Journal of Chromatography, 177 (1979) 271–277

CHROM. 11,971

CHROMATOGRAPHIC ISOLATION OF THYMIC FACTORS IMPAIRING NEUROMUSCULAR TRANSMISSION

N. KELEMEN, F. LASMOLES and G. MILHAUD

I.N.S.E.R.M. U.113, Hôpital Saint-Antoine, 184, rue du Faubourg Saint-Antoine, 75571 Paris Cedex 12 (France)

(Received April 12th, 1979)

SUMMARY

Thymopoietins I and II are chemically related peptides able to inhibit neuromuscular transmission. They were first isolated from bovine thymus by a protein-denaturating method, which may have destroyed other thymic factors displaying the same biological activity. The present investigation, based on a new gentle isolation procedure, suggests that thymopoietins are the only factors impairing neuromuscular transmission that are present in the thymus. The yield of the new procedure is at least twice as high as that of the original method.

INTRODUCTION

The discovery of the central role played by the thymus in the regulation of immune response[1] has led to the hypothesis of the existence of thymic hormones[2]. Several factors have been isolated from bovine thymus on the basis of their biological activity in *in vitro* assays involving the maturation, differentiation and function of the thymus-derived lymphocytes[3–5].

An original approach to the characterisation of thymic hormones evolved from the study of myasthenia gravis; this disorder is frequently associated with thymic hypertrophy[6] and is characterized by motor weakness due to partial deficiency of neuromuscular transmission[7]. The observation that thymectomy or haemodialysis led to an improvement in these patients[8,9] strongly suggested the involvement of thymus-secreted substances in the pathogenesis of this disorder. This hypothesis was further substantiated by the impairment of neuromuscular transmission following the injection of thymic extracts[10]. This prompted the isolation from bovine thymus of two related peptides: thymopoietins I and II[11], which were later shown to be active in the *in vitro* differentiation of the thymus-derived lymphocytes[12].

As the first step of the isolation procedure for thymopoietins included heat-induced denaturation of the homogeneized whole thymus gland[11], heat-labile compounds displaying the same biological activity may have been destroyed during the processing of the material.

The present paper describes a new isolation procedure (which avoids protein

denaturation) and an attempt to search for additional thymic factors impairing neuromuscular transmission.

MATERIALS AND METHODS

The following items were used: Amicon (Lexington, Mass., U.S.A.) ultrafiltration cartridges H1P100, H1X50, H1P10 and H1P5 (molecular weight cut-off 100,000, 50,000, 10,000 and 5000 daltons, respectively); Dextran blue 2000; Sephadex G-25, G-50 and QAE A-25 (Pharmacia, Uppsala, Sweden), Bio-Gel P-2, P-6, hydroxyapatite, and Bio Phore 12% and 7.5% pre-cast polyacrylamide gels (Bio-Rad Labs., Richmond, Calif., U.S.A.). All chemicals were of reagent grade.

Electromyographic assays were performed on two-months-old male Wistar rats following the method previously described[13]. The assay consisted in measuring, 48 h after intraperitoneal injection of the test sample, the *flexor digitorum* electrical potential following supramaximal nerve stimulation with 10 impulses at 50 Hz.

Samples to be tested were diluted in 0.9% sodium chloride solution containing 0.1% bovine serum albumin and injected from 0.1 to 10 mg per kg of body mass.

Fresh calf thymus glands were provided by the C.N.R.Z. (Jouy-en-Josas, France). All separation steps were carried out at 4°. Chromatographic eluates were monitored by absorbance at 278 nm with an LKB Uvicord III photometer. The collected fractions were stored at −20° after lyophilisation.

