Amino Acids (1991) 1: 215–223

Amino
Acids

Relationship of amino acid sequence to immunological activity – syntheses and structure-activity relationships of thymosin β_4 family*

T. Abiko and **H. Sekino**

Kidney Research Laboratory, Kojinkai, Miyagino-ku, Sendai Japan

Summary. Four peptides related to thymosin β_4 family and its six fragments were synthesized by the solution method. Among them, the four peptides related to thymosin β_4 family and its two fragments were found to have restoring activity on the impaired blastogenic response of T-lymphocytes isolated from uremic patients, but the other four fragments had no effect. Studies on the structure-activity relationships suggest that the tricosapeptide moiety corresponding to amino acids 16–38 of thymosin β_4 is found to be an important moiety on impaired immunological deficiency.

Keywords: Amino acids – Thymosin β_4 family – Immunological activity – Uremic patient – Impaired immunological deficiency – Restoring effect

Introduction

The impairement of immunological responsiveness in patients with chronic renal failure is well known. All aspects of the immune response appear to be affected by the uremic state and the depression of cell mediated immunity has been studied most extensively. The numbers, subpopulations and reactivities of circulating lymphocytes may be altered by uremia [1, 2].

In addition, severe structural changes have been reported by many investigators in the lymph nodes and thymus glands of uremic patients and animals.

---

* Amino acids and their derivatives used in this investigations were of the L-configuration. The following abbreviations are used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; Boc, tert-butoxycarbonyl; Z, benzyloxycarbonyl; NP, p-nitrophenyl; ONp, p-nitrophenyl ester; OBzl, benzyl ester; Bzl, benzyl; Troc, β, β, β-tricloroethoxycarbonly; Su, N-hydroxysuccinimide; NMM, N-methylmorpholine; OSu, N-hydroxysuccinimide ester; WSCI, l-ethyl-3; (3-dimethylaminopropyl) carbodiimide; HOBT, N-hydroxybenzotriazole; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; E-rosette, a rosette with sheep erythrocytes; EDTA, ethylenediaminetetraacetic acid; PHA, phytohemagglutinin; HPLC, high-performance liquid chromatography; ONb, p-nitrobenzyl ester; DCC, dicyclohexylcarbodiimide.

Thymus is an endocrine gland which controls the development, differentiation and maturation of T-lymphocytes and by these cells regurates the immune responses.

Many active peptides have been isolated from thymus itself or from blood and recently from spleen. They all seem to fulfil the criteria of a thymus hormone but they have completely different structures [3–5].

One of the hormones called serum thymic factor (STF) was extracted from serum, while thymosins and thymopoietins were isolated from thymus [3–5].

It is important for us to study on the structure-activity relationships of these peptides and also interesting for us to find out active centers of these peptides.

We describe here the chemical syntheses of four peptides corresponding to entire amino acid sequences of thymosin β_4 family [6–9] and its six fragments [10] and their immunologcal effect on the impaired T-lymphocytes isolated from uremic patients [9].

As you can see in Fig. 1, thymosins β_4, β_8, β_9 and thymosin β_4^{Xen}, are closely related polypeptides isolated from bovine thymus and oocytes of Xenopus laevis. All these peptides belong to thymosin β_4 family are composed of more than 40 amino acid residues and acetylated at the N-terminal amino acid. All these four purified polypeptides subsequently proved to be active in induction of early T-cell differentiation and modulation of mature lymphocytes.

