Synthesis of Thymosin β_4^{Xen} and its Comparison with the Natural Tritetraconpeptide [1])

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Abstract. Thymosin β_4^{Xen}, a 43 residue peptide recently isolated from *Xenopus laevis*, was synthesized by automatic solid phase procedure and compared with the natural product, isolated from the ovaries of *Xenopus laevis*. For the synthesis *N*-methylpyrrolidone was chosen as solvent instead of the commonly used dimethylformamide because this solvent seems to be superior for solid phase peptide synthesis due to the favorable swelling properties of the polystyrene resin in this solvent and its dissolving power against the resin-bound peptide which reduces intermolecular aggregation. With acetic anhydride/pyridine and hydroxysuccinimide acetate two different acetylation reagents were tested for the final acetylation step, which gave both comparable results as shown by analytical HPLC investigations. The crude synthetic product was purified by HPLC, confirmed by ASA and LD-MS and was identical compared with the natural thymosin β_4^{Xen}.

Thymosins are polypeptides, originally isolated from tissues of vertebrates and structurally characterized during the last decade. Because these peptides were first isolated from thymus tissue preparations, they were termed "thymosins" and postulated to possess immunomodulating or hormonal functions [1]. The main members of this peptide family are the α- and β-thymosins. α-Thymosins include prothymosin α with 109 amino acid residues, the structurally related parathymosin α with 101 amino acid residues and thymosin α_1, the *N*-terminal fragment 1–28 of prothymosin α. The term β-thymosin is restricted to a group of highly homologous peptides with an average molecular weight of 5 kDa. The phylogenetic distribution of β-thymosins is ranging from mammals down to echinoderms, but they are also found in species containing no thymus gland. Usually, two highly homologous β-thymosins are present in one species. In mammals the most commonly found β-thymosin is thymosin β_4 (Tβ_4) with 43 amino acid residues, which is accompanied by thymosin β_{10} (human, horse, cat, rat and mouse), thymosin β_9 (calf), thymosin β_9^{Met} (pig) or thymosin β_{13} (whale). Up to now no β-thymosins could be detected in avertebrates like the gypsy moth larvae, the earthworm, the mealworm or yeast [2, 3]. The parent peptide of this family, thymosin β_4, has been shown to be active in a series of biological tests, *e.g*. the terminal deoxynucleotidyl transferase activity *in vivo* and *in vitro* is induced [1] or the hypothalamic secretion of luteinizing hormone releasing factor is stimulated [4]. It was found recently that thymosin β_4 forms an 1:1 complex with G-actin, thereby sequestering its polymerisation [5] 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 [6, 7]. From the oocytes of *xenopus leavis* a thymosin β_4-like peptide was isolated by our group and after amino acid sequence determination it could be shown, that this peptide comprising also 43 amino acid residues is not identical, but highly homologous to thymosin β_4. It was found, that the positions 15, 40 and 41 (Ser, Ala and Gly) of thymosin β_4 are replaced by the amino acids Ala (pos. 15), Thr (pos. 40) and Ser (pos. 41) in the so-called thymosin β_4^{Xen} (Scheme 1). For further structural proof, an efficient synthesis was developed and performed in order to compare the synthetic material with the natural peptide. As natural thymosin β_4^{Xen} was available in only extremely low amounts, biological

Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ala(Ser)-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Thr(Ala)-Ser(Gly)-Glu-Ser-OH

Scheme 1 Comparison of the primary sequences of thymosin β_4^{Xen} with thymosin b_4 (deviated amino acids in brackets).

[1]) Abbreviations: DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; HMP-resin, 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) resin; 1-HOBt, 1-hydroxybenzotriazole; PBS, phosphate buffered saline; MPA, 3-mercaptopropionic acid; NMP, *N*-methylpyrrolidone; OPA, *o*-phthalaldehyde; PCA, perchloric acid; TFA, trifluoroacetic acid; LD-MS, matrix-assisted laser desorption mass spectrometry.

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activities and function could so far not be tested and compared with those of the parent compound, thymosin β_4.

Experimental

Materials and Methods

All Fmoc-amino acids used and the HMP resin were products of NovaBiochem (Heidelberg, Germany). DMAP and l-HOBt were obtained from Fluka and used as 0.1M 4-dimethylaminopyridine in DMF and 1M 1-hydroxybenzotriazole in NMP. DCC (used as 1M DCC in NMP), TFA, thioanisole and ethanedithiol were products from Aldrich (Steinheim, Germany). NMP, DMF, acetic anhydride, pyridine, piperidine, methanol (fresh destilled over P_2O_5), dichloromethane (fresh distilled over $CaCl_2$) and other chemicals were obtained from Merck (Darmstadt, Germany).

