The Heterogeneity of the S Peptide of Bovine Pancreatic Ribonuclease A*

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ABSTRACT: Cleavage of bovine pancreatic ribonuclease A with the bacterial proteinase Nagarse is not as selective as was the earlier cleavage with subtilisin. Selective cleavage of the S peptide of ribonuclease with cyanogen bromide led to the liberation of the octapeptide H-Asp(14)-Ser-OH(21) in addition to the expected heptapeptide H-Asp(14)-Ser-OH(20). The unexpected partial separation of the two components by gel chromatography suggests considerable conformational differences. Both peptides were purified and characterized.

The cleavage of bovine pancreatic ribonuclease with cyanogen bromide (Gross and Witkop, 1962) liberated four new NH_2-terminal amino acids expected on the basis of the number of methionine residues present in the molecule. The cleavage was both selective and quantitative and led to revisions of the primary structure of ribonuclease (Gross and Witkop, 1962; Smyth *et al.*, 1962; Potts *et al.*, 1962). Richards (1955) discovered the formation of an active complex when ribonuclease was treated, under specific conditions, with the bacterial (*Bacillus subtilis*) proteinase subtilisin. The separation of an eicosa peptide (S peptide) from a core protein (S protein) of ribonuclease was reported subsequently (Richards and Vithayathil, 1959). Once allowed to recombine, the ribonuclease S′ complex is fully enzymatically active (Richards and Vithayathil, 1959). It appeared that in the course of this digestion of ribonuclease with subtilisin only one peptide bond, *viz.*, 20–21, was cleaved.

The formation of the S peptide and its recombination with the core protein has been the subject of many investigations. Recently, subtilisin had to be replaced by a commercially available bacterial proteinase known as Nagarse,[1] which differs from subtilisin both in the amino acid sequence and in the proteolytic specificity (Hunt and Ottesen, 1961). In the present study, Nagarse was used to prepare S peptide. When the single methionyl peptide bond of this S peptide was cleaved with cyanogen bromide (Gross and Witkop, 1962, and unpublished data), the resulting COOH-terminal peptide was not homogeneous. It consisted not only of the heptapeptide containing the seven amino acids expected from the primary structure of bovine pancreatic ribonuclease (Figure 1), but was admixed with an octapeptide with a COOH-terminal serine, residue 21 of bovine pancreatic ribonuclease. The two peptides were separated by ion-exchange chromatography on 0.9 × 150 cm columns of Amberlite IR 120[2] which were operated on automatic amino acid analyzers (Spackman *et al.*, 1958), initially at pH 3.25 and later, for reasons of better separation, at pH 2.2.

Experimental Section

Ribonuclease was obtained commercially from Wilson Laboratories, Chicago, Ill., and purified by ion-exchange chromatography according to the procedure of Hirs *et al.* (1953). *S peptide* was prepared according to the method of Richards and Vithayathil (1959) with the exception that subtilisin was replaced by Nagarse. The S peptide was separated from S protein by gel filtration over Sephadex (Gross and Witkop, 1966).

The cyanogen bromide reaction on the S peptide was carried out under standard conditions (Gross and Witkop, 1962, and unpublished data): 0.1 N hydrochloric acid, 30-fold excess of reagent, at room temperature for 24 hr. Excess reagent and by-products were removed by lyophilization. The reaction products were separated by gel filtration over Sephadex G-25 (column dimensions 6 × 120 cm; 0.2 N acetic acid). The resulting resolution pattern is shown in Figure 2.

The material represented by the last of the three peaks was rechromatographed on Amberlite IR 120 columns (0.9 × 150 cm, 0.2 N sodium citrate buffer, pH 3.25 or 2.2, 50°) and resolved into two components (Figure 3) on an automatic amino acid analyzer (Spackman *et al.*, 1958). For preparative purposes the effluent was split into two streams. The smaller volume was pumped to the automatic analyzer, while the larger portion of the effluent was directed to a fraction collector.

The sodium citrate of the eluting buffer was removed

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[1] Teikoku Chemical Industry Co., Ltd. 7, Osaka, Japan, through Biddle Sawyer Corp., New York, N. Y.

[2] Drs. W. L. Haas and K. Hofmann have used these conditions for the purification of the synthetic heptapeptide which they kindly made available to us for direct comparison.

