Purification and Characterization of Carboxypeptidases A and B from the White Shrimp (*Penaeus setiferus*)†

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ABSTRACT: Carboxypeptidase A and B activity in the white shrimp, *Penaeus setiferus*, has been found to be due to two different proteins. Both have been purified to homogeneity by ion-exchange chromatography, affinity chromatography on arginyl-Sepharose, and gel filtration. Carboxypeptidase B has a molecular weight of 34,200 and is present in significantly higher concentrations than carboxypeptidase A. The latter has a molecular weight of 30,000. Comparison of the amino acid composition of each protein with carboxypeptidases from other species indicates a high degree of similarity except that the shrimp enzymes have fewer basic amino acids. Both enzymes contain zinc and each is inhibited by the metal chelator, *o*-phenanthroline, and also by β-phenylpropionic acid. In addition, carboxypeptidase B is inhibited by ε-aminocaproic acid. The results suggest a strong relationship between crustacean carboxypeptidases and those of higher organisms.

Survey studies of the complement of digestive enzymes elicited from the hepatopancreas of several crustacean species have yielded information indicating that (a) trypsin-like enzymes are the major proteolytic components, (b) all species are devoid of chymotrypsin-like activity, and (c) carboxypeptidase A and B activities appear to be associated with a single protein (Devillez, 1965; Devillez and Buschlen, 1967).

Detailed investigations of the trypsin-like enzyme in both the crayfish, *Astacus fluviatilus* (Zwilling *et al.*, 1969), and the white shrimp, *Penaeus setiferus* (Gates and Travis, 1969), have resulted in the resolution of this activity into two, unusually stable protein components which have now been purified to homogeneity and characterized. Recently, it was restated that, indeed a single carboxypeptidase did exist in the crayfish which contained both A and B activities (Kleine, 1969). In an effort to examine the mechanism by which such an enzyme might function we have investigated the carboxypeptidase activity of the white shrimp, a closely related organism. The results presented here indicate that separate enzymes, having A and B activities, exist in this species. The inability of others to separate each activity, heretofore, is probably due to the very similar ionic properties exhibited by the two enzymes.

Materials

Sources of materials, including tissue, were the same as given by Gates and Travis (1969) with the following additions. L-Arginine, hippuryl-L-arginine, hippuryl-L-phenylalanine, bradykinin, and ε-aminocaproic acid were purchased from Mann Research Laboratories. Angiotensin II was obtained from Miles Research Chemicals. β-Phenylpropionic acid was purchased from Eastman Chemicals. Bovine carboxypeptidase A and porcine carboxypeptidase B were obtained from Worthington Biochemicals Corp. Both were treated with diisopropyl fluorophosphate prior to use.

Methods

Assay of Enzymes. Enzyme activity was routinely followed by measuring the rate of hydrolysis of ester or peptide substrates by spectrophotometric methods. For carboxypeptidase A activity the substrate used was hippuryl-L-phenylalanine according to the method of Folk and Schirmer (1963). The substrate solution contained 10^{-3} M hippuryl-L-phenylalanine in 0.025 M Tris-HCl (pH 7.65)–0.1 M NaCl. The enzyme solution (0.1 ml) was added to 2.9 ml of the substrate solution and the increase in A_{254} followed at 25°. The enzyme was diluted so that the rate was linear for at least 3 min. One unit of activity was defined as the amount of enzyme which produced an absorbance change of 1 unit/min at 254 mμ. The specific activity was defined as units of enzyme per optical density unit at 280 mμ during the purification and as units of enzyme per milligram of protein for the purified material.

Carboxypeptidase B activity was followed using hippuryl-L-arginine as substrate (Folk *et al.*, 1960). The substrate solution contained 10^{-3} M hippuryl-L-arginine in 0.025 M Tris-HCl (pH 7.65)–0.5 M NaCl. A unit of activity and specific activity were defined as for carboxypeptidase A. Proteolytic activity was determined by the method of Kunitz as described by Laskowski (1955) using casein as substrate. Protein concentration was determined by the method of Warburg and Christian (1942) on crude preparations and with experimentally determined extinction coefficients for the purified proteins.

The specificity of shrimp carboxypeptidases on small polypeptide substrates was investigated by quantitatively following the release of individual amino acids with the amino acid analyzer. The enzyme (0.5 nmol) was incubated with substrate (0.20 nmol) in 0.5 ml of 0.2 M *N*-ethylmorpholineacetic acid (pH 8.5) at 37°. At the end of a given digestion period the enzyme was inactivated by the addition of 0.1 ml of glacial acetic acid. The sample was dried on a rotary evaporator and the residue was dissolved in buffer for amino acid analysis on