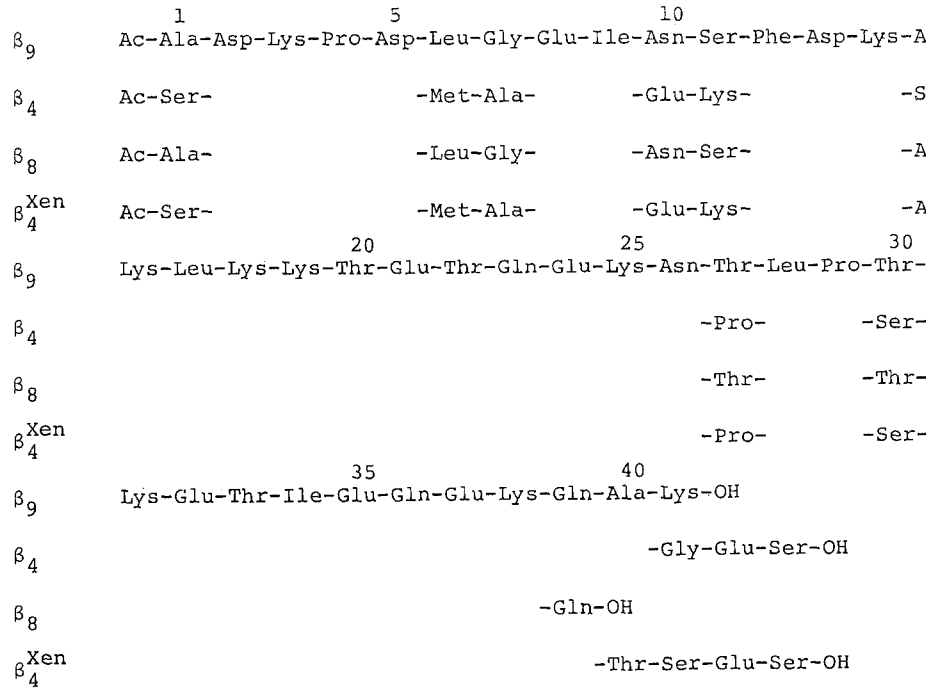

Fig. 1. Amino acid sequences of thymosins β_9, β_4, β_8 and thymosin β_4^{Xen}

To investigate whether these four peptides related to thymosin β_4 family have the restoring effect on impaired T-lymphocyte blastogenic response to PHA in uremic patients, we synthesized following four peptides related to thymosin β_4 family and its six peptide fragments.

Synthesis of deacetyl-thymosin β_4^{Xen}

As one of the examples, we describe here the synthetic route to deacetyl-thymosin β_4^{Xen} (Fig. 2). From the synthetic viewpoint, compared with our previous syntheses of deacetyl-thymosin β_4, thymosins β_8 and β_9, the thioanisole-mediated trifluoromethane-sulfonic acid (TFMSA) deprotecting procedure was applied in the final step of the present synthesis instead of hydrogen fluoride.

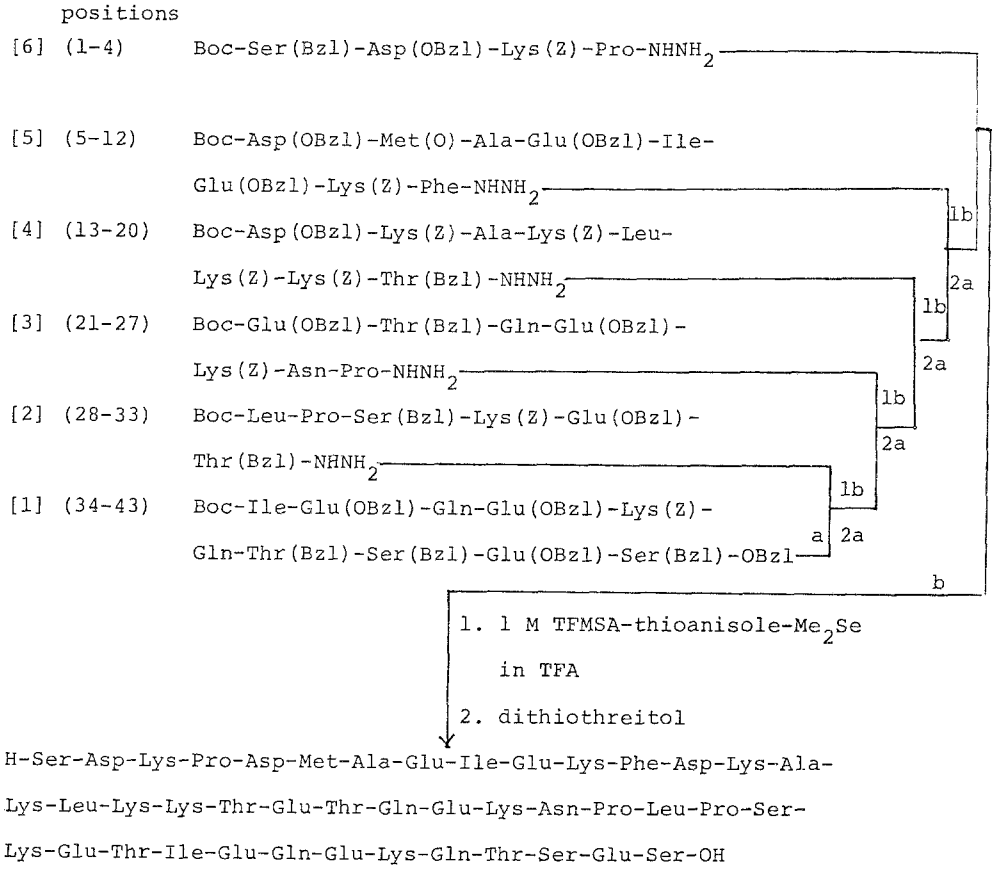

Fig. 2. Synthetic route to deacetyl-thymosin β_4^{Xen}, *a* TFA-anisole; *b* azide

