Complete Amino Acid Sequences of Bovine Thymopoietins I, II, and III: Closely Homologous Polypeptides†

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ABSTRACT: Complete amino acid sequences were determined for thymopoietins I and II (revision), isolated from bovine thymus, and for thymopoietin III, a newly identified polypeptide isolated from bovine spleen. Thymopoietin III (TP-III) is a 49 amino acid monomeric peptide that shows minor microheterogeneity at residue 34. The three thymopoietins have largely identical sequences yet some distinct differences, suggesting very recent evolution from a common gene. The complete amino acid sequences are

TP-I NH_2-Gly-Gln
TP-II NH_2-Pro-Glu-Phe-Leu-Glu-Asp-Pro-Ser-Val-Leu^{10}-Thr-Lys-
TP-III NH_2-Pro-Glu
Glu-Lys-Leu-Lys-Ser-Glu-Leu-Val^{20}-Ala-Asn-Asn-
Val-Thr-Leu-Pro-Ala-Gly-Glu^{30}-Gln-Arg-Lys-
Asp^{34} His^{43}
Asp-Val-Tyr-Val-Glu-Leu-Tyr^{40}-Leu-Gln-Ser-
Glu His
Leu-Thr-Ala-Leu-Lys-Arg^{49}-COOH

Thymopoietins I and II are two closely related polypeptides isolated from bovine thymus. The bioassay used to isolate these molecules was the detection of delayed neuromuscular impairment in mice following the injection of thymic extracts or purified polypeptides (Goldstein, 1974). This assay was derived from basic experimental studies related to myasthenia gravis, a human disease in which a thymic hormone affecting neuromuscular transmission had been detected (Goldstein & Whittingham, 1966; Goldstein, 1966, 1968; Goldstein & Hofmann, 1968, 1969; Kalden et al., 1969). Our studies demonstrated that extracts of thymus produced these effects but not extracts of control tissues (Goldstein, 1968). Two biochemically homogeneous thymic polypeptides, thymopoietins I and II, were isolated, and both were active in this system (Goldstein, 1974). These purified polypeptides subsequently proved to be active in induction of early T cell differentiation, inhibition of B cell differentiation, and modulation of mature lymphocytes (Basch & Goldstein, 1974, 1975; Scheid et al., 1975, 1978; Komuro et al., 1975; Sunshine et al., 1978). The complete amino acid sequence of thymopoietin II (TP-II) was determined (Schlesinger & Goldstein, 1975), and the biological activity was shown to reside in segment 29–41 by chemical synthesis (Schlesinger et al., 1975). Subsequently the pentapeptide TP-5, corresponding to residues 32–36 of thymopoietin II, was shown to retain the biological activity of thymopoietin II and thus probably corresponds to a biologically active site of the parent molecule (Goldstein et al., 1979). TP-5 has immunoregulatory effects in a number of model systems in animals (Lau & Goldstein, 1980; Goldstein & Lau, 1979; Lau et al., 1980; Gershwin et al., 1979; Verhaegen et al., 1980; Weksler et al., 1978) and is presently being evaluated as an immunoregulatory therapeutic agent in man (Horwitz et al., 1980).

A radioimmunoassay was developed for thymopoietin II which proved to be wholly cross-reactive with thymopoietin I (TP-I) (Goldstein, 1976). Thymopoietin was readily detected in the thymus and was absent from control tissues such as liver, kidney, thyroid, and muscle. However, the radioimmunoassay detected thymopoietin-like material in spleen and lymph node extracts. For determination of the nature of this material, which we subsequently termed thymopoietin III (TP-III), a radioimmunoassay was utilized to isolate TP-III from extracts of bovine spleen; additionally, the radioimmunoassay was utilized to devise an improved isolation method for TP-I and TP-II from thymus.

This paper reports these improved methods for isolating TP-III from spleen and TP-I and TP-II from thymus and presents the complete amino acid sequences of TP-I, TP-II (revised), and TP-III.

Materials and Methods

Materials. Fresh bovine spleen was obtained on ice from Max Cohen Inc., Livingston, NJ. After the capsules and excess fats were removed, the tissue was stored at −30 °C until further processing. Sephadex G-75, Sephadex G-25, and QAE-Sephadex were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Cation-exchange resin AG-MP-50 (50–100 mesh, H^+ form) and anion-exchange resin AG-MP-1 (50–100 mesh, Cl^- form) were from Bio-Rad Laboratories, Richmond, CA. Isoelectric focusing on agarose was performed by using an LKB Multiphor 2117 apparatus attached with an LKB 2103 power supply. Ultraviolet absorption of the effluent from the gel filtration column was monitored by an LKB Unicord-I attached to a 6-channel DC Recorder 6520. Absorbance of protein at 280 nm was measured with a Beckman Model 35 spectrophotometer.

