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# Isolation and partial characterization of prothymosin α from porcine tissues

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Prothymosin α, an immunoactive polypeptide of 12 kDa, has been isolated from porcine thymus, spleen, lung and kidney. It lacks aromatic and sulfur-containing amino acids and has a high content of glutamic and aspartic acids. Tryptic digestion of porcine thymus prothymosin α yielded peptides which on separation, amino acid analysis and alignment with the known sequence of prothymosin α from rat and man showed that the amino terminal portion of the molecule is conserved and the few differences present are confined to the carboxy terminal.

Prothymosin α; Thymic peptide

## 1. INTRODUCTION

ProTα, a highly acidic polypeptide (p*I* 3.55), has been isolated [1] and sequenced [2] from rat thymus and identified in other rat tissues [3]. ProTα has been sequenced from human thymus [4] and identified in leukocytes [5]. The sequence of 109 amino acids [6] has been deduced from clones isolated from a human spleen cDNA library [6] and from simian virus 40-transformed human fibroblasts [7].

The amino acid sequence of the amino terminal portion of ProTα from rat and man is identical with thymosin $\alpha_1$, a 28-amino acid acidic peptide isolated and sequenced from extracts of calf thymus, prepared under conditions which did not prevent or minimize proteolytic activity [8,9].

Prothymosin α is immunoactive. In the human autologous MLR rat and porcine ProTα have been shown to be effective in nanogram quantities in doubling the magnitude of the response. Furthermore, these same polypeptides restored to normal levels the suboptimal MLR, whereas thymosin $\alpha_1$ had no effect [10,11]. In in vivo tests, species differences appear to be confined to the carboxy terminal of ProTα [4].

The isolation of ProTα from mammals other than rat, especially those closer to man and potentially better sources of this polypeptide, has not been reported. In this communication we present the purification of ProTα from porcine tissues, and the isolation of tryptic fragments by HPLC for structure-function studies.

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*Abbreviations:* ProTα, prothymosin α; HPLC, high-pressure liquid chromatography; MLR, mixed lymphocyte response

## 2. MATERIALS AND METHODS

Pig tissues from male 5–6-month-old animals (85 kg or over) were excised immediately after killing, cut into small pieces, frozen in liquid nitrogen and stored at −80°C until use [1]. Synthetic thymosin $\alpha_1$ [12] was a kind gift from A.M. Felix, and fluorescamine was a gift from W.E. Scott, both of Hoffmann-La Roche, Nutley, NJ, USA. Sodium boro[$^3$H]hydride, 12.1 Ci/mmol, was supplied by New England Nuclear.

Prothymosin α was assayed by radioimmunoassay as in [1,13,14]. The assay was sensitive in the range of 5–150 ng thymosin $\alpha_1$ (2–50 pmol). Peptide concentration was deter-



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mined manually with fluorescamine [15]. Samples for amino acid analysis were hydrolysed [1] and analysed as the phenylthiocarbamyl derivatives [16]. HPLC was performed on Altex Ultrasphere ODS $C_{18}$ columns, 5 $\mu$m (250×4.6 mm; Beckman Instruments), and analytical isoelectric focusing as described [1].

Prothymosin $\alpha$ from thymus, spleen, lung and kidney was purified according to Haritos et al. [1], as modified in [17]. Briefly, 10 g of unthawed tissue was pulverized in liquid $N_2$ and immediately added to 200 ml boiling water. The homogenate was sonicated, centrifuged, the supernatant acidified, centrifuged again, the resulting supernatant (acid supernatant) concentrated, and chromatographed on a column of Sephacryl S-200, superfine, [1.5 i.d. ×90 cm, equilibrated and eluted with buffer A (1 M HCOOH/0.2 M pyridine, pH 2.8)], and by HPLC (buffer A and 1-propanol gradient).

Tryptic digests of ProT$\alpha$ from thymus were obtained as in [2], and chromatographed on HPLC at room temperature. The column was equilibrated with buffer A and eluted with a gradient of the same buffer (solvent A) and 50% acetonitrile in buffer A (solvent B) (Haritos, A.A., personal communication). The flow rate was 0.6 ml/min, fractions were collected every 30 s and 0.06 ml aliquots were analyzed by fluorescamine. For amino acid analysis, aliquots of 0.05–0.12 ml were dried directly in glass hydrolysis ampoules.

