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Large-scale chromatofocusing-based method for isolating thymosin β_4 and thymosin β_9 from bovine tissues

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Abstract

A large-scale method for the isolation of thymosin β_4 (up to 120 mg) and thymosin β_9 (up to 40 mg) from bovine lung (up to 2 kg) was developed. The isolation protocol included tissue homogenization in 0.4 *M* $HClO_4$, centrifugation, solid-phase extraction through LiChroprep RP-18 material, chromatofocusing on polybuffer exchanger PBE 94-modified Sepharose and dialysis against water. The isolated products were characterized by analytical isoelectric focusing, reversed-phase HPLC, electrospray ionization mass spectrometry and amino acid analysis. The method developed is rapid and convenient, requires no expensive equipment and can be used for the isolation of thymosin β_4 and homologous peptides from various animal tissues.

1. Introduction

Thymosin β_4 ($T\beta_4$)[1], a 43-amino acid residues peptide, was originally isolated in 1981 from a crude thymic extract termed thymosin fraction 5 [1]; since then, $T\beta_4$ has been found, in relatively large quantities, in several tissues of various animal species [2,3]. This peptide was reported to induce terminal deoxynucleotidyl transferase activity in bone marrow cells from normal and athymic mice in vivo and in vitro, to inhibit the migration of guinea pig peritoneal macrophages, to stimulate the hypothalamic secretion of luteinizing hormone releasing factor, and to induce phenotypic changes in the Molt-4 leukemic cell line [4]. Apart from the above mentioned functions, it has been recently revealed that $T\beta_4$ is an actin-sequestering peptide, which inhibits the polymerization of G-actin [5]. In addition, $T\beta_4$ might be involved in other biological processes, e.g. it may participate as

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[1] Abbreviations used: $T\beta_4$, thymosin β_4; $T\beta_9$, thymosin β_9; $T\beta_{10}$, thymosin β_{10}; IEF, isoelectric focusing; HPLC, high-performance liquid chromatography; ESI/MS, electrospray ionization mass spectrometry; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; IgG/HRP, goat anti-rabbit immunoglobulin/horseradish peroxidase conjugate; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt.

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the precursor molecule in the biosynthesis of the tetrapeptide AcSer-Asp-Lys-Pro, a negative regulator of the haematopoietic stem cell proliferation [6]. Preliminary studies indicate that $T\beta_4$ may have important prognostic and/or therapeutic properties as well [7,8].

The still not completely understood functions of $T\beta_4$ emphasize the need for the development of a rapid, convenient, and inexpensive method for its isolation from natural sources, since increased availability will help the elucidation of its functions and the assessment of any prognostic and/or therapeutic value it may have. Bovine tissues are often used as a source for $T\beta_4$, since this peptide is identical in bovine and human. On the other hand, in most mammals $T\beta_4$ is accompanied with a highly homologous peptide (Fig. 1), such as the 41-amino acid residues thymosin β_9 ($T\beta_9$), present in bovine tissues [9], or the 43-amino acid residues thymosin β_{10} ($T\beta_{10}$), present in human tissues [10]. It is therefore difficult to isolate $T\beta_4$ in a form free of contaminating homologues. On the other hand, although synthetic $T\beta_4$ and $T\beta_4$-analogues [11,12] can be used as alternative reagents in biological experiments, it is usually necessary to evaluate their effectiveness using the native isolated peptide as control material.

A few methods for the isolation of $T\beta_4$ and $T\beta_9$ from bovine tissues have been described in the literature [13–18]. Most of them require expensive laboratory equipment (e.g. a preparative isoelectric focusing (IEF) apparatus plus a semi-preparative HPLC system equipped with a fluorescence detector [16]), or they involve tedious, multi-step, and time-consuming procedures (e.g. many column-purification steps [18]). Moreover, these methods are usually only suitable for processing rather small amounts of tissue. In this work, we present a simple and inexpensive, large-scale method for the isolation of $T\beta_4$ and $T\beta_9$ from bovine tissues, which is based on chromatofocusing. This method could be applied to the isolation of β-thymosins from other animal tissues, as well.

Fig. 1. Amino acid sequences of $T\beta_4$ and $T\beta_9$.

