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The primary structure of rat parathymosin

(rat thymus/rat liver peptide/peptide isolation/prothymosin α/thymosin α_1)

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ABSTRACT **Parathymosin has been isolated from rat thymus and from rat liver. Its primary structure is reported as follows: AcSer-Lys-Ser-Glu-Val-Glu-Ala-Ala-Ala-Glu^{10}-Leu-Ser-Ala-Lys-Asp-Leu-Lys-Glu-Lys-Lys^{20}-Asp-Lys-Val-Glu-Glu-Lys-Ala-Gly-Arg-Lys^{30}-Glu-Arg-Lys-Lys-Glu-Val-Val-Glu-Glu-Glu^{40}-Glu-Asn-Gly-Ala-Glu-Glu-Glu-Glu-Glu-Glu^{50}-Thr-Ala-Glu-(Gly$_3$,Asx$_7$,Glx$_{14}$)-Glu-Gly-Pro^{80}-Val-Arg-Lys-Arg-Thr-Ala-Glu-Glu-Glu-Asp^{90}-Glu-Ala-Asp-Pro-Lys-Arg-Gln-Lys-Thr-Glu^{100}-Asn-Gly-Ala-Ser-AlaOH. The blocking group at the NH_2 terminus was identified by mass spectrometry as acetyl. Regions homologous to amino acid sequences in prothymosin α were found to be located between residues 14–20, 23–25, 33–39, 41–43, and 83–87 of parathymosin.**

Parathymosin was first isolated from rat thymus (1) as a by-product of the procedure used for the isolation of prothymosin α. It was named parathymosin because of its structural similarity to prothymosin α, the putative precursor of thymosin α_1 (2, 3). Parathymosin, like prothymosin α, was found to be devoid of aromatic and sulfur-containing amino acids. It was also similar in size, containing approximately 105 amino acid residues, as compared to 113 for prothymosin α (3). Analysis of a 30-amino acid sequence at the NH_2 terminus of parathymosin revealed a region, comprising residues 14–25, having close homology to the corresponding segment in prothymosin α. However, this homology did not extend to the first 13 amino acid residues, and parathymosin was found to react poorly, if at all, in a radioimmunoassay that used an antibody directed against an epitope that included the first 14 amino acid residues of prothymosin α (4).

The precise functions of these two polypeptides remain undetermined. Preliminary studies (see ref. 1) suggest that prothymosin α, like thymosin α_1 (5, 6), can protect mice against opportunistic infections and, indeed, may be active at lower doses than thymosin α_1 (1). Parathymosin did not exhibit this activity but it did appear to block the effects of prothymosin α. Its tissue distribution was also different from that of prothymosin α, with significantly higher concentrations found in liver and kidney than in thymus.

In the present paper, we report a simplified procedure for the isolation of both peptides from rat thymus and the nearly complete amino acid sequence of parathymosin. A procedure for the isolation of parathymosin from rat liver, a richer source for this peptide, is also described.

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MATERIALS AND METHODS

Materials. Rat tissues were collected immediately after sacrifice of the animals by decapitation, frozen in liquid N_2, and stored at −70°C. Other materials were from sources identified earlier (1, 3, 4).

Methods. Chromatography on Sephacryl S-200 was carried out as described (1). For the HPLC, a Waters Associates system with an Altex Ultrasphere ODS C_{18} column was used.

Protein and peptide analyses were carried out fluorometrically following alkaline hydrolysis and reaction with fluorescamine (7). Automated sequence analysis was as described (3). The quantitative radioimmunoassay for prothymosin α was as described by Haritos and Horecker (4).

Digestion with clostripain was carried out in 30 μl of 0.1 mM NH_4HCO_3 containing 50 μg of peptide substrate, 10 mM dithiothreitol, and 1.0 μg of clostripain preactivated by incubation in 1 mM $CaCl_2$ and 2.5 mM dithiothreitol at pH 7.6. After 15 hr at 25°C, the reaction was stopped by addition of HCOOH, and the solution was lyophilized.

