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USE OF TSK-SW COLUMNS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PROTEINS, ISOLATED FROM SYMPATHETIC NERVES AND FRACTIONATED BY FRACTOGEL TSK-HW CHROMATOGRAPHY

PURIFICATION OF L-DOPA DECARBOXYLASE

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SUMMARY

The soluble proteins isolated from sympathetic nerves were separated on Fractogel TSK-HW columns. With a mobile phase of 0.1 *M* phosphate + 0.1 *M* K_2SO_4, pH 6.8, the main fractions I–VI were obtained. These fractions were analysed by high-performance (HPLC) on TSK-SW columns. Fractogel fractions I–III showed peaks of molecular weights, M_r 670,000, as estimated by HPLC. With sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) these fractions show no bands stainable with Coomassie Blue. The protein of fraction IV was L-DOPA decarboxylase (AADC E.C. 4.1.1.28) with M_r 150,000 existing of subunits with M_r 55,000, 45,000, 27,000 and purified according to Christenson *et al.* (*Arch. Biochem. Biophys.*, 141 (1970) 356). The dopamine-β-hydroxylase (E.C. 1.14.2.1) subunits with M_r 75,000 proteins were detected in Fractogel fraction V. Fraction VI was M_r 27,000 protein. Proteins with molecular weights $M_r < 5{,}000$ were also detected. With Phenothiazine-Affigel the proteins of fraction V (M_r 75,000) showed no affinity to the phenothiazine column equilibrated with application buffer containing Ca^{2+}. 50–70% fraction IV (M_r 150,000), eluted with Tris–EGTA buffer, and 100% fraction VI (M_r 27,000) showed affinity to the Phenothiazine-Affigel column.

INTRODUCTION

The analytical separation of the proteins isolated from sympathetic bovine splenic nerves by different methods, high-performance liquid chromatography (HPLC), sodium dodecyl sulphate (SDS)-HPLC on TSK columns and electrofocusing, etc., gave similar results on the protein composition in soluble[1] and membrane-bound states[2].

The quantitative analysis of the proteins on soft gels such as Sephadex is time-consuming. Therefore, the preparative separation of the proteins by medium-performance gel filtration on Fractogel TSK-HW was used. With high flow-rates the

high contents of proteins in the samples were separated in short times. The purity of the fractions separated was examined by molecular weight (M_r) estimation with high-performance gel filtration on TSK-SW columns and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins fractionated were further analysed by affinity chromatography with Phenothiazine-Affigel.

EXPERIMENTAL

Materials

Fractogel TSK, particle size 25–40 μm, HW-50 (separation range 500–200,000 g/mol), and TSK HW-55 (separation range 10^3–10^6 g/mol) and the chemicals used were analytical grade from E. Merck (Darmstadt, F.R.G.). Protein standards were from Pharmacia and Bio-Rad. Phenothiazine-Affigel was from Bio-Rad. The buffers used for affinity chromatography were: (1) 10 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.0, and 0.5 m*M* $CaCl_2$; (2) elution buffer, 10 m*M* tris(hydroxymethyl)aminomethane (Tris) and 5 m*M* ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 8.0.

Instrumentation

Analytical separation was performed on a Varian 5000 liquid chromatograph with UV detector (280 nm) and integrator CDS 111. The columns for HPLC were TSK 3000 SW (LKB), 600 × 7.5 mm, with pre-column (7.5 × 100 mm). The mobile phase was 0.1 *M* sodium phosphate buffer, pH 6.8, and 0.1 *M* potassium sulphate; flow-rate 0.6 ml/min.

The calibration of the molecular weights of the different fractions separated by the TSK 3000 column was achieved by use of curves published previously[1].

Fractogel TSK HW columns were slurry packed with a constant flow[3,4]. Fractogel 50, 25.4 × 515 mm, and Fractogel 55, 25.4 × 870 mm and 25.4 × 430 mm (coupled system), were used. For UV detection an UV III monitor (LDC) and pump from Duramat (CFG) was used. The flow-rate for proteins separation was 0.9–1.2 ml/min with the same buffer as used in HPLC. The sample loop for injection was 5.0–7.5 ml. The Fractogel columns were loaded with 130–150 mg proteins. The main fractions I–VI were separated in 5–9 h. For SDS-PAGE on a 5% polyacrylamide gel an LKB 2117-Multiphor system was used[1].