2. Experimental

2.1. Reagents

The polybuffer exchanger PBE 94-modified Sepharose and the elution buffer PB 74 were products of Pharmacia (Uppsala, Sweden). The LiChroprep RP-18 material (40–63 μm particle size), the solvents and the other chemicals used were purchased from Merck (Darmstadt, Germany), except when otherwise indicated. Standard $T\beta_4$ and $T\beta_9$ were isolated from bovine lungs by a previously published method [19]. The anti-$T\beta_4$ antiserum was prepared in rabbits against standard $T\beta_4$ as previously described [19].

2.2. $T\beta_4$ and $T\beta_9$ isolation protocol

A 500-g amount of bovine lungs was homogenized (Ultra Torrax homogenator) in 2.5 l of ice-cold 0.4 *M* $HClO_4$. After centrifugation (13 700 *g*, 4°C, 20 min), the pH of the supernatant was adjusted to 2.5–3.5 with a 10 *M* KOH solution, and the resultant salt was removed by centrifugation (13 700 *g*, 4°C, 10 min). The supernatant was extracted with LiChroprep RP-18 material and the adsorbed material was eluted with 300 ml of 40% *n*-propanol. The eluate was concentrated approximately 10-fold in a rotary evaporator (40°C), and the residue was lyophilized and stored at 4°C until chromatofocusing.

Chromatofocusing was performed on a plexiglass column (100 × 9 mm I.D.) filled with polybuffer exchanger PBE 94-modified Sepharose, which was pre-equilibrated with a 25 m*M* imidazole-HCl buffer (pH 7.4). The lyophilized tissue extract was dissolved in 3 ml of the elution buffer PB 74 (pH 4.0), transferred to the column, and eluted at a flow-rate of 40 ml/h. Fractions of 0.8

ml were collected and their pH was measured. The fractions 22–25, with pH 6.4–6.0 (corresponding to $T\beta_9$) and 62–68, with pH 4.9–4.7 (corresponding to $T\beta_4$) were pooled, dialyzed against water (24 h, cut-off: 1000 Da), and lyophilized. The chromatofocusing column was washed with a 1 *M* NaCl solution and then stored in 24% aqueous ethanol at 4°C, until next use.

2.3. $T\beta_4$ and $T\beta_9$ identification techniques

$T\beta_4$ and $T\beta_9$ were identified as follows, using the standard peptides as control material.

Analytical IEF

This was performed on a gel column electrophoresis system (EC Apparatus Corporation, 12 columns with 105 mm height and 4 mm I.D.), using a 5.20% T, 3.85% C gel, which contained 2.5% ampholytes of pH 3.0–10.0 (Pharmacia) and 2.5% ampholytes of pH 4.0–6.5 (Pharmacia). The conditions applied were a 9-W constant power, a 20-mA maximum current, and a 3-h run time. After isoelectric focusing, the gel rods were fixed overnight in a 4% sulfosalicylic acid–11% trichloroacetic acid solution, and then the peptide bands were stained with Coomassie Brilliant Blue R-250 (USB). The staining solution contained 1.15 g of the dye in 1 l of ethanol–acetic acid–water (250:80:670, v/v). The solvent was used as the destaining solution in the peptide visualization process.

Analytical reversed-phase HPLC

HPLC was performed with a Waters HPLC system, Model 600, equipped with a Waters UV detector (214 nm), Model 484. The chromatographic column used was LiChrospher RP-18 (250 × 4.6 mm I.D., 5-μm particle size) (Merck). Solvent A was 0.05% trifluoroacetic acid (amino acid sequencing grade) in water and solvent B was 60% acetonitrile (HPLC grade) in solvent A. A linear gradient was applied from 10 to 100% B in 30 min, at a flow-rate of 1.4 ml/min.

Electrospray ionization mass spectrometry (ESI-MS)

Control protein (equine myoglobin) or test peptide ($T\beta_4$ or $T\beta_9$) solutions of 0.5 mg/ml in 50% aqueous methanol (containing 1% acetic acid) were infused at a flow-rate of 3 μl/min, using a Harvard syringe pump, into an electrospray interface mass spectrometer (MS Engine HP-5989A). In the electrospray source the spray needle was grounded: voltages of −4.5, −3.5, −3.0 kV were applied to the capillary, end plate and cylindrical electrodes, respectively. The capillary/skimmer potential difference varied between 150 and 300 V; the other source lenses were held at potentials that optimized the signal intensity. Hot nitrogen gas was used for desolvation.

Amino acid analysis

Hydrolysis of the peptides was performed in the gas phase with 6 *M* HCl at 150°C for 1 h. Free amino acids were derivatized with phenylisothiocyanate and identified as phenylthiocarbamyl derivatives by reversed-phase HPLC [20].

