Heterogeneity of Bovine Carboxypeptidase A. II. Chromatographic Purification of Carboxypeptidase A (Cox)*

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ABSTRACT: Bovine carboxypeptidase A (Cox) has been chromatographically purified on DEAE-cellulose (DE-52) using a complex gradient of both LiCl and β-phenylpropionate. Four fractions having similar specific esterase and peptidase activity have been isolated. Isolation of the carboxyl-terminal peptides obtained by cleavage with cyanogen bromide indicate that peak I is carboxypeptidase A_α^{Val}, peak II is carboxypeptidase A_α^{Leu} containing a 30% contamination of carboxypeptidase A_β^{Val}, and peak III is carboxypeptidase A_β^{Leu}. Each of these fractions as well as those isolated from carboxypeptidase A (Anson) are homogeneous by disk gel electrophoresis. As to be expected, the amino acid replacement previously found in the carboxyl-terminal region of carboxypeptidase A_β and A_γ is also present in A_α. Heat-inactivation studies at 50° reveal that both the chain length of the amino-terminal region of the enzyme and the amino acid replacements characterizing each valine and leucine variant may be important in stabilizing the conformation of the molecule.

Bovine carboxypeptidase A can be prepared by three different methods, each yielding, as a major component, a form of the enzyme which differs chemically and functionally from the others. Carboxypeptidase A_α, the main component obtained from the tryptic activation of partially purified procarboxypeptidase A (Cox *et al.*, 1964), has an additional amino-terminal heptapeptide (Figure 1) when compared with carboxypeptidase A_γ (Sampath Kumar *et al.*, 1964b), the main form of the enzyme isolated from thawing and autolyzing pancreatic tissue (Anson, 1937). Carboxypeptidase A_β, another form of the enzyme (Figure 1), is present in all preparations of carboxypeptidase A (Bargetzi *et al.*, 1964).

In addition to these demonstrable variations in the amino-terminal region, carboxypeptidase A was also found to exist in two allotypic forms, each of which contains either valine or leucine in the antepenultimate position of the polypeptide chain (Bargetzi *et al.*, 1964, and Figure 1). Two other replacements, both genetically linked to the valine–leucine replacement, have also been found in other regions of the molecule (Pétra *et al.*, 1969).

We have recently reported a chromatographic system for the separation of the allotypic variants of carboxypeptidase A_γ and A_β, using carboxypeptidase A (Anson) as starting material (Pétra and Neurath, 1969). However, early attempts to apply the same procedure to carboxypeptidase A (Cox) proved unsuccessful, in that most of the protein was eluted as a breakthrough fraction. In this communication we wish to report a reproducible system for the ion-exchange chromatography of carboxypeptidase A (Cox) as well as some of the chemical and enzymatic properties of the separated fractions.

Experimental Section

Enzyme. Bovine carboxypeptidase A was prepared from pooled pancreas glands according to the method of Cox *et al.* (1964).

Chemicals and Substrates. CbzGly-L-Phe and HPLA[1] were purchased from Fox Chemical Co. β-Phenylpropionic acid was purchased from Baker and Eastman and was recrystallized from an ethanol–water mixture. All other materials were of reagent grade quality and were used without further purification.

Standard Enzymatic Assays. Peptidase activity was measured spectrophotometrically with the use of CbzGly-L-Phe according to Whitaker *et al.* (1966). A Cary Model 16 spectrophotometer equipped with a Model 1626 recording interface and a Beckman 10-in. recorder were used for all rate and absorption measurements. The wavelength (223–224 mμ) was adjusted so that the reaction cuvet containing 3 ml of buffered substrate solution (0.45 M KCl–0.05 M Tris-Cl, pH 7.5, 25°) read 1.8 optical density units against buffer solution. A substrate concentration of 1.18×10^{-3} M was used in the standard assay in order to compare the first-order rate constants obtained in this study with that reported by Whitaker *et al.* (1966). The enzyme obeyed normal Michaelis–Menten kinetics at this particular substrate concentration (Whitaker *et al.*, 1966; Davies *et al.*, 1968).

The specific activity is expressed as a first-order rate constant, k (sec^{-1}) = V/E_0, where V is the initial velocity calculated from the linear portion of the line representing less than 5% of the total substrate hydrolyzed (10 in. = 0.1 optical density unit), and E_0 is the enzyme concentration ($1–3 \times 10^{-8}$ M) calculated from $\epsilon_{278} = 6.42 \times 10^4\ M^{-1}\ cm^{-1}$ (Simpson *et al.*, 1963). Total hydrolysis of the substrate was carried out at the same wavelength in order to convert absorption data into rate data.

Esterase activity was measured spectrophotometrically at 254 mμ using HPLA as substrate (McClure *et al.*, 1964; Whitaker *et al.*, 1966). A substrate concentration of 4.08×10^{-4} M was used in order to compare the rate constant with that reported by Whitaker *et al.* (1966) (the substrate solution

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[1] Abbreviation used is: HPLA, hippuryl-DL-β-phenyllactic acid; Cbz is carbobenzoxy.

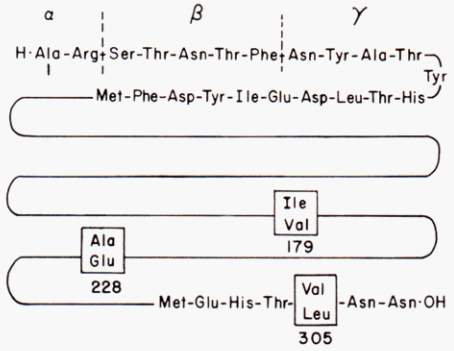

FIGURE 1: Schematic representation of the terminal regions of bovine carboxypeptidase A including the allotypic amino acid replacements.