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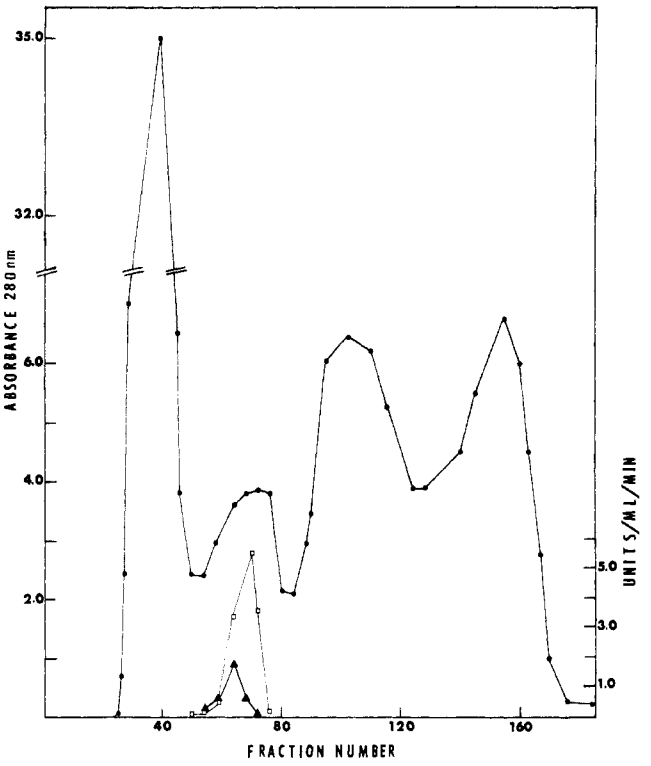

FIGURE 1: Sephadex G-75 chromatography of a 32-ml sample of extract of 5 g of acetone powder of shrimp digestive glands. The column (4.5 × 60 cm) was equilibrated with 0.01 M Tris-HCl (pH 8.0) and eluted with the same buffer. The flow rate was 28 ml/hr and 3.5-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (●) left ordinate; activity against hippuryl-L-arginine (□) and hippuryl-L-phenylalanine (▲) right ordinate.

a Beckman Model 120C analyzer. For comparison, the polypeptide substrates were also subjected to commercially available, diisopropyl fluorophosphate treated, mammalian carboxypeptidases (A and B) under the same conditions.

Polyacrylamide Electrophoresis. Disc electrophoresis in polyacrylamide gels was performed as described by Davis (1964) using both 7.5 and 15% acrylamide gels. Riboflavine was used to polymerize the gels.

Acrylamide gel electrophoresis in sodium dodecyl sulfate was performed by the method of Weber and Osborn (1969), using one-fifth the recommended amount of ammonium persulfate to catalyze the polymerization.

Metal Analyses. Carboxypeptidases A and B were analyzed for metal content using a Perkin-Elmer Model 403 atomic absorption spectrophotometer. The samples were dialyzed overnight against 10^{-3} M NaEDTA (pH 7.0), then several hours against 0.005 M Tris buffer (pH 7.0) prior to analysis. The Tris dialysis buffer was used as a blank.

Ultracentrifugation Analyses. A Beckman-Spinco Model E ultracentrifuge was used to determine both the sedimentation coefficient, $s_{20,w}$ (Schachman, 1967), and the molecular weight (Yphantis, 1964) of each carboxypeptidase. Partial specific volumes of 0.718 for carboxypeptidase A and 0.721 for carboxypeptidase B, both estimated from the amino acid composition (McMeekin and Marshall, 1952), were utilized. The analytical ultracentrifuge was also used as a differential refractometer to derive an extinction coefficient for shrimp carboxypeptidase B (Babul and Stellwagen, 1969).

All other methods were performed as previously described (Gates and Travis, 1969).

Results

Purification of Enzymes. STEP I. EXTRACTION. Acetone powders (5 g) of shrimp hepatopancreas, prepared as described earlier (Gates and Travis, 1969), were extracted for 24 hr at 4° with 35 ml of 0.1 M sodium borate buffer (pH 8.0). The extract was centrifuged at 7970*g* for 20 min and the precipitate was discarded.

STEP II. SEPHADEX G-75 COLUMN CHROMATOGRAPHY. The supernatant from step I (32 ml) was applied to a column of Sephadex G-75 (4.6 × 60 cm) previously equilibrated with 0.01 M Tris-HCl (pH 8.0). The elution profile (Figure 1) indicates that carboxypeptidase A and B activities emerged coincidentally. Trypsin esterase activity was also detected in these fractions.

STEP III. FIRST DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY. The most active fractions from step II (130 ml) were pooled and solid NaCl was added with stirring until the concentration was 0.4 M in NaCl. The solution was then applied to a DEAE-Sephadex A-50 column (1.9 × 11 cm) which had been equilibrated with 0.01 M Tris-HCl (pH 8.0)–0.4 M NaCl. Both carboxypeptidase activities were unretarded and passed directly through the column while all of the trypsin activity was retained.

STEP IV. SECOND DEAE-SEPHADEX A-50 COLUMN CHROMATOGRAPHY. The carboxypeptidase A and B fractions from step III were pooled (180 ml), diluted to 0.15 M NaCl by addition of 0.01 M Tris-HCl (pH 8.0), and applied to a second DEAE-Sephadex A-50 column equilibrated with 0.01 M Tris-HCl (pH 8.0)–0.15 M NaCl. After the passage through the column of a small quantity of protein, a stepwise gradient was initiated using 0.01 M Tris-HCl (pH 8.0), containing, in order, 0.2, 0.25, and 0.3 M NaCl. The elution profile (Figure 2) indicates the incomplete separation of the two exopeptidases from each other. However, two enriched fractions containing mainly carboxypeptidase B (0.25 M wash) and carboxypeptidase A (0.30 M wash), respectively, were obtained. Better separation of the two components using a number of other types of salt gradients was unsuccessful.