2.4. $T\beta_4$ immunoreactivity studies

Enzyme-linked immunosorbent assay (ELISA) microwells (Nunc, Module 8) were coated with 200 μl of an aqueous solution (0.1 μg/ml) of either standard $T\beta_4$ or $T\beta_4$ isolated with the method described. The microwells were dried overnight at 50°C. Then, 100 μl of buffer A [15 m*M* potassium phosphate buffer (pH 7.4) containing 0.2% bovine serum albumine (BSA), 15 m*M* NaCl, and 0.05% Tween 20] and 100 μl of an antiserum against standard $T\beta_4$, diluted 1:400–1:6400 with buffer A, were added to each microwell. After incubating (3 h, 37°C), the liquid phase was discarded and the wells were washed three times with buffer B [15 m*M* potassium phosphate buffer (pH 7.4), containing 15 m*M* NaCl, and 0.05% Tween 20]. Next, 200 μl of an goat anti-rabbit immunoglobulin/horseradish peroxidase conjugate (IgG/HRP) (Sigma) solution, diluted 1:1000 with buffer A, were added to each microwell. After incubation (2 h, 37°C), the liquid phase was removed and the

wells were washed three times with buffer B. Finally, 200 μl of an ABTS [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] (Sigma, 1 mg/ml)–H_2O_2 (Ferak, 0.003%) solution in buffer C [0.1 *M* citrate/phosphate buffer (pH 4.5)] were added to each microwell. The absorbance was measured after 30 min of development in a microtitre plate reader (Model MR 5000, Dynateck) at 405 nm.

3. Results

The purification efficiency at different stages of the isolation procedure was evaluated using the IEF and the HPLC analytical techniques described. Thus, unseparated Tβ_4 and Tβ_9 together with several other peptides were present in the tissue extract immediately before chromatofocusing (Fig. 2b). As established by the use of internal standards, Tβ_9 was isolated in the chromatofocusing fractions 22–25, with pH 6.4–6.0, while Tβ_4 was isolated in the fractions 62–68, with pH 4.9–4.7 (Fig. 3). After dialysis, highly pure Tβ_9 (Fig. 2a) and Tβ_4 (Fig. 2c) were obtained. The overall yield from 500 g of tissue was 30 mg of Tβ_4 and 10 mg of Tβ_9.

The isolated peptides were analyzed by ESI-MS. This relatively recent and gentle ionization method [21,22], in which peptide/protein solutions are sprayed in the presence of a high electric field, leads to the formation of analyte ions, often bearing multiple charges. Fig. 4A shows the electrospray mass spectra of the isolated Tβ_9 (a) and of Tβ_4, standard (b) or isolated (c). The major ions were the +7 and +6 charged states at m/z 674.7 and 787.0 for Tβ_9, or 710.2 and 828.2 for Tβ_4; other related minor ions were also present. The charge on each ion and the molecular mass of the peptides were determined by deconvolution algorithms (Fig. 4B). The Tβ_9 molecular mass obtained ($M_{r,exp}$ = 4715.5) was very close to the average molecular mass calculated ($M_{r,cal}$ = 4716.2) on the basis of the peptide primary structure. Monosodium and potassium salts adduct ions were also present in small intensities. The Tβ_4 mass spectrum was

