The complete sequences of trout (*Salmo gairdneri*) thymosin β_{11} and its homologue thymosin β_{12}

Paraskevas P. YIALOURIS,* Brian COLES,† Ourania TSITSILONI,* Bettina SCHMID,‡ Steve HOWELL,§ Alistair AITKEN,§ Wolfgang VOELTER‡ and Athanassios A. HARITOS*||

*Zoological Laboratory, Department of Biology, University of Athens, Athens, GR 157 84, Greece,

†Department of Biochemistry, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1P 6DB, U.K., ‡Abteilung für Physikalische Biochemie, Physiologische-chemisches Institut der Universität Tübingen, Hoppe-Seyler Strasse 4, D-7400 Tübingen, Federal Republic of Germany, and §National Institute for Medical Research, The Ridgeway, London NW7 1AA, U.K.

Two forms of β -thymosins, designated thymosin β_{11} and thymosin β_{12} , were isolated from trout (*Salmo gairdneri*) spleen. This suggests that the presence of two β -thymosins, previously thought to be a property of mammalian tissues only, is a more general phenomenon in vertebrate species. Both trout β -thymosins were found to be *N*-terminally blocked by a group identified as acetyl by m.s. Automated protein sequencing of tryptic, thermolytic and *Staphylococcus aureus* in 41-residue V8 proteinase fragments revealed that one of the two β -thymosins corresponds to the previously reported 41-residue-long sequence of thymosin β_{11} with two substitutions at positions 5 and 7, i.e. Asn instead of Asp, and Glu instead of Gln, whereas the other β -thymosin, designated thymosin β_{12} , was found to be a 42-residue polypeptide closely similar in sequence to thymosin β_{11} , with five substitutions (i.e. at positions 5, 7, 10, 11 and 41, with Asp, Ala, Ser, Asn and Thr instead of Asn, Glu, Ala, Ser and Ser respectively) and one addition at position 42 (Ala). Comparison of the known six sequences of β -thymosins together with the sequences reported here showed that the sequence similarity of the two β -thymosins in trout (86 %) is greater than that of the two β -thymosins in mammalian species (74 %) and that residues at 28 positions are identical in all β -thymosins, the longer conserved segments located at positions 16–26 and 31–38.

INTRODUCTION

β -Thymosins are 41–43-residue polypeptides. Two forms of β -thymosins are co-expressed in mammalian species, i.e. thymosin β_4 [1–5] and either thymosin β_9 (in calf [6] and pig [7]) or thymosin β_{10} (in man, rat, mouse and cat [2,5,8,9]). However, only one form of β -thymosin was identified in chicken, frog and fish [10], with the sequences of thymosin β_4^{Xen} in the amphibian *Xenopus* [11] and thymosin β_{11} in trout (*Salmo gairdneri*) [12] partly derived by Edman degradation.

In vertebrate tissues β -thymosins are ubiquitous and occur in high concentrations (approaching 0.1–1 % of total protein [8,13,14]). They are expressed *in situ* [5], with spleen being the richest source of thymosin β_4 in mammals [16] and spleen, thymus and lung the richest sources of the respective mRNA [15]. Blood plasma also contains thymosin β_4 [13], although this polypeptide [3] (as well as thymosin β_{10} [9]) is not synthesized with a leader peptide (or as a precursor molecule). This might suggest an alternative secretion mechanism [17] or leakage from damaged cells. Thymosin β_4 has also been reported in several cell types of lymphoid and non-lymphoid origin [14,18–20] and found to be secreted [18] or not secreted [14] by them. The intracellular localization of thymosin β_4 has been reported to be cytoplasmic [20,21].

In spite of the ubiquity and high levels of β -thymosins, their physiological role still remains unclear 10 years after their discovery. Fragmentary information, however, shows that they are most closely associated with immune-related cells or phenomena and/or cell proliferation. Thymosin β_4 has been shown to induce terminal deoxynucleotidyltransferase activity in bone-marrow cells [1], inhibit the migration of macrophages [22], depress the human autologous and allogeneic mixed lymphocyte responses [23] and to be present with reticular dendritic and

phagocytic cells in lymphoid organs [24]. Thymosin β_4 has been reported to increase severalfold in concanavalin-stimulated rat thymocytes by translational control [25], its mRNA to appear severalfold higher in thymocytes than in thymus epithelial cells [15], and recently the endogenous *N*-terminal tetrapeptide of thymosin β_4 was found to inhibit bone-marrow haematopoietic cell and hepatocyte proliferation [26,27].

Here we show that, in fish, as in mammals, two thymosins are co-expressed. The corrected complete sequence of the previously reported thymosin β_{11} is reported, as well as the complete sequence of the new second β -thymosin, designated thymosin β_{12} . The comparison of fish β -thymosins with the respective known mammalian and amphibian peptides further underlines their conserved segments which might be essential for the physiological function of the polypeptides.