Synthesis of Thymosin β_4^{Xen}

a) Desacetyl-thymosin β_4^{Xen}:

The synthesis of thymosin β_4^{Xen} was performed on an automated solid phase synthesizer model 431 A (Applied Biosystems, Weiterstadt, Germany) following the protection strategy, given in Scheme 2. For the synthesis, *N*-methylpyrrolidone was chosen as solvent instead of

Ac-Ser(*t*Bu)-Asp(O*t*Bu)-Lys(Boc)-Pro-Asp(O*t*Bu)-Met-Ala-Glu(O*t*Bu)-Ile-Glu(O*t*Bu)-Lys(Boc)-Phe-Asp(O*t*Bu)-Lys(Boc)-Ala-Lys(Boc)-Leu-Lys(Boc)-Lys(Boc)-Thr(*t*Bu)-Glu(O*t*Bu)-Thr(*t*Bu)-Gln-Glu(O*t*Bu)-Lys(Boc)-Asn-Pro-Leu-Pro-Ser(*t*Bu)-Lys(Boc)-Glu(O*t*Bu)-Thr(*t*Bu)-Ile-Glu(O*t*Bu)-Gln-Glu(O*t*Bu)-Lys(Boc)-Gln-Thr(*t*Bu)-Ser(*t*Bu)-Glu(O*t*Bu)-Ser(*t*Bu)-HMP-resin

Scheme 2 The chosen protection scheme (side chain protection groups in parenthesis) for the solid phase synthesis of thymosin β_4^{Xen}, performed on a HMP resin.

dimethylformamide commonly used in the solid phase peptide synthesis. *N*-Methylpyrrolidone seems to be superior for solid phase peptide synthesis due to the favorable swelling properties of the polystyrene resin in this solvent and its dissolving power against the resin-bound peptide, which reduces intermolecular aggregation. For attachment of the *C*-terminal amino acid to the HMP resin [8], Fmoc-Ser (*t*Bu)-OH (385 mg, 1 mmol) was first converted into its symmetrical anhydride *via* DCC (103 mg, 0.5 mmol) in 0.5 ml NMP in 23 min. This preformed symmetrical anhydride was added to the swollen HMP resin (500 mg, 0.25 mmol); and the reaction was performed in 5 ml NMP in the presence of DMAP (6.1 mg, 0.05 mmol) within one hour. After washing the derivatized solid support (6 × 10 ml NMP, 7 min), the *N*-terminal Fmoc-protecting group was removed by treatment with 20% piperidine in NMP (2 × 15 ml, 2 and 15 min). Washing with NMP (9 × 10 ml, 9 min) was followed by coupling the next amino acid, Fmoc-Glu(O*t*Bu)-OH (426 mg, 1 mmol in 10 ml NMP as 1-HOB*t* ester), in form of its active ester (72 min). The formation of the active ester was accomplished by reaction of 1 mmol Fmoc-amino acid with 1 mmol 1-HOBt (135 mg) in the presence of 1 mmol DCC (206 mg). After an additional NMP-washing step (2× 10 ml, 2 min), recoupling was carried out under the same conditions described before. All subsequent Fmoc-amino acids were incorporated by the same protocol using the following trifunctional amino acids: Fmoc-Glu(O*t*Bu)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)–OH, Fmoc-Lys(Boc)-OH. After completion of the synthesis, the *N*-terminal Fmoc-protecting group was removed as described before, and the peptide resin was washed with NMP (7 × 10 ml, 8 min). 100 mg of the N^{α}-deprotected peptide resin were removed for receiving the desacetyl-thymosin β_4^{Xen} peptide.