Methods. Extraction of Soluble Proteins from Spleen Tissue. About 2 kg of spleen were extracted in ice cold 0.1 M ammonium bicarbonate (25% wet w/v) by homogenization using a Waring blender. The insoluble materials were removed by centrifugation at 14000*g* for 40 min in a Sorvall RE5B refrigerated centrifuge. The supernatant was filtered through gauze followed by the addition of thimerosal (0.1%) and sodium azide (0.1%) as bacteriostatic agents.

Isolation and Separation of Thymopoietins by Ultrafiltration and Column Chromatography. The soluble extract was processed through an Amicon Hollow Fiber Cartridge H10 × 100 (molecular "cut off" ~100 000 daltons), and the filtrate was concentrated by using a 150-mm Diaflo UM2 membrane (molecular cut off ~1000 daltons) in a TC5E thin-channel ultrafiltration system. Approximately 40–42 L were concentrated to 3–4 L. The retentate was passed through a 450-cm-long stainless steel coil (0.5-cm diameter) immersed in a 70 °C water bath. The sample was introduced with a Masterflex standard-load pump head (size E, Cole-Parmer

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Instrument Co., Chicago, IL) at a flow rate of 60 mL/min. The effluent was collected on an ice bath. Insoluble materials were removed by centrifugation at 14000*g* for 30 min at 4 °C. The supernatant solution was adjusted to pH 5.0, and with constant stirring, the cation-exchange resin (Ag-MP-50) was added slowly at 4 °C at a concentration of 50 mL/min. After being stirred for 30 min, the suspension was filtered through Whatman No. 1 filter paper. The pH was adjusted to 9.0, and the anion-exchange resin (AG-MP-1) was added as described above. After subsequent filtration, the pH was adjusted to 8.0. The filtrate was chromatographed on bead-type Sephadex G-75 (particle size, 40–120 μm) and Sephadex G-50 (particle size 20–80 μm) at room temperature in all glass columns (5 × 150 cm for G-75 and 1.5 × 150 cm for G-50) in 50 mM ammonium bicarbonate. The flow rate was approximately 70 mL/h with the Sephadex G-75 column and 40 mL/h with the Sephadex G-50 column. The appropriate fractions were identified by radioimmunoassay (see below) and lyophilized. Anion-exchange chromatography of QAE-Sephadex was performed on a 15 × 30 cm column equilibrated with ammonium bicarbonate (0.1 M, pH 10.5) and eluted at a flow rate of 20 mL/h with a linear strength gradient of 0.1–0.8 M ammonium bicarbonate generated with two 150-mL containers. The appropriate fractions were lyophilized and desalted on a Sephadex G-50 column as described above.

Assay System. Each fraction isolated from gel filtration chromatography and ion-exchange chromatography was analyzed by radioimmunoassay for thymopoietin (Goldstein, 1976) and ubiquitin (Goldstein et al., 1975).

Analytical Polyacrylamide Gel Electrophoresis of Thymopoietin Polypeptides. Purification was monitored by polyacrylamide gel electrophoresis. Protein solution (50 μg/25 μL) was applied on 7% gel (5.5 × 150 mm) with a running pH of 8.9 or 4.3 at a current of 2.5 mA/gel. Methyl green was used as a dye marker in the acid gels which ran toward the anode. Samples were loaded in electrode buffer made up with the dye marker in 25% sucrose. Protein was detected by staining with 0.05% (W/V) Coomassie brilliant blue in 12.5% Cl_3CCOOH. After the protein was destained, the relative mobilities (R_f) were calculated with respect to the dye markers.

Isoelectric Focusing of Purified Thymopoietin Polypeptide. Isoelectric focusing on the gel-stabilized layer was performed with the LKB 2117 Multiphor apparatus according to the method of Radola (1969) by using a pH gradient between 3.5 and 9.5.

Amino Acid Composition of Purified Thymopoietin. The amino acid composition was determined from quadruple samples of polypeptide hydrolyzed with 6 N HCl at 100 °C in vacuo for 24, 48, 71 and 95 h, and value obtained were extrapolated to zero hydrolysis time. Amino acid analyses were performed on a Durrum amino acid analyzer by the method of Spackman et al. (1958).