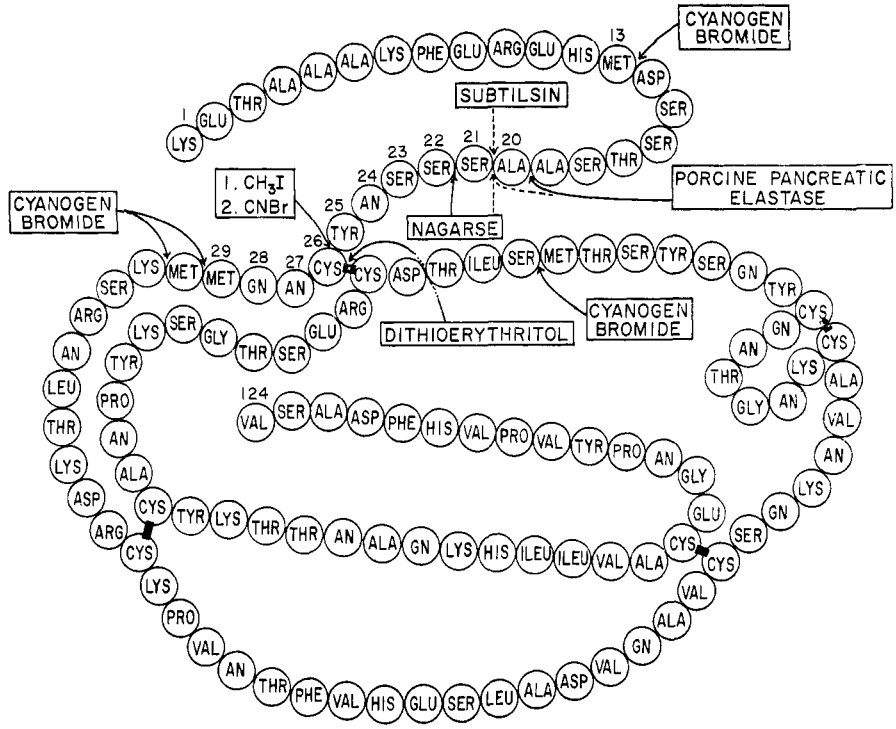

FIGURE 1: The primary structure of bovine pancreatic ribonuclease A. The Nagarse-released S peptides comprise the sequences 1–20 and 1–21.

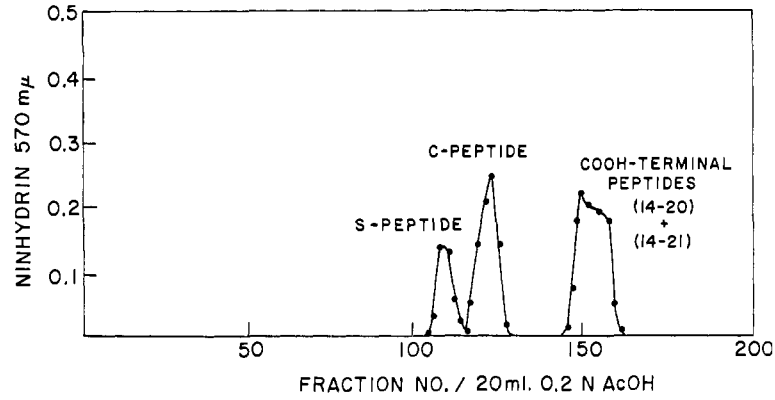

FIGURE 2: The separation on Sephadex G-25 of the fragments of RNase S peptide after cleavage with cyanogen bromide. Column dimensions 6 × 180 cm. C peptide (C for cyanogen bromide) is identical with peptide 1–13 from cleavage of RNase A with cyanogen bromide.

by desalting the pooled fractions on a Sephadex G-10 column. The salt-free peptides were chromatographed at pH 2.2 and otherwise identical conditions on the Amberlite IR 120 column that was earlier used for their separation (Figure 4).

The desalted peptides were analyzed for their amino acid composition in the automatic amino acid analyzer (Spackman *et al.*, 1958). The NH_2-terminal residues were assayed as dinitrophenyl derivatives (Sanger, 1945). The COOH-terminal hydrazinolysis was analyzed on the automatic amino acid analyzer.

Results

Cleavage with cyanogen bromide of a Nagarse-released S peptide produces *two* COOH-terminal peptides. The amino acid analysis (Table I) of the two peptides differs by one residue of serine. According to the established primary structure of bovine pancreatic ribonuclease (Gross and Witkop, 1962; Smyth *et al.*, 1962; Potts *et al.*, 1962) the Nagarse-released S peptide must, in part, consist of a second peptide incorporating residue 21. Nagarse in its attack on a single peptide

TABLE I: Amino Acid Composition[a] of COOH-Terminal Peptides of RNase S Peptide after Cleavage with Cyanogen Bromide.