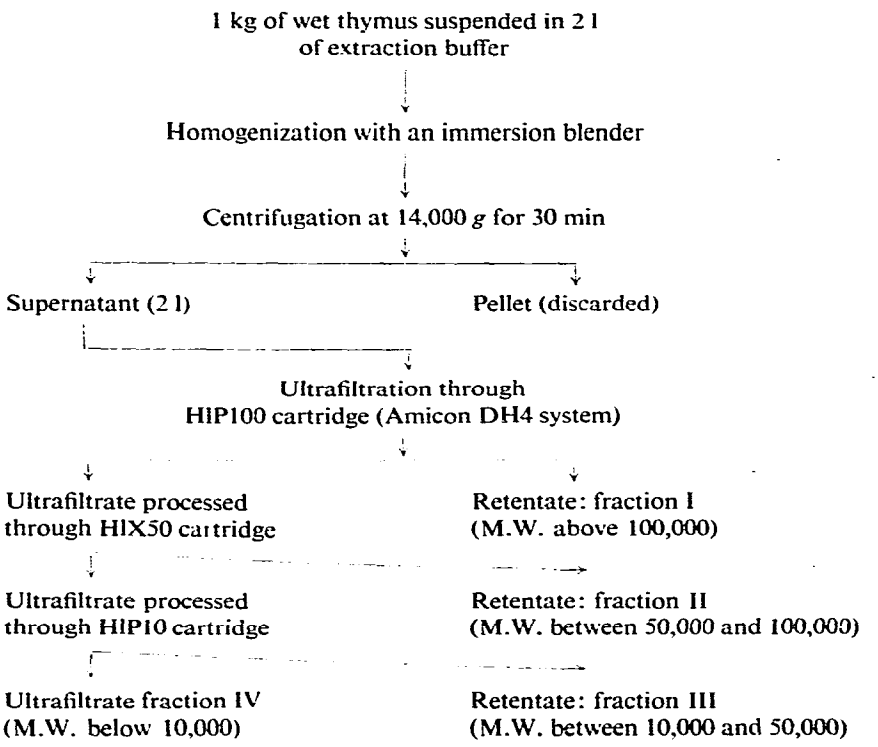
RESULTS

Non-denaturating isolation procedure

In order to protect labile factors from denaturation, ultrafiltration was selected as the fractionation method: the fractions obtained were expected to contain all the factors present in the thymus.

Thymus glands were dissected immediately after slaughter and homogeneized in pH 8.0 extraction buffer (0.1 *M* ammonium bicarbonate, 1 m*M* 2-mercaptoethanol; 30% wet weight per volume). Nuclear and membrane materials were sedimented by centrifugation (14,000 *g*, 30 min). The supernatant solution was processed by ultrafiltration through an Amicon DH4 hollow-fibre system (concentration mode) fitted successively with three different diafibre cartridges (H1P100, H1X50 and H1P10); the procedure is summarized in Scheme 1. The four fractions separated according to their molecular sizes were lyophilised and tested in the electromyographic assay. Biological activity was present exclusively in the fraction containing material with molecular weight below 10,000 (fraction IV). Further fractionation of this material through a H1P5 cartridge (molecular weight cut-off 5,000) was unsuccessful, as both ultrafiltrate and retentate were active in the bioassay.

Further purification of the active material present in fraction IV (26 g of lyophilised powder per kg of wet thymus) was achieved by molecular sieving on a 2.6 × 36 cm column of Sephadex G-25 (medium) gel equilibrated and eluted with the extraction buffer. A 5-g portion of fraction IV was dissolved in 15 ml of extraction buffer and filtered through the column; three well-defined fractions were obtained. Biological activity was present in the material eluting within the void volume of the column as determined by the Dextran blue 2000 elution volume. No activity could be



Scheme 1. Molecular sieving by ultrafiltration of calf-thymus homogenate. All steps are carried at 4°; the final four fractions are lyophilised and stored desiccated at −20°.

detected in the assay of the subsequent fractions, even if large amounts (up to 10 mg per kg of body mass) had been injected.

The final molecular sieving was performed on Bio-Gel P-6. The active material from the preceding step was dissolved in the extraction buffer and filtered through a 2.6 × 36 cm column. Two clearly distinct peaks were obtained (see Fig. 1a), the first coinciding with the void volume, and the second exhibiting activity in the electromyographic assay. This active material had an apparent molecular weight ranging from 5000 to 6000 since these values are the exclusion limits of the Sephadex G-25 and Bio-Gel P-6 gels.

