Pancreatic Enzymes of the Spiny Pacific Dogfish. II. Procarboxypeptidase B and Carboxypeptidase B*

James W. Prahl† and Hans Neurath

ABSTRACT: Procarboxypeptidase B has been isolated from acetone powders of pancreas glands of the spiny Pacific dogfish, *Squalus acanthias*, and purified by chromatographic procedures.

The zymogen has been characterized by determinations of molecular weight (44,500), end-group analysis, and amino acid composition. Activation by bovine trypsin yields carboxypeptidase B which has been crystallized and characterized by chemical and enzymatic procedures. The enzyme (molecular weight 34,500) resembles bovine and porcine carboxypeptidase B in amino acid composition, and like the bovine enzyme, dogfish carboxypeptidase B also hydrolyzes substrates for carboxypeptidase A.

In the preceding paper of this series (Prahl and Neurath, 1966), it has been reported that the pancreas of the spiny Pacific dogfish contains zymogens of proteolytic enzymes analogous to those found in the bovine. One of these, cationic chymotrypsinogen, has been isolated, purified, and characterized by chemical and enzymatic procedures. The present paper is concerned with the anionic pancreatic enzymes of the dogfish, particularly with procarboxypeptidase B and the product of its activation, carboxypeptidase B. These proteins have been chosen because they can be isolated in pure form with relative ease and because they lend themselves to a detailed comparison with analogous proteins in the bovine and porcine pancreas, respectively. This investigation has led to the isolation of procarboxypeptidase B and its chemical characterization and activation, and also reports certain molecular properties of the product of activation, carboxypeptidase B.

Experimental Section

Materials and Methods. Most of these have been described in detail in the preceding paper of the series, and hence will not be repeated here. Special methods not previously used will be described where they have been applied. The enzymatic activity of carboxypeptidase B was determined by the hydrolysis of BGA[1] as substrate, using either the spectrophotometric method of Folk *et al.* (1960) or the development of ninhydrin color (Matheson *et al.*, 1961) to measure the progress of hydrolysis. Carboxypeptidase A activity was determined using HPLA[1] (Bargetzi *et al.*, 1963) or CGP[1] (Putnam and Neurath, 1946) as substrates. Activity is expressed as per cent hydrolysis per minute (units) per milliliter, or as units per absorbance unit of 1.0 at 280 mμ. Specific activity is expressed as units per milligram of protein.

Results

Isolation and Purification of Dogfish Procarboxypeptidase B. The starting material was an aqueous extract of acetone powder of dogfish pancreatic tissue identical with that used for the isolation and purification of cationic chymotrypsinogen A. In a typical preparation, 100 g of acetone powder was extracted with 1 l. of cold distilled water and after 4 hr the insoluble material was removed by centrifugation and the supernatant solution was adjusted to pH 7.5–8.0 by addition of 1 N NaOH and to 10^{-3} M DFP by the addition of a 1 M solution in isopropyl alcohol. Solid ammonium sulfate was then added to 70% saturation at room temperature. After standing for 4 hr, the precipitate was collected by centrifugation, redissolved in a small volume of water, and dialyzed against 0.005 M potassium phosphate buffer, pH 7.0. The solution was then pumped onto a column of DEAE-Sephadex (5 × 70 cm) which had been equilibrated with the same buffer. After the breakthrough fractions had been collected, the anionic proteins were eluted by the development of a linear gradient from 0.005 M potassium

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† Taken in part from a dissertation submitted by J. W. Prahl to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Immunology, Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England. Aided by a grant for a postdoctoral fellowship from the American Cancer Society.

[1] The following abbreviations are used: BGA, benzoylglycyl-L-arginine; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-β-DL-phenyllactate; STI, soybean trypsin inhibitor; DFP, diisopropylphosphorofluoridate; FDNB, fluoro-2,4-dinitrobenzene; PTH, phenylthiohydantoin derivative.