COOH-Terminal Analysis with Carboxypeptidases A and B. COOH-Terminal analysis of bovine thymopoietins II and III was performed by digestion with diisopropyl fluorophosphate (DFP) treated carboxypeptidases A and B. Thymopoietin polypeptides (30 nmol) in 100 μL of *N*-ethylmorpholinium acetate (NEM) buffer (20 mM, pH 8.5) were treated with 0.02 nmol of carboxypeptidase B diluted in NEM buffer. The incubation was carried out in duplicate at 37 °C for 15-s, 30-s, 1-min, 2-min, 5-min, 15-min, and 30-min periods with buffer, enzymes, and thymopoietin as control. In another set of experiments, the polypeptides and carboxypeptidase B in the same ratio were incubated at 37 °C for 30 min followed by the addition of 0.05 nmol of carboxypeptidase A and further digestion for additional periods of 15 s, 1 min, 5 min, 30 min, 1 h, 2 h, and 4 h. The digestions were terminated by adding 100 μL of 6 N acetic acid, and the product was immediately lyophilized. In some experiments, norleucine (1 nmol) was included in each sample as an internal amino acid standard. The lyophilized product was dissolved in 0.2 M sodium citrate buffer, pH 2.2, and was subjected to amino acid analysis. The experiment was repeated twice with each different batch of polypeptide.

COOH-Terminal Analysis of Maleated Thymopoietin III. Maleic anhydride in 1,4-dioxane was added at a 20-fold excess over the free amino groups of the polypeptide (500 μg) which was dissolved in 0.1 M sodium borate (pH 9.3). Maleic anhydride was added stepwise over a 4-h period (pH 9.3), being maintained with 6 N NaOH. Maleated protein was then desalted on Bio-Gel P-2 in 0.1 M NH_4HCO_3 (pH 8.2) and lyophilized. Lyophilysate was dissolved in 100 μL of *N*-ethylmorpholinium acetate buffer (NEM) (20 mM, pH 8.5) and treated with 0.05 nmol of DFP-treated carboxypeptidase B diluted in NEM buffer. Incubation was carried out in duplicate at 37 °C for 15 min with enzyme and thymopoietin as control. The digestion was terminated with acetic acid, and the products were lyophilized. The lyophilysate was dissolved in 0.2 M sodium citrate buffer, pH 2.2, analyzed on a Model 119CL amino acid analyzer.

NH_2-Terminal Sequence Analysis. Analyses were performed on a Beckman Model 890 sequencer by the method of Edman & Begg (1967) by using a modified dimethylallylamine–peptide program. Coupling with phenyl isothiocyanate was performed once before initiating automated sequencing. Following sequencing, the resulting thiazolinones were converted to their phenylthiohydantoin (PTH) amino acids with 0.2 mL of 1.0 N HCl at 80 °C for 10 min. Identification of PTH-amino acid derivatives was made by gas chromatography (Pisano & Bronzert, 1969), thin-layer chromatography (Edman, 1970), and reverse-phase high-pressure liquid chromatography (Zimmerman et al., 1977; Simmons & Schlesinger, 1980). Aqueous phases were lyophilized twice from water. The complete sequence was repeated 3 times from three batches of purified protein.

Results

Ten kilograms of frozen bovine spleen, upon extraction with 0.1 M ammonium bicarbonate and subsequent ultrafiltration through H10 × 100 and UM 2, gave a total of 162 mg of polypeptides which exhibited several bands on polyacrylamide gel electrophoresis. This step was essential for desalting as well as for concentrating the whole preparation. When this preparation was heated for a short period of time, 2-fold purification was obtained (Table I). Although this represents a minor purification, this step was necessary to eliminate some contaminants which were difficult to remove by other conventional techniques. Following extraction with macroporous cation- and anion-exchange resin at 4 °C, the isolated fraction contained 87.3 mg of thymopoietin with over a 10-fold purification. To obtain this amounts of polypeptide, we observed that it was important both to maintain the pH of the solution at or above 4.5 at 4 °C and to control the addition of resin. A sudden change in pH has resulted in a drastic loss of the immunoactive polypeptides. Increasing amounts of ion-exchange resin did not give rise to a significantly higher yield. It also appears that the resin concentration may be dependent on the nature of the extract being treated since we found that isolation of thymopoietin from bovine thymus required 30% less resin concentration than spleen to attain a similar state of purity and yield.

