Development of Specific Anti-Thymosin β₁₀ Antipeptide Antibodies for Application in Immunochemical Techniques

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We present theoretical and experimental data necessary for raising specific antibodies for thymosin β₁₀, a 43-amino acid residues peptide occurring in human tissues. We postulate that thymosin β₁₀ contains three major antigenic determinants (residues 2–8, 17–25, and 35–41). For antibody development, we synthesized the N-terminal fragment thymosin β₁₀(1–16) as well as the C-terminal fragments thymosin β₁₀(31–43) and thymosin β₁₀(38–43), due to their putative antigenic properties and minimal structural similarity with the homologous peptide thymosin β₄, which also occurs in humans. The putative antigenic determinant 17–25 is present in all β-thymosins and was therefore not synthesized. All antisera raised against the above peptide fragments or the intact molecule of thymosin β₁₀ were found capable of recognizing and binding synthetic or natural thymosin β₁₀ with high specificity, showing minimal cross-reactivity with thymosin β₄ isolated from bovine tissues or synthetic thymosin β₄. Due to its easy preparation and the highly specific affinity of the antibody raised against it for the intact peptide, the fragment thymosin β₁₀(38–43) may be considered the antigen of choice for developing anti-thymosin β₁₀ antibodies, which can eventually be applied to immunochemical studies.

Thymosin β₁₀ Thymosin β₄ Antigenic predictions Antipeptide antibodies ELISA

The naturally occurring polypeptides α- and β-thymosins have been implicated in important cellular mechanisms (13,23,24), through which they can possibly influence the potential for malignant transformation of cells. These polypeptides have also been reported to provide indications, through their expression level (5,6,26,29), through that of their mRNA (11,12), or through their possible binding sites (21), as to the normal/malignant state of cells. Thus, prothymosin α, thymosin β₄, and thymosin β₁₀ have been reported to be present in elevated levels in human cancer tissues. However, exact determination of these peptide levels in human fluids and tissue extracts and subsequent evaluation of their clinical importance as diagnostic tools remain to be carried out, due to the lack of appropriate samples for the routine clinical laboratory analytical methodology. In this direction, our Laboratory has undertaken efforts to prepare basic reagent components for the immunochemical analysis of β-thymosins (18,22), and we report the development of antipeptide antibodies specific for thymosin β₁₀. In humans, thymosin β₁₀ (7,10) is always accompanied by thymosin β₄ (15); both of these peptides contain 43 amino acid residues and show higher than 75% structural similarity (Fig. 1). Previous experience in developing antibodies for thymosin β₄ and thymosin β₉, both present in bovine tissues (14), indicated the necessity of using specific antigenic peptide fragments of the corresponding intact molecule for immunization, due to the high cross-reactivity observed for the antibodies raised against the whole molecule of the above peptides (19,20). Thus, in this study we present our results concerning theoretical prediction of the major antigenic sites (2) on thymosin β₁₀, synthesis of properly selected peptide fragments, development of the corresponding antipeptide antibodies, evaluation of these antibodies by means of an ELISA system and, overall, we provide the theoretical and experimental data necessary for raising specific oligoclonal antibodies against peptide fragments of thymosin β₁₀. The term oligoclonal refers here to antibodies that are raised against a small fragment of the maternal polypeptide and are still able to recognize the corresponding intact molecule. The information reported in this work may prove to be useful for the researchers.

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Thymosin β4

SDKPDMAEIEKFDKSKLKKTETQEKKNPLPSKETIEQEQKQGES

Thymosin β10

ADKPDMEIEASFDKAKLKKTETQEKNTLPTKETIEQERKASEIS

Secondary structure

Antigenic determinants

FIG. 1. Thymosin β4 primary structure and schematic representation of its structural topology, according to theoretical prediction. Different symbols for α-helix (O), β-turn (curved arrow), and coil structure (---) are used. Thymosin β10 primary structure is also presented. Not-in-common amino acid residues are underlined.

METHOD

Materials

Fmoc-protected amino acids were obtained from CBL-Patras. Analytical grade reagents were obtained from Merck or Sigma. Trifluoroacetic acid (TFA), sequential grade, was obtained from Sigma. Water and acetonitrile for high performance liquid chromatography (HPLC) were obtained from Lab-Scan. Aminomethyl-polystyrene resin and the 4-cyano-4'-carboxy triphenyl methanol handle were prepared as previously described (32,33). HCl 6 M, phenylisothiocyanate (PITC), and amino acid standards for amino acid analysis were purchased from Pierce.