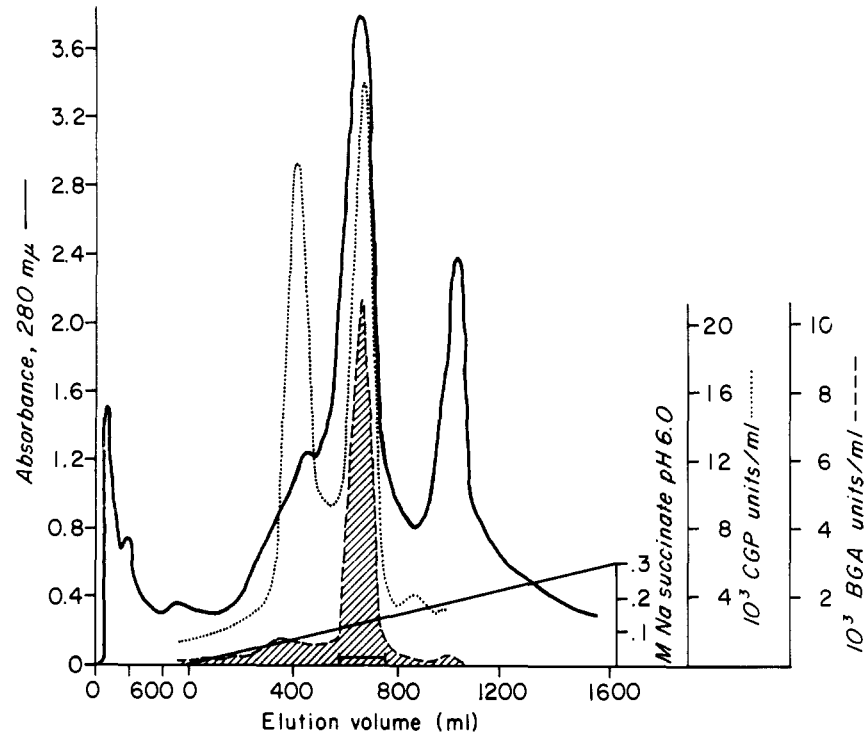

FIGURE 1: Chromatography on DEAE-Sephadex A-50 of an extract of acetone powder of dogfish pancreas. Details are given in the text. The symbols refer as follows: ———, absorbance at 280 mμ; • • • •, activity toward CGP; – – – –, activity toward BGA. The proteins were applied to the column in 0.005 M potassium phosphate buffer, pH 7.0, and a linear gradient developed with sodium succinate buffer, pH 6. The bracketed fractions were pooled and lyophilized for further purification. The shaded area represents total potential carboxypeptidase B activity.

phosphate, pH 7.0, to 0.3 M sodium succinate, pH 6.0. Figure 1 illustrates the results of such a chromatographic analysis in terms of protein (A_{280}) and exopeptidase activity, the latter measured after activation by trypsin. It will be noted that activities characteristic of bovine carboxypeptidases A and B are present in this system and that the two are well separated from each another. As in the case of the bovine, the fractions active toward BGA also show activity toward CGP.

The peak fractions containing the zymogen analogous to bovine procarboxypeptidase B were pooled (see Figure 1), and after the addition of 20 mg of STI[1,2] to inhibit tryptic activity, the solution was lyophilized. For further purification, the lyophilized powder was dissolved in 0.1 M potassium phosphate, pH 8.0, the pH was adjusted from 7.5 to 8.0 with 1 N NaOH, and DFP[1] was added to a concentration of 10^{-2} M. After equilibration against 0.005 M potassium phosphate buffer, pH 7.0, the solution was pumped onto a DEAE-cellulose column (3 × 40 cm) which had been equilibrated with the same buffer. A linear gradient of NaCl (0–0.2 M) was developed in the same buffer. The elution profile of the protein and carboxypeptidases A and B activities are shown in Figure 2. The fractions marked by the bracket in Figure 2 were pooled, again adjusted to 10^{-2} M DFP, and after dialysis against 0.005 M phosyhate buffer, pH 7.0, adsorbed directly onto a column of DEAE-cellulose. The results of the third and fourth chromatography on DEAE-cellulose under the same conditions are shown in Figures 3A and B. Nonlinear gradients were developed by the use of a varigrad (Peterson and Sober, 1960). Beyond the fourth chromatography, the specific activities of the fractions of the leading and trailing edges increased somewhat, but not the specific activity of the peak fraction itself.

Molecular Characterization of Procarboxypeptidase B. The purified material obtained after four chromatographies as described herein was subjected to characterization by the methods described below.

SEDIMENTATION ANALYSIS at 4–6° in a 0.1 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl revealed the presence of a single symmetrical boundary. The plot of the sedimentation coefficient against protein concentration gave a straight line of negative slope which extrapolated to $s_{20,w}$ = 3.67 S at zero protein concentration.

The molecular weight of the zymogen was estimated by sedimentation equilibrium analysis employing Rayleigh interference optics. Measurements were carried out in 0.1 M potassium phosphate buffer, pH 7.8, at

[2] As demonstrated in the preceding paper of this series, trypsinogen occurs as an anionic protein and is found in the ultimate peak shown in Figure 1.