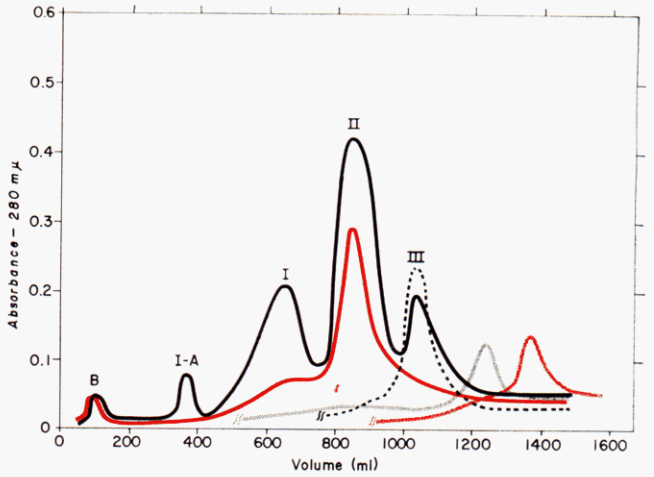

FIGURE 2: Elution patterns of different forms of bovine carboxypeptidase A on a column (1.5 × 90 cm) of DE-52 developed at 50 ml/hr at 4° using the compound gradient described in the experimental section. Black line: 150 mg of twice-crystallized carboxypeptidase A (Cox); red line: 40 mg of carboxypeptidase A_β^{Val}; black dotted line: 30 mg of carboxypeptidase A_β^{Leu}; gray line: 15 mg of carboxypeptidase A_γ^{Val}; and pink line: 15 mg of carboxypeptidase A_γ^{Leu}.

contained both racemates). Normal Michaelis–Menten kinetics prevail at this substrate concentration (McClure *et al.*, 1964; Whitaker *et al.*, 1966). The specific activity is expressed as a first-order rate constant, k (sec^{-1}) = V/E_0, where V is the initial velocity calculated from the slope, and E_0 is the enzyme concentration ($1–3 \times 10^{-9}$ M) calculated using $\epsilon_{222.5} = 5.27 \times 10^5$ M^{-1} cm^{-1} (Whitaker *et al.*, 1966). Total hydrolysis of the substrate was measured at 254 mμ in order to convert absorption data into rate data.

Chromatographic Procedure. The enzyme was chromatographed on DEAE-cellulose at 4° by using a complex gradient of both LiCl and β-phenylpropionate at pH 7.5. A Varigrad (Peterson and Sober, 1959) Model 3000 (Phoenix Co.) was used. Recycled DE-52 (microgranular DEAE-cellulose, preswollen wet powder, Reeve Angel Co.) was equilibrated with 0.05 M β-phenylpropionate–0.004 M LiCl–0.05 M Tris-Cl (pH 7.5) (the pH was adjusted with 10 N NaOH at room temperature) and packed into a Pharmacia column (1.5 × 90 cm) at a flow rate of 120 ml/hr. When the column was completely filled with the adsorbent bed, a Pharmacia polyoxymethylene end piece equipped with a 40-μ nylon bed supporting net (that normally used for the bottom of the column) was screwed onto the top. The column was then transferred to the cold room (4°) and equilibrated overnight with 0.05 M β-phenylpropionate–0.004 M LiCl–0.05 M Tris-Cl (pH 7.5) at a flow rate of 50 ml/hr. All subsequent operations were done in the cold. A crystal suspension containing 150 mg of carboxypeptidase A was centrifuged and the crystals were dissolved in 1.0 ml of 0.05 M β-phenylpropionate–0.004 M LiCl–2 M Tris-Cl (pH 7.5) (adjusted with HCl); 39 ml of 0.004 M LiCl–0.05 M β-phenylpropionate (pH 7.5) was added and the solution was centrifuged at 3000 rpm for 10 min. The supernatant was pumped onto the column at a flow rate of 50 ml/hr, and the elution was carried out at the same flow rate collecting 20-ml fractions. The complex gradient was prepared by placing 200 ml of 0.05 M β-phenylpropionate–0.004 M LiCl–0.05 M Tris (pH 7.5) in each of chambers 1, 2, 3, and 5, and 200 ml of 0.122 M β-phenylpropionate–0.04 M LiCl–0.05 M Tris (pH 7.5) in each of chambers 4, 6, 7, 8, and 9. The total volume of buffer in the Varigrad was 1800 ml. Chromatography was completed in approximately 30 hr. The appropriate fractions were pooled, concentrated, and crystallized as previously described (Pétra and Neurath, 1969). Some packing of the column takes place during the chromatography preventing reuse of the same column even after reequilibration. Experience has shown that small errors in LiCl concentrations and flow rate as well as the purity of β-phenylpropionic acid have drastic effects on the reproducibility of the elution pattern.

Disc Gel Electrophoresis. Electrophoresis was carried out according to Davis (1964) with slight modifications. The separating gel followed by the spacer gel were first polymerized in the tubes. The sample gel, prepared by mixing 0.025 ml of stock enzyme solution with 0.125 ml of concentrating gel, was then applied on top and allowed to polymerize. The concentration of enzyme stock solutions ranged from 2 to 3 mg per ml for carboxypeptidase A (Anson), A (Cox), A_γ^{Val}, and A_γ^{Leu} (for nomenclature see Pétra and Neurath, 1969) to 0.8 mg/ml for all other purified enzymes. These solutions were prepared by dissolving the crystals with 0.1 ml of 5 M NaCl and diluting with 0.05 M Tris-Cl buffer (pH 7.5) to the proper protein concentration.

Other Methods. Cyanogen bromide cleavage, purification of amino- and carboxyl-terminal cyanogen bromide fragments, peptide sequence analysis, heat-inactivation studies, and amino acid analyses were all done according to methods already described (Pétra and Neurath, 1969).