Keyhole limpet hemocyanin (KLH), glutaraldehyde, goat anti-rabbit γ-immunoglobulin conjugated to horseradish peroxidase (IgG/HRP), and, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma. Complete Freund's Adjuvant was a product of Difco. Tween 20 and H2O2 were purchased from Merck and Ferak, respectively. The ELISA microplates were obtained from Costar. Thymosin β10 used for raising the corresponding antiserum and for the ELISA experiments was synthesized as previously described (18). Thymosin β10 used for estimating the cross-reactivity of the antisera developed was isolated from bovine tissues (22). Synthetic thymosin α1, used also in cross-reactivity studies, was a product of Sigma.

Secondary Structure and Antigenicity Predictions

Conformational/antigenic predictions were performed using the PC/GENE release 6.85 package from IntelliGenetics, Inc. Secondary structure predictions for putative α-helices and β-turns were performed using the methods of Chou and Fasman (3,4) as well as that of Gamier et al. (9). Helical wheel representation was achieved using the method of Schiffer and Edmundson (25). Flexibility indices were determined using the method of Karplus and Schulz (17). Antigenic properties based on local average hydrophilicity were predicted using the method of Hopp and Woods (16).

Antigenic determinants

working in the promising area of developing new diagnostic tools for monitoring cancer.

Peptide Synthesis

Synthesis of the peptides was carried out manually by using Fmoc amino acids, onto the 4-cyano-4'-carboxethoxyl trityl resin, following the method of Fmoc solid phase synthesis (8). The side chain functional groups of aspartic acid and glutamic acid were protected as tert-butyl esters, Fmoc-Asp(OBu')-OH and Fmoc-Glu(OBu')-OH, threonine and serine as tert-butyl ethers, Fmoc-Thr(Bu')-OH and Fmoc-Ser(Bu')-OH, lysine as tert-butyloxycarbonyl derivative, Fmoc-Lys(Boc)-OH, and arginine as the 2,2,5,7,8-pentamethyloxycarbonyl derivative, Fmoc-Arg(Pmc)-OH.

Couplings were performed by using DIPC/HOBt in DMF. Coupling success was checked with the Kaiser ninhydrin test. Deprotection of the Fmoc group was achieved by repetitive treatment with 20% piperidine in DMF. The resin-bound peptide was removed from the resin and amino acid side chains were deprotected by treatment with a cocktail of TFA, CH2Cl2, water, and scavengers. After removing the organic solvents, the crude product was precipitated with cold diethyl ether and dissolved in water to be purified.

The peptides were purified by semipreparative reverse-phase HPLC on a Nucleosil RP-C18 column (250 X 10 mm i.d., 7 μm particle size, Macherey-Nagel) with 0.1% TFA/acetonitrile gradient in 0.1% TFA/water as eluent. Peptide identification was confirmed by amino acid analysis, which was performed using the PICO-TAG method (Waters). Peptide purity was higher than 99%, as assessed on a LiChrospher RP-C18 analytical column (250 X 4.6 mm i.d., 5 μm particle size, Merck) with 0.1% TFA/acetonitrile gradient in 0.1% TFA/water as eluent.

Preparation of Peptide Immunogens and Rabbit Antipeptide Antisera

The peptides were coupled to the KLH carrier protein through their amino groups using glutaraldehyde, as follows.

The peptide (1 mg in 200 μl of 0.1 M phosphate buffer, pH 7.4) was pipetted into a reaction vessel containing KLH (0.6 mg in 60 μl of 0.1 M phosphate buffer, pH 7.4) and then 12 μl of a 25% aqueous glutaraldehyde solution was added. After gentle agitation, first for 3 h at room temperature and then for 18 h at 4°C, the reaction mixture was diluted with 15 mM NaCl
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to a final concentration of 40 \( \mu \)g peptide/ml. The diluted mixture, which was used for immunization without any special purification, was stored in lyophilized form at 4°C, until injection.

