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Note

Shortened purification procedure of a spleen-derived immunosuppressive peptide

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Several studies have shown that activated T cell supernatants[1–3], sera fractions[4,5] and lymphoid organs[6–11] contain immunosuppressive agents which depress the humoral response in mice.

Working with bovine spleen extracts, we demonstrated[12] that in this case the immunosuppressive activity could be attributed to the presence of a low-molecular-weight spleen-derived immunosuppressive peptide (SDIP) which was non-covalently bound to high-molecular-weight carriers. Taking advantage of this observation, we isolated the active fraction through ultrafiltration (Amicon PM 10) of an acetic extract prepared from a bovine spleen acetonic powder[13]. Then we developed a reproducible but tedious purification procedure (Fig. 1) which led to 10^7-fold purified SDIP[14].

As a preliminary high-performance liquid chromatographic (HPLC) analysis of SDIP showed that the biologically active agents could be separated from some contaminants which were still present in the purified fraction, this technique has been developed in order to curtail the actual purification procedure. The results presented here show that, from the ultrafiltrate, a three-step purification procedure (one molecular sieving plus two HPLC steps) yields an homogeneous substance.

EXPERIMENTAL

As evaluation of the isolated substance by weighing was inpractical in routine work, the concentration and quantities were estimated by measuring the optical density of aqueous solutions at 220 nm. One $A_{220\ nm}$ unit represents the quantity of substance dissolved in 1 ml of water which has an optical density of 1 at 220 nm in a 1-ml cuvette.

Preparation of the ultrafiltrate, U

The extraction procedure and the ultrafiltration were performed in a pilot plant, using 10–100 kg spleen. An acetone extract was obtained from spleen, the solvent removed and the powder was extracted with water. The extract was concen-

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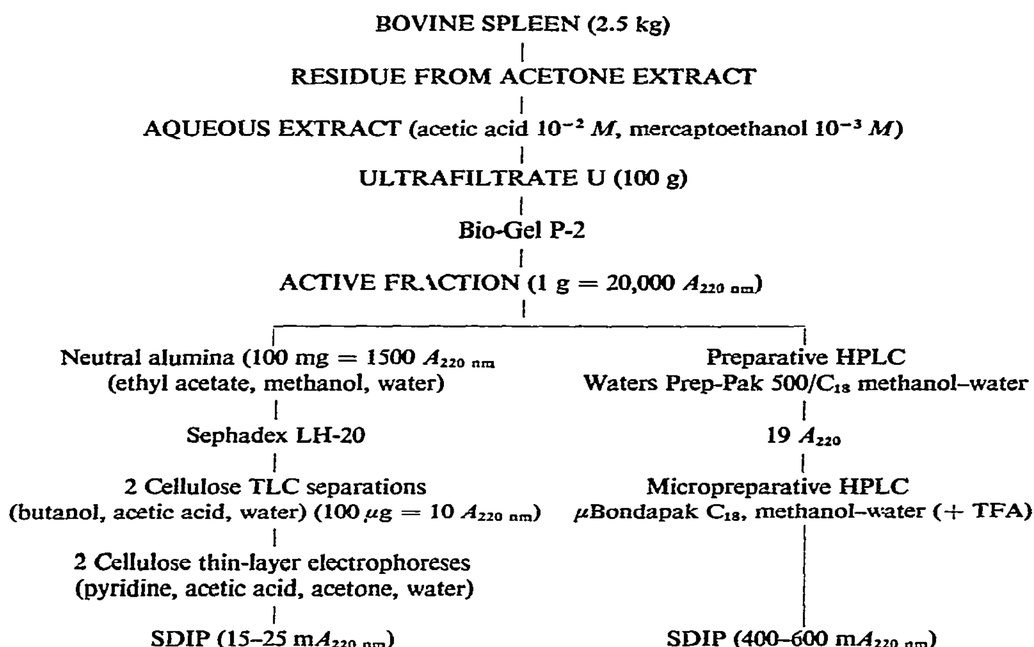

Fig. 1. SDIP purification procedures.

trated ten-fold by ultrafiltration on Sartorius membranes. After 1:50 dilution with water, the concentrate was again subjected to ultrafiltration. The successive ultrafiltrates thus obtained contained the active factor.

Chromatography of fraction U

The ultrafiltrates were chromatographed on a Bio-Gel P-2 column (750 × 95 mm) as previously described[13], using 10^{-2} M acetic acid as solvent (flow-rate 3 ml/min). The active fraction was eluted between 2.6 and 3.4 V_0, where V_0 is the void volume of the column.

Preparative HPLC

A Waters Assoc. Prep LC/system 500 and a Gilson Spectochrom UV detector were used. Two cartridges of Prep-Pak 500/C_{18} (30 × 5.7 cm, particle size 37 µm) (Waters) were used and equilibrated in the eluent prior to sample injection. A 1.6-g amount of the active fraction was dissolved in 40 ml methanol–water (80:20) and injected. The column was eluted with methanol–water (80:20) at a rate of 200 ml/min.

Microscale HPLC purification of SDIP

A Waters HPLC system equipped with a Pye Unicam LC_3 UV detector was used. Separation was carried out on a µBondapak C_{18} stainless-steel column (30 cm × 4 mm) (Waters) using methanol–water (+ 0.1% trifluoroacetic acid, TFA) (38:62) as solvent. Flow-rate was 1 ml/min. Peak heights at 215 nm were recorded. The sample

to be injected was dissolved in water (1 ml) and passed through a Millipore filter. All experiments were carried out at room temperature.