Sample preparation

The soluble proteins from sympathetic splenic nerve were prepared in 0.13 *M* phosphate buffer, pH 7.1, according to a modification of the method of Schümann and Burger[5]. The supernatant obtained after centrifugation at 12,000 *g* was ultracentrifuged at 230,000 *g* for 60 min without a sucrose gradient. The resulting supernatant was used for determination of the soluble protein content.

For the Fractogel chromatography the supernatant was concentrated on a Diaflow-filter PM-10 (Amicon) to a concentration of about 20 mg/ml protein and 7.5 ml were injected.

RESULTS

The soluble proteins isolated from sympathetic splenic nerves by HPLC on TSK 3000 SW show main fractions with apparent molecular weights, M_r, of >670,000 (I–III), 150,000 (IV), 75,000 (V), 27,000 (VI) and <5000 (Fig. 1).

Medium-performance gel filtration with Fractogel 50 and 55 was used for the preparative separation in a two-column system (see *Instrumentation*) (Fig. 3). The Fractogel fractions (I–VI) were re-analysed for molecular weight estimation by HPLC on TSK 3000 SW columns.

Fractions I–III showed peaks of M_r >670,000, fraction IV of M_r 150,000 and fraction V of M_r 75,000. Fraction VI showed a main protein of M_r 27,000 (Fig. 3).

The soluble protein of the sympathetic nerves was separated by SDS-PAGE into main fractions of M_r 75,000, 55,000, 45,000, 27,000 and <12,000 (Fig. 4). After the preparative separation the Fractogel fractions I–III analysed by SDS-PAGE showed no bands which were coloured upon treatment with Coomassie Blue. In fraction IV, peaks were found at M_r 55,000, 45,000 and 27,000. The Fractogel fraction V when re-analysed gave a protein of M_r 75,000, and fraction VI showed a main band at M_r <12,000 (Fig. 4).

In the Fractogel fraction IV the protein of M_r 150,000 showed DOPA decarboxylase (AADC E.C. 4.1.1.28) activity. This peak was found upon 10–26%

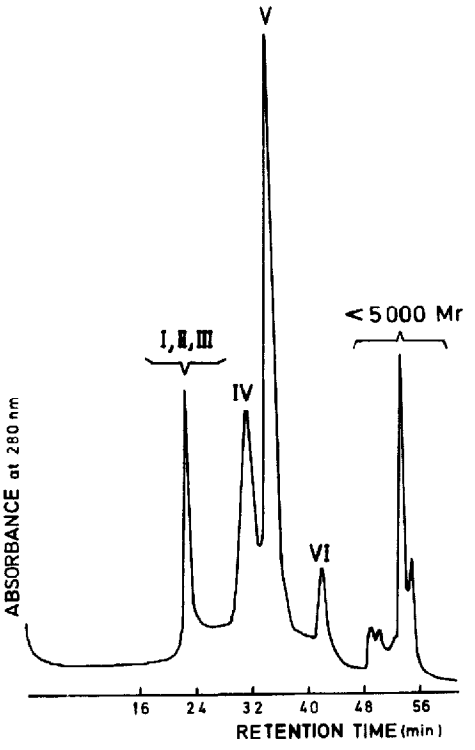

Fig. 1. Separation of soluble proteins isolated from sympathetic nerves by HPLC on a TSK 3000 SW column. Peaks: I–III, M_r 670,000; IV, M_r 150,000; V, M_r 75,000; VI, M_r 27,000; multiple peaks, M_r < 5000.