New Zealand White rabbits were immunized with 20 \( \mu \)g of peptide, in its conjugated to KLH form, emulsified in Complete Freund’s Adjuvant, according to the method of Vaitukaitis (27). The animals were boosted at first after 6 weeks and then every 4 weeks. Blood was collected 2 weeks after each booster injection. The antisera was isolated with low-speed centrifugation and stored at -35°C. Antiserum titers had a peak after the second and third booster injection.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

**Titer Estimation.** The ELISA microwells were coated with 200 \( \mu \)l of an aqueous thymosin \( \beta_{10} \) or thymosin \( \beta_4 \) solution (100 ng/ml) and dried overnight at 50°C. Two hundred microliters of the antisera at different dilutions in buffer A [phosphate-buffered saline (15 mM, pH 7.4), containing BSA (0.2%) and Tween 20 (0.05%, v/v)] was added to the microwells and incubated for 2 h at 37°C. Then the liquid was discarded and the microwells were washed three times with buffer B [phosphate-buffered saline (15 mM, pH 7.4) containing Tween 20 (0.05%, v/v)]. Next, 200 \( \mu \)l of an IgG/HRP solution, diluted 1:1000 in buffer A, was added to each microwell and incubated for 2 h at 37°C. After discarding the liquid, the wells were again washed three times with buffer B. Finally, 200 \( \mu \)l of an ABTS/H_2O_2 (0.13%/0.003%) solution in citrate/phosphate buffer (0.1 M, pH 4.5) was pipetted. The absorbance was measured as 30 min in a microtiter plate reader (Model MR 5000, Dynatech Laboratories, Torrance, CA) at 405 nm.

**Displacement Curves.** The ELISA microwells were coated with 200 \( \mu \)l of an aqueous T\( \beta_{10} \) solution (50 ng/ml) and dried overnight at 50°C. Four hundred microliters of thymosin \( \beta_{10} \), thymosin \( \beta_4 \), or thymosin \( \alpha_1 \) standard solutions (0, 12.5 ng/ml, 125 ng/ml, 0.5 \( \mu \)g/ml, 2 \( \mu \)g/ml, 10 \( \mu \)g/ml, 40 \( \mu \)g/ml) prepared in buffer A [phosphate-buffered saline (15 mM, pH 7.4) containing BSA (0.2%) and Tween 20 (0.05%, v/v)], and 400 \( \mu \)l of each antiserum, diluted 1:5000 in buffer A, were mixed in plastic tubes and allowed to preincubate for 18 h at 4°C. Two hundred microliters of these solutions were added to the microwells and incubated for 2 h at 37°C. Then the liquid phase was discarded and the wells were washed three times with buffer B [phosphate-buffered saline (15 mM, pH 7.4) containing Tween 20 (0.05%, v/v)]. Two hundred microliters of an IgG/HRP solution (diluted 1:3000 in buffer A) was added to each microwell and incubated for 2 h at 37°C. The liquid phase was again discarded and the wells were washed three times with buffer B. Finally, 200 \( \mu \)l of an ABTS/H_2O_2 (0.13%/0.003%) solution in citrate/phosphate buffer (0.1 M, pH 4.5) was added to each microwell and the optical absorbance was measured as 30 min at 405 nm.

**RESULTS**

**Prediction of Antigenic Sites**

The amino acid sequence of thymosin \( \beta_{10} \) was analyzed for its potential secondary structure based on the methods of Chou and Fasman (3,4). According to the results obtained, the peptide was found to be generally organized with two alternative \( \alpha \)-helices, at residues 5–16 and 30–40 [Fig. 2(a)]. A major \( \beta \)-turn was found at residues 3–6, providing evidence that this sequence is a surface-lying region [Fig. 2(b)]. Similar data were obtained using the method of Garnier et al. (9).

![Graphs showing theoretical data concerning secondary structure features and hydrophilicity of thymosin \( \beta_{10} \).](image)

**FIG. 2.** Theoretical data concerning secondary structure features [\( \alpha \)-helix (a), \( \beta \)-turn (b), backbone flexibility (c), and hydrophilicity (d), on which the prediction of major antigenic sites on thymosin \( \beta_{10} \) was based.

The amino acid sequence of thymosin \( \beta_{10} \) was also analyzed for identifying regions with high indices of flexibility (17). Three sequence segments (residues 2–8, 17–25, and 35–41) were found to share high values of thermal mobility, or ‘B’ factor, with the region between residues 17 and 25 corresponding to the highest value [Fig. 2(c)].

Application of the method of Hopp and Woods (16) for predicting antigenicity based on local average hydrophilicity revealed that highest hydrophilicity is observed between the residues 35 and 41 of thymosin \( \beta_{10} \) [Fig. 2(d)].