STEP V. AFFINITY CHROMATOGRAPHY ON ARGINYL-SEPHAROSE. Each of the carboxypeptidase enriched fractions obtained from step IV was applied, separately, to a column of arginyl-Sepharose (1.8 × 85 cm) which had been prepared by techniques described by Cuatrecacus (1970). Both the samples and the Sepharose derivative were equilibrated with 0.025 M Tris-HCl (pH 7.65) prior to chromatography. When a carboxypeptidase B enriched fraction (230 ml) was applied and a linear salt gradient used to develop the column, two peaks containing carboxypeptidase A and B esterase activities, respectively, were obtained (Figure 3). Identical results occurred when carboxypeptidase A enriched fractions (136 ml) were applied. If ion-exchange chromatography on DEAE-Sephadex A-50 (step IV) was omitted, poor resolution of the two exopeptidases resulted, due to the presence of other contaminating proteins.

The specific esterase activities of shrimp carboxypeptidase B preparations prepared by chromatography of either enriched or poor fractions were found to be identical and these were pooled. Similar results were obtained for carboxypeptidase A fractions and these too were pooled.

STEP VI. RECHROMATOGRAPHY ON SEPHADEX G-75. The carboxypeptidase A and B fractions from step V were concentrated by ultrafiltration and applied, separately, to Sephadex G-75 columns (4.2 × 65 cm) which had previously been equilibrated with 0.01 M Tris-HCl (pH 8.0). When the columns

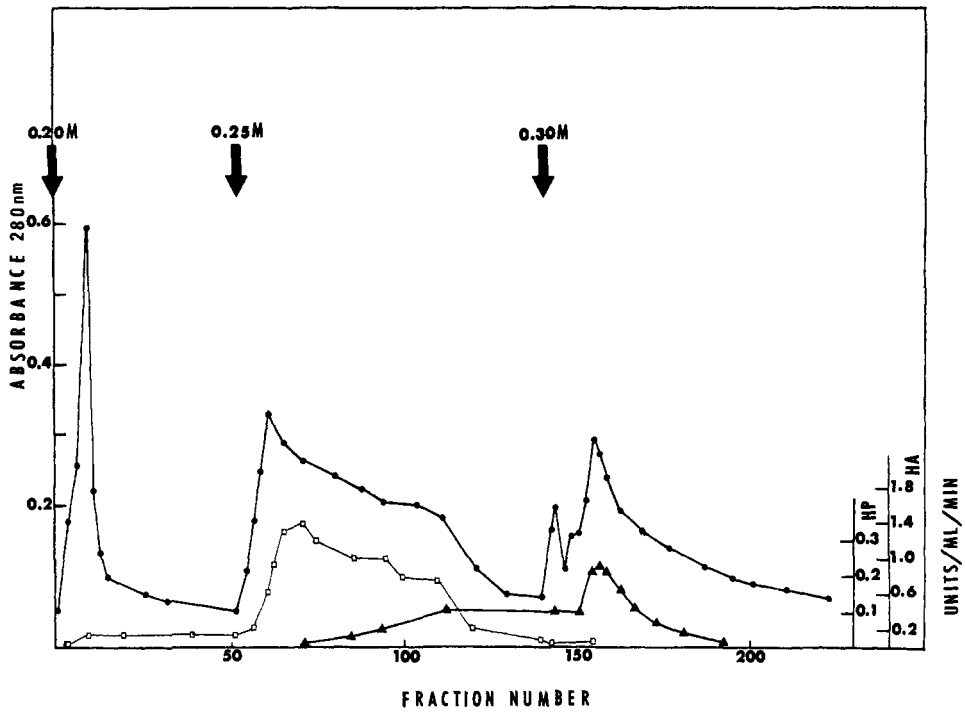

FIGURE 2: Rechromatography of shrimp carboxypeptidase fractions from step III on DEAE-Sephadex A-50. The column (1.9 × 11 cm) was equilibrated with 0.01 M Tris-HCl (pH 8) containing 0.15 M NaCl and eluted by stepwise addition of 0.2, 0.25, and 0.3 M NaCl in the same buffer. The flow rate was 31.2 ml/hr and 2.6-ml fractions were collected. Curves are designated as described in Figure 1.

were developed with the same buffer, a single component having constant specific esterase activity across the peak was obtained in each case (Figures 4 and 5). Some increase in the specific activity of each preparation over that of the applied samples was noted, particularly with the carboxypeptidase A enzyme. The active fractions from each column were pooled and used for the studies to be described below. The results of each fractionation step are summarized in Table I and represent the overall yield of carboxypeptidase A (0.7 mg) and carboxypeptidase B (8.7 mg) from 5 g of acetone powders of shrimp hepatopancreas. The final yields were based on experimentally derived extinction coefficients of the two