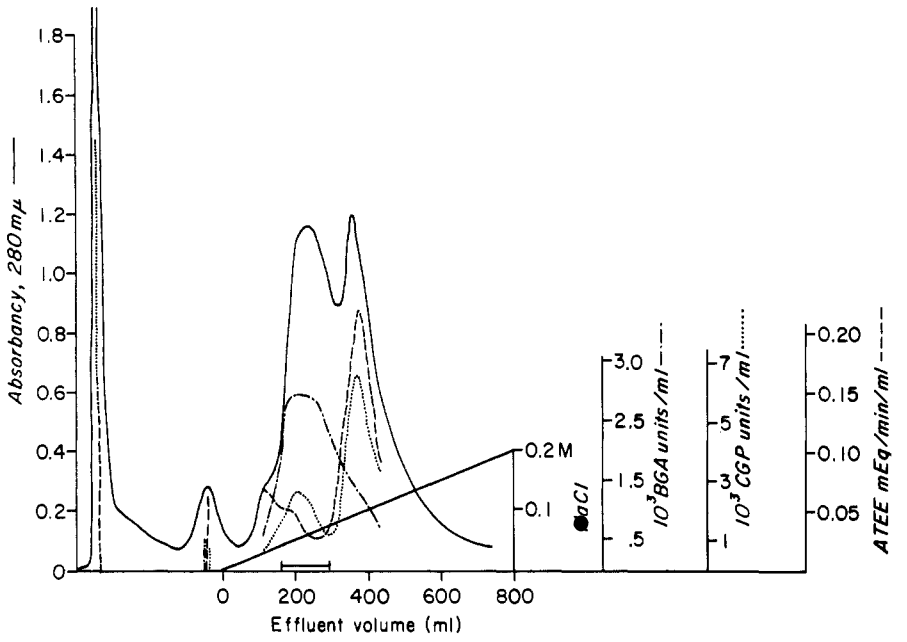

FIGURE 2: Chromatography on DEAE-cellulose of the pooled fractions of procarboxypeptidase B obtained from first chromatography of the extract on DEAE-Sephadex. Details are given in the text. The symbols refer as follows: ———, absorbance at 280 mμ; - · - ·, activity toward BGA; · · · ·, activity toward CGP; - - - -, activity toward ATEE. The protein was applied in 0.005 M potassium phosphate buffer, pH 7.0, and a linear gradient developed with NaCl. The fractions included in the bracket were pooled and concentrated for further purification.

5–7° at 6569 rpm. A plot of $1/M_w$ against protein concentration (Kegeles *et al.*, 1957) indicated that the apparent molecular weight decreased as the protein concentration approached zero, yielding an extrapolated value for the molecular weight of 41,000–42,000. At intermediate concentrations corresponding to M_w/M_z ratios approaching 1, the molecular weight was 44,000–45,000 (Table I).

TABLE I: Molecular Weights Obtained by Sedimentation Equilibrium Analysis.

Protein (mg/ml)	M_w[b] ($\times 10^{-3}$)	M_z[c] ($\times 10^{-3}$)	M_w/M_z
	Procarboxypeptidase B[a]		
12.83	49.4	67.3	0.74
6.32	45.2	54.9	0.82
6.21	44.1	46.0	0.96
4.25	45.0	54.1	0.83
3.32	36.8	47.2	0.79
2.13	33.2	46.0	0.72
	Carboxypeptidase B[d]		
3.62	36.7	50.6	0.73
2.27	37.4	40.4	0.92
4.30	37.0	65.6	0.57
4.30	35.0		

[a] Measurements in potassium phosphate buffer, $\Gamma/2 = 0.1$, pH 7.78. [b] M_w = apparent weight-average molecular weight. [c] M_z = z-average molecular weight. [d] Measurements in 0.01 M Tris–HCl–0.5 M NaCl buffer, pH 8.0.

ABSORBANCY INDEX. An absorbancy index of 19.0 ± 0.2 was determined by relating absorbance at 280 mμ to concentration of the zymogen as measured under the area of a schlieren pattern obtained in the ultracentrifuge, assuming a refractive index increment of 0.001850.

MOVING-BOUNDARY ELECTROPHORESIS. After 535 min of moving-boundary electrophoresis in the Spinco Mod 1 H electrophoresis apparatus at 1° in potassium phosphate buffers, ionic strength 0.1, pH 8.0, more than 95% of the protein remained under a single peak.

AMINO ACID COMPOSITION. Amino acid analysis was performed on the hydrolysates of a stock solution of the purified zymogen, using norleucine as internal standard (Walsh and Brown, 1962). These measurements were performed as described in the preceding communication of this series. Half-cystine was determined as cysteic acid (Moore, 1963) and tryptophan spectrophotometrically (Bencze and Schmid, 1957). The data are presented in Table II, in which the extrapolated values are also expressed as residues per mole assuming a molecular weight of 44,500. After correcting for 96% recovery of total protein and using the nearest integral number of amino acid residues, a molecular weight of 44,406 was calculated.

END-GROUP ANALYSIS. The amino-terminal residue of the zymogen was identified by reaction with FDNB (Porter, 1957). The DNP-amino acids found in the

TABLE II: Amino Acid Composition of Dogfish Procarboxypeptidase B.