Our synthetic route to deacetyl-thymosin β_4^{Xen} is illustrated in Fig. 2, which shows six fragments selected as building blocks to construct the entire amino acid sequence of deacetyl-thymosin β_4^{Xen}. Boc group, removable by TFA, was adopted as a temporary N^{α}-protecting group for every intermediate. Amino acid derivatives bearing protecting groups removable by 1 M TFMSA-thioanisole in TFA were employed, i.e., Ser (Bzl), Lys (Z), Glu (OBzl), Asp (OBzl), Thr (Bzl) and Ser(BZl)-OBzl. The Met residue was reversibly protected as its sulfoxide in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air oxydation during the synthesis. The substituted hydrazine, Troc-$NHNH_2$ was employed for the preparation of five fragments [2], [3], [4], [5] and [6] containing the Glu (OBzl) or the Asp (OBzl) residue. This Troc group

is known to be cleaved by Zn in AcOH without affecting other functional groups. Throughout the synthesis of these fragments and intermediates, the purity of every fragment and intermediate was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis after acid hydrolysis.

The six fragments were prepared stepwise starting from each C-terminal amino acid by the Su active ester procedure except for the introduction of Gln residue, which was introduced by the NP active ester procedure. Each protected fragment Troc-hydrazide thus obtained was treated with Zn powder in AcOH to remove the Troc group and zinc acetate was removed by treatment with EDTA to afford required hydrazides. The six fragments thus obtained were successively condensed by the azide procedure according to the route shown in Fig. 2. Every reaction was carried out in a mixture of DMF and DMSO and the amount of the acyl component was increased from 2 to 4 eq as the chain elongation progressed. Every product was purified by precipitation from DMF or DMSO with MeOH or gel-filtration on Sephadex LH-60.

In the final step of the synthesis, the protected tri-tetracontapeptide ester was treated with 1 M TFMSA-thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of protecting groups. The deprotected peptide was converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH at pH 8.0 to reverse a possible $N \rightarrow 0$ shift at the Ser and Thr residues. The Met(0) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se during the above acid treatment and secondly with dithiothreitol during incubation of the deprotected peptide.

The reduced product was purified by ion-exchange column chromatography on a DEAE-cellulose column with linear gradient elution using pH 7.8 NH_4HCO_3 buffer, followed by preparative TLC. Desalting on a Sephadex G-25 column gave a fluffy powder, which exhibited a single spot (ninhydrin- and chlorine-tolidine-positive) on TLC in two different solvent systems and paper electrophoresis. Its purity was further confirmed by amino acid analysis after acid hydrolysis and enzymatic digestion.

This peptide is slightly sensitive to air-oxydation. The purity of the product as estimated by HPLC was about 99%. The minor impurity (approximately 1%) seemed to be the Met(O)-derivative, since the main product was converted to this minor component by excess H_2O_2 treatment.

The other three peptides, deacetyl-thymosin β_4, thymosins β_8 and β_9 were synthesized by essentially the same manner as described for the preparation of deacetyl-thymosin β_4^{Xen} except for using hydrogen fluoride in the final step of the synthesis.

Immunological effect of the synthetic four peptides related to thymosin β_4 family

The immunological effect of the synthetic four peptides related to thymosin β_4 family was examined by means of the JIMRO (Japan Immunoresearch Laboratories Co. Ltd.) fluorometric blast-formation test according to Itoh and Kawai [11].

Responses of T-lymphocytes to mitogenic stimulation were lower in uremic patients than those of normal persons. The in vitro effect of the synthetic peptides on the impaired PHA response of T-lymphocytes from uremic patients is shown in Table 1.