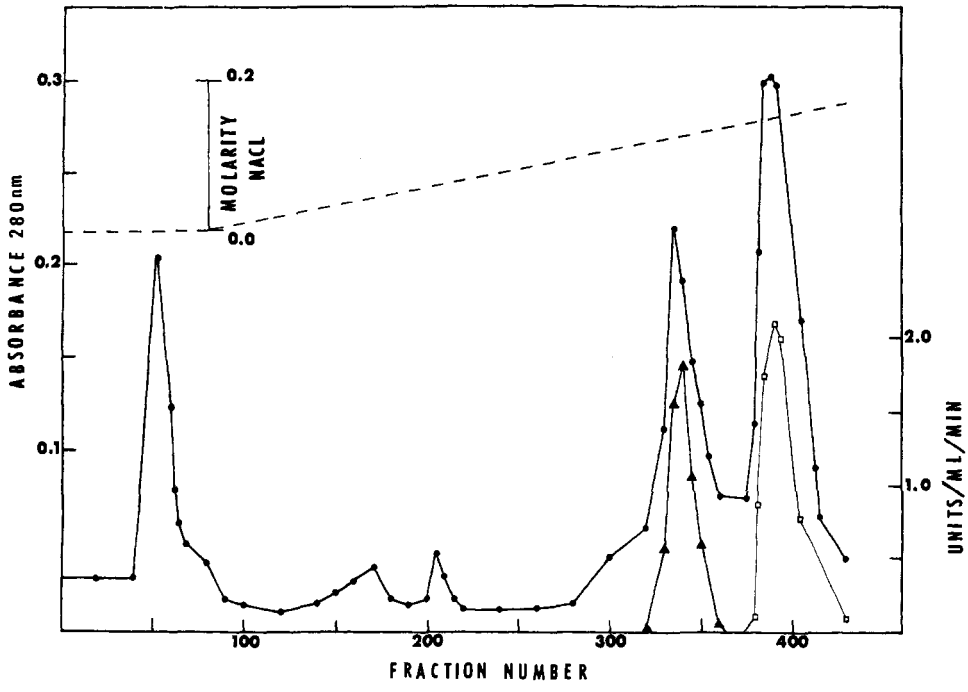

FIGURE 3: L-Arginine-Sepharose chromatography of shrimp carboxypeptidase B enriched fractions obtained from DEAE-Sephadex A-50 column. The column (1.8 × 85 cm) was equilibrated with 0.025 M Tris-HCl (pH 7.65) and eluted with a linear gradient from 0 to 0.2 M NaCl in the same buffer. The flow rate was 43 ml/hr and 3.6-ml fractions were collected. Curves are designated as described in Figure 1.

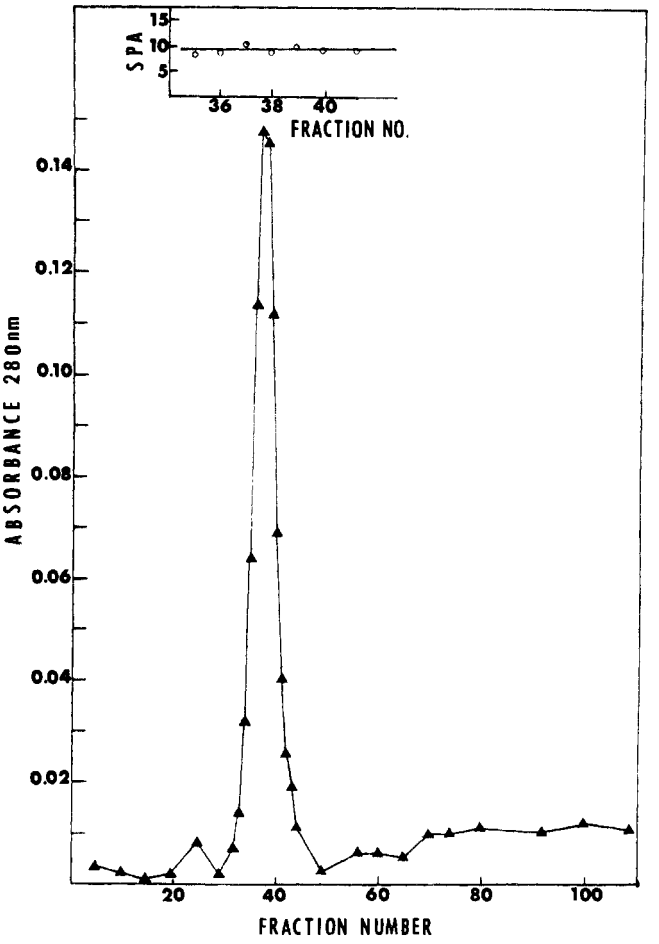

FIGURE 4: Sephadex G-75 chromatography of shrimp carboxypeptidase A fractions (4 ml). The column (4.25 × 65 cm) was equilibrated with 0.01 M Tris-HCl (pH 8.0) and developed with the same buffer. The flow rate was 30 ml/hr and 5-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (▲), left ordinate. The specific activity of each fraction is denoted above the protein peak.

