Chemical Characterization of Bovine Carboxypeptidase A Isolated from a Single Pancreas*

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Received July 3, 1964

Crystalline carboxypeptidase A has been isolated from the bovine pancreas of a single animal. This preparation, like those obtained from pooled glands, contains two closely related polypeptide chains differing from each other in the mutual replacement of a leucine and a valine residue near the C terminus. Its amino acid composition is generally similar to that of the enzyme isolated from the pooled glands but does not preclude additional possible replacements elsewhere in the molecule.

Carboxypeptidase A has been isolated from pooled glands of bovine pancreas by several procedures (Anson, 1937; Cox *et al.*, 1964; Allan *et al.*, 1964). The crystalline enzyme has been characterized on the basis of its terminal amino acid residues (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1963) and its amino acid composition (Bargetzi *et al.*, 1963). Bargetzi *et al.* (1964) have also demonstrated that preparations of the enzyme contain almost equal quantities of two similar chemical species, one containing the C-terminal sequence -Met.Glu.His.Thr.*Leu*.Asn.Asn.OH and the other the sequence -Met.Glu.His.Thr.*Val*.Asn.Asn.OH. Since all the preparations of the enzyme have utilized pooled pancreata as the starting material, the findings of Bargetzi *et al.* (1964) lead one to speculate whether the pancreas of a single animal would contain one or both of the species of polypeptide chains. The present communication describes the isolation of crystalline carboxypeptidase A from the pancreas of a single cow, its amino acid analysis, and the composition of the C-terminal peptide.

Experimental

A healthy purebred dairy cow (Holstein-Friesian) kindly provided by the Carnation Milk Farm, Washington, was slaughtered and its pancreas was frozen in dry ice within 20 minutes. An acetone powder of the gland was prepared from minced tissues according to Keller *et al.* (1956) and carboxypeptidase A was isolated from the acetone powders according to the method of Cox *et al.* (1964). The yield of the acetone powder was about 100 g and the yield of the crystalline enzyme was about 200 mg. The enzyme had the same specific activity toward hippuryl-DL-phenyllactate as the enzyme isolated from pooled pancreas (Bargetzi *et al.*, 1963).

Dinitrophenylation and subsequent hydrolysis of the enzyme yielded only two N-terminal amino acids, alanine and serine, in the ratio of 5:1 (without correction for destruction of the amino acids). Thus the preparation contains predominantly the chemical species, carboxypeptidase A_α, with N-terminal alanine, in keeping with the findings of Bargetzi *et al.* (1964).

The amino acid composition of the protein was determined according to the procedures outlined by Bargetzi *et al.* (1963). The hydrolyses were carried out at four different time intervals; two such sets of analyses were carried out on the same protein preparation. The results are summarized in Table I, which indicates that the amino acid composition of the enzyme is strikingly similar to that previously reported for the enzyme prepared from pooled glands (Bargetzi *et al.*, 1963). Nearly all amino acid residues approached integral values.

Table I
Amino Acid Composition of Carboxypeptidase A Isolated from a Single Pancreas

Amino Acid	Single Pancreas: Mean Value[a] (number of residues per molecule)	Single Pancreas: Nearest Integer (number of residues per molecule)	Pooled Glands (Bargetzi *et al.*, 1963) (number of residues per molecule)
Aspartic acid	27.9 ± 0.20	28	28
Threonine[b]	25.1	25	28
Serine[b]	30.7	31	33
Glutamic acid	24.8 ± 0.10	25	25
Proline[c]	11.0 ± 0.25	11	10
Glycine	22.7 ± 0.10	22.5–23	22.5
Alanine	20.0	20	20
Half-cystine[d]	2.0	2	2
Valine[e]	16.0	16	16
Methionine	2.8 ± 0.06	3	3
Isoleucine[e]	20.0	20	20
Leucine	22.5 ± 0.30	22–23	23
Tyrosine	17.8 ± 0.30	18	19
Phenylalanine	15.3 ± 0.10	15	16
Lysine	14.4 ± 0.30	14–15	15
Histidine	7.6 ± 0.20	8	8
Arginine	10.3 ± 0.20	10	11

[a] Mean of two sets of determinations at four different time intervals. [b] In the present experiment, serine and threonine represent values obtained at zero-time hydrolysis by linear extrapolation. A higher order of decay of these amino acids was observed with the enzyme isolated from pooled glands. [c] Cysteine which appears in the same position as proline on the amino acid analyzer may be responsible for the apparent higher values of proline. In the performic acid–oxidized protein, the value of proline was 10.2 residues. [d] Half-cystine determined as cysteic acid. [e] Values for valine and isoleucine represent maximum recovery.