Results

Chromatography of Carboxypeptidase A (Cox). A typical elution pattern of 150 mg of carboxypeptidase A is shown in Figure 2 (black line). Peak fractions I-A, I, II, and III emerged at approximately 370, 650, 850, and 1050 ml, respectively. The reproducibility of these elution volumes depends greatly upon equilibration of the column, flow rate, and purity of reagents used in the buffers. The amount of protein, based on absorption at 278 mμ, recovered from each of the crystallized fractions of Figure 2 (black line) were 54, 62, and 17 mg for fractions I, II, and III, respectively. The amount of protein in each fraction varied markedly from preparation to preparation;

TABLE I: Specific Activity of Chromatographically Purified Fractions Isolated from Carboxypeptidase A (Cox).

	CbzGly-L-Phe[a] (sec^{-1})	HPLA[b] (sec^{-1})
Pool sample[c]	19.7	339
Peak I-A	19.0	365
Peak I	19.7	336
Peak II	24.0	323
Peak III	20.6	342

[a] 0.45 M KCl–0.05 M Tris-Cl, pH 7.5, 25°; substrate concentration = 1.18×10^{-3} M. [b] 0.45 M KCl–0.05 M Tris-Cl, pH 7.5, 25°; racemate substrate concentration = 4.08×10^{-4} M. [c] Enzyme prior to chromatography.

however, the total yield (89%) of crystalline carboxypeptidase recovered after chromatography was fairly constant. Table I shows the specific activity of the isolated fractions including the unchromatographed enzyme. There are no major differences among the fractions and the pool sample. The breakthrough fraction (B) was present in very small amounts and showed no catalytic activity. The ultraviolet absorption spectrum of this material indicated that it is nonprotein and most probably constitutes β-phenylpropionate released during chromatography. Rechromatography of each active fraction gave a single component which separated at its appropriate elution volume when compared with the original chromatographic pattern. Carboxypeptidases A_γ^{Val} and A_γ^{Leu} (10–15 mg) obtained from the purification of carboxypeptidase A (Anson) (Pétra and Neurath, 1969) were each chromatographed under these new conditions and separated at elution volumes of 1240 and 1380 ml, respectively (Figure 2, gray and pink trace). Larger amounts (40–50 mg) of these two fractions could not be separated from each other because of extensive overlapping indicating that the method described here can only be used to detect small amounts of carboxypeptidases A_γ^{Val} and A_γ^{Leu} which may be present in enzyme preparations that do not contain large amounts of carboxypeptidase A_γ. Certain preparations of carboxypeptidase A (Cox) do contain trace amounts of these two forms and these have been detected by this method. The red trace and the black dotted line in Figure 2 represent the elution patterns of carboxypeptidases A_β^{Val} and A_β^{Leu}, respectively, isolated from carboxypeptidase A (Anson) (Pétra and Neurath, 1969).

Disc Gel Electrophoresis. Figure 3 shows the results obtained with unchromatographed carboxypeptidase A (Cox) (gel 1) and the isolated fractions depicted by the black line in Figure 2: fraction I-A (gel 2), fraction I (gel 3), fraction II (gel 4), and fraction III (gel 5). The small amounts of fractions I and II present in III (gel 5) could be removed by rechromatography. The data establish the homogeneity of the isolated fractions insofar as the method is able to detect. The assignment of the particular form of carboxypeptidase represented by each band in Figure 3 (gel 1) is given below. The specific peptidase and esterase activities of the isolated fractions including the unchromatographed enzyme are shown in Table I. There are only minor differences in the catalytic properties of the purified fractions.

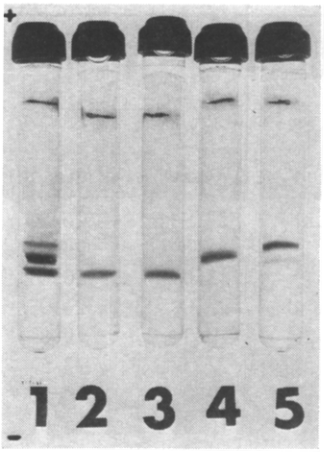

FIGURE 3: Disc gel electrophoresis of chromatographically isolated fractions obtained from carboxypeptidase A (Cox). Gel 1: unchromatographed enzyme; gel 2: fraction I-A (Figure 2); gel 3: fraction I (Figure 2); gel 4: fraction II (Figure 2); and gel 5: fraction III (Figure 2). The conditions were according to Davis (1964).

Figure 4 shows the results obtained with unchromatographed carboxypeptidase A (Anson) (gel 1) along with the separated fractions (Figure 3 in Pétra and Neurath, 1969): carboxypeptidases A_β^{Val} (gel 2), A_β^{Leu} (gel 3), A_γ^{Val} (gel 4), and A_γ^{Leu} (gel 5). The small contamination of carboxypeptidases A_γ^{Leu} in A_γ^{Val} (gel 4) could be removed by one additional chromatography in the same system. Experience has shown that pure samples of carboxypeptidases A_γ^{Val} and A_γ^{Leu} tend to streak during electrophoresis, thereby rendering the production of sharp bands a difficult task. The specific

TABLE II: Specific Activity of Chromatographically Purified Fractions[a] Isolated from Carboxypeptidase A (Anson).[b]

	CbzGly-L-Phe[c] (sec^{-1})	HPLA[d] (sec^{-1})
Carboxypeptidase A (Anson)	18.9[e]	330[e]
Carboxypeptidase A (Anson)[f]	19.0	346
Peak I (carboxypeptidase A_α^{Val})	18.7	322
Peak II (carboxypeptidase A_β^{Val})	23.6	327
Peak III (carboxypeptidase A_β^{Leu})	23.2	336
Peak IV (carboxypeptidase A_γ^{Val})	20.3	355
Peak V (carboxypeptidase A_γ^{Leu})	22.0	343