The bulk of biological activity was eluted between 34 and 40 min (Fig. 2). The active peak was collected and analysed under the same conditions and on the same column using acetonitrile–isopropanol–water (+ 0.1 % TFA) (225:75:700) as eluent, as shown in Fig. 3. Electrophoresis confirmed that the isolated substance is homogeneous.

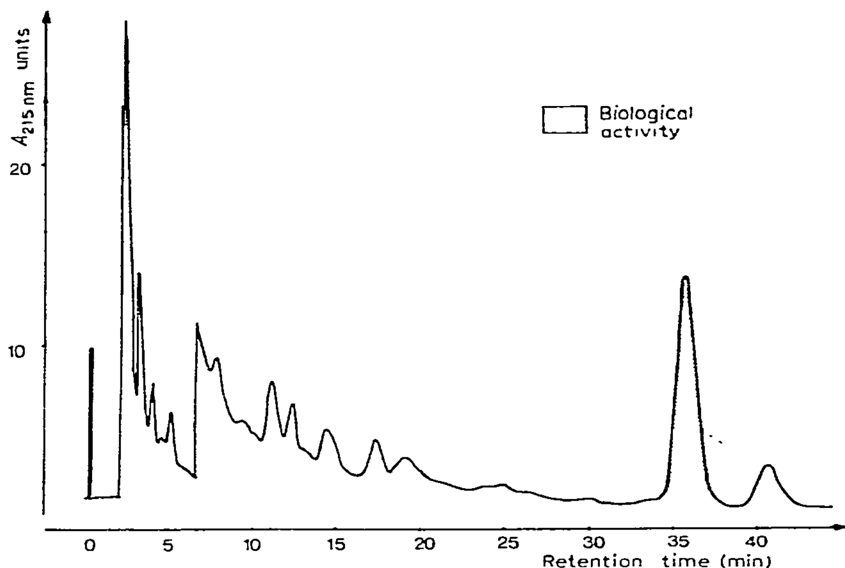

Fig. 2. HPLC chromatogram of SDIP on a μBondapak C_{18} column, with methanol–water (+ 0.1 % TFA) (38:62) as solvent. $A_{215\,nm}$ units are × 10^3.

Compared to previously established purification schemes, application of HPLC permits very rapid recovery of a highly pure substance, with a considerably improved yield. Furthermore, these analytical conditions should allow the detection of SDIP in different organ extracts, and the comparison of SDIP to the different lymphokines described in the literature which exhibit analogous biological activities.

HPLC analysis of purified SDIP

The previously described apparatus and conditions were used. The solvent was acetonitrile–isopropanol–water (+ 0.1 % TFA) (225:75:700) or methanol–water (+ 0.1 % TFA) (38:62).

RESULTS AND DISCUSSION

Bovine spleen was extracted in a pilot plant as previously described[13]. In brief, lipids and water were removed from bovine tissue by acetone extraction. The powder was extracted in acetic acid (10^{-2} *M*) in presence of 2-mercaptoethanol (10^{-3} *M*) and the aqueous extract was submitted to ultrafiltration. The ultrafiltrates were chromatographed on a Bio-Gel P-2 column, using dilute acetic acid solution as eluent.

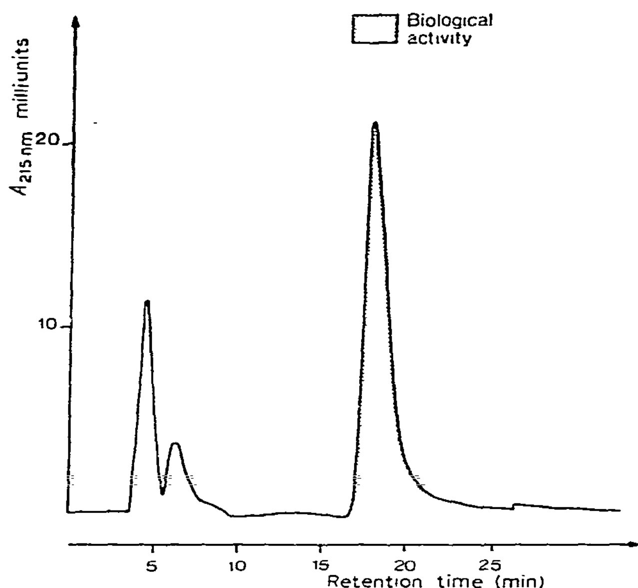

Fig. 3. HPLC analysis of purified SDIP on a μBondapak C_{18} column, with acetonitrile–isopropanol–water (+ 0.1% TFA) (225:75:700) as solvent.

The active fractions were pooled and submitted to preparative reversed-phase liquid chromatography on cartridges of Prep-Pak 500/C_{18} (Waters), using methanol–water (80:20) as solvent. This technique allowed the separation of roughly 99% of the inactive material eluted before 16 min from the active material which is retained on the column and eluted between 42 and 60 min.

These fractions were then submitted to a micropreparative separation on a μBondapak C_{18} column using as elution system the mixture methanol–water (+ 0.1% TFA) (38:62), which has recently been recommended for peptide separations[15].

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