Amino Acid	Residues/ 10^5 g of Protein	g Residue/ 10^5 g Protein[c]	Minimal Mol Wt	Residues/ 44,500 g Protein[c]	Nearest Integer/ 44,500 g	Nearest Integer × Minimal Mol Wt	Integral No. × Mol Wt of Residue
Alanine	67.4	4,792	1,425	31.2	31	44,175	2,204
Arginine	42.7	6,670	2,249	19.8	20	44,980	3,124
Aspartic acid	98.4	11.326	979	45.5	46	45,034	5,295
Glutamic acid	76.0	9.812	1,265	35.1	35	44,275	4,519
Glycine	57.3	3,272	1,683	26.4	26	43,758	1,485
Histidine	11.6	1,592	8,253	5.4	5	41,265	686
Isoleucine	51.9	5,875	1,859	23.9	24	44,616	2,717
Leucine	53.6	6,068	1,792	24.8	25	44,800	2,830
Lysine	46.5	5,961	2,076	21.4	21	43,596	2,692
Methionine[a]	17.6	2,309	5,486	8.1	8	43,888	1,050
Phenylalanine	28.2	4,151	3,397	13.1	13	44,161	1,914
Proline	37.1	3,602	2,601	17.1	17	44,217	1,651
Serine	58.9	5,130	1,634	27.2	27	44,118	2,352
Threonine	60.0	6,066	1,606	27.7	28	44,968	2,831
Tyrosine	41.9	6,838	2,295	19.4	19	43,605	3,101
Valine	58.1	5,758	1,659	26.8	27	44,793	2,676
Half-cystine[a]	18.9	1,930	5,095	8.7	9	45,855	919
Tryptophan[b]	28.1	5,232	3,420	13.0	13	44,460	2,421
		96,251			394	44,254 ± 605	44,406

[a] Determined as methionine sulfone and cysteic acid after performic acid oxidation (Moore, 1963). [b] Determined spectrophotometrically according to Bencze and Schmid (1957). [c] Recovery corrected.

ether-extractable phase of a 16-hr hydrolysate of the dinitrophenyl (DNP) protein in 5.7 N redistilled HCl are given in Table III. The major DNP-amino acid is glutamic or aspartic acid, respectively, with a smaller amount of DNP-glycine. Since the recovery of the latter was less when hydrolysis was carried out for 4 hr in 12 N HCl, this material was considered to be an artifact or possibly due to a DNP peptide. The material corresponding to DNP-glutamic or -aspartic acid was rechromatographed in pH 5.0 phthalate buffer (Fraenkel-Conrat *et al.*, 1955) and found to contain only DNP-glutamic acid. The nature of this amino-terminal residue of procarboxypeptidase B was confirmed by the phenylhydantoin method of Edman (1956). Chromatography of the PTH residues in heptane–ethylene chloride–75% formic acid (30:60:5) yielded clearly PTH-glutamine.

TABLE III: Ether-Soluble DNP-Amino Acids Isolated from DNP-Dogfish Procarboxypeptidase B.

Protein (mg)	12.8	9.45
HCl (N)	5.7	12
Hydrolyzed (hr)	16	4
DNP-Glu/Asp	0.438	0.198
DNP-Serine	0.007	0.014
DNP-Glycine	0.104	0.042
DNP-Alanine	0.021	0.024
DNP-Val/Leu	0.031	0

Recoveries reported in moles/44,500 g of protein, uncorrected.

Activation of Procarboxypeptidase B. Procarboxypeptidase B, purified as just described, is rapidly activated by bovine trypsin at 0° in the pH range of 7.0–8.0. Figure 4 shows the progress curves of activation of a 0.5% solution of the zymogen in 0.1 M potassium phosphate buffer, pH 8, at zymogen:trypsin ratios of 10:1 and 1000:1, respectively. At the higher trypsin concentration, maximum activity toward BGA was attained within 20 min. Activity measurements toward HPLA, a substrate for carboxypeptidase A, gave similar relationships.

When aliquots of the activation mixture were subjected to sedimentation analysis in the ultracentrifuge at 59,780 rpm, the sedimentation coefficient ($s_{20,w}$) decreased from 3.65 to 3.52 S after 2 hr and the same value was observed after 24 hr of activation. The product of activation appeared to be less soluble at