[a] Figure 3 in Pétra and Neurath (1969). [b] For nomenclature, see Pétra and Neurath (1969). [c] 0.45 M KCl–0.05 M Tris-Cl, pH 7.5, 25°; substrate concentration = 1.18×10^{-3} M. [d] 0.45 M KCl–0.05 M Tris-Cl, pH 7.5, 25°; racemate substrate concentration = 4.08×10^{-4} M. [e] Data of Whitaker *et al.* (1966). Five-times-crystallized carboxypeptidase A (Anson), lot no. C2560 (Mann Research Laboratories). [f] Twice-crystallized carboxypeptidase A (Anson), lot no. COA-7HA (Worthington Biochemical Corp.). This latter preparation was used for the chromatographic separation shown in Figure 3 of Pétra and Neurath (1969).

TABLE III: Comparison of Amino Acid Composition of Chromatographically Separated Fractions of Carboxypeptidase A (Cox) (Residues per Mole).

Amino Acid	A (Cox)[a]	A (Anson)[a]	A_β^{Leu}[b]	Fractions			
				I-A[c]	I	II	III
Aspartic acid	27.9	26.5	27.6	27.6	28.1	28.1	28.2
Threonine	27.8	23.4	25.0[d]	24.0	25.0[d]	25.0[d]	26.0[d]
Serine	33.0	30.0	30.5[d]	29.9	31.0[d]	31.0[d]	31.0[d]
Glutamic acid	25.0	24.9	26.0	25.4	24.7	25.4	26.3
Proline	9.7	9.8	10.1	10.2	9.5	10.0	9.5
Glycine	22.5	22.5	22.2	23.4	23.1	22.8	22.8
Alanine[e]	20.0	19.0	(19.0)	(21.0)	(21.0)	(20.0)	(19.0)
Valine	15.8	15.6	16.0[h]	14.6	15.8[h]	15.7[h]	15.8[h]
Methionine	3.0	2.7	2.6	2.5	2.7	2.9	2.8
Isoleucine	20.3	19.8	19.8[h]	16.4	18.4[h]	17.9[h]	18.1[h]
Leucine	23.2	22.9	23.0	21.5	22.9	23.9	23.4
Tyrosine	19.2	18.8	17.9	18.2	19.0	18.6	18.2
Phenylalanine	16.0	14.6	16.2	15.7	15.5	15.4	15.7
Lysine	15.0	15.0	14.7	15.6	15.5	15.1	15.0
Histidine	8.2	7.7	7.7	8.0	8.0	7.6	7.7
Arginine	11.1	9.9	9.6	10.2	10.5	10.7	9.9
Half-cystine	2	2	2.0[f]	1.8[f]	1.9[f]	1.9[f]	2.1[f]
Tryptophan	8	8	8.1[g]	8.3[g]	8.5[g]	7.8[g]	7.9[g]

[a] Bargetzi *et al.* (1963). [b] Pétra and Neurath (1969). [c] Represents an uncorrected determination. [d] Corrected by linear extrapolation to zero- time hydrolysis. [e] Reference amino acid (Walsh *et al.*, 1966). [f] Determined as cysteic acid (Moore, 1963). [g] Method of Edelhoch (1967) and corrected for 1 mole of cystine/mole of protein. [h] Values after 72-hr hydrolysis.

activity of these various fractions including the unchromatographed material is reported in Table II. These forms of the enzyme are not significantly different in their catalytic function. Carboxypeptidase A_α^{Val} (peak I in Figure 3 of Pétra and Neurath, 1969) is present to the extent of 2% in preparations of carboxypeptidase A (Anson) probably explaining its absence in the disc gel electrophoretic pattern of gel 1 (Figure 4). However, caution should be exercised when comparing the relative position of each band from one gel to another. It is entirely possible, although not likely (see Discussion), that the lower band in gel 1 (Figure 4) may be carboxypeptidase A_α^{Val} and that the band directly above it comprises both unseparated fractions of carboxypeptidases A_β^{Val} and A_β^{Leu}. For the present, however, the bands in Figure 4 (gel 1) can be tentatively assigned as follows: starting from the bottom of the gel A_β^{Val}, A_β^{Leu}, A_γ^{Val}, and A_γ^{Leu}. The results clearly establish the homogeneity of the fractions isolated according to this method.

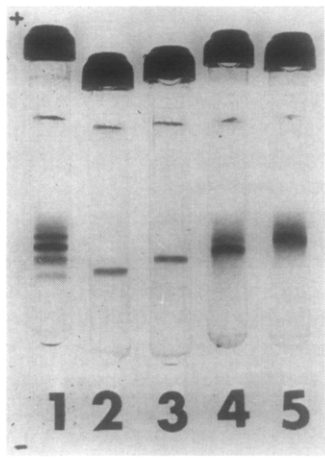

FIGURE 4: Disc gel electrophoresis of chromatographically isolated fractions obtained from carboxypeptidase A (Anson). The gels refer to Figure 3 in Pétra and Neurath (1969). Gel 1: unchromatographed enzyme; gel 2: carboxypeptidase A_β^{Val}; gel 3: carboxypeptidase A_β^{Leu}; gel 4: carboxypeptidase A_γ^{Val}; and gel 5: carboxypeptidase A_γ^{Leu}. The conditions were according to Davis (1964).