The last purification step involved ion-exchange chromatography on a QAE A-25 Sephadex gel equilibrated with 50 m*M* Tris–HCl buffer of pH 9.5. The elution pattern and details are shown in Fig. 1b. All fractions were collected and desalted on a 2.6 × 85 cm column of Bio-Gel P-2 in distilled water. Two fractions active in the electromyographic assay were eluted with 0.21 *M* and 0.32 *M* potassium chloride, respectively. Each active fraction was re-chromatographed on the same QAE A-25 column, eluting with a slower slope gradient (0.15 *M* to 0.60 *M* potassium chloride). A 250-μg load of material from this step, when analysed by electrophoresis on a 12% polyacrylamide gel at pH 4.0 and at 9.0, revealed a single band when stained with Coomassie blue. For identification purposes, the relative mobilities of the biologically active material were compared with those of thymopoietins I and II at pH 8.9 and 4.3 in 7.5% polyacrylamide gels. A 200-μg amount of the biologically

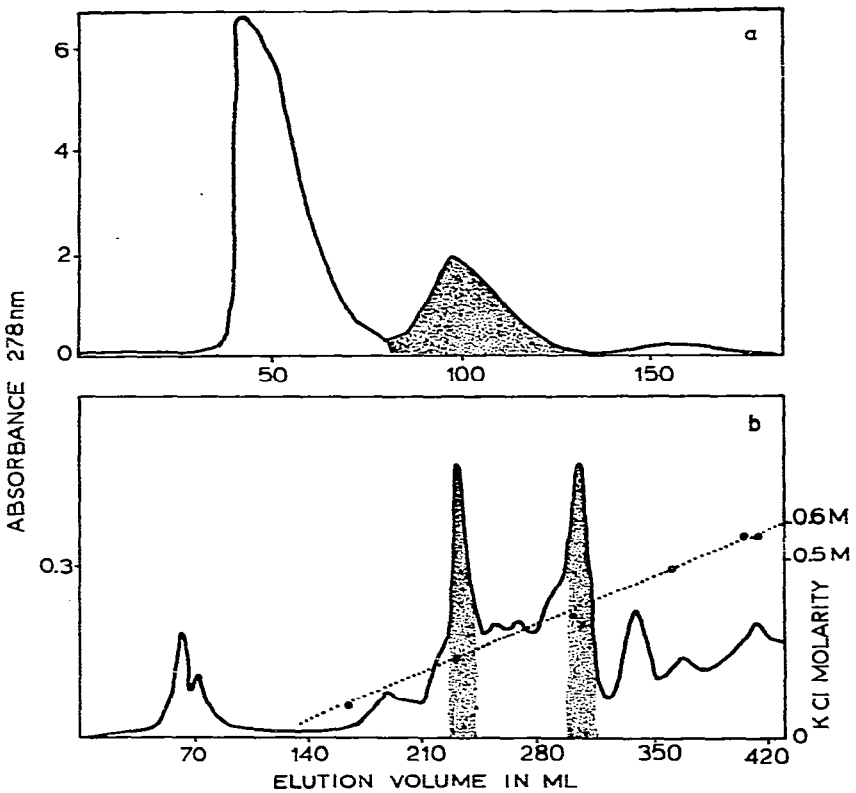


Fig. 1. (a) Exclusion chromatography on Bio-Gel P-6 (200–400 mesh). The active material from the Sephadex G-25 step is lyophilised and pooled (0.12 g/kg of wet thymus), dissolved in 10 ml of extraction buffer and loaded on a 2.6 × 36 cm column. The flow-rate is 60 ml/h. Active material elutes after the void volume. (b) Ion-exchange chromatography on QAE-Sephadex A-25. Active material from the preceeding step is dissolved in 10 ml of 50 m*M* Tris–HCl buffer (pH 9.5), which is used as equilibrating and elution medium for the 1.5 × 22-cm column. A 250–250 ml 0.05 *M* – 0.8 *M* KCl linear gradient is developed through the column when all unadsorbed material has been eluted from the gel phase. Flow-rate is 75 ml/h. Gradient elution is controlled by chloride titrimetry. Active fractions in the electromyographic assay are represented as shaded areas.

active material was loaded, together with the dyes used as reference compounds. At pH 8.9, the R_F of two fractions was 0.28 with respect to bromophenol blue; at pH 4.3, the R_F of the first fraction eluted from the QAE-Sephadex column was 0.62 and the R_F of the second fraction was 0.55 with respect to methyl green. These values are in very close agreement with those published for thymopoietins I and II. The yield of each isolation step cannot be absolutely quantitated due to the lack of precision[11,13] of the currently available biological assay. After the last chromatographic step, the yield amounts to 4–6 mg of lyophilised powder per kg of wet thymus for each thymopoietin; these values are higher than the one reported by Goldstein[11] (1–2 mg per kg of wet thymus).