Two immunologically active peaks were obtained following chromatography on Sephadex G-75 in 50 mM ammonium

Table I: Summary of Purification of Bovine Thymopoietin Polypeptides

purification step	total thymopoietin [a] (mg)	total protein [b]	thymopoietin (%)	purification (fold)	recovery (%)
ultrafiltered protein	162.40	262.50 g	0.06	1	100.0
heat-treated protein	138.60	132.10 g	0.11	2	85.3
cation-exchange treated protein	98.20	26.55 g	0.37	6	60.5
anion-exchange treated protein	87.30	13.88 g	0.63	11	53.8
gel filtration, G-75	36.30	105.05 mg	34.60	577	22.4
gel filtration, G-50	28.70	63.60 mg	45.13	752	17.7
anion-exchange, QAE-Sephadex	23.19	32.10 mg	72.24	1204	14.3
gel filtration, G-50	23.05	24.20 mg	95.20	1587	14.2

[a] Thymopoietin estimated by radioimmunoassay. [b] Total protein estimated by Folin reagent.

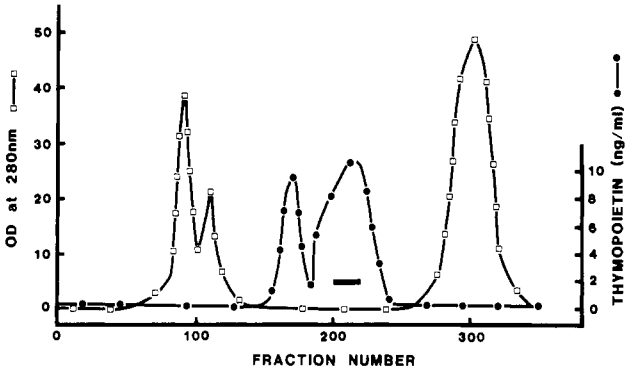


FIGURE 1: Gel filtration of bovine thymopoietin polypeptides. Proteins (1 g in 20 mL) isolated after anion-exchange treatment were applied to a Sephadex G-75 column and were eluted with 50 mM ammonium bicarbonate. Absorption was measured at 280 nm (□) and thymopoietin levels were measured by RIA (●). Thymopoietin-like material, indicated by the shaded area, were used for further experiments.

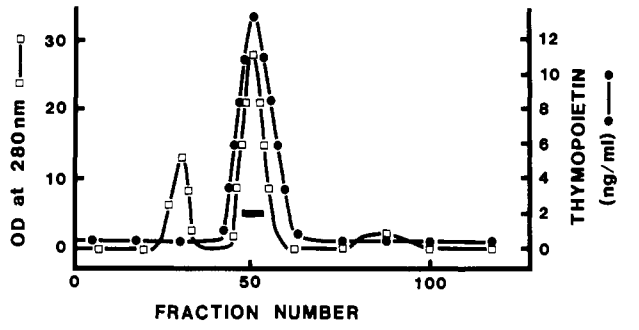


FIGURE 2: Gel permeation chromatography of bovine thymopoietin polypeptides. The thymopoietin fraction (from the Sephadex G-75 column) was applied to a Sephadex G-50 column (80 mg in 1.5 mL) equilibrated and eluted with 50 mM ammonium bicarbonate. Units are identical with those in Figure 1.

bicarbonate (Figure 1). The first peak contained 9.0 mg of protein, but radioimmunoassay of this fraction did not produce a parallel line wth bovine thymopoietin II. A further investigation of this fraction is in progress. The second peak (fractions 190–225) contained polypeptides in the molecular mass range 5000–8000 daltons. Approximately 35% of thymopoietin-like polypeptide was included in these fractions, and a nearly 600-fold purification was achieved at this step.

This thymopoietin fraction was further chromatographed on Sephadex G-50 in 50 mM ammonium bicarbonate (Figure 2). The active peak (fractions 44–56) had 28 mg of thymopoietin, giving an overall 750-fold purification. The isoelectric focusing pattern of this thymopoietin fraction showed three major acidic bands, one neutral band, and a few minor basic components. Two immunoactive peaks were obtained by further purification of a 32-mg sample in ammonium bicarbonate (100 mM, pH 10.5) by anion-exchange chromatography on QAE-Sephadex followed by elution with an ionic strength gradient up to 800 mM ammonium bicarbonate. The