Amino Acid Composition. Table III shows the amino acid composition of the purified fractions. All values (except peak I-A) represent the average of two determinations obtained after 24-hr hydrolysis. Serine and threonine were estimated at three different times of hydrolysis, 24, 48, and 72 hr, and the values were extrapolated to zero-time hydrolysis (Smith and Stockell, 1954). Valine and isoleucine are maximum values after 72-hr hydrolysis. The amino acid residues were calculated on the basis of the total alanine content per molecular weight of 34,600 (Walsh *et al.*, 1966). Fractions I (column 5) and II (column 6) have almost identical compositions and seem to resemble carboxypeptidase A_α containing one or two more residues of alanine (see Discussion) and one more arginine than carboxypeptidase A_β (column 3, Table II; Figure 1). Fraction III, however, is more similar to carboxypeptidase A_β and was subsequently proven to be identical with that fraction (*vide infra*). All valine variants have an additional alanine residue at position 228 (carboxypeptidase A_α numbering system) where one of the three linked amino acid replacements

TABLE IV: Amino Acid Compositions of the Carboxyl-Terminal Cyanogen Bromide Fragments Derived from Chromatographically Isolated Fractions (Residues per Mole).[a]

	Glu	His	Thr	Val	Leu	Asp
Carboxypeptidase A (Cox)[b]	1.01	0.99	0.94	0.61	0.39	1.92
Peak I	1.00	1.00	0.96	*0.90*	*0.04*	2.00
Peak II	1.00	1.07	0.93	*0.31*	*0.71*	2.00
Peak III	1.02	1.00	1.00	*0.07*	*0.89*	2.10

[a] Based on aspartic acid content. [b] Taken from Bargetzi *et al.* (1964).

occurs (Pétra *et al.*, 1969). Furthermore, carboxypeptidase A_α also has an extra alanine since this residue is the N-terminal amino acid (Bergetzi *et al.*, 1964, and Figure 1). These two facts were taken into consideration when chosing the proper value for the reference amino acid. Peak I-A was assumed to be carboxypeptidase A_α^{Val} for reasons described in the Discussion.

Isolation of Carboxyl-Terminal Cyanogen Bromide Fragments Derived from Fractions I, II, and III. Each fraction was treated with cyanogen bromide (Gross and Witkop, 1961) as modified for carboxypeptidase A by Sampath Kumar *et al.* (1964a) and the carboxyl-terminal peptide fragments were isolated on Sephadex G-25 in 50% acetic acid (Pétra and Neurath, 1969). A typical gel filtration is shown in Figure 5A which depicts the elution pattern of fraction I (Figure 2). The fractions corresponding to peak 2 (Figure 5A) were pooled and further purified by paper electrophoresis (Bargetzi *et al.*, 1964; Pétra and Neurath, 1969). The amino acid composition of all three carboxyl-terminal fragments is shown in Table IV. The peptide derived from peak I (Figure 2) contains valine and no leucine in contrast to that isolated from peak III which has leucine and no valine. The peptide derived from peak II, however, contains both amino acids in a different ratio than is found in the unchromatographed enzyme (see Discussion).

Isolation of Amino-Terminal Cyanogen Bromide Fragments Derived from Fractions I, II, and III. The fractions corresponding to peak I (Figure 5A) were pooled, taken to dryness, and passed through Sephadex G-50 (Pétra and Neurath, 1969). Figure 5B shows a typical elution profile representing the amino-terminal peptide fragment derived from peak I (Figure 2). The amino acid composition of the isolated peptides is shown in Table V. Peptides N_I and N_{II}, obtained from fractions I and II, respectively, have the same amino acid composition as those derived from carboxypeptidase A (Cox) (Sampath Kumar *et al.*, 1964b), while peptide N_{III}, from fraction III, has an identical amino acid composition to N_β, derived from carboxypeptidase A_β; each contains approximately one residue of alanine and no arginine. Furthermore, all three peptides, N_I, N_{II}, and N_{III}, differ markedly in aspartic acid, threonine, serine, and phenylalanine content when compared with N_γ, the peptide obtained from carboxypeptidase A_γ (Pétra and Neurath, 1969), suggesting that none of these three forms of the enzyme is carboxypeptidase A_γ (*vide infra*). The maximum yields obtained for all three peptides were 30–40%.

Sequence of Fragment N_I. The amino acid composition of

TABLE V: Amino Acid Composition of the Amino-Terminal Peptide Fragments Obtained after Cyanogen Bromide Cleavage of Chromatographically Isolated Fractions (Residues per Mole).

Amino Acid	N_γ[a]	$N_{(Cox)}$[b]	N_β[c]	N_I	N_{II}	N_{III}
Aspartic acid	2.9	4.0	4.0	4.0	3.8	3.8
Threonine	1.8	3.8	3.6	3.8	3.4	3.5
Serine		1.2	1.0	1.1	1.4	1.3
Glutamic acid	1.1	1.4	1.2	1.3	1.6[e]	1.7[e]
Alanine	1.0	1.8	1.2	1.8	1.8	1.2
Isoleucine	0.9	0.9	1.1	0.9	1.2	1.0
Leucine	1.0	1.2	1.3	1.1	1.5	1.2
Tyrosine	2.6	2.6	2.8	2.1	1.8	2.3
Phenylalanine	1.0	2.0	2.0	1.8	1.7	1.8
Histidine	1.0	1.0	1.1	1.1	1.0	1.0
Arginine		0.9		1.0	0.7	
Methionine	0.9[d]	1.1[d]	1.0[d]	0.9[d]	0.4[f]	0.5[f]

[a] Derived from carboxypeptidase A_γ (Pétra and Neurath, 1969). [b] Derived from carboxypeptidase A (Cox) (Sampath Kumar *et al.*, 1964b). [c] Derived from carboxypeptidase A_β (Pétra and Neurath, 1969). [d] Sum of homoserine and homoserine lactone. [e] Homoserine did not separate from glutamic acid. [f] Values for homoserine lactone only.