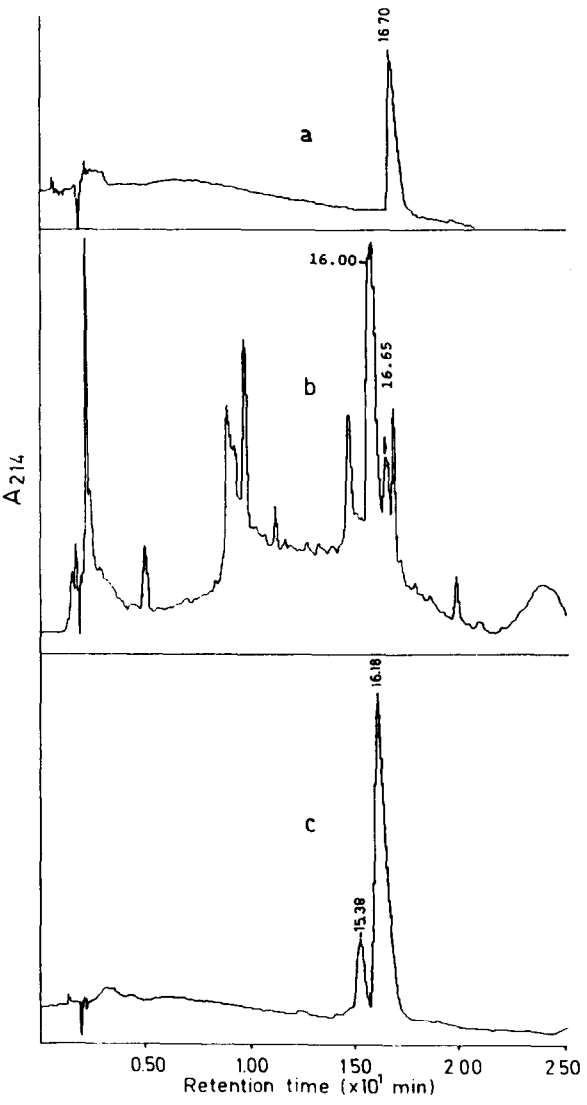

Fig. 2. Analytical HPLC of: (a) pooled chromatofocusing fractions 22–25, after dialysis (Tβ_9), (b) tissue extract before chromatofocusing, (c) pooled chromatofocusing fractions 62–68, after dialysis (Tβ_4; the minor peak preceding Tβ_4 is the oxidized form of the peptide, as established by the use of standard Tβ_4 and Tβ_4 treated with H_2O_2).

similar to that of standard Tβ_4. The molecular mass obtained for the isolated Tβ_4 ($M_{r,exp}$ = 4963.6) was in agreement with the average molecular mass calculated ($M_{r,cal}$ = 4963.5) on the basis of the proposed relevant primary struc-

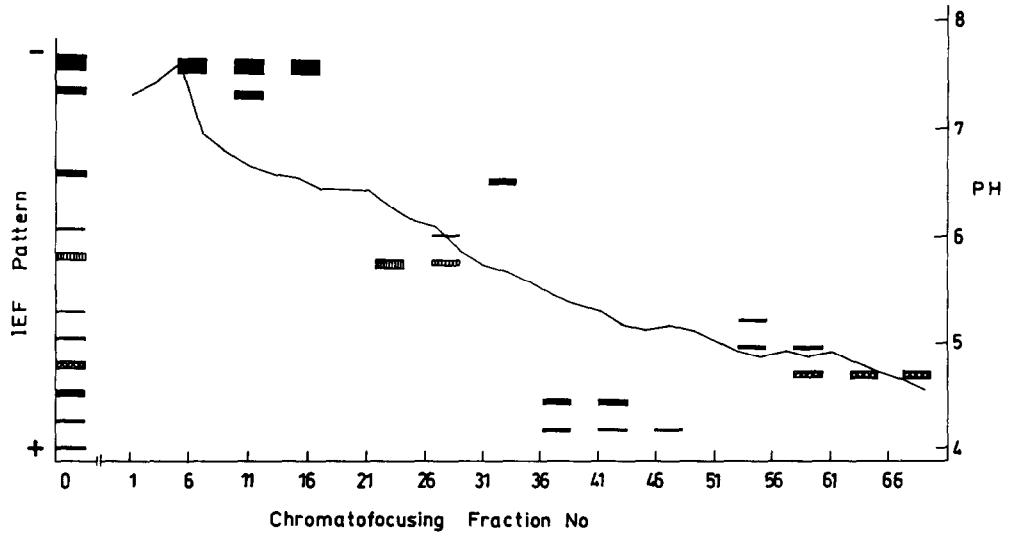

Fig. 3. Analytical IEF patterns of the chromatofocusing fractions. Fraction No. 0 refers to tissue extract before chromatofocusing. The bars correspond to $T\beta_9$ (striped), $T\beta_4$ (crossed), or unidentified peptides (filled).

ture. In addition to the monosodium and potassium salts adduct ions present in the spectra of standard and isolated $T\beta_4$ samples, ions of medium intensities followed the base ions in the spectrum of the isolated $T\beta_4$. Deconvolution of these related ions assigned them to a second peptide with the average molecular mass $M_{r,exp} = 4979.6$, i.e. 16 units higher than the M_r of $T\beta_4$. This peptide corresponds to the oxidized form of $T\beta_4$ (methionine oxidation), as established by the use of standard $T\beta_4$ treated with H_2O_2, and the results are in agreement with the HPLC profile obtained for this sample (Fig. 2c). The molecular mass of the control protein equine myoglobin was measured at 16 951.5 and the charge state distribution spanned from ions m/z 738 (+ 23) to m/z 1305 (+ 13).

