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Isolation and Identification of a New Thymic Peptide from Calf Thymus

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Abstract—Various thymic peptides (including thymulin, thymic humoral factor, thymopoietin, etc.) play important roles in the process of T cell maturation and development. We isolated a new peptide from calf thymus and named it thymus activity factor II (TAF-II). A yield of 0.92 mg of TAF-II was purified from 500 g calf thymus. Analysis by LC/MSD-Trap showed the amino acid sequence of this hexapeptide to be Glu-Ala-Lys-Ser-Gln-Gly-OH with molecular weight 618.5 daltons. We have also begun to investigate the influence of TAF-II.

Key words: thymus, TAF-II, E-rosette assay, thymic peptide

In 1961, Miller reported that removal of the thymus of newborn mice caused some immune defects [1], suggesting that the thymus is crucial also in the human body. Near the same time, Good also demonstrated that thymus was significantly related with the immune system by studying the effect of removing the rabbit thymus [2]. The discoveries of Miller and Good were thought to be a great step in biology and immunology.

The mammalian thymus, a small organ located just above the heart, is a primary lymphatic organ. It provides a specialized microenvironment for the maturation, education, and selection of developing T-lymphocytes. Shortly after birth, the human thymus begins a life-long process of evolution, whereby the net size of the thymus is not altered but the organ is replaced by adipose tissue [3]. Also reported is a process of maturation and selection of T-lymphocytes migrating from bone marrow [4].

In the microenvironment of the thymus there are a wide variety of thymic peptides that play important roles in the process of T cell maturation and development. Oscar's review [5] reports on peptides that have been discovered from the mid 1970s as follows: thymulin, thymic humoral factor, thymopoietin, thymosins, and prothymosin-α. These peptides, as well as a variety of other modulators (interleukins (IL)-1, -3, -6, granulocyte monocyte colony stimulating factor (GM-CSF)), regulate a process known as thymic selection by which prothymocytes become mature and functional T-cells [5]. Though the mechanism of the interaction of thymic peptides and T lymphocytes is not fully understood, some biological and clinical applications of these peptides have been developed, e.g., treatment of elderly patients to improve immune function or combat aging [6], and therapeutic treatment for hepatitis B virus, cancer, and human immunodeficiency virus [7].

We recently isolated and purified a new peptide, thymus activity factor II (TAF-II), from fresh calf thymus. We obtained 0.92 mg TAF-II from 500 g fresh calf thymus tissue. With the help of the Analysis Center of Tsinghua University, we determined the structure of this natural hexapeptide and its molecular weight.

MATERIALS AND METHODS

Reagents and materials. Fresh calf thymus tissue was obtained from the Beijing abattoir. Sephadex G-15, Sephadex G-10, and DEAE-Sephadex A-25 were purchased from Pharmacia (Sweden). RPMI-1640 was pur-

Abbreviations: TAF-II) thymus activity factor II; SRBC) sheep red blood cell; GM-CSF) granulocyte monocyte colony stimulating factor; CD) cluster of differentiation; APC) antigen presenting cell; RFC) rosette-forming cells; THF) thymic humoral factor; TCR) T-cell receptor.

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chased from Invitrogen (USA) and 1× liquid medium was prepared following the manufacturer's instruction. Fixative solution: 25% glutaraldehyde, 3.5% sodium hydrogen carbonate, and Hank's solution at a rate of 1 : 1 : 38 were mixed. Stain solution was prepared by mixing Giemsa Stain Solution and Hank's solution at ratio 1 : 3, then centrifuging the mixture at 1500 rpm for 10 min and retaining the supernatant.

Isolation and purification of the hexapeptide. The peptide was isolated and purified from calf thymus using the method described previously [8]. The method is described briefly as follows: calf thymus tissue (500 g) was homogenized and then it was frozen and thawed for three cycles and centrifuged. The supernatant was ultrafiltered, and then the ultrafiltrate was subjected to Sephadex G-15 gel filtration, DEAE-Sephadex A-25 ion-exchange chromatography, and reverse-phase high performance liquid chromatography (HPLC). A yield of 0.92 mg of TAF-II was obtained.