The removed peptide resin (100 mg, 11.3 µmol) was treated under a stream of nitrogen with a mixture of 25 ml TFA, thioanisole and ethanedithiol (10:2:0.5), and after 3.5 h the solution was separated from the resin by filtration with a fritted filter funnel. After evaporation of the filtrate *in vacuo* to about $^1/_{10}$ of the former volume, the peptide was precipitated by adding 40 ml of ice-cold ether, and collected by centrifugation. The precipitate was washed with cold ether several times, then first dried under a stream of nitrogen and further *in vacuo* at room temperature. Finally, the peptide was dissolved in 20 ml 0.1M acetic acid and lyophilized. Overall yield 35.9 mg (64%). Aliquots of 40–100 µg of the crude peptide were dissolved in 20 µl 0.05% TFA in H_2O, and the main product was separated by RP-HPLC. Fractions containing the major product were concentrated in a speed-vac centrifuge (Savant, New York, USA). After adding equal volumes of water the product was lyophilized. Amino acid analysis of the purified peptide: Asx 3.90 (4), Glu 10.87 (11), Ser 3.61 (4), Thr 3.75 (4), Ala 2.00 (2), Met 0.87 (1), Ile 1.98 (2), Phe 1.01 (1), Leu 1.92 (2) and Lys 10.41 (9); Pro (3), was not determined.

b) Thymosin ß$_4^{Xen}$:

The remainder amount of resin (1 g) was divided into two parts for performing different *N*-acetylation procedures:

1. 500 mg of the peptide resin were treated with 2 ml acetic anhydride in 2 ml pyridine for 30 min. According to the quantitative ninhydrin method [9, 10], the acetylation was incomplete and afforded a second acetylation with the same procedure.
2. 500 mg of the remaining peptide resin were treated with 10 equivalents hydroxysuccinimide acetate (200 mg, 1.3 mmol), dissolved in 7 ml NMP, for 40 min. Though the ninhydrin reaction indicated almost complete acetylation the reaction was also repeated.

202 mg of the obtained acetylated peptide resins were treated in an analogous manner as described for the des-acetyl-thymosin β_4^{Xen}-polymer. After removing the protection groups (see above), both products obtained from the different acetylation methods showed consistent amino acid analysis values. Asx 3.97 (4), Glx 10.60 (11), Ser 3.33 (4), Thr 3.11 (4), Ala 2.00 (2), Met 1.11 (1), Ile 2.02 (2), Phe 1.16 (1), Leu 2.12 (2) and Lys 10.24 (9); Pro (3), was not determined.

RP-HPLC

For routine analytical HPLC, we used a Rheodyne 7125 sample injector (Rheodyne, Berkeley, USA), a low-pressure gradient former M 2500 (Gynkotek, Munich, Germany), a constant-flow pump M 600/200 (Gynkotek, Munich, Germany), a variable wavelength detector BT 3030 (Eppendorf-

Biotronik, Maintal, Germany) and a LiChrospher 100 RP-18 column (250 × 4 mm, 5 µm particle size, Merck, Darmstadt, Germany, conditions see Fig. 1A).

For semipreparative HPLC isolation of native thymosin β_4^{Xen} and comparative HPLC-studies of the synthetic and natural thymosin β_4^{Xen}, we used a Beckmann 420 HPLC system (Beckman, Munich, Germany) with an Altex Ultraspere IP column (250 × 4.6 mm, 5 µm particle size, Beckman, Munich, Germany), an FS 950 fluorometer (Kratos, Berlin, Germany) with 365 nm excitation from an FSA 110 lamp (no excitation filter) and an FSA 427 emission filter cut-off at 440 nm for fluorescamine derivatives detection. The fluorometer was connected to a single-channel chart recorder and an D-2000 integrator (Merck-Hitachi, Darmstadt, Germany, conditions see Fig. 2).

Amino Acid Analysis

Amino acid compositions were determined on a BT 8100 HPLC system (Eppendorf-Biotronik, Maintal, Germany), equipped with a RF-535 fluorometer (Shimadzu, Duisburg, Germany) and a LiChrospher 100 RP-18e (250 × 4 mm, 5 µm particle size) column. The peptides (1–2 mmol) were hydrolyzed in 0.1 ml redistilled 6M HCl at 155 °C for 45 min. The samples were lyophilized, subjected to precolumn derivatization with *o*-phthalaldehyde/3-mercaptopropionic acid and separated by RP-HPLC. The samples were dissolved in 100 µl of 1M sodium borate buffer (pH 9.3), and an aliquot of 20 µl was used for derivatization with 10 µl OPA/MPA (10 µl OPA/ml methanol and 10 µl MPA/ml methanol). After 2.5 min, the reaction was terminated by addition of 20 µl 1M KH_2PO_4. A 20 µl sample was injected *via* a 20 µl loop into the HPLC-system. At a flow rate of 1.5 ml/min the amino acid derivatives were eluted by a linear gradient from buffer A (12.5 mM Na_2HPO_4/12.5 mM NaH_2PO_4/2% tetrahydrofuran/3% acetonitrile) to 100% B (30% acetonitrile in 12.5 mM Na_2HPO_4/12.5 mM NaH_2PO_4) within 12 min.