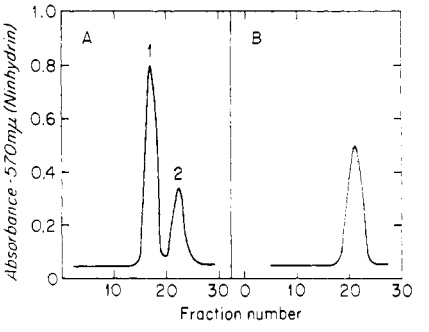

FIGURE 5: Elution pattern for the isolation of amino- and carboxyl-terminal cyanogen bromide fragments derived from fraction I. (A) Gel filtration on a column (0.9 × 100 cm) of Sephadex G-25 of 15 mg of peptide fraction. (B) Gel filtration on a column (0.9 × 100 cm) of Sephadex G-50 of the pooled fraction from peak 1 of Figure 5A. Both elutions were carried out in 50% acetic acid at a flow rate of 5 ml/hr; 1.5-ml fractions were collected.

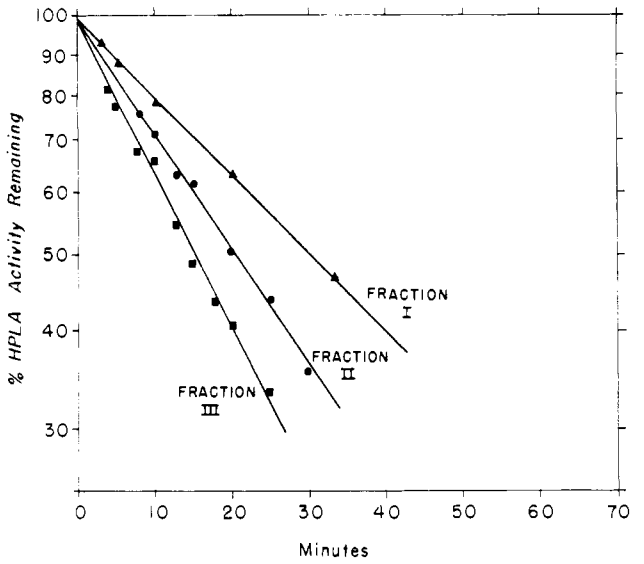

FIGURE 6: Heat inactivation of isolated forms of carboxypeptidase A at 50 ± 0.1°. Protein concentration was 0.2 mg/ml in 1 M NaCl–0.005 M sodium phosphate (pH 7.5). Activity was measured using 3 ml of a standard esterase assay solution: 1×10^{-2} M HPLA–5 × 10^{-3} M sodium Veronal, and 4.5×10^{-2} M NaCl (pH 7.5).

this peptide is given in Table V. The modified subtractive Edman procedure (Konigsberg and Hill, 1962) as applied to carboxypeptidase A (Bradshaw *et al.*, 1969; Pétra and Neurath, 1969) was used.

FIRST DEGRADATION: Asp, 3.8; Thr, 3.7; Ser, 1.1; Glu, 1.3; *Ala, 1.2;* Ile, 1.1; Leu, 1.2; Tyr, 1.8; Phe, 1.6; His, 1.1; Arg, 1.0; and Met,[2] 0.8.

SECOND DEGRADATION: Asp, 4.0; Thr, 3.8; Ser, 1.2; Glu, 1.3; Ala, 1.2; Ile, 1.2; Leu, 1.2; Tyr, 2.3; Phe, 2.0; His, 1.1; *Arg, 0.3;* and Met,[2] 0.8.

THIRD DEGRADATION. Asp, 3.7; Thr, 3.5; *Ser, 0.7;* Glu, 1.3; Ala, 1.1; Ile, 1.1; Leu, 1.1; Tyr, 1.8; Phe, 1.8; His, 1.0; Arg, 0.3; and Met,[2] 0.7.

These data indicate the sequence H·Ala-Arg-Ser for the peptide. On the basis of the determined partial sequence and amino acid composition, this peptide can then be placed at the amino-terminal region of carboxypeptidase A_α (Figure 1). Furthermore, on the basis of the data in Table IV, peak I (Figure 2), from which the peptide is derived, is therefore carboxypeptidase A_α^{Val}.

Amino-Terminal Sequence of Fragment N_{II}. The amino acid composition is given in Table V. The subtractive Edman procedure yielded the following data.

FIRST DEGRADATION: Asp, 3.8; Thr, 3.3; Ser, 1.2, Glu,[3] 1.5; *Ala, 1.2;* Ile, 1.2; Leu, 1.4; Tyr, 2.1; Phe, 1.8; His, 1.0; Arg, 0.8; and Met,[4] 0.8.

SECOND DEGRADATION: Asp, 3.8; Thr, 3.4; Ser, 1.3; Glu,[3] 1.5; Ala, 1.2; Ile, 1.2; Leu, 1.4; Tyr, 2.0; Phe, 1.8; His, 0.9; *Arg, 0.3;* and Met,[4] 0.5.

The deduced partial sequence is H·Ala-Arg. These results seem to indicate that peak II (Figure 2) is also carboxypeptidase A_α. However, the data in Table IV show that both the valine and leucine forms of the enzyme must be present in this fraction (*vide infra*). Fraction II was rechromatographed twice on the same system described in Figure 2; the reisolated material still contained the same ratio of valine to leucine in the carboxyl-terminal region indicating that the two forms of the enzyme existing in this fraction cannot be separated by this chromatographic method. Furthermore, fraction II also appears homogeneous by disc gel electrophoresis (Figure 3, gel 4).

The problem was finally clarified when a pure sample of carboxypeptidase A_β^{Val} isolated according to Pétra and Neurath (1969) (Figure 4, gel 2) was chromatographed using the system described in this report. One peak emerging at the same elution volume as fraction II was obtained (Figure 2, red line). The data in Table IV indicate that this latter fraction is composed of 70% carboxypeptidase A_α^{Leu} and 30% carboxypeptidase A_β^{Val}.