## 3. RESULTS AND DISCUSSION

Acid supernatants from porcine thymus were found to contain 124 $\mu$g ProT$\alpha$ per g wet tissue,

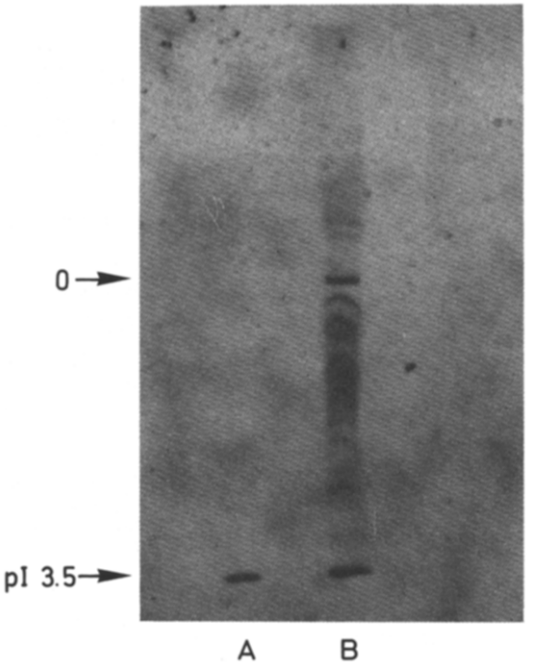


Fig.2. Isoelectric focusing of pig thymus ProT$\alpha$. (A) ProT$\alpha$ (20 $\mu$g) purified on HPLC (fig.1). (B) Material before HPLC (45 $\mu$g). O, origin.

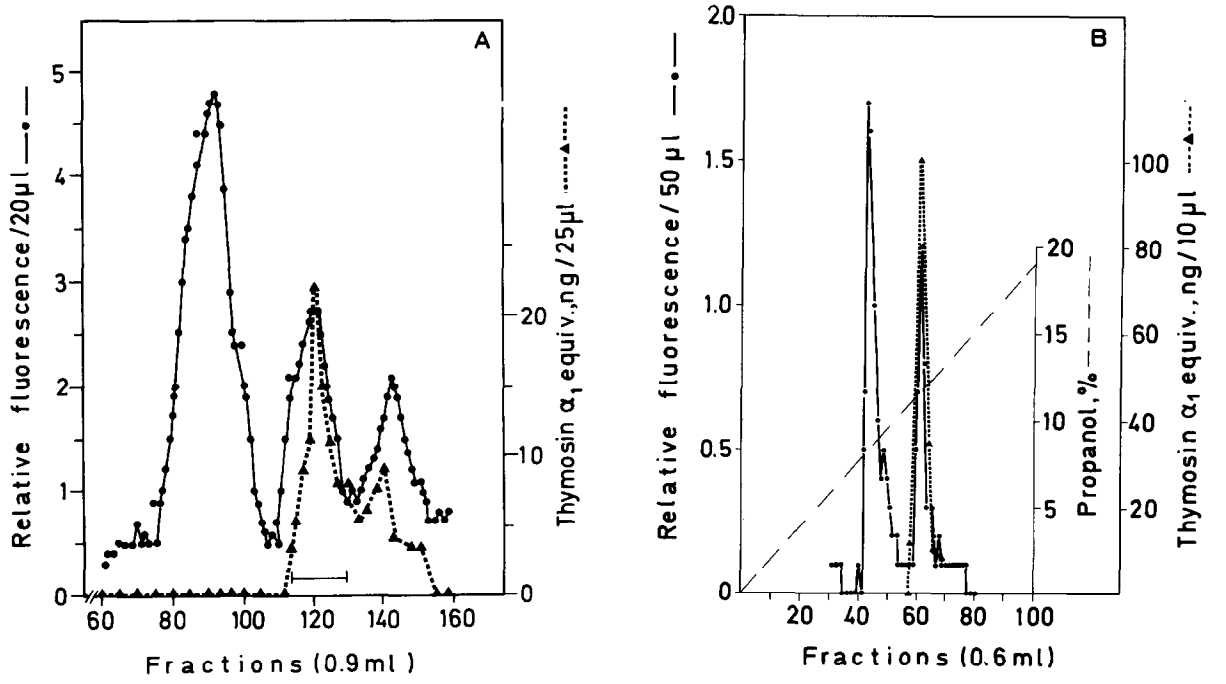


Fig.1. Purification of ProT$\alpha$ from pig thymus. (A) Gel filtration on Sephacryl S-200 (superfine) column (10 g tissue). (B) HPLC of the underlined peak in panel A (equivalent to 5 g tissue).