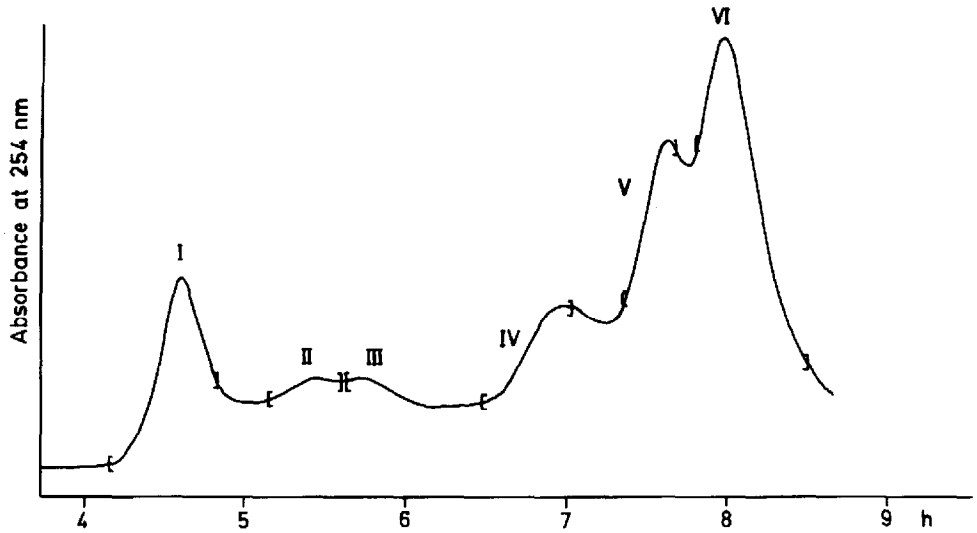

Fig. 2. Preparative separation of soluble proteins isolated from sympathetic nerves by Fractogel TSK HW 55 (S) and 50 (S) on a two-column system. Flow-rate: 1.0 ml/min of 0.1 *M* phosphate buffer, pH 6.8, +0.1 *M* K_2SO_4. Load: 150 mg protein in 7.5 ml and separated in fractions I–VI. Experimental time: 4–9 h.

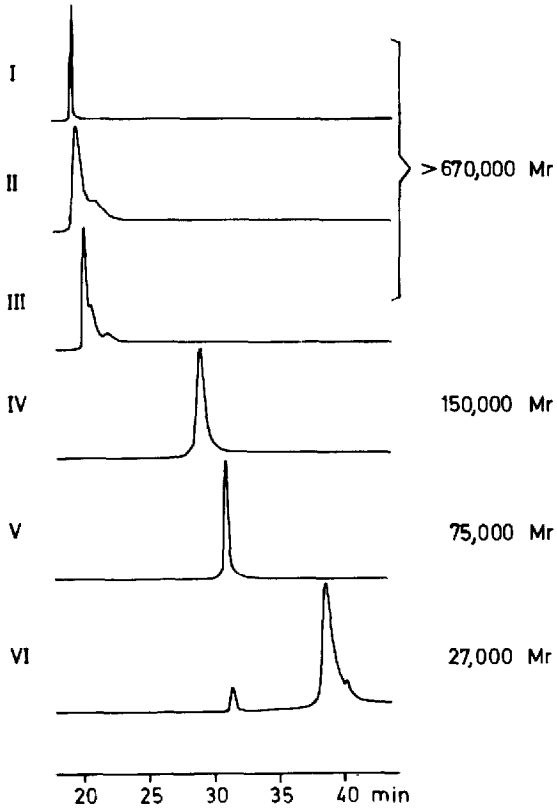

Fig. 3. Fractions from the Fractogel TSK HW columns analysed by HPLC (TSK 3000 SW columns). Absorption at 280 nm.

$(NH_4)_2SO_4$ saturation to be a single protein. The DOPA decarboxylase activity was estimated by the method of Laduron and Belpaire[6]. It increased twelve-fold when the protein was treated according to Christenson *et al.*[7] (Fig. 5).

The proteins were further characterized by Phenothiazine-Affigel affinity chromatography (Fig. 6). The columns were equilibrated in Ca^{2+}-containing application buffer (see Experimental). The column was loaded with 1.0–5.0 mg proteins. The proteins without affinity were eluted with the application buffer (1). The proteins with affinity were eluted with the elution buffer (2) containing EGTA. Fig. 6 illustrates the affinity chromatography of the total soluble protein. The M_r 75,000 protein showed no affinity and was found only in the application buffer (1). The M_r 27,000 protein was quantitatively eluted in the EGTA buffer (2) and showed the highest affinity for the Phenothiazine-Affigel. The same affinities as in Fig. 6 were found in the protein fractions IV–VI after Fractogel separation.

DISCUSSION

The separation of the proteins from sympathetic bovine splenic nerves by different methods yielded new analytical data[1]. The purification and quantitative analysis of the proteins was achieved by gel filtration on Fractogel-TSK HW using a two-column system. The high protein content was separated in short time.

The Fractogel-filtration method yielded fractions I–VI in 5–9 h. These proteins were re-analysed by HPLC (TSK 3000 SW columns) and SDS-PAGE and the results were found to be in good agreement with those obtained upon separation of the total protein by quantitative electrofocusing[1]. When re-analysed by HPLC the Fractogel fractions I–III showed peaks at $M_r > 670{,}000$. The same fractions re-analysed by SDS-PAGE showed no bands coloured by Coomassie Blue. Nevertheless, these fractions show UV absorption at 280 nm.