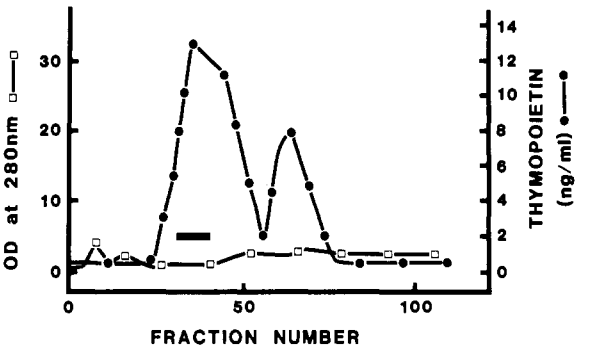


FIGURE 3: Dissociation of bovine thymopoietin III from nonrelated proteins by ion-exchange chromatography. The QAE-Sephadex column was charged with thymopoietin polypeptide mixture (32 mg) dissolved in 100 mM ammonium bicarbonate (pH 10.5). The protein was eluted with a linear ionic strength gradient up to 800 mM. Units are identical with those in Figure 1.

first major peak eluted at 460 mM ammonium bicarbonate (midpoint of elution volume of 29–51 mL) and the second at 550 mM ammonium bicarbonate (midpoint of elution volume of 54–65 mL). The fractions from the first peak indicated in Figure 3 were collected, pooled, and lyophilized. After being desalted on a Sephadex G-50 column, this fraction, which was subsequently identified as pure thymopoietin III (see below), contained 23 mg of thymopoietin, giving a total of 1600-fold purification. The overall recovery of thymopoietin by this procedure was approximately 14%. The second immunoactive peak had at least one major and two minor bands on isoelectric focusing on gel. The major band, corresponding to the p*I* value of 6.7, was eluted from the gel and found to be cross-reactive with ubiquitin antibody. The N-terminal sequence of the first six amino acids of this major protein fraction was Met-Gln-Ile-Phe-Val-Lys, the N-terminal sequence of ubiquitin. The other two minor bands could possibly be breakdown products related to thymopoietin. Purified thymopoietin III has an isoelectric point of 6.9 ± 0.15. Analytical disc gel electrophoresis at pH 8.9 and 4.3 gave a single sharp band. The protein was completely inactivated below pH 4.0 and by 4 M urea and 6 M guanadinium chloride but was stable up to 1.8 M NaCl. The amino acid composition is shown in Table II. The protein is comprised of 49 amino acids and is devoid of any sulfur-containing amino acids, tryptophan, and isoleucine and, unlike bovine thymopoietin II, possesses one residue of histidine. In radioimmunoassay, the purified protein gave a displacement slope parallel with bovine thymopoietin II.

Native bovine thymopoietin III (4 mg) was subjected to automated liquid phase sequence analysis by Edman degradation and contiguous identifications were made through the NH_2-terminal 46 residues. The average yield per cycle was 94.1%, and the quantitated recoveries of selected residues are shown in Figure 4. At position 34, we recovered 27 nmol of Asp and 45 nmol of Glu with a background of 18 nmol. These results provided a four amino acid sequence overlap with

Table II: Amino Acid Composition of Purified Bovine Thymopoietin III[a]

amino acids	found[b]	mean ± SD	hydrolysis time (h) 24	48	71	95
C_4SO_3H			ND	ND	ND	ND
aspartic acid	4	3.97 ± 0.30	4.31	3.64	4.12	3.81
threonine	3	3.05 ± 0.23	3.09	3.34	2.81	2.96
serine	2	2.29 ± 0.12	2.35	2.49	2.26	2.14
glutamic acid	8	8.34 ± 0.58	9.02	8.60	7.75	7.99
proline	3	3.42 ± 0.22	3.13	3.40	3.66	3.49
glycine	1-2	1.62 ± 0.20	1.51	1.82	1.40	1.75
alanine	3	3.38 ± 0.24	3.31	3.08	3.64	3.49
valine	5	4.55 ± 0.22	4.57	4.76	4.25	4.62
methionine			ND	ND	ND	ND
isoleucine			ND	ND	ND	ND
leucine	9	9.31 ± 0.69	10.08	8.51	9.03	9.62
tyrosine	2	1.66 ± 0.19	1.63	1.86	1.74	1.41
phenylalanine	1	1.00 ± 0.10	1.15	0.97	0.95	0.93
lysine	5	4.47 ± 0.41	3.99	4.92	4.67	4.30
histidine	1	0.81 ± 0.08	0.80	0.84	0.71	0.89
arginine	2	1.66 ± 0.13	1.75	1.51	1.60	1.78

[a] Expressed as molar ratio of amino acid. [b] Average of four determinations (24, 48, 71, and 95 h). Serine increased by 10% and threonine increased by 5% to compensate for destruction by acid. Hydrolysis was performed with constant boiling HCl at 110 °C in vacuo. ND, not detected or present only in trace amounts.