Table 1

Amino acid composition of prothymosin $\alpha$ from porcine tissues

| Amino acid | Thymus $\bar{x} \pm SD$ ($n=4$) | Spleen $\bar{x}$ ($n=3$) | Lung $\bar{x}$ ($n=3$) | Kidney $\bar{x}$ ($n=2$) |
|---|---|---|---|---|
| Asx | 27.2±0.6 | 25.9 | 23.2 | 26.8 |
| Glx | 37.5±0.9 | 36.3 | 34.4 | 36.9 |
| Ser | 2.5±0.4 | 2.4 | 3.3 | 2.5 |
| Gly | 8.9±0.6 | 9.4 | 10.5 | 9.3 |
| Thr | 5.0±1.6 | 4.8 | 4.0 | 5.9 |
| Ala | 11.2±0.3 | 11.7 | 12.5 | 11.1 |
| Arg | 1.9±0.2 | 2.1 | 2.2 | 1.6 |
| Pro | 1.4±0.1 | 2.1 | 2.8 | 1.3 |
| Val | 4.4±0.1 | 4.5 | 4.4 | 4.4 |
| Ile | 1.0±0.1 | 1.3 | 1.3 | 0.9 |
| Leu | 1.0±0.1 | 1.3 | 1.7 | 0.8 |
| Lys | 7.0±0.3 | 7.0 | 7.6 | 7.3 |
| Total | 109.0 | 108.8 | 107.9 | 108.8 |

followed by spleen, lung, kidney and liver (75, 66, 48 and 26 $\mu$g, respectively). These results were calculated using purified goat thymus ProT$\alpha$ as the standard in the radioimmunoassay (Frillingos, S., unpublished).

Purification of ProT$\alpha$ from porcine thymus by Sephacryl S-200 (fig.1A) followed by HPLC (fig.1B) gave a pure polypeptide with a p$I$ of 3.5, as judged by isoelectric focusing (fig.2). The yield from 10 g of thymus was 160 $\mu$g of pure ProT$\alpha$. ProT$\alpha$ was also purified from spleen, lung and kidney. The amino acid analysis of the top fraction from HPLC eluates, including those of thymus, is shown in table 1. The analyses show a composition rich in glutamic and aspartic acids, poor in hydrophobic amino acids and the absence of tyrosine, phenylalanine, histidine, tryptophan and