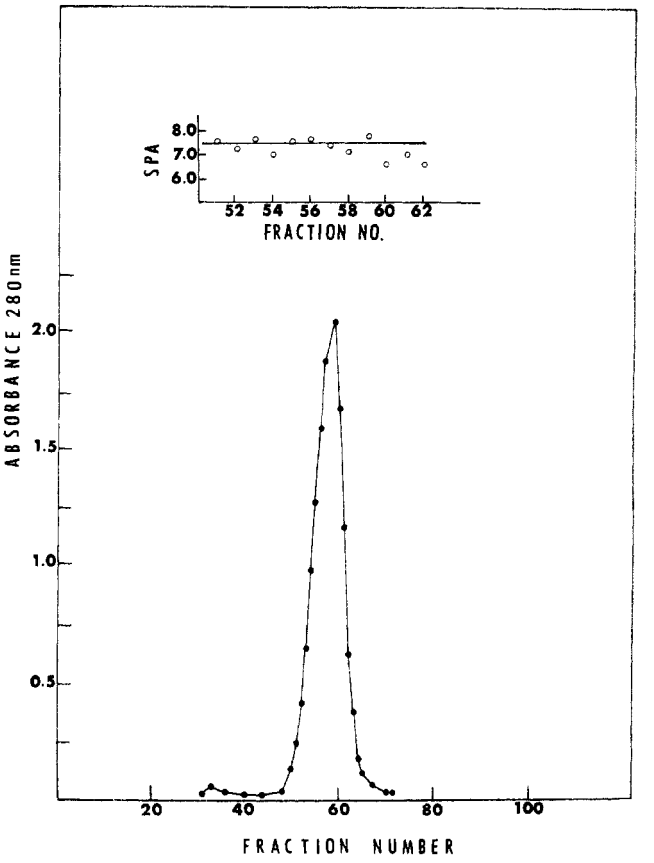

FIGURE 5: Sephadex G-75 chromatography of shrimp carboxypeptidase B fractions (6.6 ml). The column (4.25 × 65 cm) was equilibrated with 0.01 M Tris-HCl (pH 8.0) and developed with the same buffer. The flow rate was 24 ml/hr and 4-ml fractions were collected. Curves are designated as follows: optical density at 280 mμ (●), left ordinate. The specific activity of each fraction is denoted above the protein peak.

proteins ($E^{1\%}_{280\,nm}$ = 25.8 for carboxypeptidase A as determined by amino acid analysis; $E^{1\%}_{280\,nm}$ = 27.8 for carboxypeptidase B as determined using the analytical ultracentrifuge).

Homogeneity of Shrimp Carboxypeptidases A and B. Although the individual carboxypeptidases eluted from Sephadex G-75 columns with constant specific activity, indicating that the preparations were homogeneous, the purity of each enzyme was also established by other criteria.

TABLE I: Purification of Shrimp Carboxypeptidases.

Step	Carboxypeptidase A					Carboxypeptidase B				
	Total Protein (A_{280})	Total Act. (Units)	Sp Act. (Units/A_{280})	Recovery (%)	Purifcn -fold	Total Protein	Total Act. (Units)	Sp Act. (Units/A_{280})	Recovery (%)	Purifcn -fold
Extract	4389	132	0.03	100	1	4389	363	0.08	100	1
Sephadex G-75 column	591	128	0.21	97	7.0	591	363	0.61	100	7.7
First DEAE-Sephadex A-50 column	330	74	0.22	56	7.3	330	314	0.95	86	11.9
Second DEAE-Sephadex A-50 column	77.4	74	0.97	56	32.3	100	270	2.7	75	33.8
L-Arginine Sepharose column	3.8	25	6.6	19	216.7	39.1	210	5.4	58	67.5
Sephadex G-75[b] column	1.8	18	10.0	16	333.3	24.0	190	7.9	53	98.8

[a] From 5 g of acetone powder. [b] Final recoveries are based on pooled samples as described in text.

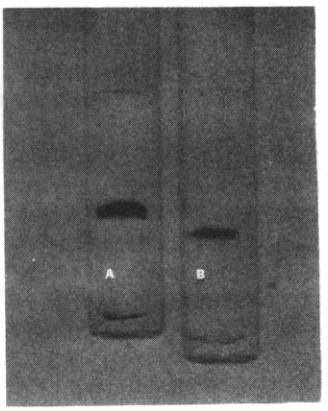

FIGURE 6: Polyacrylamide disc electrophoresis of shrimp carboxypeptidases A and B (100 μg). Conditions of electrophoresis: running pH 7.9; 15% gel. Direction of migration is from cathode (top) to anode (bottom). Patterns were obtained by staining in 1% Amido Schwarz in 75% acetic acid: (A) carboxypeptidase A; (B) carboxypeptidase B.

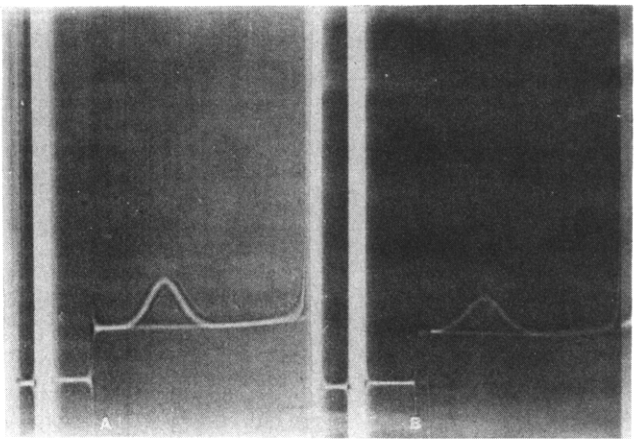

FIGURE 8: Sedimentation velocity patterns of shrimp carboxypeptidase A (4.5 mg/ml) and B (6.3 mg/ml) in 0.01 M Tris-HCl (pH 8)–0.1 M NaCl, taken 128 min after maintaining maximum speed of 60,000 rpm at 4°. Bar angle 60°. Sedimentation is from left to right: (A) carboxypeptidase A; (B) carboxypeptidase B.

Polyacrylamide Gel Electrophoresis. As shown in Figure 6, both enzymes migrated as single components during polyacrylamide gel electrophoresis in 15% gels. The use of 7.5% gels was found to be uninterpretable because partially purified preparations (after step IV) which appeared as single components in this system could be shown to be heterogeneous when 15% gels were utilized.

Sodium Dodecyl Sulfate Gel Electrophoresis. Shrimp carboxypeptidases A and B were each subjected to gel electrophoresis after incubation in sodium dodecyl sulfate solutions in order to determine both the homogeneity and molecular weights of each enzyme. In each case (Figure 7) a single component was detected. Using proteins of known molecular weights as standards, carboxypeptidase A and B preparations were estimated to have molecular weights of 26,000 and 31,000, respectively.