Cleavage of Asn-Gly bonds with H_2NOH was by a modification of the method of Bornstein and Balian (8), as follows. A lyophilized sample of parathymosin (200 μg) was dissolved in 60 μl of 2 M H_2NOH previously adjusted to pH 9.0 with K_2CO_3. After 4 hr at 50°C, the solution was acidified with 26 μl of concentrated HCOOH, diluted with 10 ml of buffer A (1 M HCOOH/0.2 M pyridine, pH 2.8) and pumped through a Sep-Pak C_{18} cartridge. After the cartridge was washed with 10 ml of buffer A, the peptides were eluted with 3 ml of 40% acetonitrile in buffer A, and the eluate was lyophilized.

Digestion with *Staphylococcus aureus* V8 protease was in 25 μl of 0.1 M NH_4HCO_3 containing 50 μg of peptide, 2 mM EDTA, and 1.67 μg of protease. After 13 hr at 37°C, the solution was lyophilized.

For the isolation of peptides in the proteinase digests and after treatment with H_2NOH, the lyophilized solutions were dissolved in buffer A and separated by HPLC using the ODS C_{18} columns and a gradient of 0–40% acetonitrile in buffer A.

Hydrolysis of Asp-Pro bonds with HCOOH was by the procedure of Landon (9), with 50 μg of peptide dissolved in 26 μl of water and 100 μl of 88% HCOOH. After incubation for 24 hr at 40°C, the reaction was stopped by the addition of 33 μl of pyridine and 100 μl of 0.1% trifluoroacetic acid, and

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the solution was lyophilized. Peptides formed with this procedure were separated by HPLC as described above with a gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid.

Isolation of Prothymosin α and Parathymosin from Rat Thymus. A modification of the published procedure (10, 11) has been developed that avoids the requirement for desalting on Sep-Pak cartridges and is more convenient for larger scale preparations. The recoveries of both prothymosin α and parathymosin are significantly improved. In this procedure, the pulverized frozen thymus tissue was added to boiling water (1 g per 14 ml) in place of the phosphate buffer used previously. The boiled tissue suspension was homogenized with a Polytron homogenizer as described (2), and the extract obtained after centrifugation was treated at 4°C with an equal volume of buffer A. The precipitate was removed by centrifugation at 16,000 × *g* for 30 min, and the clear supernatant solution was concentrated on a Speed Vac (Savant). The peptides were then separated by chromatography on Sephacryl S-200 followed by HPLC as described (1, 10, 11).

For experiments on the biological activities of parathymosin, the product obtained after the first HPLC separation with a gradient of 1-propanol in buffer A (1) was rechromatographed on the same column with a gradient of 0–40% acetonitrile in buffer A. This procedure was not required for samples used for sequence analysis.

Isolation of Parathymosin from Rat Liver. Because the content of parathymosin is higher in rat liver than in rat thymus, liver was usually used for the isolation of this peptide. However, substantial modification of the isolation procedure was required as follows: 10 g of frozen, pulverized rat liver was added to 100 ml of boiling water, and the boiling was continued for 5 min after the final addition. The cooled suspension was homogenized with a Polytron homogenizer at top speed for 2 min. Insoluble material was removed by centrifugation, and the supernatant solution was adjusted to pH 5.2 with HCOOH (*ca.* 15 μl). The precipitate that formed was removed by centrifugation, and the clear supernatant solution was concentrated by ultrafiltration with a Diaflo YM2 filter. The retentate was washed on the filter with 10 ml of buffer A, and the insoluble material was removed by centrifugation. The clarified concentrate (2.2 ml) was chromatographed on a Sephacryl S-200 column (1.6 × 92 cm) as described (1). The rate of elution was 0.13 ml/min, and fractions were collected every 10 min. Fractions 68–92, containing the major peptide peak, were pooled. Prothymosin α, assayed by using the RIA for this peptide (see below), was present in fractions 77–92. The pooled fractions were lyophilized with a Speed Vac, and the residue was dissolved in 0.3 ml of buffer A. Separation of the peptides in this solution was accomplished by HPLC (Fig. 1). Peaks C and D were found to contain prothymosin α and parathymosin, respectively, as determined by amino acid analysis. For the experiment shown, the amino acid composition of the peptide in peak D was: $Asp_{13.2}Thr_{3.5}Glu_{36}Gly_{6.9}Val_{10.3}Ala_{4.1}Leu_2$-$Lys_{12.5}Arg_{4.8}$ (proline was not determined). Except for glutamic acid, the values correspond closely to those reported for parathymosin isolated from rat thymus (1).

