THE PRIMARY STRUCTURE OF GOAT AND SHEEP PANCREATIC RIBONUCLEASES

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Received 28 January 1974

1. Introduction

The comparative study of pancreatic ribonuclease structure was started by Anfinsen [1]. With semi-quantitative data obtained upon digestion of oxidised sheep ribonuclease with trypsin and chymotrypsin, the primary structure was compared with that of bovine ribonuclease [2]. Since then, many pancreatic ribonucleases from different mammals have been isolated, and the primary structures of the ribonucleases from rat [3], pig [4, 5], horse [6], red deer, roe deer [7], reindeer, European elk and fallow deer (Gita Leijenaar and J. J. Beintema, unpublished) have been determined.

Recently, Kobayashi and Hirs [8] determined the amino acid sequence of sheep ribonuclease from pancreatic secretion. Here we present the primary structure of goat and sheep ribonucleases* isolated from pancreatic tissue after sulfuric acid extraction and subsequent ammonium sulfate precipitation. Differences with previous results of Anfinsen et al. [1] and a different amide assignment by Kobayashi and Hirs [8] are discussed.

2. Materials and methods

Trypsin-DCC was a product of Miles-Seravac (Maidenhead). α-Chymotrypsin was from Worthington Biochemical Corp. Thermolysin, A and B grade was obtained from Calbiochem. Aminopeptidase (hog kidney) was from P.L. Biochemicals Inc. (Milwaukee). Subtilisin Carlsberg was a gift from Novo Industri (Copenhagen). Phenylisothiocyanate was sequencer grade from Beckman Instruments. Trifluoroacetic acid (A.R.) was obtained from Baker.

Sephadex G-25 (fine) and G-50 (superfine) were from Pharmacia (Uppsala). Dowex 50 W-X2 (200–400 mesh), Fluka, was fractionated by a hydraulic method [9]; the 80–120 μm fraction was used. Dowex AG1-X2 (Biorad) was fractionated in the same way, the 30–50 μm fraction was used. Polyamide sheets were from Cheng Chin Trading Co Ltd. (Taiwan) or from Schleicher and Schüll (Dassel). All other reagents were A.R. grade.

Goat and sheep ribonuclease were isolated by affinity chromatography according to Wierenga et al. [5] preceded by ammonium sulfate fractionation. Polyacrylamide gel electrophoresis, amino acid analysis, dansylation, dansyl–Edman degradation, tryptic and aminopeptidase digestion were performed as described [5, 7].

Subtilisin digestion was carried out on 4 mg samples of goat ribonuclease in 225 μl 0.1 M Tris–HCl, pH 8.0 at 0°C (E:S = 1:100 by wt.). After 90 min 250 μl 0.5 M HCl was added and the mixture was applied to a Sephadex G-50 (superfine) column (150 × 1 cm). Elution was carried out with 0.05 M HCl at a flow rate of 10 ml/hr, collecting 1.5 ml fractions. The S-peptide (1-20), separated from S-protein (21–124) and undigested RNase, was further purified by preparative paper electrophoresis at pH 3.5.

Thermolysin digestion of ribonuclease was performed in 0.2 M ammonium acetate pH 8.5 (E:S = 1:40, by wt.) at 37°C for 2 hr.

Chymotryptic cleavage of tryptic peptides was performed in 0.1 M ammonium carbonate pH 8.0

* Preliminary sequence information was given in the 'Atlas of protein sequence and structure 1972' Vol. 5, p. D-130.

North-Holland Publishing Company – Amsterdam

(molar ratio 1:50) at 37°C for 2 hr.

Thermolysin cleavage of tryptic peptides was performed according to Matsubara et al. [10].

High voltage paper electrophoresis at pH 6.5 and 3.5 was performed as described [5, 7]. High voltage paper electrophoresis at pH 4.8 (pyridine–acetic acid–water, 3:3:94, v/v) for $2\frac{1}{2}$ hr at 1500 V, was also used for preparative purposes.