Analysis of amino acid composition. The amino acid content of TAF-II hydrolyzates was analyzed after hydrochloric acid hydrolysis. The degradation of TAF-II to free amino acids was accomplished by acid hydrolysis under vacuum and heat (110°C) for 24 h. The free amino acid composition and the percentage of each amino acid were determined using a Beckman 121 MB System (USA).

Determination of the amino acid sequence of TAF-II. The hexapeptide TAF-II purified from calf thymus was analyzed using a 1100 Series LC/MSD-Trap (Agilent, USA) and the amino acid sequence was obtained.

Human lymphocyte preparation. Heparinized peripheral blood (5 ml) was obtained from healthy volunteers (age from 20 to 26 years) and the lymphocytes were isolated at room temperature on gradients of lymphocyte separation medium (density 1.077 ± 0.002 g/ml). The lymphocyte-rich layer was collected and washed twice in phosphate buffer solution (PBS). The cells were counted under a microscope and finally the cells were resuspended at concentration $5 \cdot 10^6$ viable cells/ml in RPMI-1640 medium. Viability assessed by the trypan blue test was always greater than 95%.

Assay for E-rosette forming lymphocytes. A procedure modified from Thomas et al. [9] was used to determine the impact of the peptide on E-rosette formation. Briefly, lymphocytes in RPMI-1640 medium (concentration $5 \cdot 10^6$ viable cells/ml) were incubated at 45°C for 30 min to delete the sheep red blood cell (SRBC) receptor, then the cells were resuspended at $5 \cdot 10^6$ cells/ml. Lymphocyte suspension (100 µl) was incubated with 50 µl of TAF-II solution (at various concentrations) for 1 h (see Table 2). Finally, each solution was mixed with 0.2 ml of 0.5% suspension of SRBC in 12 × 75 mm plastic tubes and incubated at 37°C for 60 min. The mixture was centrifuged at 800 rpm for 5 min and incubated at 4°C overnight. Each tube was supplemented with fixative solution for 10 min the next day. The cell pellets were gently resuspended by rolling the tubes, and the stain solution was added with incubation for 15 min. Finally, the rosette forming cells (RFC) were counted under a microscope. The control is RFC production of normal lymphocytes that were not incubated at 45°C for 30 min with SRBC. All tests were performed in triplicate and at least 200 lymphocytes were counted. Lymphocytes with three or more attached SRBC were considered as RFC.

To calculate the sample unit the following formula was used: activity unit (U) = (sample positive rate – control positive rate)/(control positive rate).

RESULTS

Isolation and purification of the new thymic peptide. Calf thymus tissue (500 g) was homogenized, then froze and thawed through three cycles, centrifuged, and ultrafiltered. The ultrafiltrate was then gel-filtered through Sephadex G-15 gel (1.5 × 100 cm). Six peaks were eluted (Fig. 1a). The E-rosette assay showed that the second peak possessed the ability to generate RFC, hence the second peak was pooled and concentrated. After DEAE-Sephadex A-25 anionic ion-exchange chromatography (1.5 × 10 cm), seven peaks were eluted using 0.05-0.5 M ammonium acetate buffer (pH 5.8) (Fig. 1b). The sixth peak that showed ability in generation of RFC was concentrated and desalted using Sephadex G-10, and was then purified by reverse-phase HPLC. We obtained a peak that had bioactivity (see Fig. 1c). After lyophilization, the final yield was 0.92 mg powder, which we named TAF-II.

Amino acid composition of the thymic peptide. TAF-II was degraded to its free amino acids by acid hydrolysis under vacuum and heat, at 110°C for 24 h. Under such hot acidic conditions, the peptide bond between amino acids is hydrolyzed, resulting in free amino acids. Hydrolysis is followed by chromatographic separation of the free amino acids. Analysis by LC/MSD showed that TAF-II is composed of Ala, Gly, Glu, Gln, Lys, and Ser.

Determination of the sequence of the TAF-II. To determine the amino acid sequence of the thymic peptide TAF-II, we used a 1100 Series LC/MSD-Trap apparatus to determine its structure. As shown in Fig. 2, the sequence is Glu-Ala-Lys-Ser-Gln-Gly.