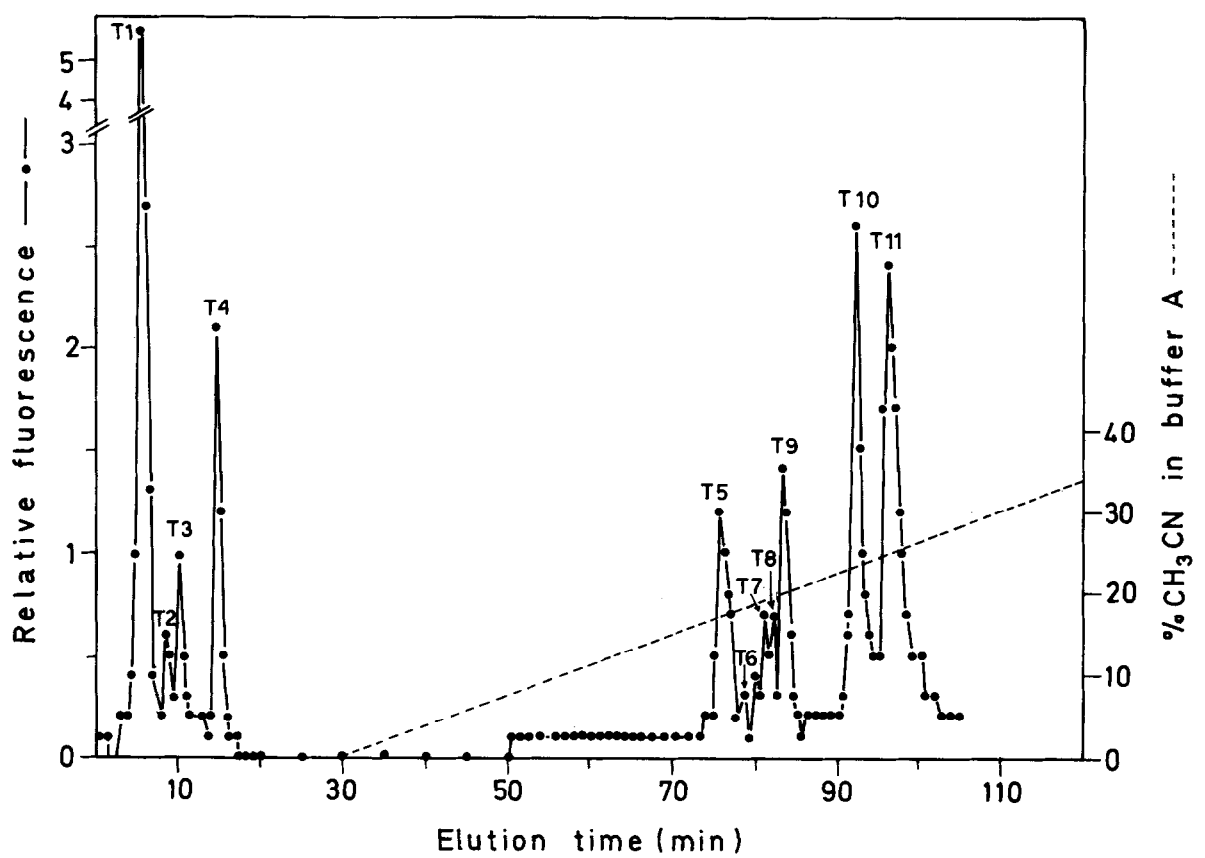


Fig.3. HPLC of a digest of pig thymus ProT$\alpha$ (250 $\mu$g) with TPCK-trypsin (10 $\mu$g).

Table 2

Amino acid analysis of peptides from tryptic digestion of porcine thymus ProT$\alpha$[a]

| cDNA: | 1–14 | 15–17 | 18–20 | 21–30 | 31–87 | 88 | 89–102 | 103–109 | |
|---|---|---|---|---|---|---|---|---|---|
| Tryptic peptide: | T10 | T4 | T1[b] | T8, T9 | T11 | T1[b] | T5 | T3 | Sum |
| Asx | 2.2(2) 2 | 1.3(1) 1 | 1.1(1) 1 | 1.1(1) 1 | 14.7(15) 12 | | 6.3(6) 6 | 2.9(3) 3 | (28) |
| Glx | 1.1(1) 1 | | | 3.6(4) 4 | 22.9(23) 26 | | 1.9(2) 2 | 2.0(2)2 | (33) |
| Ser | 2.5(3) 3 | | | | 0.3(0) 1 | | | | (3) |
| Gly | | | | 1.3(1) 1 | 8.9(9) 8 | | | | (10) |
| Thr | 2.9(3) 3 | | | | 0.5(1) 1 | | 1.4(1) 1 | (1)[c]1 | (6) |
| Ala | 2.2(2) 2 | | | 1.2(1) 1 | 7.2(7) 6 | | 1.9(2) 2 | | (12) |
| Arg | | | 0.5(0) | 1.0(1) 1 | | (1) 1 | | | (2) |
| Pro | | | | | 1.7(2) 1 | | | | (2) |
| Val | 1.2(1) 1 | | | 1.7(2) 2 | 1.3(1) 1 | | 1.1(1) 1 | | (5) |
| Ile | 1.1(1) 1 | | | | | | | | (1) |
| Leu | | 1.1(1) 1 | | | | | | | (1) |
| Lys | 1.0(1) 1 | 1.0(1) 1 | 1.5(2) 2 | | 1.0(1) 1 | | 1.8(2) 2 | 0.6(1)1 | (8) |
| | (14) 14 | (3) 3 | (3) 3 | (10) 10 | (59) 57 | (1) 1 | (14) 14 | (7)7 | (111) |