Analytical Ultracentrifuge Studies. Sedimentation velocity experiments in the ultracentrifuge gave single symmetrical schlieren peaks for both shrimp carboxypeptidases A and B (Figure 8). Sedimentation constants ($s_{20,w}$) of 3.20 S for carboxypeptidase A and 3.21 S for carboxypeptidase B were calculated from these data.

Molecular weights for shrimp carboxypeptidases A and B were determined by boundary depletion sedimentation equilibrium experiments. Protein concentrations of 0.3 mg/ml in 0.01 M Tris-HCl (pH 8.0)–0.1 M NaCl at 20° were used for both enzymes at rotor speed of 36,000 rpm. From the data

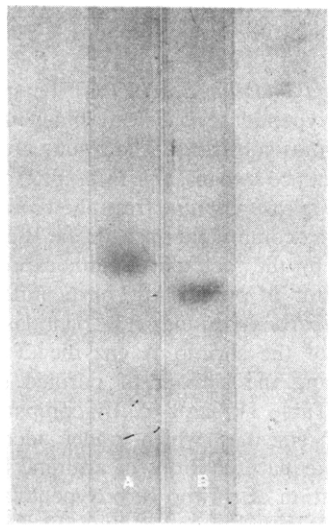

FIGURE 7: Polyacrylamide disc electrophoresis of shrimp carboxypeptidases A and B after incubation in sodium dodecyl sulfate solution. Direction of migration is from cathode (top) to anode (bottom). Gels were stained with Coomassie Brilliant Blue: (A) carboxypeptidase A; (B) carboxypeptidase B.

TABLE II: Amino Acid Composition of Shrimp Carboxypeptidases.

	Carboxypeptidase A (Residues/ 30,000 Daltons)	Nearest Integer	Carboxypeptidase B (Residues/ 34,200 Daltons)	Nearest Integer
Lys	4.2	4	11.0	11
His	6.6	7	5.5	6
Arg	9.2	9	9.0	9
Asp	32.2	32	45.2	45
Thr	20.9	21	22.3	22
Ser	25.3	25	23.3	23
Glu	19.9	20	22.9	33
Pro	14.8	15	13.5	14
Gly	24.4	24	25.8	26
Ala	22.0	22	22.0	22
Half-Cys	6.8[a]	7	4.1[a]	4
Val	17.8[c]	18	17.3[c]	17
Met	3.8[a]	4	5.0[a]	5
Ile	12.8[d]	13	15.3[d]	15
Leu	14.9	15	21.1	21
Tyr	16.1	16	20.0	20
Phe	9.2	9	10.6	11
Trp	3.1[b]	3	6.6[b]	7

[a] Determined after performic acid oxidation. [b] Determined by the method of Matsubara and Sasaki (1969). [c] 72-hr values. [d] Average of 48- and 72-hr hydrolysis.

TABLE III: Comparative Amino Acid Composition[a] of Several Carboxypeptidases.

Amino Acid	Carboxypeptidase B					Carboxypeptidase A		
	Bovine[b]	Porcine[c]	Dogfish[d]	Lungfish[e]	Shrimp[f]	Bovine[g]	Dogfish[h]	Shrimp[f]
Lys	17	18	15	15	11	15	14	4
His	7	6	4	4	6	8	7	7
Arg	13	10	14	13	9	11	14	8
Asp	26	32	31	33	45	29	27	32
Thr	26	30	25	24	22	26	21	21
Ser	26	18	25	32	23	32	25	25
Glu	24	25	20	21	23	25	29	20
Pro	12	13	14	13	14	10	17	15
Gly	21	23	20	22	26	23	30	24
Ala	22	25	24	27	22	20	21	22
Half-Cys	7	8	7	8	4	2	4	7
Val	14	11	17	13	17	16	17	18
Met	6	5	8	4	5	3	9	4
Ile	16	17	20	15	15	20	20	13
Leu	20	23	17	19	21	23	16	15
Tyr	22	20	20	22	20	19	19	16
Phe	12	12	9	12	11	16	11	9
Trp	10	9	10	8	7	7	11	3
Total	301	305	299	305	302	305	312	263

[a] Residues per molecule. [b] From Cox *et al.* (1962). [c] From Folk *et al.* (1960). [d] From Prahl and Neurath (1966). [e] From Reeck and Neurath (1972). [f] From Table II. [g] From Bradshaw *et al.* (1969). [h] From Lacko and Neurath (1970).

obtained, molecular weights of 30,000 and 34,200 were calculated for carboxypeptidases A and B, respectively. No evidence of heterogeneity could be observed in either sample during these experiments.

The molecular weights determined by this procedure were utilized in obtaining other physical and chemical properties of each carboxypeptidase.

Amino Acid Composition. The amino acid composition of shrimp carboxypeptidase A and B are presented in Table II. These data were collected by analysis of 24-, 48-, and 72-hr hydrolysates of each protein. In Table III a comparison of carboxypeptidases from other species with the shrimp enzymes is made. The low content of basic amino acids in the latter seems to be the major consistent differences in these compositions.

Metal Content. Purified preparations of shrimp carboxypeptidases A and B are, apparently, metalloenzymes since upon dialysis of each against 1×10^{-3} M *o*-phenanthroline at pH 7.0, 86% of the carboxypeptidase A, and 60% of the carboxypeptidase B esterase activity was inhibited. The most common metal found in mammalian carboxypeptidases is zinc and analysis for this element indicated significant quantities in both enzymes. Owing to the paucity of material available, other metal ions, which might also form an intrinsic part of the structure of either shrimp carboxypeptidase, were not determined.