Modification of the isolation procedure

The main difference between the procedure of Goldstein and ours lies in the omission of incubation of the homogeneized thymus at 70° for 30 min. This could

account for differences in yields, if thymopoietins were heat-sensitive peptides, but this is not the case, as incubation of the purified thymopoietins at 70° for 30 min did not result in detectable loss of activity.

The isolation procedure was then modified as follows: the supernatant solution after centrifugation at 14,000 *g* was incubated at 70° for 30 min, and most of the precipitated protein was removed by centrifugation (10,000 *g*; 20 min). The ultrafiltration was achieved in a single step through the H1P10 cartridge, and the ultrafiltrate was then processed either according to the preceding procedure or to the sequence of chromatographic separations used by Goldstein[11]; results and experimental data are given with Fig. 2. Both procedures gave similar yields amounting to 1–2 mg of each thymopoietin per kg of wet thymus.

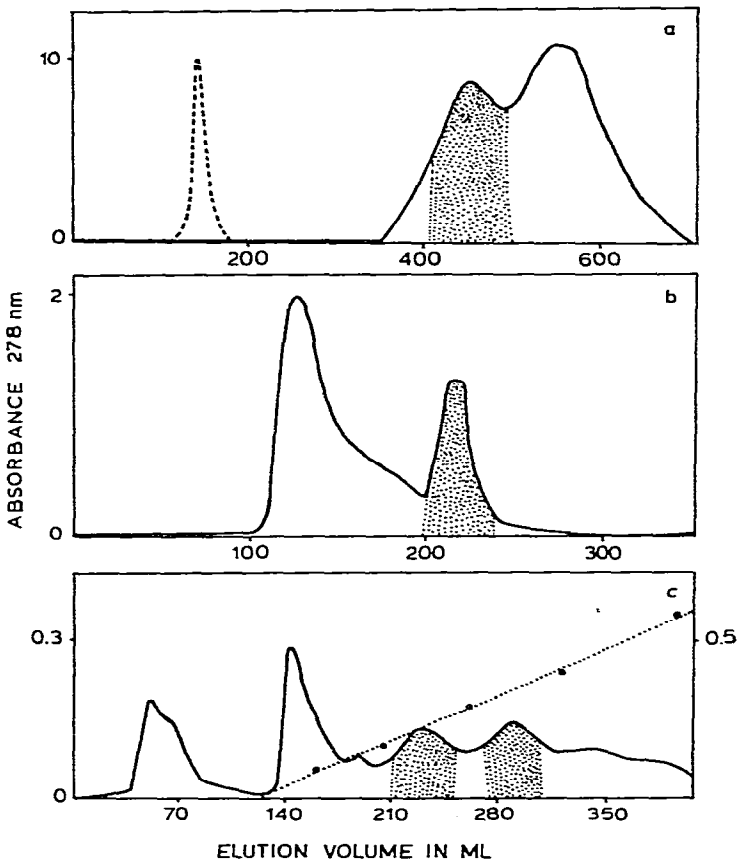


Fig. 2. (a) Exclusion chromatography on Sephadex G-50. A 5-g portion of lyophilised 0–10,000-dalton ultrafiltrate is dissolved in 15 ml of extraction buffer and loaded on a 2.6 × 90-cm column. Flow-rate 150 ml/h; absorbance measured in a 0.5-mm cell. Dashed lines represent elution of Dextran blue 2000 and gives position of void volume. Active material is found in the first peak eluting after the void volume. (b) Adsorption chromatography on hydroxyapatite. Lyophilised active material from the preceding step is dissolved in 10 ml of 5 m*M* phosphate buffer (pH 6.8) and loaded on a 2.6 × 35 cm column equilibrated with the same buffer; elution flow-rate 40 ml/h. The active material, included in the second peak, is collected and de-salted on a 2.6 × 85 cm column of Bio-Gel P-2 (50–100 mesh) in distilled water and lyophilised. (c) Ion-exchange chromatography on QAE-Sephadex A-25. Conditions as in Fig. 1b.

