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A Novel β-Thymosin from the Sea Urchin: Extending the Phylogenetic Distribution of β-Thymosins from Mammals to Echinoderms

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Abstract: The study of the phylogenetic distribution of the β-thymosin family is important to elucidate its biological function further. A new thymosin, designated as thymosin β_{14}, consisting of 40 amino acid residues and with a molecular weight of 4537 Da as determined by ion spray mass spectrometry, was isolated from the sea urchin. The N-terminus of this polypeptide is blocked by an acetyl group as found by matrix-assisted laser desorption mass spectrometric and amino acid analysis. The primary structure was elucidatd by Edman degradation of the HPLC-purified thymosin β_{14} fragments produced by digestion with endoproteinase Asp-N and trypsin. Sequence comparison reveals that thymosin β_{14} is 73% homologous to thymosin β_4, obtained from calf thymus. By isolating and characterising the structure of thymosin β_{14} from the sea urchin, an invertebrate, substantial knowledge about the phylogenetic distribution and evolution of β-thymosins is gained.

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INTRODUCTION

Thymosins are a group of peptides, first isolated from calf thymus tissue [1], and efficient methods for their isolation have now been developed [2–4]. During the past few years it has been found that their distribution is not limited to the thymus but that they are also present in numerous tissues [5, 6]. Recent experiments provided evidence for their extranuclear localization in the cells [7]. Different β-thymosin peptides have been isolated from many species of the animal kingdom, ranging from amphibia to mammals [8–15]. Thymosin β_4 was found to be present in most vertebrate classes [5, 11]. It is well established that in vertebrate tissues β-thymosins are ubiquitous and occur in high concentrations (300 μg/g protein). Furthermore, in many species, a second thymosin β_4-like peptide exists [8, 11–16]. These peptides show a high sequence homology to thymosin β_4. Thymosin β_4 has been shown to be active in a series of biological tests, e.g., the terminal desoxynucleotidyl transferase activity *in vivo* and *in vitro* is induced [1] or the hypothalamic secretion of luteinizing hormone releasing factor is stimulated [17]. It was found recently that thymosin β_4 forms a 1:1 complex with G-actin, thereby sequestering its polymerization [18] and playing a significant role in the regulation of the actin polymerization in many cell types. The endogenous N-terminal tetrapeptide of thymosin β_4 was found to inhibit bone-marrow haematopoietic cell and hepatocyte proliferation [19, 20]. Some members of the β-thymosin family as well as their fragments have been synthesized using different approaches [21–25]

Abbreviations: LDMS, laser desorption mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; TPCK, L-*p*-tosylaminophenylethylchloromethyl ketone.

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and used for immunization and development of specific antibodies [24, 26, 27]. In order to extend the phylogenetic distribution of this peptide family, regulating fundamental cell processes, for the first time a β-thymosin from an invertebrate, the sea urchin, has been isolated and its sequence determined.

MATERIALS AND METHODS

Materials

Sea urchins (*Echinus esculentus*) were purchased from the sea station, Helgoland. TFA and acetonitrile were obtained from Merck, Darmstadt, Germany. Trypsin-TPCK and endoproteinase Asp-N were purchased from Boehringer, Mannheim, Germany. All other chemicals were of reagent grade and were used without further purification.

Isolation of Thymosin β_{14} from Sea Urchin

Thymosin β_{14} was isolated from 20 sea urchins after removing their intestines by homogenization in 0.4 M perchloric acid. The mixture was then adjusted to pH 3.5 with 2 M KOH. The extract was centrifuged at 30,000 *g* for 30 min at room temperature. The peptides of the supernatant solution were absorbed on 40 g RP-18 material (LiChroprep 40–63 μm, Merck, Darmstadt, Germany; glass funnel, diameter 8 cm). After removing the salts by washing with distilled water, the peptides were eluted with 30% *n*-propanol. The eluates were concentrated under reduced pressure, then the crude extract material was lyophilized [3]. Finally, the desired peptide was isolated by RP-HPLC to high purity.