Amino-Terminal Sequence of Fragment N_{III}. The amino acid composition is given in Table V.

FIRST DEGRADATION: Asp, 3.8; Thr, 3.3; *Ser, 0.8;* Glu,[3] 1.6; Ala, 1.2; Ile, 1.0; Leu, 1.1; Tyr, 1.9; Phe, 1.5; His, 1.1; and Met,[4] 0.5.

SECOND DEGRADATION: Asp, 3.6; *Thr, 2.8;* Ser, 0.9; Glu,[3] 1.6; Ala, 1.3; Ile, 1.0; Leu, 1.1; Tyr, 1.9; Phe, 1.5; His, 1.0; and Met,[4] 0.6.

THIRD DEGRADATION: *Asp, 3.0;* Thr, 2.5; Ser, 0.8; Glu,[3] 1.6; Ala, 1.2; Ile, 1.0; Leu, 1.1; Tyr, 1.8; Phe, 1.5; His, 0.9; and Met,[4] 0.4.

These experiments suggest the sequence H·Ser-Thr-Asx which is identical with the amino-terminal region of carboxypeptidase A_β (Figure 1). The amino acid composition and the data in Table IV establish that fraction III is carboxypeptidase A_β^{Leu}.

Heat-Inactivation Studies. These experiments were carried out using previously described conditions (Pétra and Neurath, 1969). Figure 6 shows the loss of esterase activity as a function of time at 50° for fractions I, II, and III. The differences in heat stability among the fractions correlate well with those previously reported for the purified fractions of carboxypeptidase A (Anson) (Pétra and Neurath, 1969).

Discussion

A chromatographic method has been developed which can resolve carboxypeptidase A (Cox) into four fractions. The results confirm earlier observations indicating heterogeneity in these preparations of bovine carboxypeptidase A (Bargetzi *et al.*, 1964; Walsh *et al.*, 1966). Four different preparations of carboxypeptidase A (Cox) were tested and all gave similar elution profiles with nearly the same total recovery of enzyme. One preparation contained small amounts of carboxypeptidase A_γ^{Val} and A_γ^{Leu}. The total amount of protein found in each fraction, however, varied from preparation to preparation, suggesting that the conditions used for zymogen activation are such that the ratio of carboxypeptidase A_α to A_β varies. The present preparations contained 42% carboxypeptidase A_α^{Val} (peak 1), 34% carboxypeptidase A_α^{Leu} with 15% carboxypeptidase A_β^{Val} separating in the same fraction (peak II), and 12% carboxypeptidase A_β^{Leu} (peak III) confirming the 60:40 distribution of valine to leucine enzymes in nature (Pétra and Neurath, 1969). Proof for the presence of carboxypeptidase A_β^{Val} in peak II was obtained by the unusual ratio of

[2] Sum of homoserine and homoserine lactone.
[3] Homoserine did not separate from glutamic acid.
[4] Homoserine lactone value only.

valine to leucine in the carboxyl-terminal peptide (Table IV), chromatography of a pure sample of carboxypeptidase A_β^{Val} (Figure 2), rechromatography of fraction II using the method for the purification of carboxypeptidase A (Anson) (Pétra and Neurath, 1969), and the fact that both fraction II and carboxypeptidase A_β^{Val} gave one band in disc gel electrophoresis (Figures 4 and 5). It is reasonable to conclude from these observations that both carboxypeptidase A_β^{Val} and A_α^{Leu} must have nearly the same charge distribution in their native conformation since they cannot be separated under the conditions just described. This point is supported by the recent observation that two additional amino acid replacements both linked to either the valine or leucine replacement exist in carboxypeptidase A_γ (Pétra *et al.*, 1969). The two allotypic forms are Ile_{179}-Ala_{228}-Val_{305}, and Val_{179}-Glu_{228}-Leu_{305}, using the numbering system (Bradshaw *et al.*, 1969) for carboxypeptidase A_α (Figure 1). Although the identifications of these amino acid replacements were made on carboxypeptidase A_γ, there is no reason to doubt their existence in carboxypeptidase A_α and A_β since these are products of zymogen activation all arising from the same precursor, procarboxypeptidase A. In fact, the distribution of the allotypic variants obtained from carboxypeptidase A_α and A_β in the population follows the 60:40 ratio (valine to leucine enzyme) already established for carboxypeptidase A_γ (Pétra and Neurath, 1969). Furthermore, recent experiments have shown that carboxypeptidase A_α can be converted into carboxypeptidase A_β which in turn can be converted into carboxypeptidase A_γ, thereby providing the necessary information to suggest strongly that the linked amino acid replacements must be present in all three forms of the enzyme.[5] It follows, therefore, that all leucine variants have an extra negative charge (Glu-228) which is located on the surface of the molecule (Lipscomb *et al.*, 1968), explaining the chromatographic separation of each variant present in carboxypeptidase A_α, A_β, and A_γ. The resulting net charge of carboxypeptidase A_β^{Val} and A_α^{Leu} is the same since the extra positive charge due to the arginine residue situated in the amino-terminal region of the latter enzyme (Figure 1) is compensated by the glutamic acid residue at position 228 in the former, thus providing a rational explanation for the chromatographic and electrophoretic behavior of these two forms of carboxypeptidase. It is therefore not possible to obtain a pure preparation of carboxypeptidase A_α^{Leu} by the present chromatographic method. Instead, this form of the enzyme must be isolated from a single animal homozygous for the leucine trait and chromatographed by use of the present system to separate carboxypeptidase A_β^{Leu} from A_α^{Leu}. Such experiments have recently been successfully performed.[5] Carboxypeptidase A_β^{Val}, on the other hand, can be obtained by chromatography of pooled carboxypeptidase A (Anson) but the preparation will be contaminated with carboxypeptidase A_α^{Leu} to the extent of 1% since the unchromatographed preparation contains only about 2% of carboxypeptidase A_α.