RESULTS

Comments on the Parathymosin Procedures. The modified procedures described here make available larger quantities of parathymosin and prothymosin α than the methods previously available. The recoveries from 10 g of rat thymus were approximately 700 μg of prothymosin and 300 μg of parathymosin. Processing of 10 g of rat liver yielded approximately 500 μg of parathymosin, but the quantity of prothymosin recovered from liver was too small to be of interest (see Fig. 1). Both products were homogeneous when evaluated by

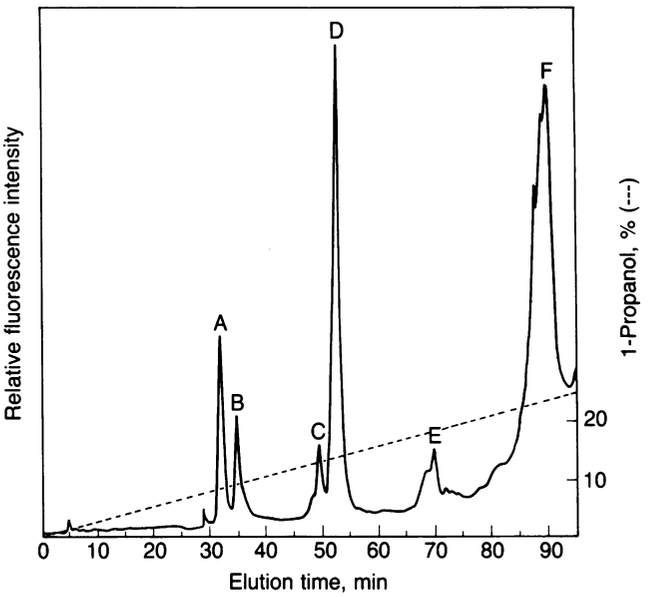

FIG. 1. Final step in the purification of parathymosin from rat liver by HPLC. An aliquot (0.15 ml) of the concentrated material in pooled fraction 68–92 from the Sephacryl S-200 column was applied to an ODS C_{18} column and eluted with a gradient of 0–40% 1-propanol in buffer A.

HPLC with the 1-propanol/buffer A system and by isoelectric focusing. However, parathymosin recovered from rat thymus was resolved into two peaks on HPLC using a gradient of acetonitrile (data not shown). This was not observed with parathymosin from liver, which was free of impurities in every test. The absence of isoleucine, aromatic amino acids, and sulfur-containing amino acids confirmed the purity of the rat liver parathymosin preparation.

Sequence Analysis. Automated sequence analysis of parathymosin, purified from either thymus or liver, confirmed the presence of a blocked NH_2 terminus (2, 3). Digestion with clostripain yielded six major peaks, one of which, designated C6, had the amino acid composition previously reported for residues 1–29 of parathymosin (1). The structure of this peptide, including the presence of the NH_2-terminal acetyl group, was confirmed by mass spectrographic analyses. The observed molecular mass was 3216.9, compared with a theoretical value of 3216.4 calculated from the amino acid sequence for residues 1–29 as shown in Fig. 2. The theoretical value for the *N*-formyl derivative would be 3202.4. The observed molecular weight also confirms the assignment of glutamyl and aspartyl residues at the positions shown.