Table 1. Effect of the synthetic four peptides related to thymosin β_4 family on the impaired PHA-stimulation of T-lymphocytes of uremic patients

Peptides	Dose (μg/ml)	SI[a,b]
—[c]	—	278.1 ± 51.6
—[d]	—	107.2 ± 53.2[g]
H-Leu-Gly-Gly-OH[d-f]	10	104.3 ± 54.3
Deacetyl-thymosin β_4[d,f]	10	216.6 ± 50.5[h]
Deacetyl-thymosin β_4^{Xen}[d,f]	10	223.1 ± 54.3[h]
Thymosin β_8[d,f]	10	215.4 ± 52.1[h]
Thymosin β_9[d,f]	10	220.9 ± 55.9[h]

[a] Each value represents the mean ± S.D. of triplicate measurements.

[b] SI (stimulation index) was calculated according to the following formula: $SI = \frac{I_2 - I_0}{I_1 - I_0} \times 100$, where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated lymphocytes and I_0 = fluorescence intensity of ethidium bromide.

[c] Normal venous lymphocytes. [d] Patient's venous lymphocytes.

[d] Patient's venous lymphocytes.

[e] Control: This peptide was purchased from the Peptide Institute, Inc., Osaka, Japan.

[f] Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 in air for 12 h.

[g] $p < 0.05$, when compared to the normal persons by using Student's test.

[h] $p < 0.02$, when compared to the uremic patients by using Student's t test.

In the case of the uremic patients, all the four peptides exhibited a restoring effect at the same concentration.

On the other hand, in the case of normal subjects, in vitro addition of these peptides had no effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown).

Syntheses of six common amino acid sequence fragments of thymosin β_4 family

Next, six common amino acid sequence fragments of thymosin β_4 family (Fig. 3), H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-OH (positions 16–26),

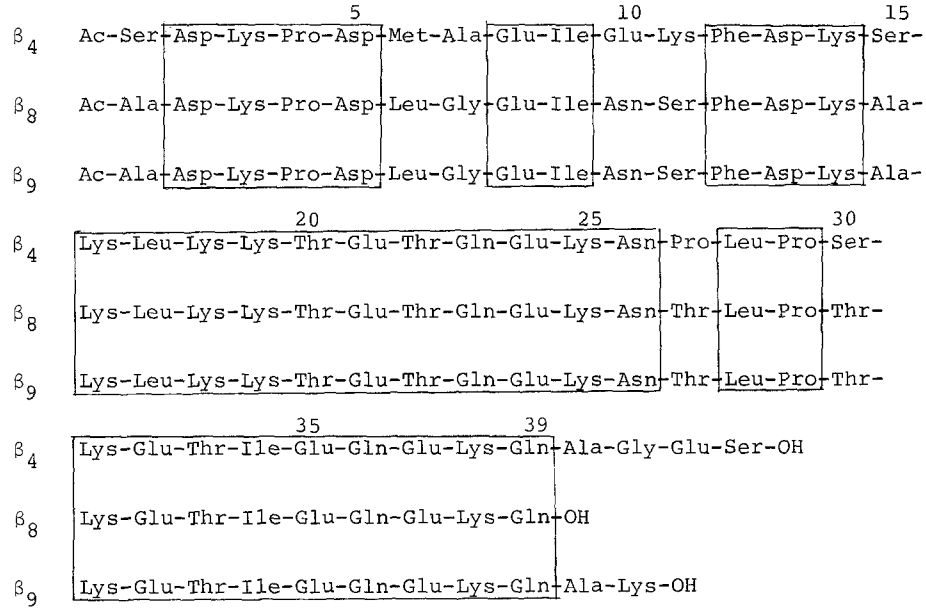

Fig. 3. Comparison to amino acid sequences of thymosin β_4, thymosin β_8 and thymosin β_9. Identical sequences are shown in boxes

H-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-OH (positions 31–39), H-Asp-Lys-Pro-OH (positions 2–5), H-Phe-Asp-Lys-OH (postions 12–14), H-Leu-Pro-OH (positions 28–29) and H-Glu-Ile-OH (positions 8–9), were synthesized by the solution method and were tested to determine their effects on the impaired blastogenic response of T-lymphocytes isolated from uremic patients.

In the present synthesis, as illustrated in Figs. 4 and 5, amino acid derivatives bearing protecting groups, i. e., Asn-ONb, Lys (Z)-ONb, Ile-OBzl, Gln-OBzl, Asp (OBzl)-OBzl, Lys (Z), Glu (OBzl), Asp (OBzl) and Z-Asp (OBzl), which could be removed by catalytic hydrogenation were used.