The Fractogel fraction IV contained a single protein with a molecular weight of M_r 150,000 when analysed by HPLC. Upon re-analysis by SDS-PAGE this fraction showed bands of M_r 55,000, 45,000 and 27,000. This difference between HPLC and SDS-PAGE was interpreted in an earlier paper[1] as due to the monomeric subunits of the L-DOPA decarboxylase estimated by SDS-PAGE.

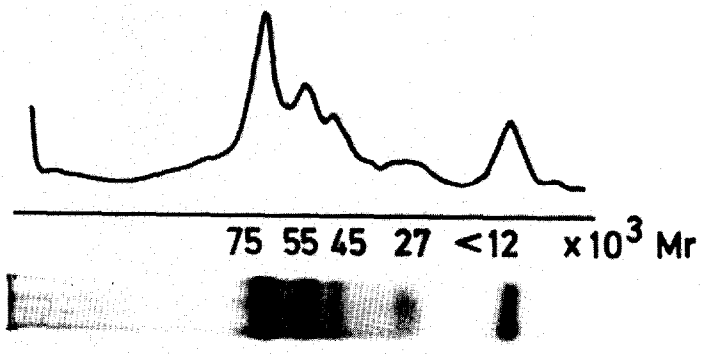

Fig. 4.

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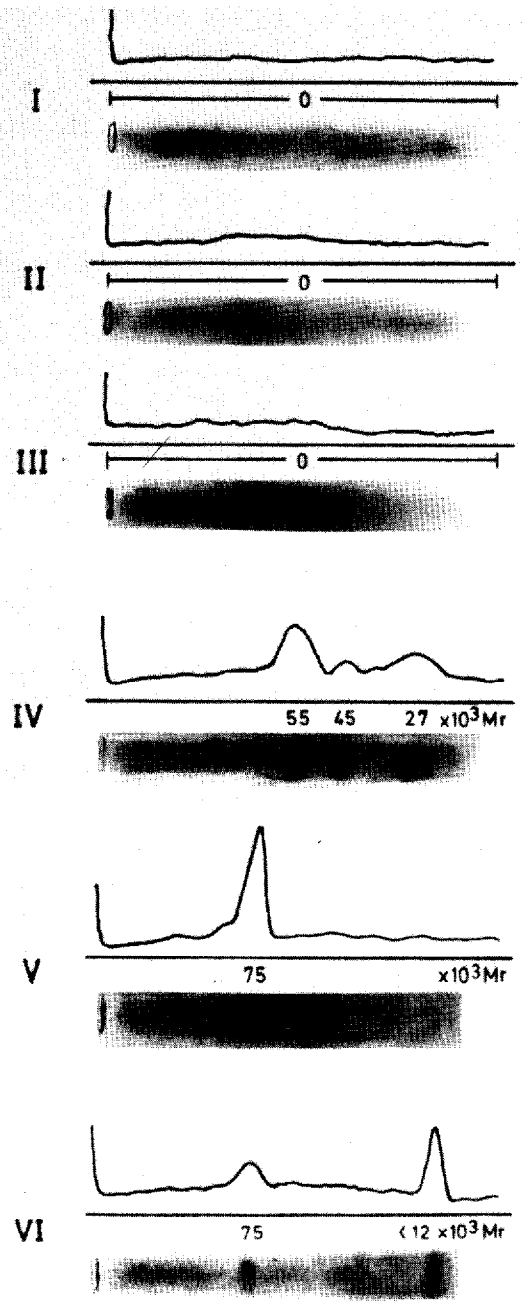

Fig. 4. Total proteins and fractions from the Fractogel TSK HW columns analysed by SDS-PAGE. Scan profile on a 5% slab gel of the Coomassie Blue (Serva) stained fractions using a Quick-Scan densitometer at 595 nm. Fractions I III show no stained bands.