Activity (total and specific) of purification products and effect of TAF-II in E-rosette assay. After each purification step, all the fractions were evaluated using the E-rosette assay, and their activity and specific activity were calculated. As described above, the positive control in the E-rosette assay was thymomodulin. In the E-rosette assay, 200 lymphocytes were counted and the lymphocytes with three or more attached SRBC were calculated. Comparing the number of RFC with number of lymphocytes (200), we got the positive rate. In Table 1, we can clearly see the activity and the specific activity of the purification product after each step.

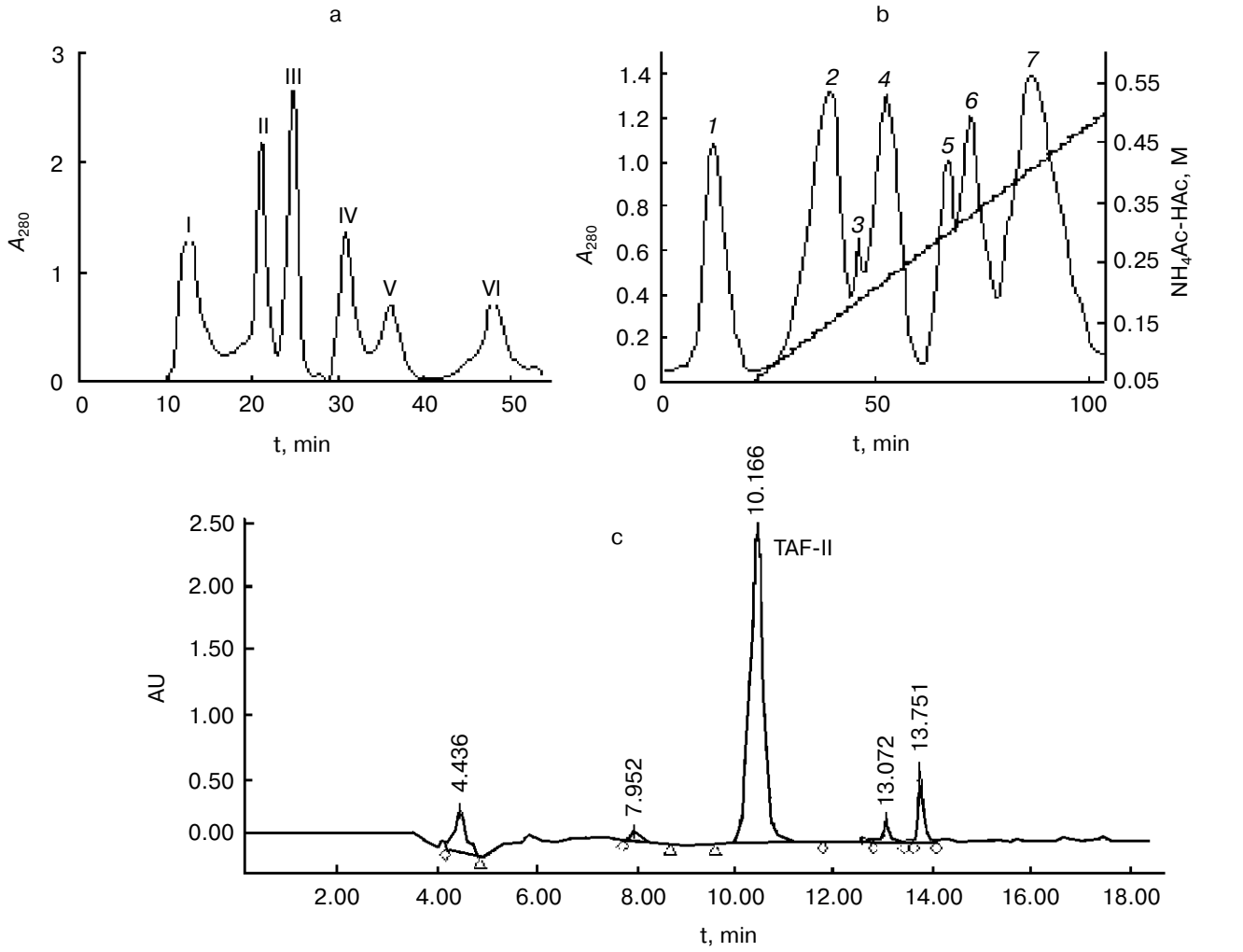

Fig. 1. Isolation and purification the new thymic peptide through Sephadex G-15, DEAE-Sephadex A-25, Sephadex G-10, and reverse-phase HPLC. a) Six peaks eluted on Sephadex G-15 filtration, in which peak II showed biological activity in the E-rosette assay. b) DEAE-Sephadex A-25 anionic ion-exchange chromatography (1.5 × 10 cm) using 0.05-0.5 M ammonium acetate buffer (pH 5.8) as eluent gave seven peaks, in which the sixth peak showed bioactivity. c) Reverse-phase HPLC gave a peak eluted at 10.46 min, which was the new thymic peptide named TAF-II. Mobile phase: a) the liquid phase containing 0.1% trifluoroacetic acid (TFA); c) acetonitrile phase containing 0.1% TFA. The column was eluted with a linear gradient at 0.6 ml/min.