Stability. It was found that at pH 6.0 or higher both enzymes remained stable for several months at concentrations up to 6.0 mg/ml. Below this pH rapid loss of esterase activity occurred and at very acid pH (3.0 or less) the enzymes precipitated. Although each enzyme could be resolubilized by adjustment to pH 8.0, no activity was regained.

The pH optima of shrimp carboxypeptidases A and B were determined over the pH range of 4.35–8.80 using 0.025 M sodium acetate buffers and 0.025 M Tris-HCl buffers. Both enzymes exhibited maximum esterase activity over a wide range, pH 6.0–8.0 for carboxypeptidase A, and pH 6.5–8.0 for carboxypeptidase B.

At low concentrations of protein (0.05 mg/ml) each enzyme was found to be stable for at least 1 month. Freezing and thawing also had no effect although lyophilization of salt-free samples usually resulted in partial loss of esterase activity.

Kinetic Parameters. K_m values for carboxypeptidases A and B were determined from eight different concentrations (1.0×10^{-3}–0.05 M) of substrate. The K_m for carboxypeptidase B using hippuryl-L-arginine was 2.0×10^{-4} M while that of carboxypeptidase A using hippuryl-L-phenylalanine was 3.3×10^{-4} M.

Substrate and Inhibitor Specificity. The specificity of each shrimp carboxypeptidase was determined using angiotensin and bradykinin as substrates. The results are given in Tables IV and V. As can be seen in Table IV (expt 2) carboxypeptidase A was able to release arginine from the bradykinin substrate suggesting some contamination with the B enzyme. This was substantiated by the fact that ϵ-aminocaproic acid, a competitive inhibitor of porcine carboxypeptidase B, effectively removed all carboxypeptidase B peptidase activity from preparations of the shrimp A enzyme (Table IV, expt 8) while decreasing the activity of purified shrimp carboxypeptidase B (Table IV, expt 6). By comparison of the concentration of enzymes which under identical conditions release nearly equal quantities of arginine from bradykinin, the contamination of shrimp carboxypeptidase A with the B enzyme was estimated to be less than 0.1% (Table IV, expt 7 and 9). No carboxypeptidase A releasing activity could be detected in shrimp carboxypeptidase B preparations under the experimental conditions used (Table V) indicating no cross contamination of the type described above.

TABLE IV: Amino Acids Obtained from Digestion of Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)[a] with Carboxypeptidases.[b]

Expt No.	Carboxypeptidase (nmol)	Digestion Time	Amino Acids Released (Residues/Mol)
1	Porcine B (0.5)	1 hr	Arg (0.76)
2	Shrimp B (0.5)	1 hr	Arg (1.06), Phe (trace)
3	Shrimp A (0.5)	2 hr	Arg (1.13), Phe (0.91)
4	Bovine A (1.0)	2 hr	None
5[c]	Shrimp B (0.03)	5 min	Arg (0.53)
6[c,d]	Shrimp B (0.03) with ε-amino-caproic acid	5 min	Arg (0.15)
7	Shrimp A (0.1)	5 min	Arg (0.29)
8[d]	Shrimp A (0.1) with ε-amino-caproic acid	5 min	Arg (trace)
9	Shrimp B (0.001)	5 min	Arg (0.31)

[a] Dayhoff (1969). [b] Conditions of digest; 34 nmol of bradykinin in 0.5 ml of 0.2 M *N*-ethylmorpholineacetic acid (pH 8.5) were incubated with enzyme at 37°. [c] Incubated at 4°. [d] Inhibitor: substrate ratio, 20:1.

TABLE V: Amino Acids Obtained from Digestion of Angiotensin (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)[a] with Carboxypeptidases.[b]

Carboxypeptidase	Amino Acids Released (Residues/Mol)
Bovine A	Phe (0.78)
Shrimp A (after step V)	Phe (0.93), Tyr (0.86), Val (0.80)
Shrimp A (after step VI)	Phe (1.10)
Bovine B	None
Shrimp B	None

[a] Dayhoff (1969). [b] Conditions of digest: 20 nmol of angiotensin and 5 nmol of enzyme in 0.5 ml of 0.2 M *N*-ethylmorpholineacetic acid (pH 8.5) were incubated for 10 hr at 37°.

It should be noted that if purified preparations of shrimp carboxypeptidase A were not subjected to Sephadex G-75 chromatography (step VI of the purification), endopeptidase activity could be detected as judged by the release of both tyrosine and valine from digests of angiotensin with such preparations (Table V). Monitoring of the column for the contaminating endopeptidase activity, using casein as substrate, failed to detect this protein. However, it is probably the "low molecular weight protease" referred to by Linke *et al.* (1969), since it could be removed by gel filtration chromatography (step VII).

β-Phenylpropionic acid was found to be a potent inhibitor of shrimp carboxypeptidase A ($K_i = 1.8 \times 10^{-4}$ M). A similar inhibitory effect was noted when ε-aminocaproic acid and shrimp carboxypeptidase B were mixed ($K_i = 1.7 \times 10^{-4}$ M). In addition, shrimp carboxypeptidase B was inhibited by very high concentrations of β-phenylpropionic acid.