After removal of the N^{α}-protecting Boc group with TFA-anisole, the peptide chain was elongated from *C*-terminus to *N*-terminus by the HOBT-DCC procedure except for Gln residues, for which the NP active ester procedure was employed. Each protected product was purified by batchwise washing followed by precipitation from appropriate solvents. The homogeneities of these protected intermediates were assessed by paper chromatography and elemental analysis. Following the final condensation, the Boc group was removed by TFA-anisole treatment and then other protecting groups were removed by catalytic hydrogenation over 10% Pd-charcoal catalyst.

Deprotected peptides were purified by recrystallization from appropriate solvents or gel-filtration on a Sephadex column or partition column chromatography.

These purified peptides obtained were found to be homogenious by paper chromatographies in two different solvent systems. The purities of these peptides were further assessed by amino acid analyses of the 6 N HCl hydrolysates and the enzymatic digests.

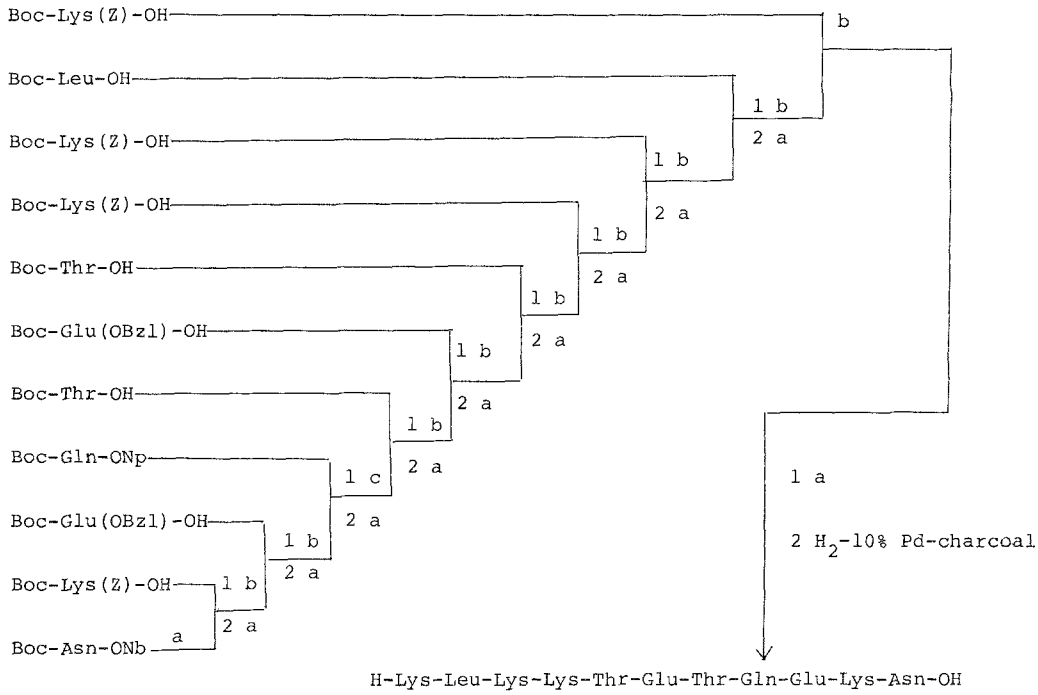

Fig. 4. Synthetic scheme for H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Gln-Lys-Asn-OH (Positions 16–26). *a* TFA-anisole; *b* HOBT-WSCI; *c* active ester