Overall, thymosin \( \beta_{10} \) is predicted to contain three major antigenic determinants (around the sequences 2–8, 17–25, and 35–41), which are located in regions supposed to contain a \( \beta \)-
turn or showing the highest hydrophilicity or flexibility indices (Fig. 1).

**Development and Evaluation of Antibodies**

The peptide fragments thymosin β₁₀(1–16), thymosin β₁₀(31–43), and thymosin β₁₀(38–43) were synthesized, purified by semipreparative reverse-phase HPLC, and their purity was verified by analytical HPLC and amino acid analysis. The whole molecule of thymosin β₁₀ was synthesized as previously described (18). The corresponding immunogens were prepared by coupling the peptides synthesized to KLH and then injected to rabbits for developing antibodies. The antisera obtained were evaluated for their titer and cross-reactivity using an enzyme-linked immunosorbent assay (ELISA), which was developed especially for that purpose. All the antisera developed were able to recognize the corresponding peptide (data not shown) as well as the intact molecule of thymosin β₁₀, with titers approximately equal to 1:15,000–1:20,000 (Fig. 3). Antiserum cross-reactivities with thymosin β₄ were estimated from titer curves obtained by incubating various dilutions of each antiserum with thymosin β₄ immobilized on the ELISA microwells as well as from the corresponding displacement curves. According to the data obtained by the titer curves, the antisera raised against thymosin β₁₀(31–43) or thymosin β₁₀(38–43) showed practically undetectable cross-reactivities, whereas those raised against thymosin β₁₀ or thymosin β₁₀(1–16) exhibited marginal cross-reactivities with thymosin β₄ at high antibody concentrations (Fig. 3). According to the results obtained by the displacement curves, none of the antisera exhibited cross-reactivity with thymosin β₄, even in the presence of 5 µg of that peptide per microwell. Displacement curves were also performed using synthetic thymosin α₁ as a putative cross-reacting material, providing no displacement even in the presence of 10 µg of the peptide per microwell. The capability of the antisera developed to recognize and bind endogenous thymosin β₁₀ was tested by measuring this peptide, through an appropriate displacement curve, in an extract of homogenized rabbit tissues, which was prepared following a protocol previously described for bovine tissues (22). A value of 9.4 µg per g of wet tissue weight was determined, which is considered reasonable according to relevant literature data (10).

**DISCUSSION**

Thymosin β₁₀ (7,10) is a natural polypeptide that has recently raised significant interest in its biological functions (13,30), especially due to its possible association to cell proliferation mechanisms or even malignant transformation of cells (11,12,29). Nevertheless, scientific information concerning this peptide is scattered and needs to be brought together, analyzed, interpreted, and clinically evaluated. Due to the high structural similarity of thymosin β₁₀ with thymosin β₄, which accompanies the former in human tissues, and previous experience in developing antibodies for thymosin β₄ and...
thymosin $\beta_4$ (19,20), we considered it necessary to define highly antigenic areas on the intact molecule with distinct differences in their primary structure compared with the corresponding areas of thymosin $\beta_1$. These defined peptide areas were synthesized and administered in rabbits for developing antibodies, postulating that the combination of their putative highly antigenic properties and unique structural features could stimulate the production of very specific antibodies recognizing the intact molecule of thymosin $\beta_1$. For optimal results we designed our synthetic approach to include a single putative antigenic site in each synthetic peptide.

To predict which peptidic sequences are exposed on the molecule surface, being probably antigenic, the amino acid sequence of thymosin $\beta_{10}$ was analyzed for its potential secondary structure using the methods of Chou and Fasman (3,4) and the results were confirmed by the method of Garnier et al. (9). The data obtained are similar with those previously reported for the highly homologous peptide thymosin $\beta_4$, based on 2D-NMR experiments (31).

Surface-oriented peptide sites expected to be antigenic were identified as those combining the highest indices of flexibility and hydrophilicity using the method of Karplus and Schulz (17) and that of Hopp and Woods (16), respectively. Three sequence segments of thymosin $\beta_{10}$ (i.e., 2–8, 17–25, and 35–41) showed high values of thermal mobility, or ‘‘B’’ factor, indicating high flexibility, with the region between residues 17–25 corresponding to the highest value. The latter region, present in all $\beta$-thymosins, is postulated to be the site of binding to G-actin (28). On the other hand, the putative antigenic determinant $\gamma$–8 and 35–41, respectively, were anticipated to contain three major antigenic determinants (residues 18–26 and 29–37).