The C-terminal hexapeptide was cleaved from the purified protein at the methionyl–glutamic acid bond with cyanogen bromide, and isolated by the following modification of the procedure of Bargetzi *et al.* (1964): The crystalline enzyme (65 mg) was dissolved in 5 ml of 75% trifluoroacetic acid and incubated with 150 mg of cyanogen bromide at room temperature for 24 hours. The solution was diluted 15-fold with water and stirred for 1 hour, and insoluble fragments were removed by centrifugation. Contaminating small peptide fragments were extracted from the precipitate by dissolving it in 4 ml of trifluoroacetic acid and reprecipitating the large fragments by dilution with water to 5% acid concentration. The precipitate was washed once

* This investigation was supported in part by the U. S. Public Health Service (GM-04617), by the American Cancer Society (P-79), and by the Office of Naval Research (NONR 477-04).

TABLE II
COMPOSITION OF THE C-TERMINAL FRAGMENT LIBERATED BY CYANOGEN BROMIDE

Amino Acid	Single Pancreas (residues per molecule)	Pooled Glands[a] (residues per molecule)
Histidine	1.01	0.99
Aspartic acid	1.96	1.92
Threonine	0.95	0.94
Glutamic acid	1.01	1.01
Valine	0.53 } = 0.96	0.61 } = 1.00
Leucine	0.43 }	0.39 }

[a] Taken from Bargetzi *et al.* (1964).

again with 5% trifluoroacetic acid and centrifuged. The three supernatants were combined, extracted three times with ether, and lyophilized. The dry product was extracted four times with small volumes of 0.5 M pyridine-acetate buffer, *p*H 6.5. The soluble material was subjected to paper electrophoresis at *p*H 2.1 for 1 hour at 2000 volts. The only major ninhydrin-positive area was located with the aid of a guide strip, eluted with water, and analyzed for its constituent amino acids in a Spinco amino acid analyzer. The results are compared in Table II with the composition of the analogous peptide previously isolated from the enzyme prepared from the pooled glands (Bargetzi *et al.*, 1964). It is evident that the two compositions are similar and that both leucine and valine are present in these peptides. The absence of homoserine in both cases confirms the identity of these peptides as being C-terminal. Hence, it may be concluded that the preparation from the single pancreas also contains two different but related C-terminal sequences.

DISCUSSION

The availability of crystalline carboxypeptidase A from the pancreas of a single animal has permitted a chemical characterization of this enzyme. The amino acid composition of the protein, given in Table I, corresponds closely to integral number of residues for most amino acids, provided 20 residues of alanine are assumed to be present as in the pooled preparations. Thus there is general agreement with the composition of the enzyme isolated from pooled glands except for tyrosine, phenylalanine, arginine, and possibly lysine. The apparent differences in the contents of serine and threonine must be taken with some reservation because a zero-order extrapolation was used in the case of the enzyme from the single pancreas, whereas a higher order of destruction of these hydroxyamino acids was observed in the case of the enzyme of the pooled glands. Closer scrutiny of the amino acid composition reveals that the enzyme of the single pancreas appears to be about 9 residues smaller (including threonine and serine). It should be emphasized that the amino acid composition is determined only as ratios of the amino acids, and if the alanine content is assumed to be 20.5 residues there is even better agreement between the two proteins in the total number of residues, but differences in aspartic acid, glutamic acid, and other amino acids become evident. It can only be inferred that the amino acid composition of the protein, while generally similar to that of the enzyme of the pooled glands, is suggestive of several replacements in the molecule.

The main purpose of this investigation was to examine whether the preparation from the single pancreas resembled that obtained from pooled glands in containing two species of nearly identical polypeptide chains differing in the mutual replacement of a leucine and a valine residue at a specific locus at the C terminus. This unusual replacement was discernible mainly by its fortuitous location in a small fragment of the molecule which could be readily liberated by the action of cyanogen bromide on the protein. The results in Table II indicate that even in the case of the enzyme isolated from a single animal the same pair of C-terminal peptides was found. Separation and chemical characterization of the individual chains of the enzyme would be required to ascertain whether additional replacements, suggested by the amino acid composition, exist elsewhere in the molecule. In addition it would be of interest to establish whether the two chemical species thus far found in all the preparations are equally active enzymatically.

The precursor of carboxypeptidase A, procarboxypeptidase A, has been shown to exist in the pancreas in two different forms (Brown *et al.*, 1963; Yamasaki *et al.*, 1963). It is plausible that the different polypeptide chains of carboxypeptidase A are derived from different precursors. While the characterization of the enzymes derived from pure precursors could answer this question, alternatively, there may be a genetic explanation of both the heterogeneity at the C terminus and the variations in the amino acid composition. Thus it is possible that the heterogeneity of the enzyme is an expression of two independent pieces of genetic information found in all cattle, or that in the present case the individual animal from which the enzyme was derived was heterozygous and that the two species of the protein were produced by two alleles of the same gene. The present investigation illustrates the need for and feasibility of further experimentation on the origin of heterogeneity of the enzyme, and focuses attention on a problem that may have severe consequences in the determination of the primary sequence of this enzyme.

ACKNOWLEDGMENT

The authors wish to thank Mr. Richard Granberg and Mr. Peter Schneider for their technical assistance.

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