Laser Desorption Mass Spectrometry

The mass spectrometer used, was a matrix-assisted laser desorption mass analyzer Kratos MALDI I (Shimadzu, Duisburg, Germany). Spectra from multiple laser shots (5–75) were summed until an acceptable signal-to-noise ratio was achieved at a repetition rate of 0.5 Hz. α-Cyano-4-hydroxycinnamic acid was used as matrix, in which the peptides were dissolved in 70% CH_3CN, 30%H_2O and 0.1% TFA to give an approximate concentration of about 5×10^{-2} mol. Insulin was used as external standard. Results [theoretical masses in bracketts]: Desacetyl-thymosin β_4^{Xen}: 4966.5 Da [4965.1 Da ($M+H^+$)]; Thymosin β_4^{Xen}: 5009.3 Da [5008.1 Da ($M+H^+$)].

Isolation of Thymosin β_4^{Xen}

A female *Xenopus laevis* was anaestethised with MS 222 (Sandoz, Nürnberg, Germany), the ovaries were removed, immersed in ice-cold PBS, weighed and homogenized after addition of 4 volume ice-cold 0.4M PCA in a Polytron homogenizer. The homogenate was stored for 30 min on ice and after centrifugation at 30000 g for 30 min at 4 °C the supernatant was adjusted to pH 2.8 by KOH. The precipitate of $KClO_4$ was removed by low-speed centrifugation, and the supernatant solution was transferred to an RP-18 column (40–63 µm, 1.5 × 1 cm, Merck, Darmstadt, Germany). Salts were eluted with 30 ml H_2O and peptides with 10 ml 30% *n*-propanol. After lyophilization, the residue was dissolved in 1 ml buffer A, pyridine (110 mM)/formic acid (76 mM), and a 0.5 ml aliquot of the sample was applied to an Altex Ultrasphere – I.P. column (250 × 4.6 mm, 5 µm partice size) and eluted at a flow rate of 0.75 ml/min with buffer A and a linear gradient to 100% buffer B (40% *n*-propanol in buffer A) within 120 min. At 15 sec intervals 2 µl samples were diverted to the fluorescamine peptide detector, and fractions were collected every 1 min. The over all yield of thymosin β^{Xen} was 2 mg with the following amino acid composition: Asx 4.04 (4), Glx 10.50 (11), Ser 3.29 (4), Thr 3.36 (4), Ala 1.97