The identification of fraction 1-A remains unresolved due to scarcity of material. Although this material represents only a small percentage of the total amount of protein applied on the column, it is a homogeneous form of carboxypeptidase A on the basis of its amino acid composition (Table III), disc gel electrophoresis (Figure 3, gel 2), and specific enzymatic activity (Table I). Some chromatographic elution patterns reveal an inverse relationship between the amount of protein present in fractions I and I-A, suggesting that fraction I-A may be an artifact of chromatography and that both fractions are in fact carboxypeptidase A_α^{Val}. Disc gel electrophoresis (Figure 3, gels 2 and 3) supports this hypothesis. However, the final assignment must be deferred until enough material becomes available for a systematic chemical analysis similar to that reported in this communication.

The assignment of carboxypeptidase A_β^{Val} to the lower band in the electrophoretic pattern of gel 1 (Figure 4) is not conclusive. It is based on the chromatographic purification data which indicate the presence of four major fractions and only a 2% contamination of carboxypeptidase A_α^{Val} (Figure 3 in Pétra and Neurath, 1969). If this lower band were carboxypeptidase A_α^{Val}, the one directly above it should be a mixture of carboxypeptidase A_β^{Val} and A_β^{Leu} (the two upper bands undoubtedly represent the allotypic variants of carboxypeptidase A_γ which are the major fractions obtained in the purification). This is unlikely since these two latter fractions can in fact be separated electrophoretically under the same conditions (Figure 3, gels 1 and 5; the upper band is pure carboxypeptidase A_β^{Leu}).

In agreement with previous observations (Pétra and Neurath, 1969) heat-inactivation experiments indicate that the length of the amino-terminal region as well as the three amino acid replacements play a role in the conformational stability of the molecule.

The demonstration of heterogeneity in fraction II illustrates an important point toward the understanding of the general problem of microheterogeneity. This material appears to be homogeneous by methods which today are thought to be among the most sensitive ones (experiments using isoelectric focusing are in progress). Nevertheless, the experiments reported here show that fraction II is in fact composed of two chemically distinct proteins which differ by a total of five amino acids. The detection of this heterogeneity was only made possible as a result of a fortunate genetic event which produced an amino acid replacement at a position in the polypeptide chain that could be readily detected following degradation with cyanogen bromide (residue 305, Figure 1). If this particular mutation had not occurred the microheterogeneity of fraction II would not have been detected. The same argument can be applied to the chromatographic separation of the valine and leucine variants which is made possible by the presence of glutamic acid at position 228 in the latter. Such a situation may not exist in other enzymes, thereby rendering the problem of heterogeneity an open issue until the complete amino acid sequence is determined.

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Monoterpene Biosynthesis. I. Occurrence and Mevalonoid Origin of Gentiopicroside and Loganic Acid in *Swertia caroliniensis**

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ABSTRACT: Gentiopicroside (**1**), a secoiridoid monoterpene glucoside, and loganic acid (**2**), a new naturally occurring iridoid monoterpene glucoside, have been isolated from the same higher plant, *Swertia caroliniensis*. These congeners have been characterized by chemical and spectral means. Double-resonance nuclear magnetic resonance studies corroborated a recently revised structure **1** for gentiopicroside, isomeric to the previously designated one **1a**. Loganic acid has been identified by methylation to loganin (**3**), and acetylation to pentaacetate derivatives. Tracer experiments with acetate-2-^{14}C, mevalonate-2-^{14}C (**6**), and mevalonate-2-^{3}H have established the mevalonoid origin of the aglucone moieties of the glucosides. Conversion of mevalonate-labeled gentiopicroside into gentianine (**4**) has revealed all of the radioactivity occurs in the aglucone. Decarboxylation of the gentianine has afforded barium carbonate from C-11 with 20% of the total activity. Loganic acid derived from mevalonate-2-^{3}H-2-^{14}C has been converted to 7-oxologanin (**5**) with the loss of 63% of total tritium. Kuhn–Roth oxidation of the methyl ester (loganin (**3**)) of loganic acid which was derived from mevalonate-2-^{14}C has afforded acetic acid from C-10 and C-8 possessing only 0.7% of the total radioactivity. These results are consistent with a biogenetic scheme wherein mevalonate (**6**) is converted *via* geranyl pyrophosphate (**7**) into loganic acid (**2**) which then undergoes ring cleavage to afford gentiopicroside (**1**) (Scheme I).

Gentiopicroside (**1**) and loganic acid (**2**) belong to a class of highly oxygenated monoterpene glucosides referred to as secoiridoids and iridoids, respectively. (For a review of the iridoids (cyclopentanoid monoterpenes), see Bobbitt and Segebarth, 1969.) Chemical studies on this group of plant metabolites has led to the recognition of a structural resemblance to the nontryptamine-derived moiety of the indole alkaloids (Thomas, 1961; Wenkert 1962) and subsequent tracer work has implicated them in indole alkaloid biosynthesis. Scott and collaborators were first to succeed in incorporating mevalonate-2-^{14}C into vindoline (**12**) in studies with *Vinca rosea* (Money *et al.*, 1965). His group and Arigoni's found that the distribution of label from mevalonate-2-^{14}C (**6**) in the *Aspidosperma* alkaloid suggested that its C_{10} segment was of cyclopentanoid monoterpenoid origin (McCapra *et al.*, 1965; Goeggel and Arigoni, 1965). The first cyclopentanoid mono-

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