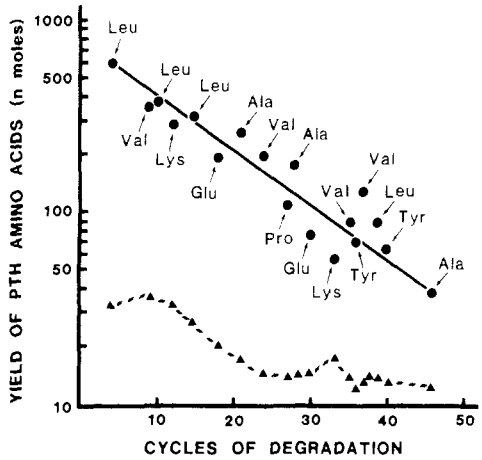


FIGURE 4: Repetitive yields of phenylthiohydantoin (PTH) amino acids during automated degradation of TP-III through the first 46 steps. (---) Background (average yield of all PTH amino acids) at selected cycles of degradation.

C-terminal heptapeptide sequence elucidated by timed release of amino acids following digestion with carboxypeptidase A (Figure 5) and B.

The time-dependent release of amino acids from the COOH terminus of thymopoietin III by digestion with carboxypeptidase A and B was as follows. At times 15 s, 1, 5, 30, 60, 120, and 240 min, the following amounts of amino acids were detected: arginine, 12.7, 13.4, 18.3, 21.4, 23.0, 27.6, and 27.3 nmol; lysine, 11.3, 17.4, 20.9, 28.2, 30.1, 35.4, and 35.7 nmol; leucine, 0.8, 8.4, 12.4, 14.5, 16.4, 24.5, and 39.3 nmol; alanine, 0.1, 5.9, 18.4, 20.4, 21.4, 25.6, and 27.1 nmol; threonine, 0, 0, 2.2, 2.7, 3.1, 10.3, and 12.9 nmol; histidine, 0, 0, 0.89, 4.3, 5.0, 5.6, and 6.2 nmol. Upon finding that arginine was released first and that eventually greater quantities of lysine were released, we concluded that the C-terminal sequence was probably -Lys-Arg-COOH, with possible truncation of some arginine residues accounting for the subsequent increase in lysis obtained. When 30 nmol of thymopoietin III was digested with carboxypeptidase B alone under similar conditions as described above, the time-dependent release of amino acids was as follows. At times 5 s, 15 s, 1 min, 5 min, and 30 min, the following amounts of amino acids were detected: arginine, 8.8, 12.0, 13.7, 14.4, and 19.6 nmol; lysine, 4.8, 10.1, 15.4, 18.3, and 25.3 nmol. These findings clearly established that