The amino acid analyses of $T\beta_4$ and $T\beta_9$ (data not shown) indicated that the peptides isolated had amino acid compositions identical to those reported in the literature [1,16].

Standard $T\beta_4$ and $T\beta_4$ isolated using the proposed protocol were immobilized onto ELISA microwells and left to react with anti-$T\beta_4$ antiserum in different dilutions. The titre curves obtained were almost superimposed, indicating that the immunoreactivity characteristics of the peptides tested were very similar (Fig. 5).

4. Discussion

A simple large-scale method for the isolation of highly pure $T\beta_4$ and $T\beta_9$ from bovine tissues was developed. A 500-g amount of bovine lungs was homogenized in 0.4 *M* $HClO_4$, in which $T\beta_4$ and $T\beta_9$ were solubilized; the above peptides were further purified by solid-phase extraction through LiChroprep RP-18 material and completely separated from each other by a chromatofocusing technique. Chromatofocusing was selected because it offers the high resolution obtained for separations based on differences in isoelectric points, together with the high capacity of ion-exchange techniques, being in addition simple and inexpensive. The dimensions of the chromatofocusing column depended on the amount of tissue to be handled. Thus, for relatively small amounts of tissue (up to 500 g) a column with 100 mm length and 9 mm I.D. was used, as described in the isolation protocol. However, larger amounts of tissue (up to 2 kg)