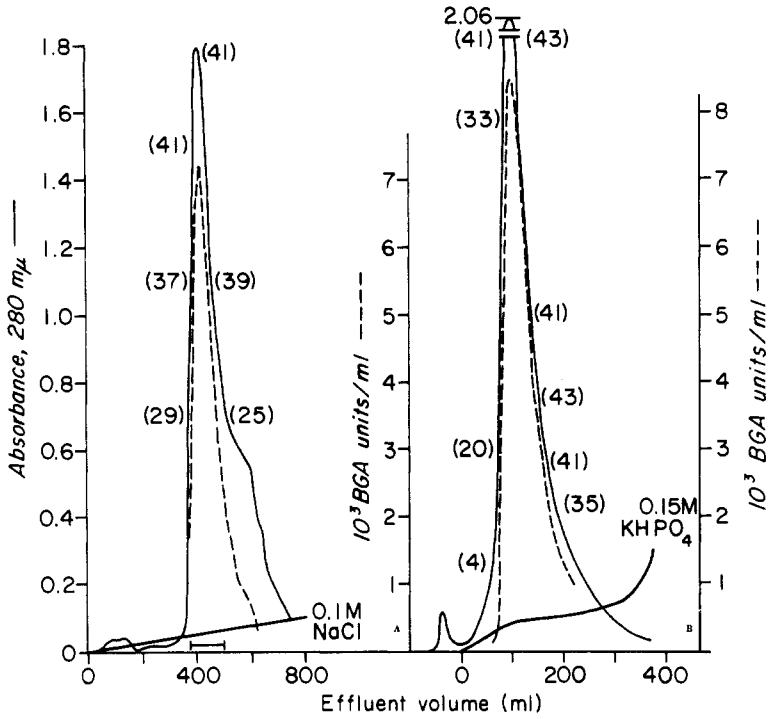

FIGURE 3: Second (left) and third (right) chromatography on DEAE-cellulose of dogfish procarboxypeptidase B. The conditions of the second chromatography were similar to those in Figure 2, except that the pH was increased from 7.0 to 8.0. The third chromatography was similar to the second except that elution was accomplished by use of a nonlinear gradient developed with potassium phosphate buffer, pH 8 (0.005–0.15 M). The figures in parentheses denote activities of the fractions, after activation, toward BGA (expressed as units per absorbance unit of 1.0 at 280 mμ)

pH 8.0 in solutions of low ionic strength than the zymogen. Thus, if after 24 hr of activation (5 mg of zymogen and 0.5 mg of trypsin/ml) the solution was dialyzed against 0.005 M potassium phosphate buffer, pH 8, crystallization of the enzyme ensued. These crystals shown in Figure 5 were readily soluble in NaCl solutions above 0.2 M. A second crystallization of the enzyme did not increase the specific activity toward BGA and such material was, therefore, used for further characterization.

Characterization of Carboxypeptidase B. The molecular weight of the crystalline enzyme was estimated by sedimentation equilibrium analysis in 0.01 M Tris–HCl buffer, pH 8, containing 0.5 M NaCl. The partial specific volume of 0.735 was assumed for the calculations given in Table I. The apparent weight-average molecular weight varied from 35,000 to 37,000.

The amino acid analysis of the crystalline enzyme was performed on aliquots of a solution containing 3.06 mg of protein/ml, in the manner described for the zymogen. The results are given in Table IV. Composition is expressed on the basis of a molecular weight of 34,000. When these data are compared to those for procarboxypeptidase B (Table II), it is seen that the enzyme contains one more tyrosine residue than the zymogen. This inconsistency may have resulted from excessive destruction of tyrosine during hydrolysis of the zymogen.

End-Group Analysis. The amino-terminal residue of the enzyme was identified by use of FDNB. To this end, 6.30 mg of two-times-crystallized carboxypeptidase B was taken up in 1 ml of 8 M urea containing 0.1 M β-phenylpropionate. After the addition of 50 mg of solid sodium bicarbonate, the protein was allowed to react with FDNB for 4 hr at room temperature and the DNP protein was precipitated by the addition of 9 ml of 5% trichloracetic acid. The precipitate of DNP protein was washed and hydrolyzed in 5.7 N redistilled HCl at 104° for 16 hr. The ether-extractable phase was found to contain 0.082 μmole of DNP-serine (0.44 mole/34,000 g of protein uncorrected) and traces of DNP-glycine, -alanine, and -glutamic or -aspartic acids. Only ε-DNP-lysine was found in the aqueous phase.

To determine the carboxyl-terminal residues of carboxypeptidase B, digestion with bovine carboxypeptidase A was attempted. In order to minimize autolysis during end-group analysis, dogfish carboxypeptidase B was first denatured by the method of Ando *et al.* (1959). To this end, 0.2% solution of the enzyme containing 0.2% sodium dodecyl sulfate and 0.005 M 1,10-phenanthroline was adjusted to pH 7.5–8.0 with sodium hydroxide and held at room temperature for 4 hr. During this time, the initially opalescent solution became water clear. The solution was then dialyzed overnight against 0.5 M NaCl and 0.1 M potassium

TABLE IV: Amino Acid Composition of Crystalline Dogfish Carboxypeptidase B.