The remainder of the sequence was deduced from automated sequence analysis of the peptides formed by digestion with clostripain, *S. aureus* V8 protease, and hydrolysis with hydroxylamine or formic acid (Fig. 2). The overlap from residues 29–35 had been established previously by analysis of thermolysin peptides (ref. 1 and unpublished data). Identical results were obtained with peptides derived from rat thymus parathymosin or from rat liver parathymosin for peptides C5, H2, C4, and C1. Digestion of rat liver parathymosin with thermolysin as described by Haritos *et al.* (1) yielded peptides with amino acid compositions corresponding to residues 5–10, 11–15, 11–35, and 16–35. The sequences of these peptides were confirmed by automated sequence analysis. The results indicate that the same peptide is present in thymus and liver.

A sequence of 24 residues with the presumed composition $Asx_7Glx_{14}Gly_3$, deduced from the estimated amino acid

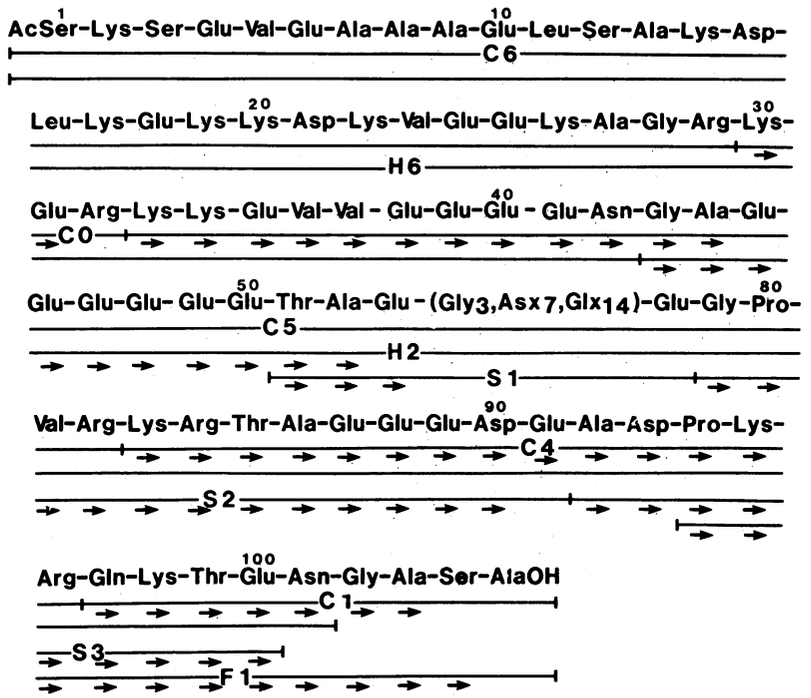

FIG. 2. The amino acid sequence of parathymosin. Peptides formed by treatment with clostripain, *S. aureus* V_8 protease, hydroxylamine, and formic acid are designated C, S, H, and F, respectively. The numbers refer to their order of elution in HPLC or, in the case of *S. aureus* protease, the elution positions of the peak fraction used for sequence analysis. The amino acid content of the missing segment ($Gly_3Asx_7Glx_{14}$, residues 54–77) was calculated from the composition of peptides C5, H2, and S1 and must be considered as approximate. The numbering of residues 78–105 is also tentative.

compositions of peptides C5, H2, and S1, could not be determined by methods currently available.