Proteolytic Digestion of Thymosin β_{14}

Asp-N Endoproteinase. Asp-N digestion of 500 μg thymosin β_{14} was performed in 250 μl 50 mM sodium phosphate, pH 8.0 for 7 h at 37°C with an enzyme-to-substrate ratio of 1:500 (w/w). The reaction was stopped by adding 5 μl of TFA. The enzyme digest was separated by RP-HPLC. The fractions were collected, lyophilized and subjected to amino acid, sequence and LDMS analysis.

Trypsin. Tryptic digestion of 500 μg thymosin β_{14} was performed in 1 ml 1% NH_4HCO_3 buffer (w/v) pH 7.6 for 4 h at 37°C with an enzyme-to-substrate ratio of 1 : 50 (w/w). The reaction was terminated by the addition of 10 μl TFA. After freeze-drying the enzyme digest was redissolved in 500 μl 0.1% TFA/H_2O and fractionated by RP-HPLC.

RP-HPCL

An Eppendorf quaternary HPLC system of the series BT 9000 (Eppendorf/Biotronik, Maintal, Germany) with an RP C 18 LiChrospher column (250 × 4 mm, 5 μm; Merck, Darmstadt, Germany) was used. In all RP-HPL; chromatographies solution A was 0.1% TFA in water and solution B 60% acetonitrile/0.1% TFA in water. Thymosin β_{14} and its fragments after proteolytic digestion were purified by RP-HPLC using a linear gradient of 0–60% B in 60 min and a flow rate of 1.0 ml/min.

Amino Acid Analysis

Samples were hydrolysed in 5.7 N HCl containing 1% phenol at 110°C *in vacuo* for 24 h and analysed in a LC 3000 amino acid analyser (Eppendorf/Biotronik, Maintal, Germany). Amino acids were quantified with post-column ninhydrin detection.

Automated Protein Sequencing

Approximately 30–120 pmol of peptide was subjected to automated Edman degradation by using an Applied Biosystems 473A peptide sequencer (Weiterstadt, Germany), with analysis of amino acids as their phenylthiohydantoin derivates.

Mass Measurements

Laser Desorption Mass Spectrometry. The mass spectrometer used in this work was a laser desorption mass analyser (Lasermat, Finnigan MAT Ltd, Bremen, Germany). Spectra from mutliple laser shots (5–30) were summed until an acceptable signal-to-noise ratio was achieved at a repetition rate of 0.5 Hz. Operation conditions and procedures are modelled in the works of Hillenkamp and coworkers [28–30]. The preferred matrices for peptides and proteins are sinapinic acid and α-cyano-4-hydroxycinnamic acid, which were dissolved in 70% CH_3CN, 30% H_2O and 0.1% TFA to give an approximate concentration of 5×10^{-2} molar.

Ion Spray Mass Spectrometry. Ion spray mass spectra were recorded on a triple-quadrupole mass spectrometer (TAGA 6000 E) equipped with an ion spray source (Sciex, Toronto, Canada). The operat-

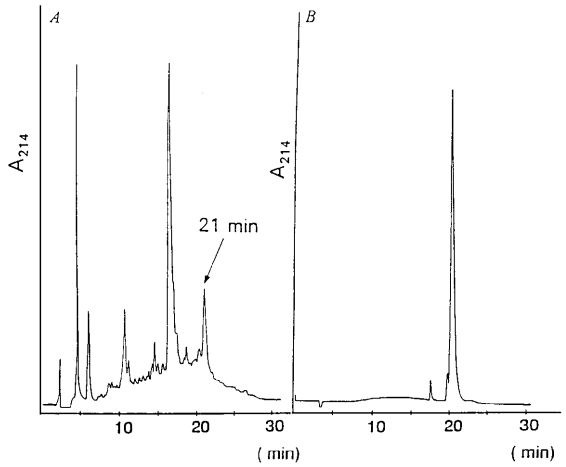

Figure 1 HPLC separation of the crude extract from sea urchin (A) and the purified thymosin β_{14} (B). The column used was LiChrospher RP-18, 5 μm (4 × 250 mm; E. Merck, Darmstadt, Germany). The solvents used were: (A), 0.05% TFA/H_2O; (B) 60% CH_3CN/0.05% TFA/H_2O. The peptides were eluted with a linear gradient of 5–95% B in 35 min and a flow rate of 1.0 ml/min. Detection was performed at $\lambda = 214$ nm.