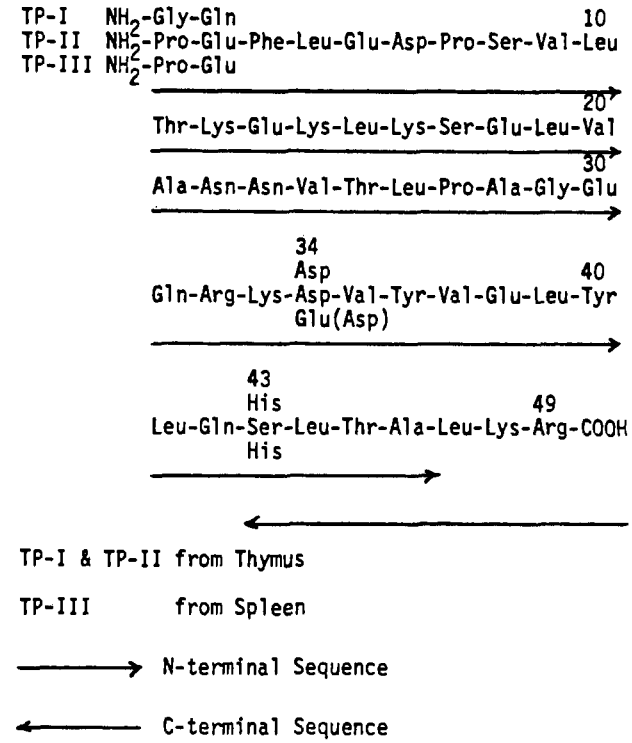


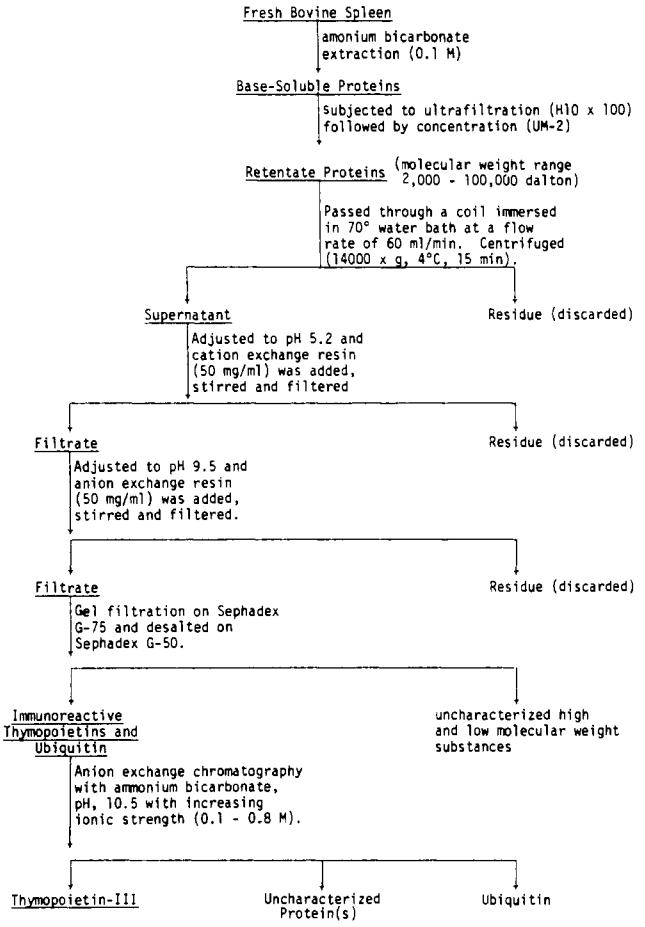
FIGURE 5: Bovine thymopoietin sequences.

lysine and arginine were C terminal to leucine, and leucine must be placed at the C-terminal side of alanine since this was identified at position 46 by automated sequence analysis. Carboxypeptidase B digestion of 500 μg of maleated thymopoietin III produced 48 nmol of arginine and only traces of lysine (0.4 nmol), establishing that arginine must be C-terminal to lysine. These results, taken with the independently established NH_2-terminal sequences of thymopoietin III, are consistent, with the COOH terminal sequence as -His-Leu-Thr-Ala-Leu-Lys-Arg-COOH.

Residues 1–46 of native thymopoietin I (160 nmol) were identified by automated Edman degradation. The average repetitive yield was 97% through the first 28 residues and 93% through residue 46.

The new isolation methods provided TP-II of greater purity than had been available previously. We had previously reported the entire amino acid sequence of TP-II (Schlesinger

Scheme I: Flow Sheet of the Isolation Procedure



& Goldstein, 1975) and now confirm this except for residues 1 and 2, which we now have corrected to Pro-Glu-, and residue 47, which is Leu rather than Val.

Discussion

Our modified method for isolating thymopoietin III from spleen offered a number of improvements over our previous method and provided higher overall yield of homogeneous polypeptide in a shorter span of time. A schematic representation of the entire procedure is given in Scheme I. Initial purification consisted of a brief heat treatment which not only removed proteolytic enzymes but also 50% of additional impurities. Cation- and anion-exchange resins further eliminated 90% of the residual inactive proteins plus most of the pigmented nonprotein materials in the extract. Combination of these two steps gave a relatively high 54% recovery of thymopoietin. Additionally, a shallow ionic strength gradient on a strongly basic anion exchanger was used to separate thymopoietin from ubiquitin and other polypeptides. This step gave a good recovery with 60-fold purification. Thymopoietin polypeptide isolated by this procedure was 95% homogeneous as demonstrated by high-pressure liquid chromatography with a reverse-phase system (using a μBondapak C_{18} column, Waters Associates, Milford, MA; the solvent system used was 50% acetonitrile in 0.01 M ammonium acetate, pH 5.7), electrophoresis, radioimmunoassay, and amino acid composition.

The amino acid sequences of thymopoietins I, II, and III are summarized in Figure 5. Because TP-III had minor differences in the N- and C-terminal residues compared with the previously published TP-II sequence, we rechecked the entire sequence of TP-II; the N-terminal sequence of thymopoietin II puified by the new procedure was NH_2-Pro-Glu- ... rather than NH_2-Ser-Gln-, and the C-terminal sequence of TP-II was -Leu-Lys-Arg-COOH rather than -Val-Lys-Arg-COOH. Thus the amino acid sequence of thymopoietins III and II are identical except for amino acid substitutions at position 43, Ser (TP-II) vs. His (TP-III), and position 34, Asp (TP-II) vs. Glu (TP-III), albeit with some microheterogeneity between Asp and Glu at this position in TP-III. Similarly, TP-I is closely related in that it differs from TP-II in only three positions, positions 1 (Gly), 2 (Gln), and 43 (His), the latter residue being identical with residue 43 of TP-III. It should be noted that manual Edman degradation was done initially to confirm polypeptide purity, and subsequently, automated runs were made to achieve longer sequence evaluation. No differences in sequence were obtained, and the data displayed were from automated runs.