Co-incubation of pure TAF-II with human peripheral blood lymphocytes and using the E-rosette assay to examine the biological activity of TAF-II. Table 2 shows that TAF-II can significantly enhance the rate of RFC production. Compared with thymomodulin, 2 μg/ml TAF-II provides a high RFC production rate in the E-rosette assay. We also synthesized the hexapeptide with the same structure and examined it in the E-rosette assay. The results of the synthetic hexapeptide (not shown here) were similar to the results shown in Table 2.

DISCUSSION

During the last century, a great number of thymic hormones have been isolated, purified, and not only their amino acid compositions but also their biological activities have been studied [11-15]. The roles of these hormones are believed to be important in T cell maturation and differentiation, though the detailed influence of these hormones is not clear due to the complex microenvironment in the thymus. To evaluate the biological activities of these hormones, the E-rosette assay, which was used based on the changes induced by thymic hormones on the minority of T cells that form spontaneous rosettes with SRBC [13, 15].

The CD2 molecule is expressed on almost all T-cells and is the receptor of SRBC. After the identification of both of CD2 and CD58 glycoproteins, the function of CD2 was thought to afford the recognition of T-cell and antigen presenting cell (APC) [16]. In another study, it was surprising that CD2 knockout mice demonstrated no abnormality not only in T-cell maturation but also in T-