MATERIALS AND METHODS

Materials

Trout (*Salmo gairdneri*) were purchased from a trout farm in the Orchomenos area of Greece.

Proteolytic digestions of thymosins

Trypsin. A 100 μg portion of each β -thymosin was dissolved in 75 μl of 0.4 M-pyridine, pH 7.5. To this was added 7.5 μg of tosylphenylalanylchloromethane ('TPCK')-treated trypsin (purchased from Sigma) dissolved in 6 μl of 1 mM-HCl. The sample was incubated for 15 h at room temperature. The reaction was terminated by the addition of 7.6 μl of formic acid. Water was added to a final volume of 100 μl . After freeze drying, the sample was redissolved in 100 μl of 0.1 % trifluoroacetic acid (TFA) and subjected to reverse-phase (r.p.) h.p.l.c.

Thermolysin. A 100 μg portion of each β -thymosin was dis-

Abbreviations used: TFA, trifluoroacetic acid; r.p., reverse phase.

|| To whom correspondence should be sent.

solved in 25 μ l of 0.1 M-ammonium bicarbonate, pH 7.5. To this was added 8 μ g of thermolysin (purchased from Sigma) dissolved in 5 μ l of the same solution. The sample was incubated for 2 h at 37 °C. After freeze-drying the sample was redissolved in 100 μ l of 0.1 % TFA and subjected to r.p.-h.p.l.c.

***Staphylococcus aureus* V8 proteinase.** An 80 μ g sample of each β -thymosin was dissolved in 50 μ l of 0.1 M-ammonium acetate. The pH was adjusted to 4.0 by K_2 EDTA. To this was added 5 μ g of *S. aureus* V8 proteinase (purchased from Boehringer Mannheim) dissolved in 5 μ l of the same buffer. The sample was incubated for 15 h at room temperature. After freeze-drying the sample was redissolved in 100 μ l of 0.1 % TFA and subjected to r.p.-h.p.l.c.

R.p.-h.p.l.c.

The h.p.l.c. system consisted of two Waters 501 h.p.l.c. pumps, a Waters gradient controller, an LKB 2151 variable-wavelength u.v. monitor and a Varian 4290 integrator. RP C18 Lichrospher or Lichrosorb h.p.l.c. columns purchased from Merck (250 mm $\times$ 4 mm, 5 μ m, 10 nm pore diameter) were employed. In all r.p.-h.p.l.c. chromatographies solution A was 0.1 % TFA in water and solution B was 60 % acetonitrile/0.1 % TFA in water. For the isolation of the fragments after proteolytic digestion, the gradient consisted of solution A for the first 10 min, followed by a linear gradient of 0–70 % B from 10 min to 120 min.

Amino-acid-composition analyses

These were performed according to the Waters Picotag amino-acid-composition-analysis method.

Automated protein sequencing

Approx. 100–1000 pmol of peptide was subjected to automated Edman degradation by using an Applied Biosystems 470A gas-phase peptide sequencer, with analysis of amino acids as their phenylthiohydantoin derivatives. Peptides in which the *N*-terminus was blocked were treated with TFA [28] at 48 °C for 4–7 days before sequence analysis.

M.s.

Fast-atom-bombardment spectra of the *N*-terminal peptides of thymosins β_{11} and β_{12} were obtained using a VG 70-250 SE spectrometer operated in the positive-ion mode with a 30 kV caesium-ion gun. Approx. 3 nmol of the peptide was applied to a thioglycerol/1 % TFA matrix on the probe. Repeated scans showed a consistent set of peaks in the *m/z* range 1600–1750 (see Fig. 6 below).

Radioimmunoassay

Rabbit polyclonal antiserum against synthetic segment 1–15 of rat thymosin β_4 coupled to haemocyanin by glutaraldehyde, and the analogue [Tyr¹²]thymosin β_4 -(1–15)-peptide labelled with ¹²⁵I as tracer, were employed for the radioimmunoassay [29].

Isolation of β -thymosins in trout spleen

Spleen tissue was immediately excised after the fish had been killed and dropped into liquid N₂. In this form it was carried to the laboratory and pulverized over solid CO₂. The resulting powder, corresponding to 28 g of tissue, was dropped into 10 vol of boiling water (boiling continuing for 5 more min) for the complete inactivation of endogenous proteinases [30], converting prothymosin α to thymosin α_1 and thymosin β to thymosin β_8 . After homogenization at full speed in a blender (Sorvall Omni-Mix) and centrifugation at 10000 *g* for 10 min, the supernatant

Fig. 1. Separation of proteins in trout spleen extract by Sephacryl S-200 gel filtration

The horizontal bar indicates fractions collected and pooled.