Peptides obtained upon tryptic and thermolysin digestion were fractionated on Sephadex G-25 (fine) columns (150 X 1.45 and 190 cm X 0.9 respectively). Elution was carried out at a flow rate of 5 ml/hr and 2.5 ml fractions were collected. High voltage paper electrophoresis of fractions eluting after the void volume was used to decide how to pool fractions for further purification. Purification was achieved by paper electrophoresis at pH 4.8 or by ion–exchange chromatography [6]. For one particular peptide (99–104) a second ion–exchange chromatography (Dowex AG1-X2) was used with a series of buffers ranging from pH 9.2 to 3.5, gradually rising in pyridine and acetic acid concentration and ending with 25% acetic acid.

Amide positions were assigned using amino-peptidase digestion and the method of Offord [11] at suitable stages of the Edman-degradation.

Automatic degradation of oxidised sheep ribonuclease was performed in a Beckman 890C sequencer using a standard protein program.

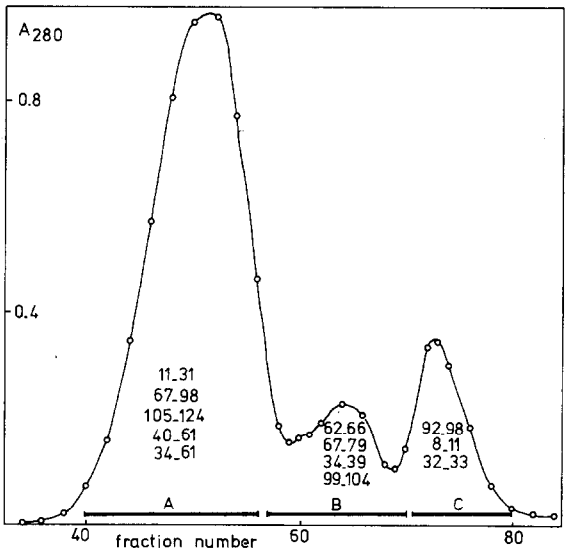

Fig. 1. Gel filtration on Sephadex G-25 (fine) (1.45 X 150 cm) of a tryptic digest of goat ribonuclease (the corresponding digest of sheep ribonuclease showed similar results). Elution with 0.1 M acetic acid. Flow rate 4.6 ml/hr; 2.3 ml fractions, (o–o–o) A_{280}. The peptides found in pools A, B and C (indicated by bars) are denoted by their positions in the sequence.

3. Results and discussion

Goat and sheep ribonuclease appeared homogeneous by the following criteria: (i) only enzymatically active bands were visible upon polyacrylamide gel electrophoresis; (ii) only di-DNS-Lys was found after dansylation of the two proteins.

Tryptic peptides accounting for the whole sequence of sheep and goat ribonuclease were isolated (fig. 1) and purified, except for sheep peptide 11–31, in which we could not determine the exact number of serine and alanine residues. For this reason, the N-terminal sequence (1–25) of sheep ribonuclease has been determined with an automatic sequencer.

Overlapping peptides were obtained by partial cleavages with trypsin DCC and from a thermolysin digest. (fig. 2). Chymotrypsin and thermolysin were also used for secondary digests of tryptic peptides.

No overlaps were obtained at three points in the sequence, viz. 61–62, 66–67 and 98–99, leaving 4 fragments. The locations of these fragments were inferred from homology with other ribonucleases.

The amino acid sequences of all peptides differing in amino acid composition from the corresponding bovine peptides, and of several other peptides, have been determined as indicated by arrows in Fig. 2. No differences were found between sheep and goat ribonuclease. Goat and sheep ribonuclease exhibit only four differences with bovine ribonuclease (Table 1), assuming maximum homology in some undetermined amide positions. In bovine ribonuclease these positions are located at the surface of the molecule. The three substitutions proposed by Anfinsen et al. [1] based on semiquantitative data