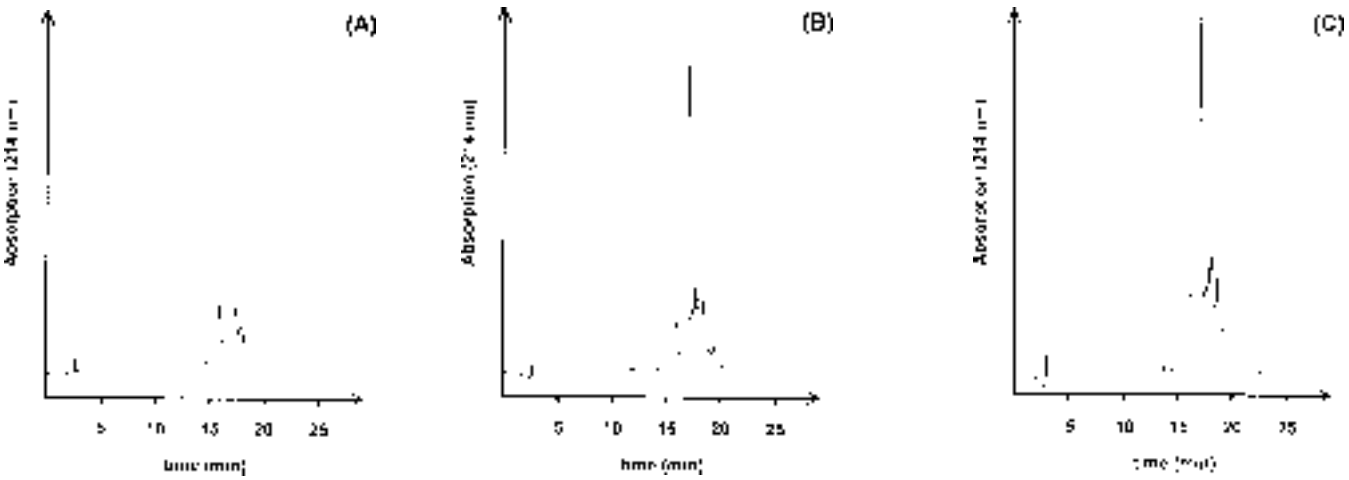

Fig. 1 Analytical HPLC-profiles of crude desacetyl-Tβ_4^{Xen} (**A**) and Tβ_4^{Xen} ((**B**), using acetylation method 1; (**C**) using acetylation method 2). Conditions: column: LiChrosper 100 RP-18 (250 × 4.6 mm, 5 µm), gradient elution: 5% B – 90% B in 30 min (buffer A: 0.05% TFA in H_2O, buffer B: 0.05% TFA in CH_3CN/H_2O = 60:40), flow rate: 1.1 ml/min, detection: (UV) λ = 214 nm.

(2), Met 0.93 (1), Ile 1.96 (2), Phe 1.07 (1), Leu 2.21 (2) and Lys 10.52 (9); Pro (3), was not determined.

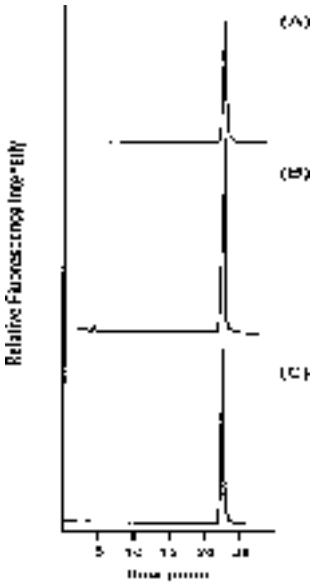

Fig. 2 Comparison of analytical HPLC-profiles of purified synthetic thymosin β_4^{Xen} (**A**), natural thymosin β_4^{Xen} (**B**) and an 1:1-mixture of the synthetic and the natural peptide (**C**). Conditions: column: Altex Ultrasphere IP (250 × 4.6 mm, 5 µm), gradient elution: 0% buffer B – 100% buffer B in 30 min (buffer A: 110 mM pyridine/76 mM formic acid, buffer B: 40% *n*-propanol in buffer A), flow rate: 0.75 ml/min, detection: fluorescence (365/440 nm cut-off filter fluorescamine).

Results and Discussion

Thymosin β_4^{Xen} was synthesized on an HPM resin using the N^{α}-Fmoc/*tert.* butyl strategy. During the development of a model containing clustered peptide chains to evaluate coupling difficulties associated with interchain interactions in solid phase peptide synthesis, Tam and Lu found out that *N*-methylpyrrolidone-swollen peptidyl resins with different substitutions levels (0.1–0.8 mmol/g) showed the best swelling properties in comparison to the solvents DCM, DMF and DMSO. They further estimated a strong correlation between solvent permeability and coupling yields and showed that NMP is the solvent of choice to overcome interchain interaction and to improve coupling efficiency [8]. Therefore NMP was chosen as solvent for the synthesis. Scheme 2 shows the primary sequence of the peptide including the chosen protecting groups for the trifunctional amino acids and the *C*-terminal peptide resin binding. The *C*-terminal amino acid Fmoc-Ser(*t*Bu)-OH was converted to its symmetrical anhydride by means of DCC and treated in this form of an active ester with the HMP resin. For elongation all further Fmoc-amino acids were coupled as 1-HOBt-esters, preformed by 1-HOBt and DCC [9], in form of a double-coupling procedure. The Fmoc-groups were removed with 20% piperidine in NMP. After completion of the synthesis and removal of the final Fmoc-group, part of the peptide resin was removed and treated with TFA and thioanisole and ethanedithiol as scavengers to yield the crude desacetyl-thymosin β^{Xen}.

For acetylation of the *N*-terminal amino group of the peptide resin two methods were applied. Therefore half of the amount of the peptide resin was treated with acetic anhydride/pyridine (method 1) and the other half with hydroxysuccinimide acetate (method 2) leading to synthetic thymosin β_4^{Xen}.