	Residues/ 10^5 g Protein	g Residue/ 10^5 g Protein	Minimal Mol Wt	Residues/ 34,000 g Protein	Nearest Integer/ 34,000 g	Nearest Integer × Minimal Mol Wt	Integral No. × Mol Wt of Residue
Alanine	70.2	4,991	1,410	23.9	24	33,624	1,706
Arginine	40.6	6,342	2,438	13.8	14	34,132	2,187
Aspartic acid	91.8	10,566	1,078	31.2	31	33,418	3,586
Glutamic acid	60.0	7,746	1,649	20.4	20	32,980	2,582
Glycine	58.2	3,323	1,701	19.8	20	34,020	1,141
Histidine	12.1	1,660	8,180	4.1	4	32,720	549
Isoleucine	57.6	6,520	1,718	19.6	20	34,360	2,263
Leucine	49.7	5,626	1,991	16.9	17	33,847	1,924
Lysine	42.9	5,500	2,307	14.6	15	34,605	1,923
Methionine	24.1	3,162	4,107	8.2	8	32,856	1,050
Phenylalanine	27.1	3,989	3,652	9.2	9	32,868	1,325
Proline	40.9	3,971	2,420	13.9	14	33,880	1,360
Serine	73.2	6,376	1,352	24.9	25	33,800	2,177
Threonine	74.1	7,492	1,336	25.2	25	33,400	2,528
Tyrosine	57.6	9,400	1,718	19.6	20	34,360	3,262
Valine	49.4	4,896	2,004	16.8	17	34,068	1,686
Half-cystine[a]	21.2	2,165	4,669	7.2	7	32,683	722
Tryptophan[b]	28.2	5,251	3,510	9.6	10	35,100	1,862
		98,976			300	33,707 ± 512	33,816

[a] Determined as cysteic acid after performic acid oxidation (Moore, 1963). [b] Determined spectrophotometrically according to Bencze and Schmid (1957).

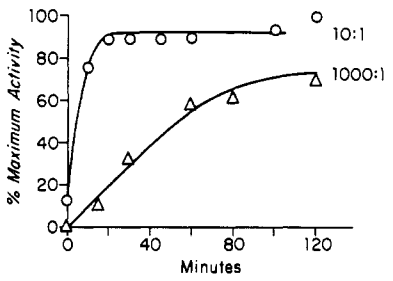

FIGURE 4: Activation of dogfish procarboxypeptidase B by bovine trypsin at 0°. The zymogen concentration was 5 mg/ml. Mole ratios of procarboxypeptidase B to trypsin were 10:1 (○) and 1000:1 (△). Activity toward BGA was measured to follow the course of activation.

phosphate buffer, pH 8. The dialyzed protein was then digested at room temperature with bovine carboxypeptidase A (which had been previously treated with 10^{-3} M DFP) using a substrate:enzyme weight ratio of 60:1. After suitable times, aliquots were removed, the pH was lowered with glacial acetic acid, and the solution was passed through a column of Dowex 50 (0.5 × 6 cm, hydrogen form). The amino acids were eluted with 1 N NH_4OH, taken to dryness in an evaporator, separated (Richmond and Hartley, 1959), and quantitated (Connell *et al.*, 1955). The results are shown in Table V. Digestion with bovine carboxypeptidase A was also performed on performic acid oxidized dogfish carboxypeptidase B (Moore, 1963), at room temperature, at a substrate:enzyme ratio of 70:1, for 2 hr. Quantitation of the free amino acids was performed in the same manner, and the results are also included in Table V.

It is evident from these data that the rates of release of carboxyl-terminal residues of denatured dogfish enzyme by bovine carboxypeptidase A were not sufficiently differentiated to permit the assignment of a specific sequence to the carboxyl-terminal region of the protein. Attempts to repeat the digestion by removing aliquots after shorter periods of time did not improve the resolution in this regard. The data suggest but do not prove that leucine is the carboxyl-terminal amino acid residue of dogfish carboxypeptidase B.

For comparison, heat-denatured procarboxypeptidase B (prepared by rapidly bringing a solution of the protein at neutral pH to 100° and holding there for 10 min) was digested at room temperature with DFP-treated bovine carboxypeptidase A (substrate:enzyme weight ratio 100:1). The liberated amino acids were identified and quantitated as just described for carboxypeptidase B. The amounts liberated after 120 min of digestion are included in Table V. The identity of the amino acids released by the zymogen and by the enzyme

TABLE V: Amino Acids Released from Dogfish Carboxypeptidase B and Procarboxypeptidase B by Bovine Carboxypeptidase A.[a]

	Carboxypeptidase B				Procarboxypeptidase B
	Denatured[b]			Performic Acid Oxidized	
Time (min)	20	40	60	60	120
Leucine	0.33	0.66	0.87	0.83	0.86
Valine	0.10	0.21	0.63	0.57	0.80
Tyrosine	0.20	0.23	0.80	0.11	0.82
Alanine	0.20	0.57	0.74	0.58	0.36
Glycine	0.20	0.40	0.47	0.17	0.38
Serine	0.10	0.22	0.40	0.39	0.54
Threonine	0.10	0.25	0.38	0.39	0.40

[a] Reported as moles of amino acid found per 34,000 and 45,000 g for the enzyme and zymogen, respectively. [b] Denatured by the method of Ando *et al.* (1959).