Discussion

The molecular weight of shrimp carboxypeptidase B calculated by ultracentrifugation experiments is essentially the same as that determined for other species while that for carboxypeptidase A is slightly lower. In all gel filtration experiments carboxypeptidase B and carboxypeptidase A activities could be partially resolved indicating differences in the molecular weights of each protein. The molecular weights calculated by electrophoresis after treatment with sodium dodecyl sulfate were substantially lower than those determined by ultracentrifugation. These values seem likely to be in error since shrimp trypsin, in similar experiments, migrated as a protein component of mol wt 13,500. Presumably, the very acidic nature of these proteins interferes with detergent binding and subsequent electrophoresis as has been suggested by Nelson (1971).

The carboxypeptidases isolated from the shrimp bring to a total of three the number of peptidases found in this organism which function at neutral or alkaline pH and yet are highly acidic in character. The amino acid compositions of the carboxypeptidases, given in Table III, suggest that the major difference between the shrimp enzymes and those from other species are in the relative content of basic amino acids. Many of these differences are probably simple replacements in the primary structure of these proteins of basic residues in higher organisms for acidic residues in the shrimp enzymes.

The fact that two carboxypeptidases have been isolated from the shrimp refutes the possibility that divergence of carboxypeptidases A and B occurred after the crustaceans had evolved. On the contrary, it suggests that evolution of these two enzymes from a single precursor probably occurred long before the arthropods, since nearly all of the properties of the shrimp exopeptidases clearly associate them with similar enzymes in mammals.

Physiologically, it has been suggested that the shrimp is a coprophagous organism, digesting some of the fecal products produced by the oyster. In such a case the requirements for a full complement of proteolytic enzymes, including chymotrypsin, would be obviated if predigestion of nutrients ingested by the shrimp had occurred. Preliminary experiments, however (Gates, 1972), indicate that several diisopropyl fluorophosphate insensitive proteolytic enzymes with alkaline pH optima are present in crude extracts of shrimp hepatopancreas. Until these enzymes are isolated and fully characterized protein digestion processes in this organism will remain unclear.

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Interaction of a Spin-Labeled Analog of Acetyl Coenzyme A with Citrate Synthase. Paramagnetic Resonance and Proton Relaxation Rate Studies of Binary and Ternary Complexes†

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ABSTRACT: A spin-labeled analog of acetyl coenzyme A (CoA), R·CoA (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA thio ester), has been prepared and shown, by magnetic resonance techniques, to bind to pig heart citrate synthase. Paramagnetic resonance studies indicated that the bound R·CoA was "partially immobilized," with a dissociation constant of 10^{-4} M and 2.2 mol bound per enzyme dimer. The bound radical enhanced the longitudinal relaxation rate of water protons by a factor of 3.4. Acetyl-CoA and CoASH displaced R·CoA from the enzyme. The dissociation constants for both, as shown by competition studies, were approximately equal to that of R·CoA. Paramagnetic resonance and proton relaxation rate studies showed that oxaloacetate formed a ternary complex, R·CoA–enzyme–oxaloacetate. The affinity of the enzyme for R·CoA was not affected by oxaloacetate binding, but the paramagnetic resonance spectrum of the bound radical changed to one of a spin-labeled substrate covalently bound to chymotrypsin by electron paramagnetic resonance. Weiner (1969) and Mildvan and Weiner (1969a,b) studied the noncovalent interaction of a spin-labeled analog of NAD^+ with alcohol dehydrogenase by paramagnetic resonance and by measurements of the relaxation rates of water and substrate protons. The information that may be obtained by such studies includes: (1) determination of the number of binding sites and the binding constant of the analog; (2) detection of ternary complexes of the analog with enzyme substrates or substrate analogs, and their thermodynamic properties; and (3) kinetic and structural information concerning binary and ternary complexes containing the analog.

This paper concerns the application of these techniques to characteristic of a "fully immobilized" nitroxide. The enhancement of the relaxation rate of water protons by the bound radical in the ternary R·CoA–enzyme–oxaloacetate complex increased by about 40% over that in the R·CoA–enzyme complex. From a large series of mono-, di-, and tricarboxylic analogs of oxaloacetate and citrate, ternary R·CoA–enzyme–ligand complexes were observed only with citrate, (*R*)-malate, and (*R*,*S*)-tartrate by the paramagnetic resonance method. The paramagnetic resonance spectra of the bound R·CoA in these ternary complexes were similar and slightly more "fully immobilized" than that in the oxaloacetate–enzyme–R·CoA system. The dissociation constants of citrate, (*R*)-malate, and (*R*,*S*)-tartrate from their corresponding ternary complexes were 0.5, 0.4, and ~2 mM, respectively. Stereochemical arguments are presented to account for the selectivity of R·CoA–enzyme–ligand complex formation.

Probing active sites of enzymes by magnetic resonance techniques using paramagnetic ions or spin-labeled substrate analogs has been exploited by several groups (Mildvan and Cohn, 1970). McConnell (1967) first investigated the motion of a spin-labeled substrate covalently bound to chymotrypsin by electron paramagnetic resonance. Weiner (1969) and Mildvan and Weiner (1969a,b) studied the noncovalent interaction of a spin-labeled analog of NAD^+ with alcohol dehydrogenase by paramagnetic resonance and by measurements of the relaxation rates of water and substrate protons. The information that may be obtained by such studies includes: (1) determination of the number of binding sites and the binding constant of the analog; (2) detection of ternary complexes of the analog with enzyme substrates or substrate analogs, and their thermodynamic properties; and (3) kinetic and structural information concerning binary and ternary complexes containing the analog.

This paper concerns the application of these techniques to

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