RP-HPLC analysis of the crude peptide products (Fig. 1) indicated that 73% of the desired desacetylthymosin β_4^{Xen} and 53% thymosin β_4^{Xen} (method 1), respectively 48% thymosin β_4^{Xen} (method 2) were obtained. As expected, the desacetylated thymosin β_4^{Xen} showed a minimal but significantly shorter retention time (R_t = 15.9 min) compared to the acetylated product (R_t = 16.3 min). In all chromatograms a small peak eluting before the main peak could be detected. This compound can also be found in the natural material and was identified as (methionine sulfoxide)^5-thymosin β_4^{Xen}. Using fluorescamine derivative detection as an alternative RP-HPLC investigation method, the chromatograms of the purified synthetic (2A), natural (2B) and a 1:1-mixture of natural and synthetic **(**2C) thymosin β_4^{Xen} (Fig. 2) document that the two peptides of synthetic and natural sources have identical chromatographic properties. The results of our synthesis of thymosin β_4^{Xen} demonstrate that the synthetic protocol and the applied performance are suitable to produce β-thymosins in high purity and, as further important advantage, without traces of biological contaminations, which are often difficult to separate from peptides isolated from natural tissues. Our described production procedure looks further promising for an up-scale production [11], so that sufficient synthetic material is available to examine physical and biological properties of β-thymosins. As the naturally occurring β-thymosins, so far determined in their structures [3], are highly homologous to the parent compound Tβ_4 (Tβ_4: 100%, Tβ_4^{Xen}: 93%, Tβ_9: 77%, Tβ_{4Met}: 79%, Tβ_{10}: 79%, Tβ_{11}: 74%, Tβ_{12}: 79%, Tβ_{12p}: 79%, Tβ_{13}: 77%, Tβ_{14}: 69%), the described synthetic procedure should allow their production in similar yield and purity.

As shown in Scheme 1, both sequences of thymosin β_4 and thymosin β_4^{Xen} are identical concerning their *N*-terminus up to residue 15. So, both peptides may function as precursor molecules for the tetrapeptide Ac-Ser-Asp-Lys-Pro-OH, which was identified as an inhibitor of bone-marrow haematopoietic stem cells or hepatocyte proliferation [6, 7].

Actually, Tβ_4 and homologous β-thymosins have raised a lot of scientific interest since Safer *et al.*

observed that Tβ_4 forms a 1:1 complex with muscle G-actin [5] under physiological ionic conditions thus preventing its polymerization to F-actin. Therefore, Tβ_4 seems to be involved in the microfilament system acting as an actin-sequestering peptide like gelsolin or profilin. Tβ_4 competes with the same actin binding site as profilin and actobindin. Like actobindin, the *N*-terminus of Tβ_4 is important for the binding on the actin molecule, especially part 17–22 with the sequence LKKTET. This hexamotive is directly involved in actin binding and is also present in thymosin β_4^{Xen}, indicating a similar biological function of the *Xenopus laevis* peptide. The binding site of the closely homologous β-thymosins must be located close to the *C*-terminus of actin, since it was possible to crosslink the thiol group of Cys^{374} of actin with the thiol group of a cysteine residue introduced into position 6 of thymosin β_4 by use of the short-distance cross-linker *m*-maleimidobenzoyl-*N*-hydroxy-succinimidate [12]. The cross-linking reaction did not proceed when Cys^{374} in actin was blocked by *N*-methyl-maleimide [13]. Besides the hexamotive LKKTET, the sequence between residues 1–16 of Tβ_4 extents a secondary effect which is necessary for the inhibitory activity of actin polymerization. Comparing this sequence region of Tβ_4 with that of thymosin β_4^{Xen}, only one amino acid residue is exchanged at position 15 (Ser by Ala).

Based on NMR and CD studies [14, 15], all β-thymosins are highly hydrophilic and flexible peptide structures. It could be shown, that Tβ_4 adopts random coil in water solution, whereby two helical regions were estimated, extending from residues 4–16 and 30 to 40, in fluorinated alcohols, which are in agreement with the prediction by a Chou/Fasman plot [16]. According to Chou/Fasman Ser residues are indifferent moieties concerning α-helix formation while Ala is a strong α-helix inducer. Therefore an exchange of Ser 15 (Tβ_4) by Ala 15 (Tβ_4^{Xen}) should have no influence on the conformational status of the *Xenopus laevis* peptide related to Tβ_4. A similar binding property of both homologues can therefore be predicted and can be proved with the synthetic material now available.

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