{2+}\) and Mg\(^{2+}\) increased the rate of hydrolysis about 50% while Ca\(^{2+}\) and Cd\(^{2+}\) had little effect. None of the metals tested resulted in restoration of proteolytic activity.

**Substrate Specificity**—A rather large number of substrates known to be hydrolyzed by esterases of other sources as well as some common substrates for proteases were tested (Table IV). The substrates BAEE, N-benzoyl-L-arginine methyl ester, p-toluenesulfonyl-L-arginine methyl ester, and p-nitrophenyl acetate were hydrolyzed by the venom esterase. When the arginine residue was replaced with alanine, lysine, or tyrosine, no hydrolysis took place. No hydrolysis could be detected on the amide bonds of L-lysine-p-nitroanilide, N-benzoyl-L-arginine amide, p-toluenesulfonyl-L-arginine amide, or N-benzoyl-L-arginine p-nitroanilide, even with 100-fold excess of enzyme. The synthetic substrate, indophenyl acetate, readily hydrolyzed by acetyl cholinesterase (13), was not hydrolyzed by the venom esterase. In like manner, none of the proteolytic enzyme substrates (casein, hemoglobin, Congocoll, Azocoll) were hydrolyzed by the purified esterase.

### FIG. 8

Reciprocal plot of reaction velocity against concentration of BAEE. The reaction mixture consisted of 3.0 ml of substrate to which 25 \(\mu\)l of enzyme (0.4 \(\mu\)g per ml) were added. The amount of 3.45 \(\times\) \(10^{-4}\) M NaOH required to maintain the pH at 8.35 was recorded.

**Effect of Inhibitors**—The effect of a number of group-specific inhibitors is shown in Table V. DFP, phenylmethylsulfonyl fluoride, and N-bromosuccinimide all strongly inhibited the enzyme, whereas thioglycolic acid and p-chloromercuribenzoic acid showed partial inhibition. The chelating agents, EDTA and 1,10-phenanthroline, exhibited only minor inhibition.

**Maximum Velocity and $K_m$**—$V_m$ and maximum velocity numbers were calculated for the substrates BAEE, N-benzoyl-L-
arginine methyl ester, p-toluenesulfonyl-L-arginine methyl ester, and p-nitrophenyl acetate. These values, as calculated from Lineweaver-Burk plots, are presented in Table IV. An example of these plots is shown in Fig. 8 where 1/v is plotted against 1/s using BAEE as substrate.

**DISCUSSION**

The results presented herein indicate that a reproducible procedure for the isolation of an esterase from the venom of *A. contortrix laticinctus* has been achieved. This procedure results in the isolation of an esterase of a high degree of purity as evidenced by four criteria: electrophoresis, ultracentrifugation, chromatography on Sephadex G-75, and isoelectric focusing.

Three methods of determining molecular weight (combination of sedimentation velocity and diffusion coefficient, Sephadex gel filtration, and amino acid composition) gave molecular weights of 27,000, 31,000, and 31,000, respectively. It can be concluded that the molecular weight of the purified esterase is near 30,000.

There has recently been some controversy as to whether BAEE and p-toluenesulfonyl-L-arginine methyl ester can be used as substrates for the assay of proteolytic enzymes in snake venoms (4, 14, 15) and whether the enzyme or enzymes responsible for the hydrolysis of BAEE and p-toluenesulfonyl-L-arginine methyl ester is actually proteolytic in nature. The evidence from this investigation strongly supports the view that the hydrolysis of the synthetic substrates by snake venom is not caused by a proteolytic enzyme. Thus the conclusions drawn by Delphiere (15) from work on partially fractionated venom, and those of Wagner, Spiekerman, and Prescott (14) based on studies of a purified protease, are confirmed in this investigation.

From the results reported herein, it appears that an esterase quite different from any esterase yet reported in literature has been isolated and characterized from snake venom. This amino acid esterase appears to be quite specific, as shown by the fact that only esters of arginine are acted upon. However, the alkyl group does not appear to play a major role, as shown by the fact that BAEE and N-benzoyl-L-arginine methyl ester exhibited almost identical $K_m$ and $V$ values.

This newly isolated esterase appears to be specific for ester bonds as illustrated by the fact that even with a 100-fold excess of enzyme, no hydrolysis could be detected on any of the synthetic substrates containing amide bonds, or with any of the peptide bonds of the various protein substrates tested. Like other esterases, serine appears to be at the active center as both diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride inhibit the enzyme. Unlike enzymes which act either as proteases or esterases depending on the cation present (16), this enzyme showed no proteolytic activity when tested in the presence of a number of divalent cations. Further chelating agents showed only slight inhibition of hydrolysis. Under the experimental conditions employed, the strong inhibition by N-bromosuccinimide suggests that tryptophan also plays an important role in the activity of the enzyme.

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