ing parameters for the measurements were as follows: ion spray voltage 4.7–5.0 kV, orifice voltage 70–100 V, argon gas thickness at the collision experiments 210–3,001,012 atoms/cm^2. All data were acquired and processed with a Sciex MacSpec data system.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC chromatogram of the crude extract from the sea urchins. Three fractions at time intervals 17–19, 20 and 21 min were collected. By ion spray mass measurements two components with molecular masses of 738 Da and 2636 Da were obtained in the 17–19 min fraction. The molecular masses of the 20 min and 21 min fractions were 916 Da (not shown) and 4537 Da (Figure 2), respectively, as found by ion spray mass spectrometry. Analysis of the intact polypeptide chain of the 21 min fraction by automatic sequencing did not yield any PTH amino acid, indicating that the N-terminus is blocked. This information and the molecular weight of 4537 Da gave the first evidence

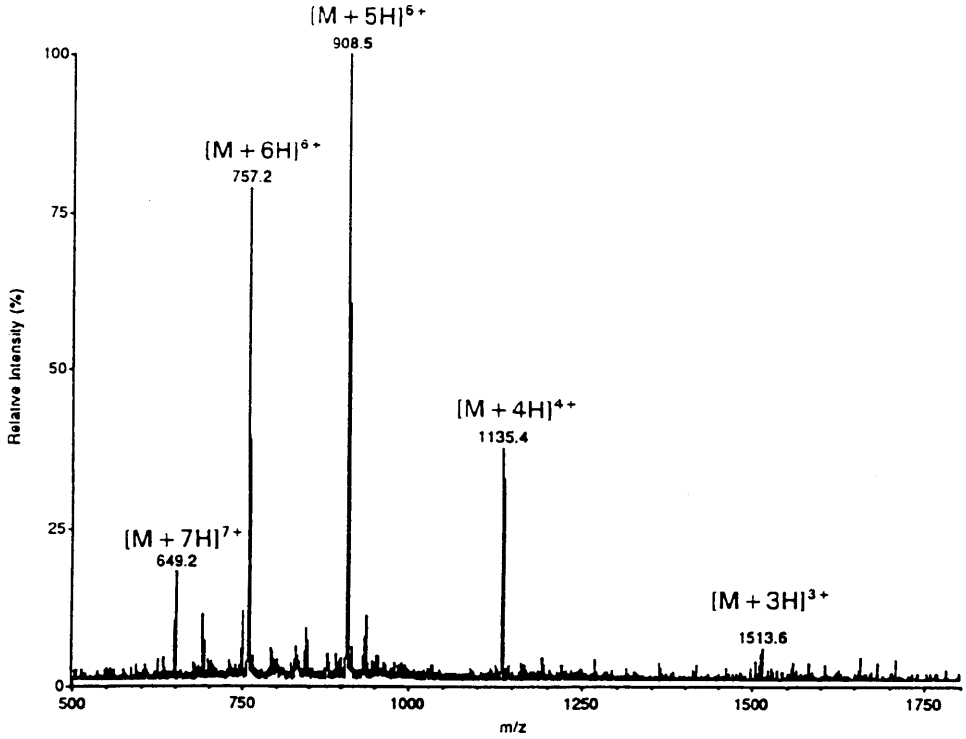

Figure 2 Ion spray mass spectrum of thymosin β_{14}, the HPLC purified fraction with RT 21.38 min (Figure 1) from the sea urchin crude extract. The labelled peaks arise from a single molecular species (thymosin β_{14}), differing in the number of excess protons (3–7). All data points were included in the calculations and no mathematical filtering was performed. The observed molecular mass is 4537 Da.