Fig. 2. Separation by r.p.-h.p.l.c. of proteins contained in the pooled fractions in Fig. 1

The linear gradient was 0–30 % solution B in 10 min and 30–60 % B in the subsequent 60 min. Solution A was 0.1 % TFA in water and solution B was 60 % acetonitrile/0.1 % TFA in water. Fractions were analysed by the radioimmunoassay for the *N*-terminus of thymosin β_4 . Fractions corresponding to thymosins β_{12} and β_{11} respectively were pooled, freeze-dried and rechromatographed (as shown in Fig. 3).

was mixed with an equal volume of buffer A (1 M-formic acid/0.2 M-pyridine, pH 2.8) and the sample was left overnight at –10 °C. After centrifugation the supernatant was dried in a Savant Speed Vac centrifugal concentrator. The residue was redissolved in 3.5 ml of buffer A. After centrifugation at full speed in a Beckman/Spinco 152 Microfuge, the supernatant was subjected to gel filtration on a Sephacryl S-200 high-resolution column (95 cm $\times$ 2.5 cm internal diam.) equilibrated and eluted in buffer A. The flow rate was 25.62 ml/h and fractions of volume 4.27 ml were collected. Aliquots (150 μ l) of each fraction were freeze-dried and analysed by the radioimmunoassay for the *N*-terminus of thymosin β_4 . Fractions 75–86, corresponding to the major immunocross-reactivity peak, were collected and pooled (Fig. 1). Five aliquots of 100 μ l of the pool were subjected to r.p.-h.p.l.c. (Fig. 2) and aliquots of 100 μ l of each fraction were analysed by the radioimmunoassay.

Fig. 3. Rechromatography by r.p.-h.p.l.c. of thymosins β_{11} and β_{12} peaks of Fig. 2

The gradient was the same as that mentioned in Fig. 2. The two independent chromatograms are shown superimposed with a perpendicular displacement of the baseline.

Fig. 4. Amino acid sequences of trout spleen thymosin β_{11}

The peptides used to establish the primary structure are shown by letters, designated according to the cleavage method used as follows: T, cleavage by tosylphenylalanylchloromethane-treated trypsin; Th, cleavage by thermolysin; and S, cleavage by *S. aureus* V8 proteinase. The one-letter code for amino acids is used; Ac is *N*-acetyl.

Fig. 5. Amino acid sequences of trout spleen thymosin β_{12}

Other details are as for Fig. 4.

RESULTS

The thymosin β_1 immunocross-reactive material obtained after trout spleen extraction was eluted in a single major peak by gel filtration (Fig. 1). This immunocross-reactive material was separated by r.p.-h.p.l.c. into two major absorbance peaks at 48.4 min and 50.9 min (approx. 30% acetonitrile), which also shared the major immunocross-reactive material (Fig. 2). The polypeptide of the peak on the right was identified as thymosin β_{11} , whereas the polypeptide of the left peak was found to correspond to a new β -thymosin designated thymosin β_{12} . Upon rechromato-

Fig. 6. Fast-atom-bombardment mass spectra of *N*-terminal peptides of T18 (a) and T29 (b).

The left-hand Table indicates the calculated molecular masses of a series of ions; the observed mass is given on the spectrum itself.

graphy, under the same conditions, the two β -thymosins were separated and eluted at 48.6 min and 51.2 min respectively (Fig. 3).

Both trout β -thymosins were found to be *N*-terminally blocked. However, after treatment with TFA, several *N*-termini were evident. Although the sequence was not readily assignable, these limited sequences confirmed that the peptides belonged to the β -thymosin family and suggested that the *N*-terminus was an acylated serine residue which partially rearranged by *N*-O acyl shift under acid conditions. In contrast with other reports [28], we did not find that the *N*-terminus was more susceptible to *N*-O acyl shift than other serine residues in the polypeptide chain. Subsequently, the complete amino acid sequence of each thymosin was established by analysis of appropriate overlapping proteolytic fragments (Figs. 4 and 5). The *N*-terminal peptides were readily identified by their amino acid composition and by their failure to undergo Edman degradation. Treatment of these peptides with TFA and subsequent sequence analysis unequivocally established the amino acid sequence of the *N*-terminus.

The composition of the tryptic *N*-terminal peptides (residues 1–14), including the nature of the substitution at the *N*-terminus, was confirmed by fast-atom-bombardment m.s. The spectrum consisted of a series of ions in which the $[M+Na]^+$ ion was a prominent one in both cases (Fig. 6). The observed pseudo molecular ions 1628.4 and 1642.5 Da correspond to $[M+Na]^+$ for the first 14 amino acids of thymosin β_{12} and thymosin β_{11} respectively, with the addition of a single acetyl group, for which the calculated molecular masses are 1628.7 and 1642.7 Da. Other ions in the spectrum, including $[M+H]^+$, and a series of ions due to the sodium and potassium salts of the peptide ion, confirm the

molecular mass. The C-terminal segments were assigned from the trypsin fragments not containing lysine.