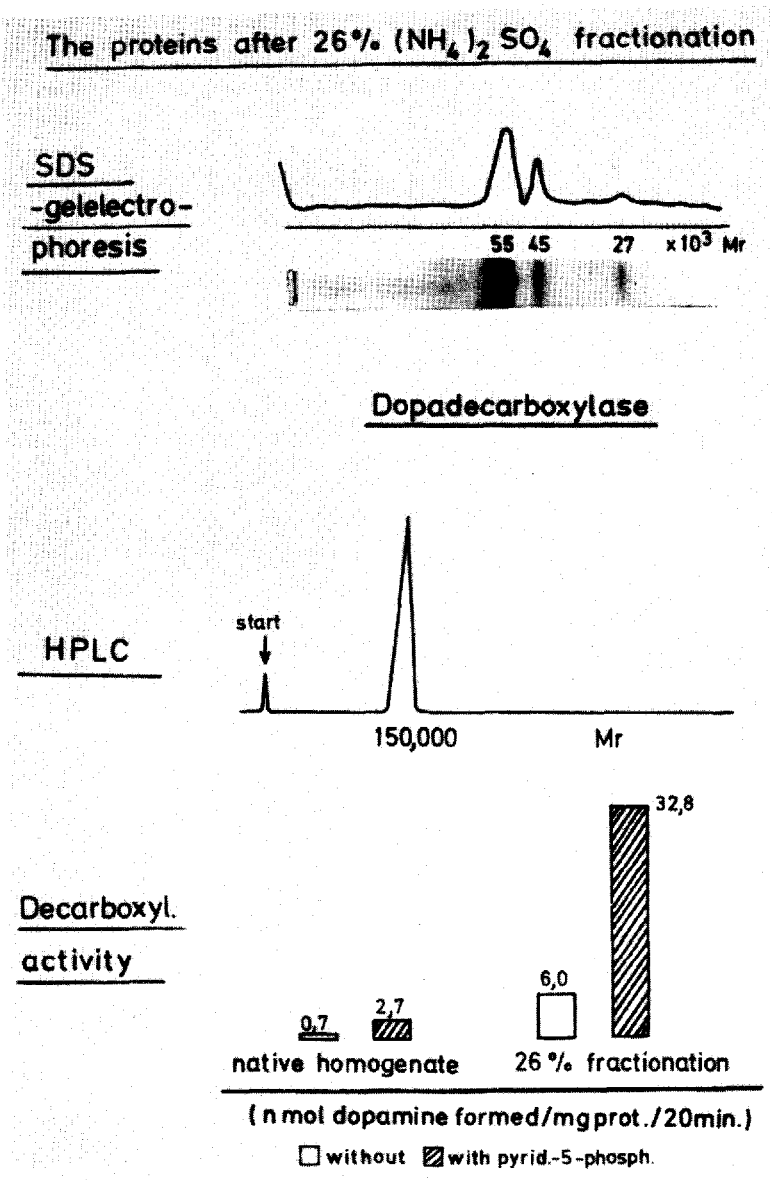

Fig. 5. DOPA decarboxylase isolated from sympathetic nerve proteins after 26% $(NH_4)_2SO_4$ saturation and treatment of the protein according to Christenson *et al.*[7]; detected by SDS-PAGE and HPLC (TSK 3000 SW). The enzyme activity was estimated by the methods of Laduron and Belpaire[6] without and with pyridoxal-5-phosphate.

A M_r 150,000 protein was not found by SDS-PAGE in the form of a dimer of M_r 75,000 dopamine-β-hydroxylase (DBH; E.D. 1.14.2.1) subunits as mentioned by Winkler[8].

In experiments with $(NH_4)_2SO_4$ fractionation (10–26% saturation) the M_r 150,000 protein showed DOPA decarboxylase activity. The activity increased twel-