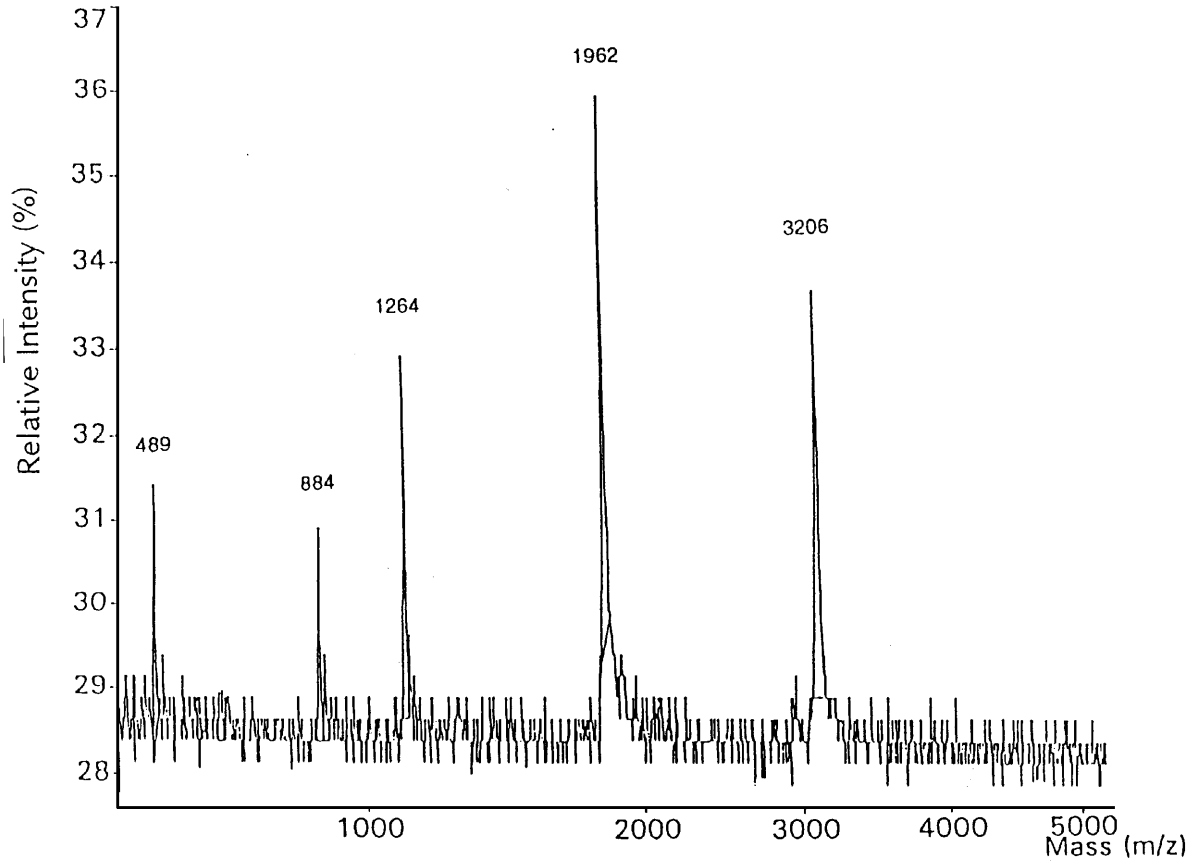

Figure 3 LDMS spectrum of the total fragment mixture obtained from thymosin β_{14} when digested with Asp-N proteinase; matrix: α-cyano-4-hydroxycinnamic acid; number of shots, 10; laser power, 33. The labelled peaks arise from a single charged Asp-N fragments of thymosin β_{14}. The observed molecular masses correspond to fragments 1–4 (489 Da), 5–12 (884 Da), 13–23 (1264 Da), 24–40 (1962 Da), 13–40 (3206 Da).

Table 1 Molecular Masses of Asp-N Fragments (A) of Thymosin β_{14} Determined by LDMS (Lasermat, Finnigan, MAT, Bremen, Germany)