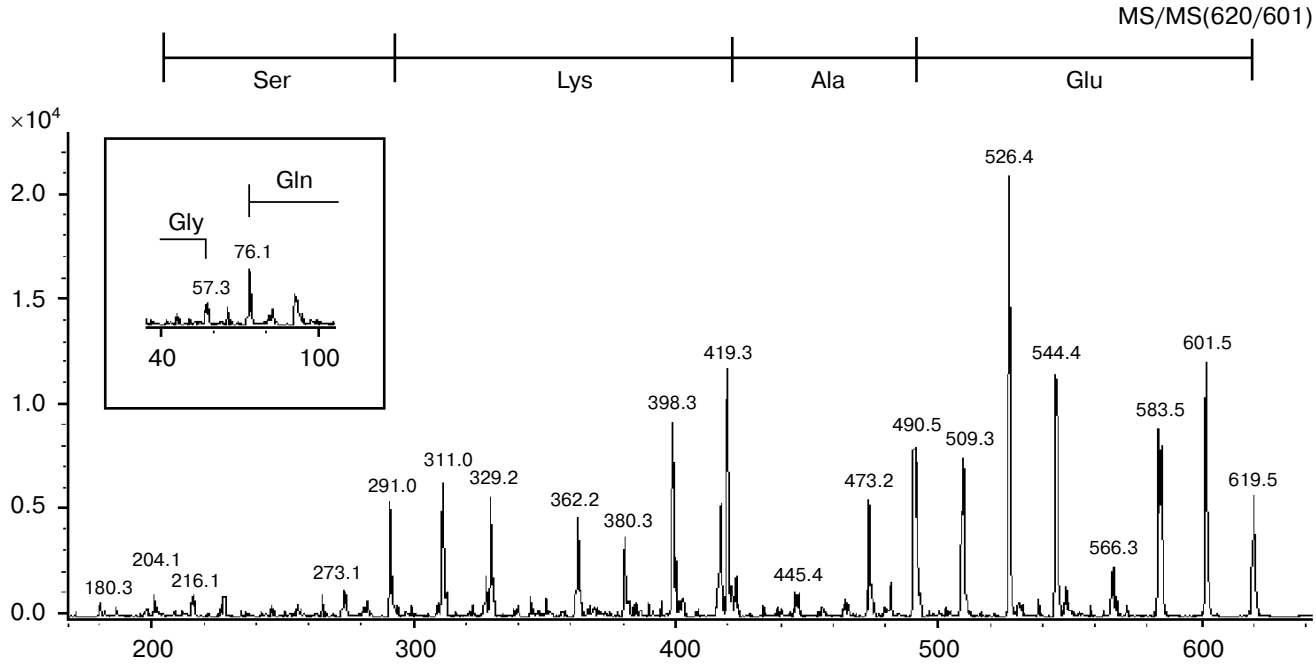

Fig. 2. LC/MSD analysis of the thymic peptide TAF-II. From the analysis by LC/MSD-Trap, the amino acid sequence of the natural peptide is N<Glu-Ala-Lys-Ser-Gln-Gly>C. The molecular weight of the hexapeptide is 618.5 daltons.

cell recognition with antigen [17]. Recently, more has been learned about the function of this molecule, a member of the Ig superfamily, in the activation and selection of T-cells. Besides the function of activating T-cells, it was found that the presence and absence of CD2 dramatically affects the repertoire selection in both CD4 and CD8 T-cells, and CD2 plays an important role in thymic pre-TCR function [18].

Oscar's review points out that since the mid 1970s thymic hormones such as thymulin, thymic humoral factor, thymopoietin, thymosins, and prothymosin-α have been discovered [5]. By using the E-rosette assay, we found that a small peptide which molecular weight is below 1000 could enhance the production of RFC in the E-rosette assay. To investigate the character of this peptide, we isolated and purified it and finally prepared one

Table 1. Purification of TAF-II

Step	Total protein, mg	Total activity, U	Specific activity, U/mg
Crude extract	61 450	141 335	2.3
Ultrafiltration	1950	46 800	24
Sephadex G-15	278.6	30 646	110
DEAE-Sephadex A-25	8.3	5170.9	623
RP-HPLC	0.92	1749.8	1902

Note: Crude extract is the suspension of the centrifugation after frozen and thawed three cycles. To evaluate the protein concentration, the Lowry method [10] was used.

Table 2. Effect of TAF-II activity in E-rosette assay ($n = 3$)

Concentration, mg/ml	RFC, %	Increase in rate	p
Control (without TAF-II)	29.4 ± 3.3		
Thymomodulin* (1.0)	48.5 ± 1.7	65.0	<0.01
TAF-II (0.4)	33.3 ± 3.2	13.3	<0.05
TAF-II (0.2)	36.7 ± 2.1	24.8	<0.01
TAF-II (0.01)	46.5 ± 4.3	58.2	<0.01
TAF-II (0.002)	55.7 ± 5.2	89.5	<0.01
TAF-II (0.001)	38.1 ± 6.7	29.6	<0.01

* The optimum concentration for the thymomodulin in E-rosette assay (thymomodulin was used as positive control) is 1 mg/ml, which was validated by our laboratory.

pure thymic peptide to which we gave the name TAF-II. By using the E-rosette assay to explore the biological activity for each purification fraction, we found that either the crude or the pure TAF-II can enhance the RFC rate. Also, as we can see in Table 1, the effect of TAF-II is significant. This phenomenon showed that TAF-II can exert stimulating effects on the T-cells, and this may have clinical applications.

The identification of the amino acid sequence of the hexapeptide showed that this peptide had not been previously reported. The question how TAF-II can influence lymphocyte activation should still be further studied. This depends on the comprehensive understanding of the microenvironment of the thymus. Furthermore, we are ready to investigate the influence of TAF-II on the immune system as the next step. And we will also examine whether a metal ion is necessary for TAF-II to play its biological roles.

The thymus has been investigated for a long time, and actually it has a vital role in the immune system, but the molecular interactions provided by the thymic microenvironment that predicates T-cell development remain obscure. Considering the complexity of the developing process of the thymus, further studies to understand the role of the thymus in the immune system and aging process will be carried out.

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