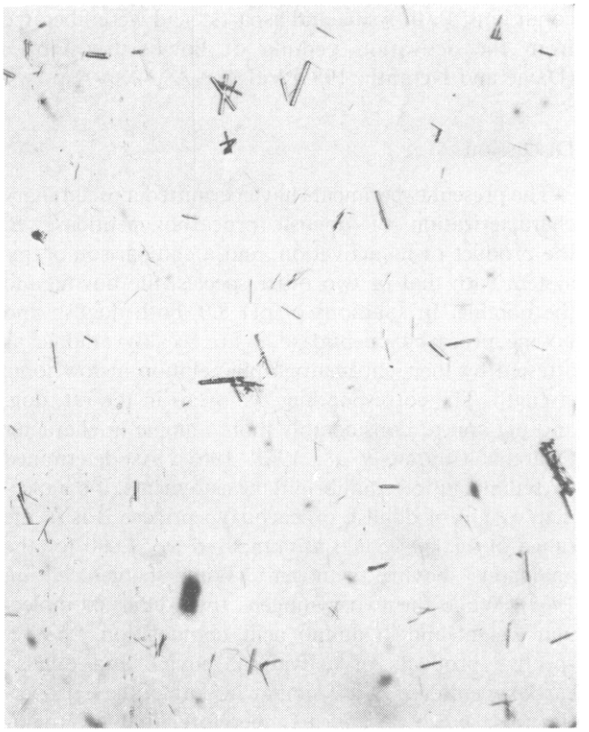

FIGURE 5: Crystalline habit of dogfish carboxypeptidase B.

was also proven by "fingerprinting." Separation was carried out by high-voltage electrophoresis at pH 3.1 in one direction and by chromatography on butanol–acetic acid–water (4:1:5) (Richmond and Hartley, 1959) in the other. The chromatograms were found to be completely identical, indicating that bovine carboxypeptidase A had released the same amino acids from the zymogen and the active enzyme. Since, moreover, the amino-terminal residue of the zymogen is glutamine and that of the enzyme serine, it appears that, as in the case of the bovine, activation of dogfish procarboxypeptidase B involved the release of a peptide from the amino-terminal region of the zymogen and that the carboxyl-terminal sequences remain the same.

Taking into account the known specificity of bovine trypsin, it seemed most likely that activation of the dogfish procarboxypeptidase B involved hydrolysis of a peptide bond at the carboxyl group of a basic amino acid residue. The specificity of the carboxypeptidase B formed in the activation mixture was employed in an attempt to establish the identity of this residue (Cox *et al.*, 1962). After 10 min, the activation of 5 mg of the dogfish zymogen (in 1.0 ml of 0.1 M potassium phosphate, pH 8.0) by 0.5 mg of bovine trypsin at 0° was stopped by the addition of 1.0 mg of STI. Approximately 80% of the maximal carboxypeptidase B activity was reached in this time. The activation mixture was incubated 1 hr longer in the presence of the STI, and the pH of the solution was then lowered to 4 by the dropwise addition of glacial acetic acid. The amino acids liberated were adsorbed onto Dowex 50, eluted with 1 N ammonia solution, taken to dryness on the rotary evaporator, and subjected to high-voltage electrophoresis at pH 6.5. Elution and quantitation of the arginine (Connell *et al.*, 1955) yielded 0.076 μmole, equivalent to 0.68 mole of arginine/45,000 g of zymogen. Only a trace amount of lysine was observed. Although neutral amino acids were present, no attempt at their identification or quantitation was made. These data indicate that "rapid" activation of the zymogen appears to involve the hydrolysis of an arginyl bond by trypsin. Conclusive evidence must await the isolation of the activation peptide.

Enzymatic Activity. Several-times-recrystallized dogfish carboxypeptidase B contains 8350 ± 200 units of activity/mg of enzyme when assayed for BGA (0.001 M, 0.005 M sodium veronal, 0.045 M NaCl, pH 7.5). If HPLA (0.01 M, 0.005 M sodium veronal, 0.045 M NaCl, pH 7.5) is employed as substrate, a specific activity of 0.03 mequiv of substrate hydrolyzed/min per mg of enzyme was observed. With CGP (0.025 M, 0.02 M sodium veronal, 0.1 M NaCl, pH 7.5) as substrate, a proteolytic coefficient of 0.90 min^{-1}/mg of protein per ml was calculated.

The specificity of dogfish carboxypeptidase B was tested toward a number of peptides[3] in 0.005 M sodium veronal buffer, pH 7.5, containing 0.05 M NaCl at 25°. The enzyme liberated serine from the tripeptide Leu-Asp-Ser. In contrast, the tetrapeptide Ser-Tyr-Pro-Asn appeared to be resistant to the enzyme under the same

[3] These peptides, derived from enzymatic digests of trypsinogen, were kindly placed at our disposal by Dr. K. A. Walsh.

conditions. Both lysine and aspartic acid were liberated from the activation peptide of bovine trypsinogen (Davie and Neurath, 1955) Val-Asp-Asp-Asp-Asp-Lys.