Fraction	Mass [MH^+] (experimental)	Thymosin β_{14} fragment	Mass [M] (theoretical)
AI	1264	13–23	1262.47
AII	489	1–4	488.50
AIII	1962	24–40	1960.18
AIV	3206	13–40	3204.63
AV	884	5–12	882.93

of thymosin presence. To elucidate the primary structure of thymosin β_{14}, it was digested with Asp-N proteinase and trypsin. The total peptide mixture obtained by Asp-N cleavage was analysed by LDMS (Figure 3 and Table 1) and separated by preparative RP-HPLC (Figure 4). LDMS measurements of the collected individual fractions (Al–AV) showed that all peptides are homogeneous. The thymosin β_{14} fragments thus isolated were characterized by amino acid analysis and sequencing (Table 2 and Figure 5). On the basis of the combined interpretation of the value for the molecular mass of fraction 1–4, its amino acid composition and the homology to the known structures of other β-thymosins (β_4 [1], β_9 [8], β_{11} and β_{12} [15]), the N-terminal sequence of thymosin β_{14} was deduced to be Ac-Ser-Asp-Lys-Pro (Figure 5). To confirm the sequence of the first four residues a synthetic

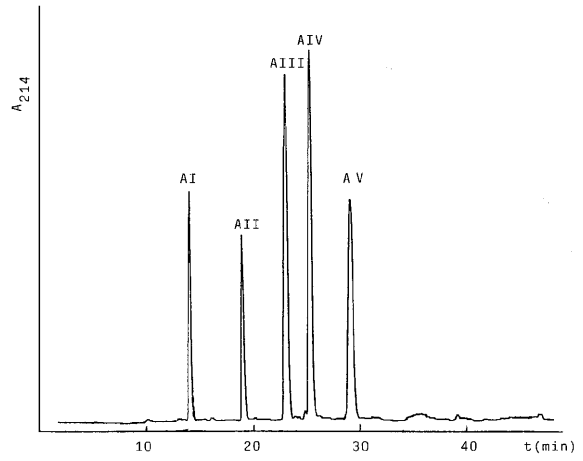

Figure 4 HPLC fractionation of the fragment mixture obtained after Asp-N cleavage of thymosin β_{14}. The column used was LiChrospher RP-18, 5 μm (4 × 250 mm; E. Merck, Darmstadt, Germany). The solvents used were: (A) 0.05% TFA/H_2O; (B) 60% CH_3CH/0.05% TFA/H_2O. The peptides were eluted with a linear gradient of 0–60% B in 60 min and with a flow rate of 1.0 ml/min. Detection was performed at $\lambda = 214$ nm.

tetrapeptide AcSDKP was run by HPLC together with the corresponding Asp-N fragment of thymosin β_{14}. Both peptides coelute at RT 20.70 min, have the same molecular weight of 489 Da and identical amino acid analysis. The complete primary structure of thymosin β_{14} was obtained from Asp-N fragments 5–12 (fraction AV), 13–23 (fraction AI), 13–40 (fraction AIV) and 24–40 (fraction AIII). Although fraction AIV (fragment 13–40) served as overlap for residues 23–24, tryptic digestion was used to confirm the amino acid sequence of thymosin β_{14} derived from the Asp-N fragments. Nine tryptic fractions were isolated by RP-HPLC (Figure 6) and each of them was analysed by LDMS and Edman degradation (Figure 5). The N-terminal sequence of the acetylated peptide was also confirmed by the molecular mass of 1597.0 Da for the tryptic fraction TIX (1–14) (Figure 7), corresponding to the value of 1595.69 Da calculated on the basis of the sequence data from the Asp-N digestion.

The primary structure of thymosin β_{14} thus determined has a chain length of 40 amino acid residues. The molecular mass for the intact polypeptide chain was found to be 4537 Da as measured to ion spray mass spectrometry (Figure 2), which corresponds to the calculated one on the basis of the sequence and the N-terminal acetyl group (4539.03 Da). Thymosin β_{14} lacks sulphur-containing, argininyl, histidyl and aromatic residues, apart from a single phenylalanine in position 12. Notable are the high numbers of lysine (8) and dicarboxilic acid residues (Asp-3, Glu-6), 16 of which are conserved.

Figure 8 shows the homology alignments of the 11 members of the β-thymosin family. The comparison of the amino acid sequence of thymosin β_{14} with all known β-thymosin structures reveals the identity of the amino acid residues at 27 positions. Two of the longest conserved sequences of β-thymosins, corresponding to positions 16–26 and 31–38, except the substitution of Gln in position 23 with Ala, are also present in the structure of β_{14}. This observation suggests that these regions might be the most essential ones for the physiological function of the β-thymosins. The N- and C-terminal segments, on the contrary, are variable and species specific.