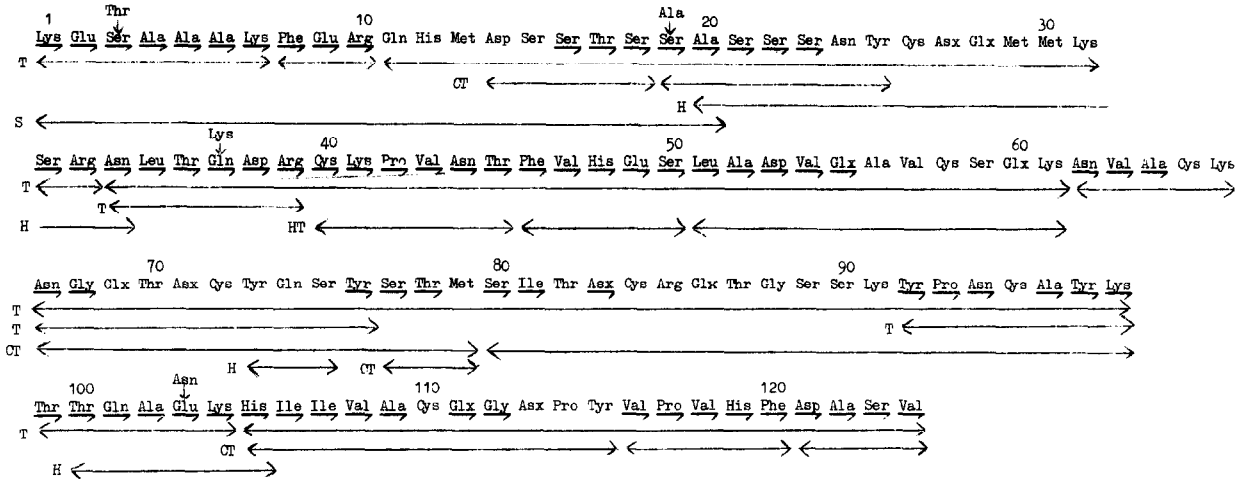

Fig. 2. Amino acid sequence of sheep and goat ribonuclease. The isolated peptides indicated by (⟷) are from the goat enzyme. From sheep ribonuclease only the tryptic peptides have been isolated. Goat RNase peptide sequences determined with the dansyl-Edman technique are indicated by arrows (⟶). The four substitutions compared to bovine RNase are also shown (↓). T = peptides from primary tryptic digest; H = peptides from primary thermolysin digest; CT = peptides from secundary chymotryptic digest (of tryptic peptides T); HT = peptides from secundary thermolysin digest (of tryptic peptides T); S = S-peptide.

Table 1
Differences between goat/sheep ribonuclease and bovine ribonuclease

Position	3	19	37	103
Goat/sheep RNase	Ser	Ser	Gln	Glu
Bovine RNase	Thr	Ala	Lys	Asn

have now been proven. However, it was demonstrated that residue 37 is Gln rather than Glu as suggested by Anfinsen et al. An additional replacement was found at position 19.

These replacements agree with the results of Kobayashi and Hirs [8] except for the amide assignment of residue 103, where they found a glutamine residue. Our results and those from Anfinsen [1] indicate that the tryptic peptide Thr-Thr-Glx-Ala-Glx-Lys (99–104) is neutral at pH 6.5, which means that it contains one Glu and one Gln. Aminopeptidase digestion yielded the sequence Thr-Gln-Ala-Glu, and electrophoresis at pH 6.5 [11] at several stages of the Edman degradation confirmed this amide assignment. No partial deamidation was found to occur. The contradictory assignments of residue 103 are probably due to a difference in the method of isolation. All pancreatic ribonucleases sequenced to date, except for the pig enzyme and the sheep enzyme already mentioned above, have been isolated from tissue after sulfuric acid extraction. Except for the bovine enzyme, these ribonucleases have glutamic acid at position 103, in contrast to the pig and sheep enzymes obtained from pancreatic secretion, which both have glutamine at this position. The difference between sheep pancreatic ribonuclease from tissue and secretion probably means that complete deamidation may have occurred at a single position in this part of the sequence. Apparently, one out of two glutamines (101 and 103), which both are located near each other at the surface of the molecule, is particularly sensitive to sulfuric acid.

A plot according to Dickerson [12] for the pancreatic ribonucleases of hoofed mammals shows a constant and high evolution rate for this class of proteins [7]. The data obtained from the sequences of goat and sheep ribonuclease correspond quite well

with this constant evolution rate, in contrast to the hemoglobins of these species, which differ much more from bovine hemoglobin than expected from the evolution rate of mammalian hemoglobins [7].

Acknowledgements

Part of this work has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.). We thank Miss Gerda Groen for her skilful technical assistance. We also thank Dr. A. Sussman and Mr. G. Sordée of Beckman Instruments and Drs. Wim Gaastra for performing the automatic Edman degradation in the Beckman training centre in Geneva.

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