The antibody developed against the peptide fragment thymosin $\beta_{10}(1–16)$ showed low cross-reactivity with thymosin $\beta_4$, although antisera raised against the intact molecule of thymosin $\beta_4$ (19) or thymosin $\beta_6$ (20) have been reported to show high cross-reactivity values with thymosin $\beta_4$ or thymosin $\beta_4$, respectively. The specificity of the antisera raised against intact thymosin $\beta_{10}$ might be related to the high flexibility and hydrophilicity predicted for the C-terminal area of the peptide, which may render this highly nonhomologous region to be the dominant antigenic determinant of thymosin $\beta_{10}$.

The antibody developed against the peptide fragment thymosin $\beta_{10}(38–43)$ could recognize the intact peptide showing titer comparable with those of the other antigenic antisera.

As revealed by evaluating the cross-reactivity with thymosin $\beta_4$, all the antisera raised proved to be specific for thymosin $\beta_{10}$. Nevertheless, optimal specificities (Fig. 3) were obtained for the antisera raised against the fragments thymosin $\beta_{10}(31–43)$ or thymosin $\beta_{10}(38–43)$. This could be expected, because the above peptides contain the most nonhomologous region on the thymosin $\beta_{10}$ molecule comparing the primary structure of $\beta$-thymosins. Thus, the C-terminus seems to be the predominant immunogenic epitope of the thymosin $\beta_{10}$ molecule, with strong hydrophilicity properties.

The antiserum raised against intact thymosin $\beta_{10}$ showed low cross-reactivity with thymosin $\beta_4$, although antisera raised against the intact molecule of thymosin $\beta_4$ (19) or thymosin $\beta_6$ (20) have been reported to show high cross-reactivity values with thymosin $\beta_4$ or thymosin $\beta_4$, respectively. The specificity of the antiserum raised against intact thymosin $\beta_{10}$ might be related to the high flexibility and hydrophilicity predicted for the C-terminal area of the peptide, which may render this highly nonhomologous region to be the dominant antigenic determinant of thymosin $\beta_{10}$.

The antibody developed against the peptide fragment thymosin $\beta_{10}(1–16)$ showed low cross-reactivity with thymosin $\beta_4$, similar to that developed against the intact molecule of thymosin $\beta_{10}$. According to the secondary structure prediction, this fragment seems to include a $\beta$-turn region followed by an $\alpha$-helix. A two-dimensional, so-called helical wheel representation (25) of the thymosin $\beta_{10}$ putative $\alpha$-helix, lying between residues 5 and 16 (Fig. 4), reveals that all the not-in-common with thymosin $\beta_4$ residues lie on the one side of the wheel. Thus, although the primary structure differences in the N-terminal region of thymosin $\beta_{10}$ and thymosin $\beta_4$ are not so pronounced, the hydrophilic side of the $\alpha$-helix combined to the $\beta$-turn may form a nonlinear epitope, which is probably

FIG. 4. Helical wheel representation of the putative $\alpha$-helix lying between residues 5 and 16 of thymosin $\beta_{10}$. The wheel is the projection of all side chain positions along the helix axis onto a plane. An incremental angle of 100° of arc on the wheel is used, corresponding to 3.6 residues per turn. Not-in-common with thymosin $\beta_4$ amino acid residues are underlined.
composed of different amino acid residues in these two \( \beta \)-thymosins. The presence of such a nonlinear epitope is postulated to be the reason of the minimal cross-reactivity with thymosin \( \beta_2 \), shown by the antibody developed against thymosin \( \beta_1 \) (1–16).

None of the antisera developed cross-reacted with thymosin \( \alpha \), a thymosin peptide present in human tissues, as could be expected from the primary structures of the corresponding antigen peptides.

In conclusion, alternative possibilities are recommended for developing antisera for thymosin \( \beta_1 \), that is, either using specific antigenic peptide fragments, such as thymosin \( \beta_1 \) (1–16), thymosin \( \beta_1 \) (31–43), and thymosin \( \beta_1 \) (38–43), or the intact molecule. Due to its easy preparation and the highly specific affinity of the corresponding antibody for intact thymosin \( \beta_1 \), the fragment thymosin \( \beta_1 \) (38–43) may be considered the antigen of choice for developing antibodies for thymosin \( \beta_1 \), which can be used for immunochemical studies.

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