Table 2 Amino Acid Compositions[a] of Thymosin β_{14}, Isolated from the Sea Urchin and its Asp-N Fragments (A)

Amino acid	AI (13–23)	AII (1–4)	AIII (24–40)	AIV (13–40)	AV (5–12)	Thymosin β_{14} (1–40)
Asx	1.1 (1)	1.1 (1)	0.9 (1)	1.9 (2)	1.2 (1)	3.9 (4)
Thr	2.7 (3)	–	3.9 (4)	6.9 (7)	–	6.6 (7)
Ser	–	0.8 (1)	–	–	2.7 (3)	3.6 (4)
Glx	1.0 (1)		4.9 (5)	5.8 (6)	1.0 (1)	7.1 (7)
Pro	–	1.0 (1)	1.1 (1)	1.1 (1)	–	2.2 (2)
Ala	1.1 (1)	–	1.0 (1)	2.2 (2)	–	2.1 (2)
Val	–	–	–	–	0.9 (1)	1.0 (1)
Ile	–	–	1.1 (1)	1.0 (1)	0.9 (1)	1.9 (2)
Leu	0.8 (1)	–	1.0 (1)	1.9 (2)	–	1.8 (2)
Phe	–	–	–		1.1 (1)	1.1 (1)
Lys	3.9 (4)	1.0 (1)	3.1 (3)	6.9 (7)	–	7.9 (8)

[a]The data are presented as assumed numbers of residue per molecule. Residues were from 5.7 N HCl hydrolysates at 110°C for 24 h. Aspartic and glutamic acid values are the sum of their acids and amides. Values in parentheses are calculated from the established sequence of thymosin β_{14} as shown in Figure 5. Glycine, half-cystine, methionine, tyrosine, histidine and arginine were not found in any of the peptide fragments generated with the endoproteinase Asp-N.


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                    5         10        15        20        25        30        35        40
          Ac- S D K P D I S E V S S F D K T K L K K T E T A E K N T L P T K E T I E Q E K T A -OH

AII       -- 489 Da--
AV                  D I S E V S S F
TV                                T K L K
AI                              D K T K L K K T E T A
TIV                                     K T E T A E K
TVIII                                             N T L P T K
AIV                             D K T K L K K T E T A E K N T L P T K E T I E Q E K T A
TVII                                                        E T I E Q E K T A
AIII                                                  E K N T L P T K E T I E Q E K T A

TIX       --------------1597,0 Da---------
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Figure 5 Amino acid sequence of thymosin β_{14}. The Asp-N (A) and tryptic (T) peptides are marked. The peptides used to establish the primary structure are shown by using the one letter code for amino acids; Ac is N-acetyl. Amino acid sequences determined by automated sequencing procedures are indicated by letters. The N-terminal fragments blocked by an acetyl group are analysed by LDMS and marked by dashes.

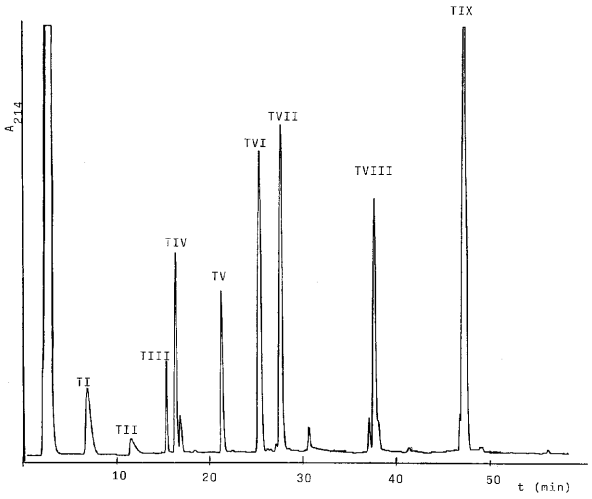

Figure 6 HPLC separation of the peptide mixture obtained after cleavage of thymosin β_{14} with trypsin. Other experimental details are as for Figure 4. By using LDMS and Edman degradation the isolated fractions are identified as follows: TI, fragments 15–16; 39–40; TII, fragment 17–18; TIII, fragment 20–25; TIV, fragment 19–25; TV, fragment 15–18; TVI, fragment 32–38; TVII, fragment 32–40; TVIII, fragment 26–31; TIX, fragment 1–14.