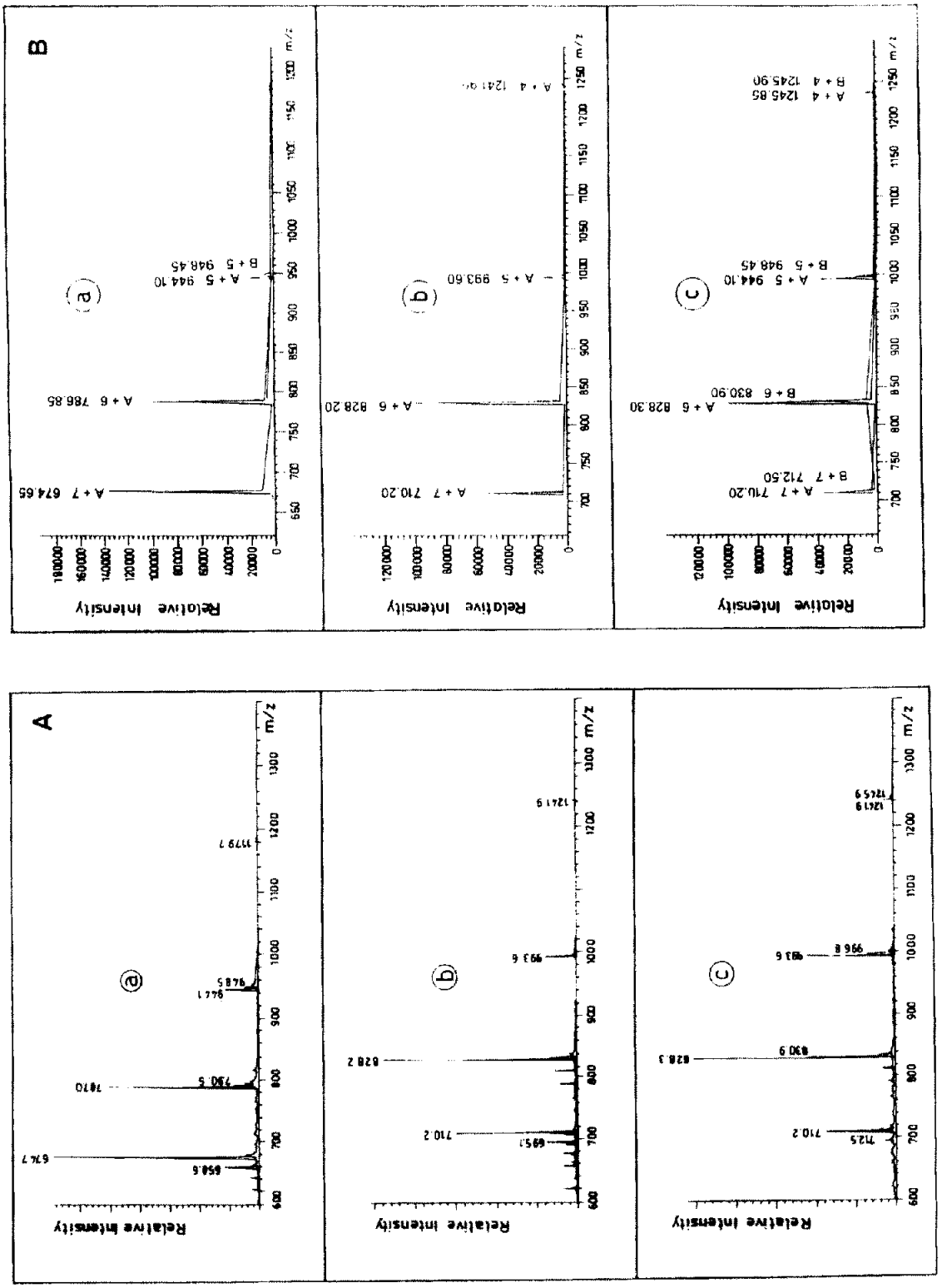

Fig. 4. Mass spectra (A) and computer deconvolution spectra (B) of: (a) isolated $T\beta_9$, (b) standard $T\beta_4$, (c) isolated $T\beta_4$. The ions used for the molecular mass determinations and their charge distribution are shown in each computer deconvolution spectrum.

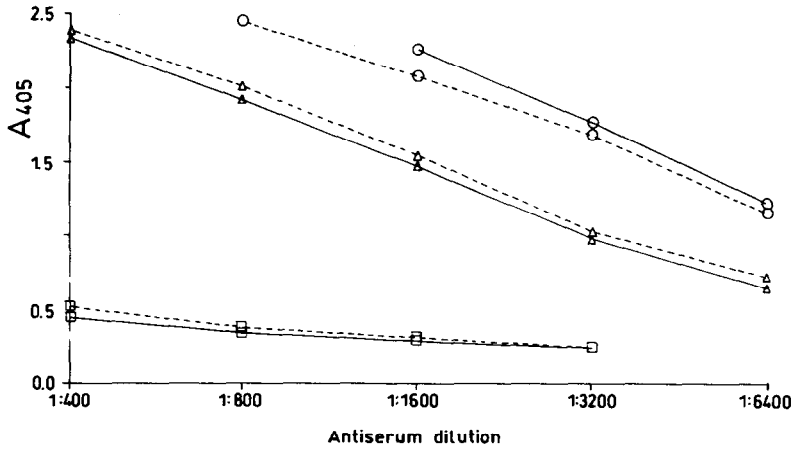

Fig. 5. Anti-Tβ_4 antiserum titre curves obtained with standard (- - -) or isolated (——) Tβ_4. Antisera from different bleedings were used (□, first bleeding; △, second bleeding; ○, third bleeding). In all cases, the titre curves were very similar.

could be treated in a single run by using a column with 50 mm length and 26 mm I.D. Any impurities present in the pooled fractions of interest could be removed by a second chromatofocusing step through a column with 100 mm length and 9 mm I.D. The ampholytes present in the chromatofocusing fractions were removed by dialysis against water. Alternative techniques, e.g. elution of the fractions through LiChroprep RP-18 material, led to only partial removal of the ampholytes.

Tβ_4 was mainly isolated as the non-oxidized form. The Tβ_4-sulfoxide was also obtained (Fig. 2c), probably due to the exposure to alkaline conditions during the first isolation steps [23]. Nevertheless, the Tβ_4-sulfoxide represented only a small fraction of the final product, smaller than what corresponds to the relevant peak area shown in Fig. 2c, since the sulfoxide absorbs more strongly than the native peptide at 214 nm. On the other hand, according to our preliminary studies the presence of the sulfoxide form in the final product does not seem to affect the Tβ_4 immunoreactivity characteristics.

5. Conclusions

Apart from leading to highly pure Tβ_4 and Tβ_9 the method proposed offers the following advantages compared with previously described ones: It is inexpensive and easily applicable to any biochemical laboratory, since it requires no special or expensive laboratory equipment, it is fast and simple, needing only a few working days for its accomplishment, and it can be used for handling as much as 2 kg of bovine lungs in a single run. The above method may be applied to the isolation of similar peptides from a great variety of natural sources.

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