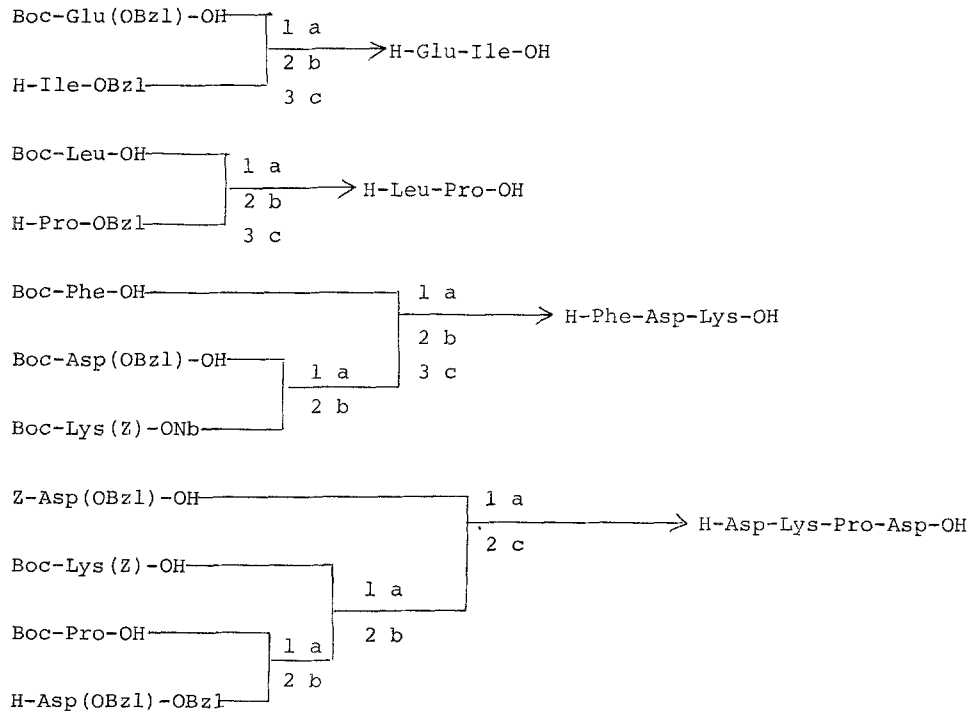

Fig. 5. Synthetic scheme for H-Asp-Lys-Pro-Asp-OH (Positions 2–5), H-Phe-Asp-Lys-OH (Positions 12-14), H-Leu-Pro-OH (Positions 28–29) and H-Glu-Ile-OH (Positions 8–9). *a* HOBT-WSCI; *b* TFA-anisole; *c* H_2 – 10% Pd-charcoal

Immunological effect of the synthetic six fragments of thymosin β_4 family

Two of six fragments, H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-OH and H-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-OH, have restoring effect on the impaired blastogenic response of T-lymphocytes isolated from uremic patients though they were less effective than their parent molecule thymosin β_9, but the other four peptide fragments had no effect under the same conditions (Table 2).

Table 2. Effect of the synthetic thymosin β_4 family and its six common fragments on the impaired PHA stimulation of T-lymphocytes of uremic patients

Peptides	Restoring effect on the impaired PHA stimulation of T-lymphocytes[a]
Deacetyl-thymosin β_4	+ + + +
Thymosin β_8	+ + + +
Thymosin β_9	+ + + +
Deacetyl-thymosin β_4^{Xen}	+ + + +
H-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-OH (16–26)	+ +
H-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Glu-OH (31–39)	+
H-Asp-Lys-Pro-Asp-OH (2–5)	−
H-Phe-Asp-Lys-OH (12–14)	−
H-Glu-Ile-OH (8–9)	−
H-Leu-Pro-OH (28–29)	−

[a] + = active; + + + + > + + > +; − = inactive

Conclusion

The results of the immunological assay seem to suggest that the four peptides related to thymosin β_4 family that conserve two immunologically active sites, -Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn- (positions 16–26) and -Lys-Glu-Thr-Ile-Glu-Gln-Lys-Gln- (positions 31–38), within their molecules have an equipotent immunological activity, and the tricosapeptide moiety corresponding to amino acids 16–38 of thymosin β_4 family seem to be an important moiety for restorative activity on impaired immunological deficiency.

References

1. Wilson WEC, Kirkpatrick CH, Talmage DW (1975) Ann Intern Med 62: 1
2. Boulton-Jones JM, Vick R, Cameron JS, Black PJ (1973) Clin Nephrol 1: 351
3. Bach JF, Dardenne M (1972) Transplant Proc 4: 345
4. Goldstein G (1975) Ann NY Acad Sci 249: 177
5. Low TLK, Goldstein AL (1982) J Biol Chem 257: 1000
6. Abiko T, Sekino H (1982) J Appl Biochem 4: 449
7. Abiko T, Sekino H (1983) Chem Pharm Bull 31: 1320
8. Abiko T, Sekino H (1984) Chem Pharm Bull 32: 4497
9. Abiko T, Sekino H (1989) Chem Pharm Bull 37: 2467

10. Abiko T, Sekino H (1984) Chem Pharm Bull 32: 228
11. Itoh Y, Kawai T (1983) Rinsho Kensa 27: 982

Authors' address: T. Abiko, D. Ph., Kidney Research Laboratory, Kojinkai, 1–6, Tsutsujigaoka 2-chome, Miyagino-Ku, Sendai 980, Japan.