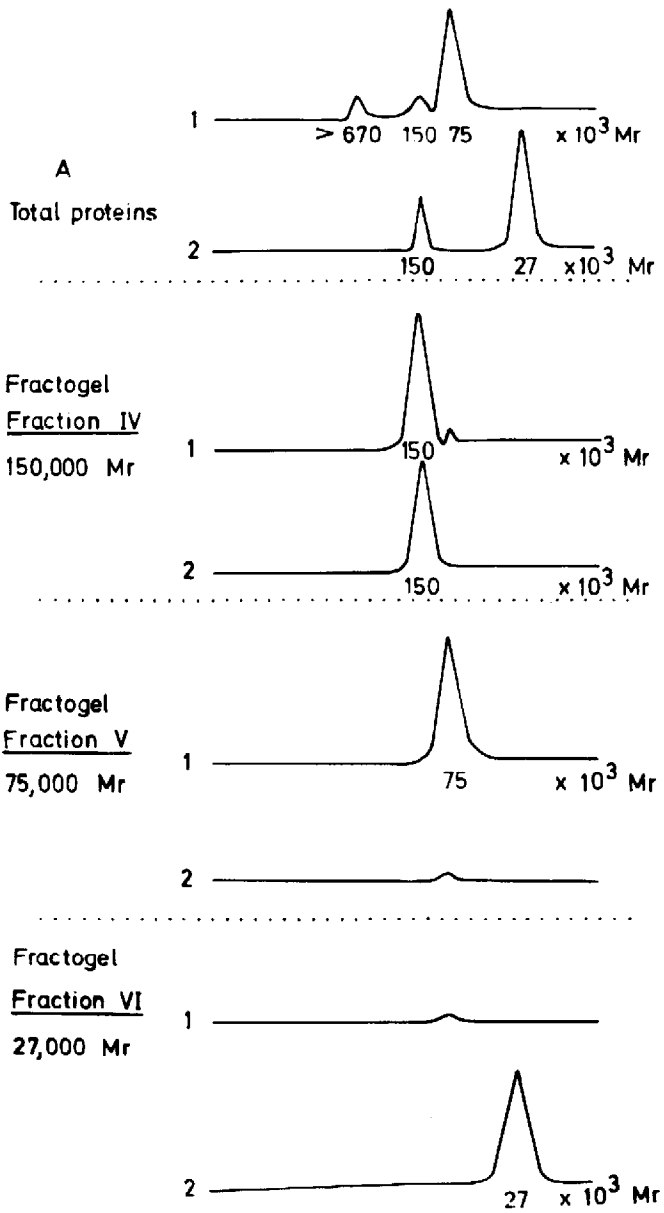

Fig. 6. Phenothiazine-Affigel affinity chromatography of the soluble protein isolated from the sympathetic nerves (A) and the Fractogel fractions IV VI. 1, Application buffer, 10 m*M* HEPES, pH 7.0, + 0.5 m*M* $CaCl_2$; 2, elution buffer, 10 m*M* Tris, pH 8.0, + 5.0 m*M* EGTA.

ve-fold compared to the total protein content (Fig. 6). The decarboxylation was less when 5-hydroxytryptophan was used as substrate than with DOPA (3,4-dihydroxyphenylalanine). The decarboxylase activity increased when the soluble protein was incubated in the presence of pyridoxal-5-phosphate, but decreased when the mem-

brane-bound form of the protein was used in the same incubation[9].

The Fractogel fraction V yielded a M_r 75,000 protein upon re-analysis by HPLC as well as by SDS-PAGE. It is well known[10] that DBH, a copper-containing enzyme[11] with M_r 290,000, resulted in subunits of M_r 75,000 upon SDS-PAGE.

Subunits of M_r 75,000 were found by quantitative electrofocusing in the p*I* 4.5–5.2 range and re-analysed by HPLC and SDS-PAGE[1]. After Fractogel filtration, DBH was only found in fraction V in form of its subunits with molecular weights of M_r 75,000 as also shown by HPLC and SDS-PAGE. It seems that the DBH was only detected in subunits of M_r 75,000 by these methods.

The Fractogel fraction VI contained protein with M_r 27,000 as shown by HPLC, and a single band of M_r < 12,000 as shown by SDS-PAGE. Proteins of M_r < 5000 were also found by the Fractogel separation under other experimental conditions. Proteins were identified by Klein *et al.*[12] as opioid peptides. Small peptides were also detected by the HPLC-TSK SW method (see Fig. 1).

In the Phenothiazine-Affigel affinity chromatography with Ca^{2+} in the application buffer and with EGTA in the elution buffer, the M_r 75,000 protein was not coupled to the Ca^{2+}–Phenothiazine-Affigel. A protein with a similar molecular weight was found in preparations of adrenal chromaffin granules and other glands by Winkler[8]. This protein is known as chromogranin A. Definitive conclusions about this protein fraction and chromogranin A cannot be made from the different methods used in this study. A relationship between the M_r 75,000 protein and chromogranin A is not evident.

50–70% of the M_r 150,000 protein (DOPA decarboxylase) was found to have affinity for the Ca^{2+}–Phenothiazine-Affigel and 30–50% was without affinity. The highest affinity for the Ca^{2+}–Phenothiazine-Affigel was found with the fraction of M_r 27,000. This protein detected by HPLC was found always as a single band with M_r < 12,000 by SDS-PAGE.

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