Amino Acid	Octapeptide 14–21	Heptapeptide 14–20[b]
Aspartic acid	0.122 (1)	0.214 (1)
Threonine	0.120 (1)	0.194 (1)
Serine	0.466 (4)	0.554 (3)
Alanine	0.228 (2)	0.375 (2)

[a] Nearest integral values in parentheses; μmoles/0.25 mg. [b] The heptapeptide was contaminated by a trace of glutamic acid.

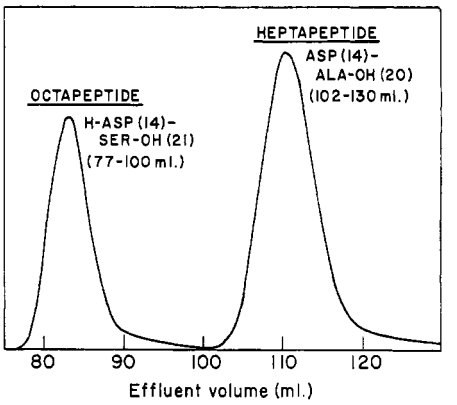

FIGURE 3: Separation of the octa- and heptapeptide on Amberlite IR 120 (0.9 × 150 cm, 0.2 N citrate buffer, pH 2.2, 50°).

bond in ribonuclease is less specific than were the subtilisin preparations from the Carlsberg Laboratories (Richards, 1955; Richards and Vithayathil, 1959). These new observations were confirmed by separation and characterization of the two peptides in the following way.

The octapeptide (14–21) containing the COOH-terminal serine is first eluted from the Amberlite column (Figure 3, effluent volumes 77–100 ml). The heptapeptide (14–20) was eluted at effluent volumes 102–130 (Figure 3). After desalting and rechromatography under identical conditions the two peptides occupy essentially the same positions on a comparable ion exchange column (Figure 4).

As expected, both peptides have aspartic acid as NH_2-terminal residues. The COOH-terminal residue of the octapeptide is serine, that of the heptapeptide alanine. These findings are in agreement with the partial sequences 14–21 and 14–20, respectively, of ribonuclease. Likewise, hydrazinolysis of the Nagarse-released S peptide[3] before cleavage with cyanogen bromide showed the presence of both COOH-terminal alanine and serine.

The experimental conditions of the cleavage of ribonuclease with Nagarse were carefully controlled. Identical results were obtained with a number of S-peptide preparations from ribonuclease samples from different sources.

So far Nagarse preparations have all come from the same source. *B. subtilis* protease preparations from several manufacturers have now been secured and will be tested for their capacity to release homogeneous S peptide from bovine pancreatic ribonuclease.

It is possible that initially Nagarse cleaves only one peptide bond, *viz.*, that between residues 21 and 22, and that the resulting COOH-terminal serine is partially lost subsequently. The experimental data presented here do not exclude this assumption. There is experimental evidence which supports the initial cleavage of a single peptide bond. The peptide next to the S peptide in the primary structure of ribonuclease and terminating in the original residue 29 of the enzyme was prepared in the following way (Gross *et al.*, 1965). S protein was cleaved with cyanogen bromide. After separation and purification of the reaction products the modified S protein was reduced with dithioerythritol (Cleland, 1964). The reaction mixture was treated with methyl iodide in order to convert cysteine to *S*-methylcysteine (Gross *et al.*, 1965). The desired peptide was isolated by gel chromatography on Sephadex G-25 and analyzed. The peptide contained glutamic acid (theory, one residue) and serine (theory, two or three residues) in a ratio of 1:1.48 which supports the assumed cleavage by Nagarse of the peptide bond 21→22 in ribonuclease. Porcine pancreatic elastase is capable of cleaving two peptide bonds in ribonuclease, *viz.*, between residues 19 and 20,

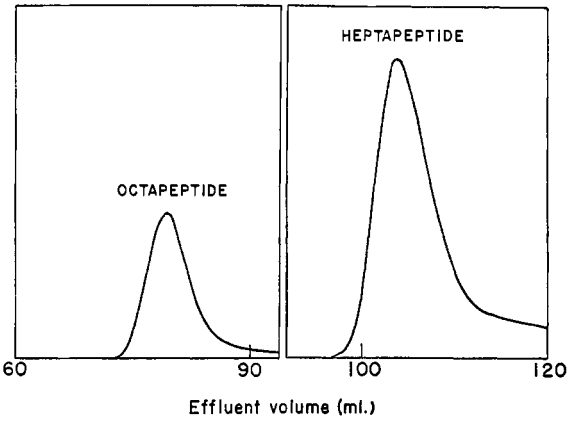

FIGURE 4: Rechromatography of the octapeptide and heptapeptide on Amberlite IR 120 (0.9 × 150 cm, 0.2 N citrate buffer, pH 2.2, 50°).