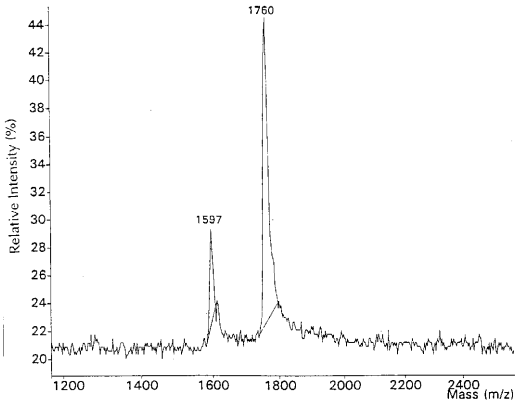

Figure 7 LDMS spectrum of fragment TIX obtained from thymosin β_{14} when digested with trypsin; matrix, α-cyano-4-hydroxycinnamic acid; number of shots, 5; laser power, 17; peak at m/z 1760, renin as internal standard; intensity versus m/z. The molecular ion of m/z 1597 represents the mass of peptide TIX with acetyl at N-terminal end.

Though a whole series of biological activities have been reported for β-thymosins (see above), the most prominent one seems to be their inhibiting capacity of actin polymerization by interacting with monomeric actin [18]. Therefore, β-thymosins seem to play an important role in fundamental cell processes such as cell locomotion or cytoskeleton formation or remodelling. Human thymosin β_4 [18] and β_{10} [37] and bovine thymosin β_9 [38] inhibit actin polymerization with similar K_d values (between 0.7 and 1.0 μM). According to Vancompernolle *et al.* [39], all of these homologues contain the conserved hexapeptide motif LKKTET, postulated to be the major contact site with actin. This motif is also present in the β-thymosin sequence of the sea urchin as demonstrated by this paper. Another functional