DISCUSSION

Trout spleen was selected for the isolation of β -thymosins because this tissue has been reported to be the richest source of thymosin β_4 in mammalian species (see the Introduction). A previously reported approach for the co-isolation of α - and β -thymosins [31] was employed. It showed the presence of two β -thymosins in spleen, in contrast with the presence of a single form in the liver of the same species [12], which may reflect a differential tissue expression of β -thymosins in trout. This situation occurs, for example, in the rabbit, where the concentrations per g of tissue of thymosin β_4 (β_4^{ala}) to thymosin β_{10} for spleen were reported to be 88:75 mg, whereas for liver the value is 87:22 mg [2]. Our results show that the assumption that mammals possess two β -thymosins, and lower vertebrates possess only one [12], is incorrect.

Both β -thymosins of trout were found to be N-terminally modified. Therefore they were sequenced by chemically unblocking the N-termini and automated sequencing of proteolytic digestion fragments. The sequence of one of the two β -thymosins revealed that it corresponded to the previously reported thymosin β_{11} with two differences at positions 5 and 7: Asn instead of Asp, and Glu instead of Gln, respectively. These differences appear to represent errors in the assumptions made for the residues in these positions by Erickson-Viitanen &

Horecker [12], as these positions were not sequenced directly but deduced by amino-acid-composition analysis and comparison with the known sequence of thymosin β_4 . Therefore the sequence reported here for thymosin β_{11} should replace the one previously reported.

The sequencing of the second β -thymosin, designated thymosin β_{12} (numbers by tradition indicate the order of discovery of β -thymosins) revealed, as compared with the corrected thymosin β_{11} sequence, the following: (a) five substitutions, i.e. at positions 5, 7, 10, 11 and 41, with Asp, Ala, Ser, Asn and Thr instead of Asn, Glu, Ala, Ser and Ser respectively; all substitutions but one are conservative and the relative distribution of hydrophilic, hydrophobic and polar residues remains unchanged; and (b) one addition at position 42 (Ala), which makes thymosin β_{12} the only 42-residue-long β -thymosin, the other β -thymosins being either 41 (β_9 , β_{11}) or 43 (β_4 , β_{10}) residues long. Six differences in all between the two β -thymosins correspond to a much higher sequence similarity (86%) than the 11 differences (74% similarity) among the two β -thymosins in mammalian species (either β_4 and β_9 , or β_4 and β_{10}). A similar degree of similarity to that of thymosin β_{11} /thymosin β_{12} occurs only among thymosins β_9 and β_{10} (84%), which, however, appear in different mammalian species (Fig. 7).

Thymosins β_{11} and β_{12} lack sulphur-containing residues, arginine and aromatic residues, apart from a single phenylalanine residue. The high proportion of lysine and dicarboxylic acid residues is notable, and this reaches the level of 16 such residues in the 28 positions conserved in all β -thymosins (Fig. 7). M.s. of the N-termini of both thymosin β_{11} and thymosin β_{12} showed that the blocking group was an acetyl one. This is in agreement with the previously reported blocking group for bovine thymosin β_4 [1] and thymosin β_9 [6]. The molecular masses calculated for the corrected thymosin β_{11} and for thymosin β_{12} are 4731 and 4802 Da respectively. Thymosin β_{12} minus the C-terminal alanine residue has been synthesized recently in the laboratory of one of us (W. V.) [32].

The comparison of all known structures of β -thymosins, including those deduced in the present study, revealed the identity of the amino acid residues at 28 positions. Two of the longest conserved sequences corresponded to positions 16–26 and 31–38. These regions may be essential to the physiological role of these thymosins. In contrast, N-terminal and C-terminal regions appear to be variable and therefore species-specific. It would be interesting to know whether β -thymosins appear in invertebrate species which lack lymphoid tissues rich in these polypeptides, such as thymus and spleen tissues, and, if so, whether one or more forms are present. By knowing the structure of β -thymosin(s) in invertebrate species, substantial knowledge of the evolution of β -thymosins will be gained. Moreover, the boundaries of the conserved segments of β -thymosins will be better defined.

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Fig. 7. Sequence similarities between thymosin β_{11} and β_{12} as well as to the known β -thymosins

(a) Double lines under residues indicate positions which show variation in the known sequences. Asterisks indicate positions of difference only among thymosin β_{11} and β_{12} . BRHC, bovine (Bov.), rat, human, cat; Rab., rabbit; Xen, *Xenopus*; Por., porcine; x, an unknown group. (b) Extents of sequence identity among bovine, rat, *Xenopus* and trout β -thymosins are expressed as percentages.

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