[3] Preparations of Nagarse-released S peptide have been resolved on ion-exchange columns into the two components by Drs. C. H. W. Hirs and M. S. Dosher, to whom we are indebted for communicating these results prior to publication [*Federation Proc. 25*, 527 (1966)]. *Cf. Biochemistry 6*, 304 (1967).

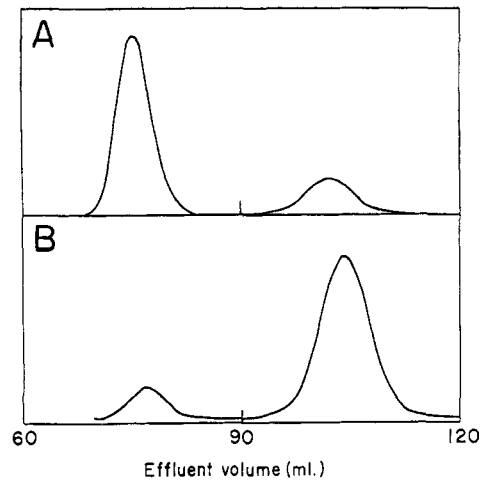

FIGURE 5: Rechromatography of the octa- and heptapeptide after partial separation on Sephadex G-25 (*cf.* Figure 2); the column is Amberlite IR-120, 0.9 × 150 cm, pH 2.2, 0.2 N citrate buffer, 50°. (A) Octapeptide, effluent volume 69–84 ml. (B) Heptapeptide, effluent volume 95–115 ml.

and 20 and 21, respectively (Klee, 1965). The experimental evidence indicates that the peptide bond Ala(19)-Ala(20) is cleaved first. The newly formed NH_2-terminal alanine is removed subsequently. According to the evidence presented above, the Nagarse preparation used removes the *COOH-terminal* serine (21) of S peptide. Klee offers a model for the ready attack of the Ala-Ala-Ser sequence and attributes the stability of the peptide bonds to the unique regions of ribonuclease rich in serine and threonine. This model fails to rationalize the accessibility of the peptide bond Ser(21)-Ser(22).

The heterogeneity of the S peptide may be the result of the existence of more than one tertiary structure for ribonuclease. The high selectivity of subtilisin and porcine pancreatic elastase, enzymes which are usually rather unspecific, must be the result of conformational factors which in turn depend on hydrogen bonding and interaction of polar groups in native ribonuclease. The initial points of attack of both proteases are between the two doublets Ala(19)–Ala(20) and Ser(21)–Ser(22), respectively.

The two peptides of ribonuclease [Asp(14)→Ala(20)] and [Asp(14)→Ser(21)] differ sufficiently, presumably in conformation, to separate partially upon gel chromatography on an improved Sephadex column with bead-shaped particles (E. Gross and J. L. Morell, unpublished results). When the two partially separated peptides were rechromatographed on Amberlite IR-120 under the conditions under which the octa- and the heptapeptide are separated, the chromatographic patterns of Figure 5 were obtained. The major peak in Figure 5A represents the octapeptide, the minor peak the heptapeptide. The octapeptide had been eluted first from the Sephadex column. The major peak in Figure 5B occupies the position of the heptapeptide and only a small amount of the octapeptide is present in this fraction.

The interesting sequence 15–20 appears to contribute little to the activity of the enzyme: De(15–20)- and de(16–20)-ribonucleases S′ were as active as native ribonuclease (Hofmann *et al.*, 1966a,b).

The significance of the histidine residue (Hofmann *et al.*, 1963; Finn and Hofmann, 1965) in position 12 has been newly demonstrated (Hofmann and Bohn, 1966). The partial S-peptide sequences 1–12 with β-(pyrazolyl-1)-L-alanine and β-(pyrazolyl-3)-L-alanine, respectively, in position 12 do not produce enzymatic activity upon combination with S protein in ratios as high as 5500:1 and 1500:1, respectively.

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