Comparison with Sequence of Prothymosin α. The primary structures of parathymosin and prothymosin α (3) are compared in Fig. 3. We have shown previously (1) that parathymosin contains sequences, including residues 14–20 and 23–25, that are identical to the corresponding residues in prothymosin α (Fig. 2). We now have identified two additional sequences in parathymosin that are homologous to sequences in prothymosin α. The sequence Lys-Lys-Glu-Val-Val-Glu-Glu-Xaa-Glu-Asn-Gly is present at positions 33–43 of parathymosin and at positions 19–29 of prothymosin α. The sequence Lys-Arg-Thr-Ala-Glu in parathymosin, including residues 83–87, is homologous with the sequence Lys-Arg-Val-Ala-Glu at positions 90–94 of prothymosin α. In addition, both peptides contain stretches of consecutive glutamyl residues, a result to be expected in view of their high content of glutamic acid. It is of interest that the sequence of residues 14–25 of prothymosin α is related to two different sequences (14–25 and 33–43) in parathymosin (see Fig. 4).

DISCUSSION

We have confirmed our previous finding (1) that parathymosin is found in higher concentrations in liver than in thymus and report here that liver is a better source for the isolation of this peptide. The results of sequence analysis with preparations of parathymosin from both thymus and liver have failed to reveal any differences between the peptides present in these tissues; therefore, it is assumed that they are encoded by a single gene. Despite the remarkable similarity in size and amino acid composition of parathymosin and prothymosin α, sequence homology was found only in a region close to the NH_2 terminus. Other observed sequence homologies were found to be displaced by 14 amino acid residues in one case

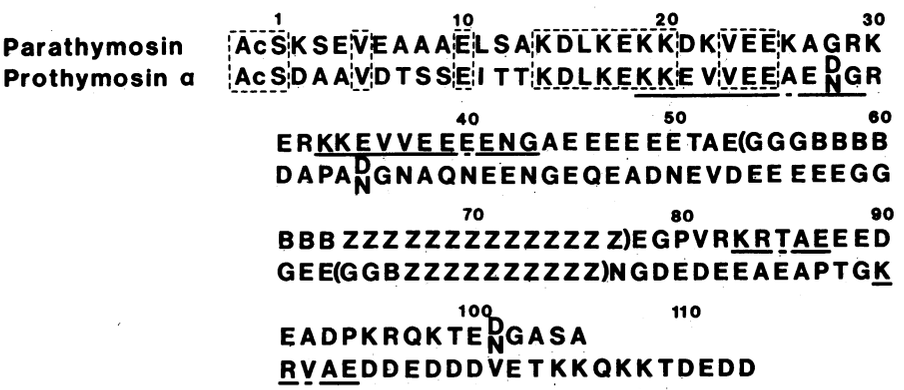

FIG. 3. Comparison of the known amino acid sequence of prothymosin (upper lines) with the reported sequence of prothymosin α (lower lines). The standard 1-letter amino acid code is used. The identical sequences are shown within the dashed boxes. Homologous sequences that occur in different regions of the two molecules are underlined. The undetermined segments are indicated within the parentheses.

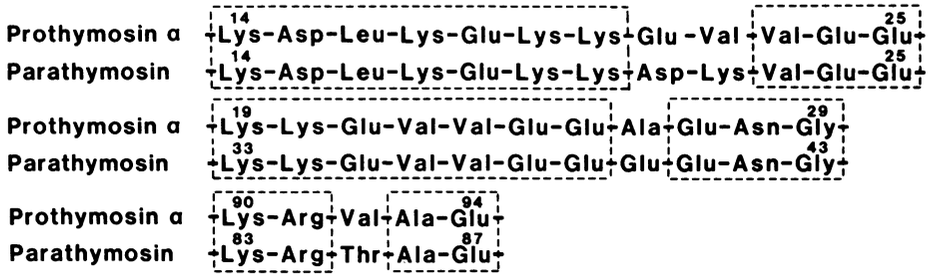

FIG. 4. Regions of sequence homology in prothymosin α and parathymosin.

and by 7 in another case, with the displacements occurring in opposite directions.

The distribution of the two peptides, with prothymosin α predominating in thymus and spleen and parathymosin present in higher concentrations in nonlymphoid tissues, provides important clues to their biological function.

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