Discussion

The present experiments have permitted a preliminary characterization of dogfish procarboxypeptidase B, the product of its activation, and a comparison of this system with that of two other species, the bovine and the porcine. In solutions of pH 8.0, both dogfish and bovine procarboxypeptidase B are weakly anionic as attested by their chromatographic elution at low ionic strength. The corresponding zymogens in the rat, dog, and pig appear considerably more anionic in character (Marchis-Mouren *et al.*, 1961, 1963). As determined by sedimentation equilibrium measurements, the molecular weight of dogfish procarboxypeptidase B is in the range of 44,000–45,000 as compared to 57,000 for the analogous bovine zymogen (Wintersberger *et al.* 1962). While the two zymogens thus differ in molecular weight and in amino acid composition, the respective products of activation, bovine and dogfish carboxypeptidase B, are similar in both of these properties (*vide infra*). It appears, therefore, that the major difference between the two zymogens relates to the length and composition of the amino-terminal fragment which is released during activation. This pattern is similar in kind to that by which bovine chymotrypsinogen and trypsinogen are related to each other (Walsh and Neurath, 1964), the activation peptides comprising 15 and 6 amino acid residues, respectively.

The mechanism of activation of dogfish procarboxypeptidase B thus resembles that of the bovine zymogen. Activation is mediated by trypsin and involves the cleavage of a peptide bond in the amino-terminal region of the zymogen. As in the case of the bovine system, an arginyl peptide bond appears to be cleaved as evidenced by the release of free arginine presumably originating from the activation peptide by the action of the newly formed enzyme carboxypeptidase B. The amino-terminal residue of the zymogen has been established as glutamine, whereas the active exopeptidase contains serine in this position. No change in the composition of the carboxyl-terminal region was detected as indicated by the similarity of the amino acids released by bovine carboxypeptidase A from dogfish procarboxypeptidase B and carboxypeptidase B, respectively.

Crystalline enzymes having the specificity of carboxypeptidase B have now been isolated from the bovine (Folk and Gladner, 1958; Wintersberger *et al.*, 1962), from the porcine (Folk *et al.*, 1960), and from the dogfish. The molecular weight of all of these enzymes has been estimated to be approximately 34,000–35,000. A comparison of the amino acid compositions of these three carboxypeptidases is given in Table VI. The three enzymes show striking similarities in amino acid composition, which are particularly significant in regard to the low content of histidine, methionine, tryptophan, and half-cystine. In the case of the bovine and the porcine enzymes, the presence of 1 g-atom of zinc/mole of protein has been reported, but similar data for the dogfish enzyme are as yet lacking. Since bovine and porcine carboxypeptidase B also show striking similarities in composition to carboxypeptidases A, except for the distribution of sulfur-containing amino acids (Cox *et al.*, 1962), taken together these data suggest a common evolutionary origin for all of these exopeptidases. In this regard, it may be of significance that dogfish carboxypeptidase B, as the bovine enzyme, exhibits dual specificities toward substrates containing basic as well as aromatic amino acid residues. In contrast, carboxypeptidase A of any species does not act on substrates originally believed to be characteristic of carboxypeptidase B. Since, according to Folk and co-workers, substrate specificity of porcine carboxypeptidase B is absolute, limited only to compounds containing basic amino acid residues, it would be of interest to determine whether another enzyme possessing dual specificity, as in the case of the bovine and the dogfish, may exist in porcine pancreatic juice as well.

The present description of dogfish procarboxypeptidase B and carboxypeptidase B is as yet incomplete. It remains for future work to elucidate more completely the detailed mechanism of activation of the proenzyme, including the structure of the activation peptide, and to delineate more precisely the substrate specificity and mechanism of action of the active enzyme, dogfish

TABLE VI: Comparison of Amino Acid Composition of Bovine, Porcine, and Dogfish Carboxypeptidase B.

	Bovine Carboxypeptidase B[a]	Porcine Carboxypeptidase B[b]	Dogfish Carboxypeptidase B
Alanine	22	25	24
Arginine	13	10	14
Aspartic acid	26	32	31
Glutamic acid	24	25	20
Glycine	21	23	20
Histidine	7	6	4
Isoleucine	16	17	20
Leucine	20	23	17
Lysine	17	18	15
Methionine	6	5	8
Phenylalanine	12	12	9
Proline	12	13	14
Serine	26	18	25
Threonine	26	30	25
Tryptophan	10	9	10
Tyrosine	22	20	20
Valine	14	11	17
Half-cystine	7	8	7

[a] Data of Cox *et al.* (1962). [b] Data of Folk *et al.* (1960).

carboxypeptidase B. Such investigations are now in progress in this laboratory.

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