The close amino acid sequence similarities between thymopoietins I, II, and III clearly imply a recent common ancestral gene, with at least three copies giving rise to the three distinct amino acid sequences described. It is intriguing that two of these genes are isolated from the thymus (TP-I and TP-II) whereas the third (TP-III) is isolated from the spleen. For TP-I and TP-II, it remains to be determined whether these represent polymorphism of a single functional hormone in cattle, since our preparations were of necessity made from a number of thymuses, or whether both genes exist and are isolated from individual cattle. If the latter case were true, the functional significance is still uncertain, but the parallelism of TP-I and TP-II in diverse bioassays (Goldstein, 1974; Basch & Goldstein, 1974) and their sharing of residues 32–36 (Arg-Lys-Asp-Val-Tyr), which has been shown to be biologically active in a number of assay systems (Weksler et al., 1978; Lau & Goldstein, 1980; Goldstein et al., 1979; Gershwin et al., 1979; Verhaegen et al., 1980; Lau et al., 1980), suggest that they are variants of a single functional hormone. This would be analogous to insulin in the rat wherein genes exist in an individual rat that encode two insulins with a single amino acid difference between them (Smith, 1966). Presumably findings such as these represent early evolution of new proteins, with gene replication permitting the essential drift of one sequence and evolution of new functional forms.

For TP-III, which is isolated from spleen, our findings suggest further progression of this process, with the new gene product being isolated from a new anatomical site and perhaps having a variation of its active site. The finding that Glu predominates at position 34 in TP-III (albeit with a small microheterogeneity with Asp) is particularly provocative since this occurs in the putative active site region of TP-I and TP-II.

There is precedent for the isolation of identical or closely related polypeptides at different anatomical sites. This would include cholecystokinin, found in both pancreas and hypothalamus, (Buchan et al., 1978), and the variants of Met and Leu enkephalin and the endorphins, with their correspondingly precise anatomical localizations (Polak & Bloom, 1980). The association of subtle amino acid sequence differences and differing anatomical sites of production is similar both for the endorphins and the thymopoietins. In both cases, the functional significance of these variations remains to be elucidated.

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Proteolytic Processing in the Biogenesis of the Neurosecretory Egg-Laying Hormone in *Aplysia*. 1. Precursors, Intermediates, and Products†

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ABSTRACT: The neurosecretory bag cells of the mollusk, *Aplysia*, produce a peptide egg-laying hormone, ELH, via a multistep proteolytic processing sequence analagous to those which have been demonstrated for secretory peptides in other systems. The goals of the present study were to identify the major members of this processing sequence by sequential sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing of bag cell proteins synthesized in the presence of labeled precursors and to elucidate the precursor–product relationships between these proteins in pulse–chase experiments. Eight major members of the processing sequence were identified. The ultimate precursor is a 29 000-dalton, pI = 7.7, protein which gives rise to a pI = 7.2 protein with an apparent M_r of 6000 as well as heterogeneous species of M_r 16 000–20 000. The latter protein or proteins is/are processed to apparent end products of 13 000–14 500 daltons, while the pI = 7.2 species yields precursors to the final secretory products. These include a pI = 7.5 peptide which is cleaved to ELH (M_r 4385, pI > 9) and a M_r 4500, pI = 4.1 species which yields the other secretory product, AP (M_r 4500, pI = 4.9). Therefore, it appears that a single precursor is processed to yield three products, two of which are known to be secreted, and that each product is generated via at least one intermediate form.

The egg-laying hormone, ELH,[1] of the marine mollusk, *Aplysia californica*, is a 4385-dalton peptide which induces oviposition when injected into recipient animals. ELH is produced and secreted by the bag cells, bilateral clusters of apparently homogeneous neurosecretory cells located at the junction of the pleuroabdominal connective nerves and the

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[1] Abbreviations used: AP, acidic peptide; ELH, egg-laying hormone; M_r, relative molecular mass; MSH, melanocyte stimulating hormone; $NaDodSO_4$, sodium dodecyl sulfate.

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