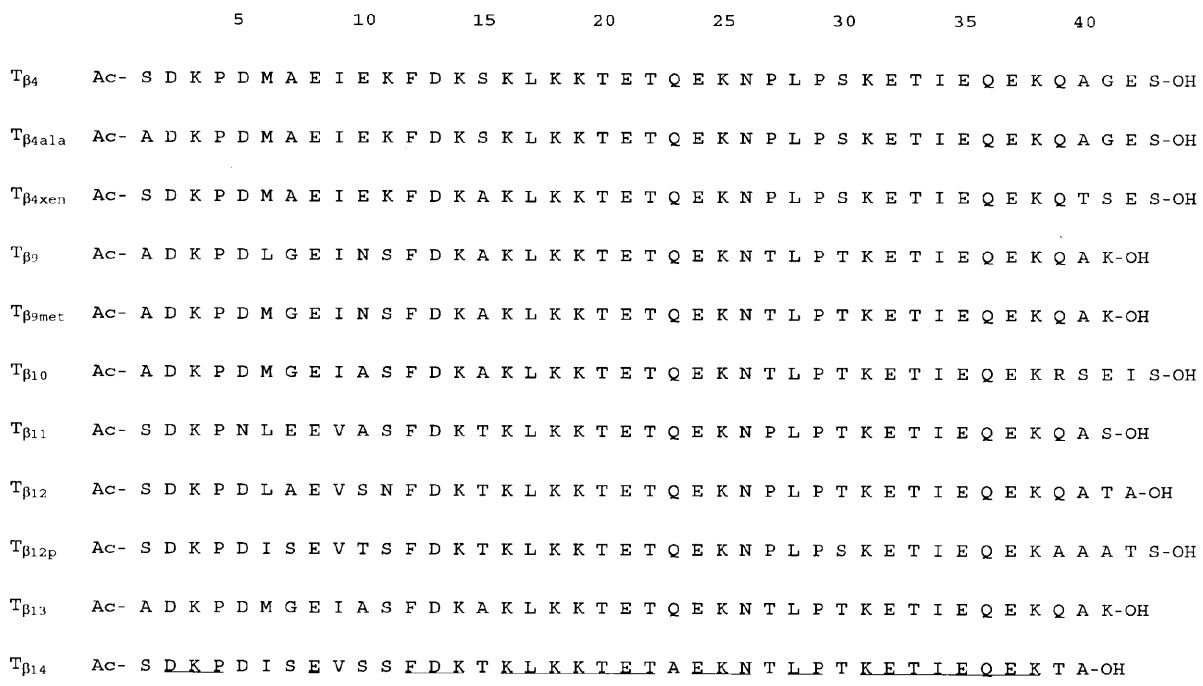

Figure 8 Comparison of amino acid sequences of ten members in β-thymosin family and thymosin β_{14}. Sequences are aligned to maximize homology. Identical amino acid residues are underlined. The sequence sources and the distribution of β-thymosins are as follows:

$T_{\beta4}$: human, chicken, cat [11], calf [1], rat, mouse [5], gecko [13], horse [31], whale [32], guinea pig [33], pig, ovine, murine [34]
$T_{\beta4ala}$: rabbit [11]
$T_{\beta4xen}$: *xenopus laevis* [9]
$T_{\beta9}$: calf [8]
$T_{\beta9met}$: calf [8]
$T_{\beta9met}$: pig [10, 16]
$T_{\beta10}$: human, cat, rat, mouse [12], rabbit [14], horse [31][x]
$T_{\beta11}$: trout [13, 15]
$T_{\beta12}$: trout [15][xx]
$T_{\beta12p}$: perch [35][xx]
$T_{\beta13}$: whale [32]
$T_{\beta14}$: sea urchin (from this study)
[x] $T_{\beta10}$: in the original sequencing studies of Erickson-Viitanen *et al.* [12] arginine-39 is missing. However, subsequent studies [36] indicated that thymosin β_{10} has 43 amino acid residues including arginine 39
[xx] $T_{\beta12}$ and $T_{\beta12p}$: from trout spleen, $T_{\beta11}$ and $T_{\beta12}$ were isolated in 1992 [15]. In the same year another $T_{\beta12}$, designated $T_{\beta12p}$ and different from that found in trout spleen, was isolated from perch liver [35].

entity of β-thymosins was postulated to be located in the non-conserved HN_2-terminus, as the thymosin β_4 fragment 7–30 completely loses its actin binding activity while the fragment 1–30 inhibits actin polymerization like the intact thymosin β_4, but at higher concentrations. Comparing the primary sequences of all known β-thymosins, including that of the sea urchin, it is striking that they all have a tripeptide sequence (2–4) as a common structural element and it could be postulated that this segment is responsible for the second functional entity. The high homology of the β-thymosins throughout the animal kingdom (Figure 8), and especially the conserved segments DKP (2–4) and LKKTET (17–

22) make it highly probable that the mechanism of action and biological function of this peptide family is similar from mammals to invertebrates.

CONCLUSIONS

A new thymosin, designated as thymosin β_{14}, consisting of 40 amino acid residues and with a molecular weight of 4537 Da was isolated. The amino-terminus of this polypeptide is blocked by an acetyl group as found by matrix-assisted laser desorption mass spectrometric and amino acid analysis. The primary structure was elucidated by automated Edman degradation of thymosin β_{14} fragments generated by the action of endoproteinase Asp-N: Ac-Ser-Asp-Lys-Pro-Asp-Ile-Ser-Glu-Val-Ser-Ser-Phe-Asp-Lys-Thr-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Ala-Glu-Lys-Asn-Thr-Leu-Pro-Thr-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Thr-Ala-OH.

Further digestion with trypsin and the identification of the thymosin β_{14} tryptic fragments by both Edman degradation and matrix-assisted laser desorption mass spectroscopy confirmed these structural data.

Thymosin β_{14} is 73% homologous to thymosin β_4, the first known member of the β-thymosin family, obtained from calf thymus.

By the isolation and structure characterization of thymosin β_{14} from the sea urchin, an invertebrate, new important information on the phylogenetic distribution and evolution of β-thymosins has been obtained.

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