[a]ProT$\alpha$ 250 $\mu$g. Average of at least two analyses; the last number in each column indicates the number of residues found from the sequence of human cDNA

[b]T1: before hydrolysis, Arg 282 pmol

[c]Thr was present in the composition of T2 (105–109): Asx 3.0, Glx 1.3, Thr 0.81

Note. Partial or overlapping peptides: T6: Asx 0.7, Glx 0.9, Lys 1.0. T7: Asx 1.1, Glx 1.8, Ala 0.9, Arg 0.9, Val 1.0, Lys 0.4. Peak to left of T7 had similar composition to T7

the sulfur containing amino acids, thus agreeing with the results from other species [1–7]. The molecular mass for the monomer of 109 amino acids was calculated as 12 kDa.

Tryptic digestion of thymus ProT$\alpha$ and separation of the resulting peptides on HPLC gave 11 peaks (fig.3). Amino acid analysis (table 2) showed that each peak contained a single peptide, with the exception of peak T1 which in addition contained a free arginine.

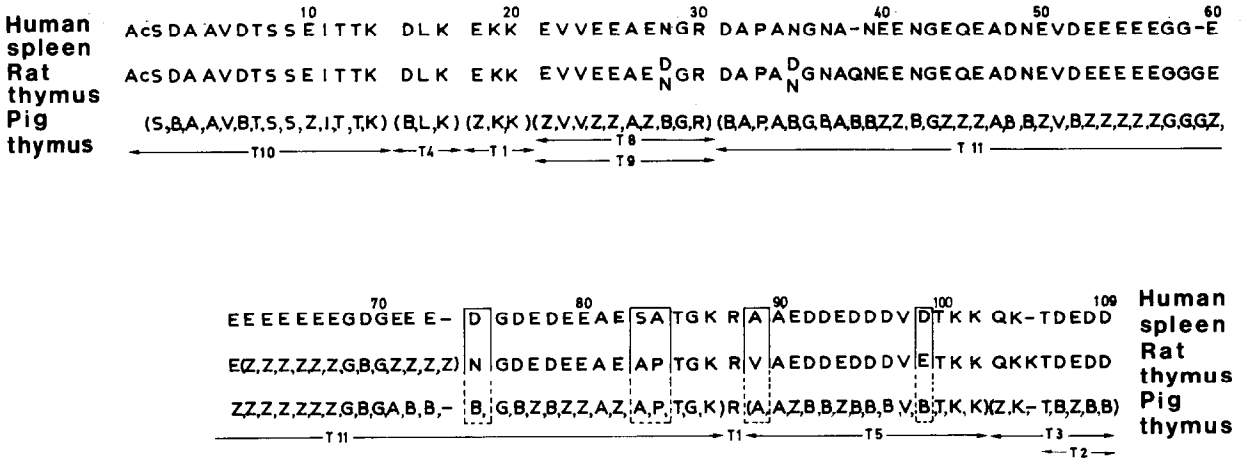


Fig.4. Alignment of the amino acid composition of tryptic peptides from pig thymus ProT$\alpha$ with the sequences of ProT$\alpha$ from human spleen [6] and rat thymus [2].

The alignment and comparison of the amino acid composition of tryptic peptides with the published sequences of ProT$\alpha$ from rat [2] and man [4,6,7] is shown in fig.4. It indicates an agreement in the sequence of ProT$\alpha$ in residues 1–30, with a heterogeneity in position 28 where peptides T8 and T9 have identical composition. Asparagine in position 28 is partially deamidated to an aspartic residue, thus giving rise to two peptides with identical composition on hydrolysis and amino acid analysis [2,4,18]. In the sequence 31–109, minor but potentially significant [4] changes are observed.

The present study shows that ProT$\alpha$ is widely distributed in pig tissues, as has already been shown in rat [3] and in the human [4–7], demonstrating that this immunomodulating polypeptide is found in lymphoid and non-lymphoid tissues of a species closely related to man. It provides a source of the polypeptide for in vitro [10,19] and in vivo [4] experiments on the maturation and modulation of the immune system.

The conservation in the sequence must have a functional significance, since the secondary structure of the molecule in aqueous media is a random coil [20], a conformation which would not impose structural constraints. The fragments obtained by tryptic digestion might prove to be of importance in understanding this and other relationships between structure and function in this molecule.

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