The isolation procedure reported here was first designed as an analytical approach and can be simplified for preparative purposes: the 14,000-*g* supernatant solution can be ultrafiltered directly through the H1P10 cartridge with an average flow-rate of 100 ml per h.

DISCUSSION

By using denaturating steps, one can isolate from bovine thymus two related peptides similar in physical properties (molecular weight, elution behaviour on QAE ion-exchange chromatography, electrophoretic migration) and in biological properties as assayed by activity on neuromuscular transmission. The physical and biological data presented here suggest that these peptides are identical with thymopoietins[11].

The final yield of thymopoietin isolation is increased if the heat-denaturation step is avoided. As thymopoietins are heat-stable peptides, the binding of thymopoietins to a cytoplasmic structure or to a larger protein precipitating during heating is likely. The main purpose of heating is to inactivate proteases. It is possible that ultrafiltration through a 10,000-dalton retaining filter achieved the same goal, since most proteases have molecular weights above 10,000. The proteolytic activity is also reduced by the low temperature (4°) used throughout the extraction procedures.

In the original procedure[11], the whole homogeneized thymus is heat-denaturated without prior removal of cell structures. During the incubation at 70°, solubilisation of such cell structures containing peptides might have occurred. The isolation of thymopoietins from a 14,000-*g* supernatant of homogeneized thymus is in favour of the cytoplasmic origin of these factors.

Among all thymic hormones so far isolated, Facteur Thymique Sérique[14] (FTS) is the only one for which increased levels have been reported in the sera of *myasthenia gravis* patients[18]. But FTS was extracted from blood and not from the thymus itself. The thymic origin of FTS is still controversial[15–17]. Synthetic FTS lacked activity in the electromyographic assay used for thymopoietin isolation[13].

CONCLUSION

The use of a highly efficient isolation procedure for thymopoietins suggests that they are the only peptides in the cytoplasm of the thymus capable of impairing neuromuscular transmission as measured by an electromyographic assay.

REFERENCES

1 J. F. A. P. Miller, *Lancet*, ii (1961) 748.
2 D. Osoba and J. F. A. P. Miller, *Nature (London)*, 199 (1963) 653.
3 A. I. Kook, Y. Yakir and N. Trainin, *Cell. Immunol.*, 19 (1975) 151.
4 A. L. Goldstein, T. L. K. Low, M. McAdoo, J. McClure, G. B. Thurman, J. Rossio, C. Y. Lai, D. Chang, S. S. Wang, C. Harvey, A. H. Ramel and J. Meienhofer, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 725.
5 W. Robey, W. Ceglowski, T. Luckey and H. Friedman, *Advan. Exp. Med. Biol.*, 29 (1973) 295.
6 B. Castelman, *Ann. N.Y. Acad. Sci.*, 135 (1966) 496.
7 J. E. Desmedt, *Arch. Int. Pharmacodyn.*, 125 (1960) 224.
8 R. W. Hedger, F. A. Davies, F. D. Schwartz and T. S. Ing, *Ann. Intern. Med.*, 74 (1971) 749.
9 G. Genkins, A. E. Papatestas, S. H. Horowitz and P. Kornfeld, *Amer. J. Med.*, 58 (1975) 517.

10 G. Goldstein, *Lancet*, ii (1968) 119.
11 G. Goldstein, *Nature (London)*, 247 (1974) 11.
12 R. S. Basch and G. Goldstein, *Proc. Nat. Acad. Sci. U.S.*, 71 (1974) 1474.
13 F. Lasmoles, N. Kelemen and G. Milhaud, *Biomedecine*, 27 (1977) 326.
14 J. F. Bach, M. Dardenne, J. M. Pleau and M. A. Bach, *Ann. N.Y. Acad. Sci.*, 249 (1975) 186.
15 A. J. S. Davies, *Nature (London)*, 266 (1977) 13.
16 A. Brand, D. G. Gilmour and G. Goldstein, *Nature (London)*, 269 (1977) 597.
17 N. Kelemen, F. Lasmoles, and G. Milhaud, *Nature (London)*, 272 (1978) 65.
18 J. F. Bach, M. Dardenne, M. Papiernik, A. Barois, P. Levasseur and H. Le Brigand, *Lancet*, ii (1972) 1056.