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Simultaneous purification of the neuroproteins synapsin I and synaptophysin

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ABSTRACT

A procedure for the simultaneous purification of synapsin I and synaptophysin from calf brain was developed. Demyelinated membranes were extracted with 2% Triton X-100 and 2 *M* KCl. The extracted proteins were separated by weak cation-exchange chromatography on carboxymethyl-Sepharose Fast Flow. Synaptophysin was finally purified by preparative sodium dodecyl sulphate–polyacrylamine gel electrophoresis and synapsin I by affinity chromatography using a calmodulin-Sepharose column. The recovery obtained was 40 μg/g in brain for synaptophysin and 25 μg/g in brain for synapsin I.

INTRODUCTION

During the 1980s, enormous progress was made in the isolation and characterization of small synaptic vesicles (SSV) from mammalian brain and Torpedo electrical organ [1,2]. Several membrane proteins of SSV have been identified [3–7], of which the synapsin family (synapsin Ia and b, IIa and b) and synaptophysin are structurally and functionally the best characterized [8,9].

Synapsin I was discovered by Johnson *et al.* [10] in 1972 as an endogenous substrate for protein kinases. The protein has been extensively studied [11,12]. It is exclusively present in neuronal tissue, where it is localized on SSV. Owing to its ability to bind different elements of the cytoskeleton and by the other hand synaptic vesicles, it is believed to play a major role in the regulation of neurotransmitter release [13,14].

Synaptophysin is the most abundant integral membrane protein of SSV [15–17]. The protein was identified by means of the monoclonal antibody SY 38, produced by immunization with intrinsic membrane proteins of SSV from mammalian brain. It is present in virtually all nerve terminals and it has also been associated with vesicles of neuroendocrine cells. However, synaptophysin is not present in other cell types [18].

The currently available procedures for purifying synapsin I involve a series of time-consuming chromatographic steps, generally resulting in low recoveries. In this paper we describe a simplified method that allows the simultaneous purification of synapsin I and synaptophysin, both with high recoveries.

EXPERIMENTAL

Materials

All reagents were of analytical-reagent grade. Phenylmethylsulphonyl fluoride (PMSF) was purchased from Sigma (St. Louis, MO, USA). All chromatographic media used in the isolation procedure were purchased from Pharmacia (Uppsala, Sweden). Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate was obtained from Bio-Rad Labs. (Richmond, CA, USA). [^{125}I]Protein A was purchased from ICN Biomedicals (Ir-

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vine, CA, USA). For the immunodetection of synaptophysin we used the commercial monoclonal antibody from Boehringer (Mannheim, Germany). The antiserum against synapsin I was kindly provided by Dr. P. De Camilli (New Haven, CT, USA) and Dr. P. Greengard (New York, NY, USA).

Isolation of proteins

The procedure described was designed using published methods [19–21] as guidelines. The purification procedure is outlined schematically in Fig. 1. Calf brain was obtained from the local slaughterhouse. The cortex was cleaned, cut into small pieces and frozen in liquid nitrogen prior to transport to the laboratory. The day after, the tissue (ca. 250 g wet weight) was thawed, minced and homogenized with a glass–PTFE homogenizer in ice-cold buffer A, 320 m*M* sucrose–5 m*M* NaH_2PO_4 (pH 7.0)–1 m*M* [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)–0.3 m*M* PMSF. All subsequent steps

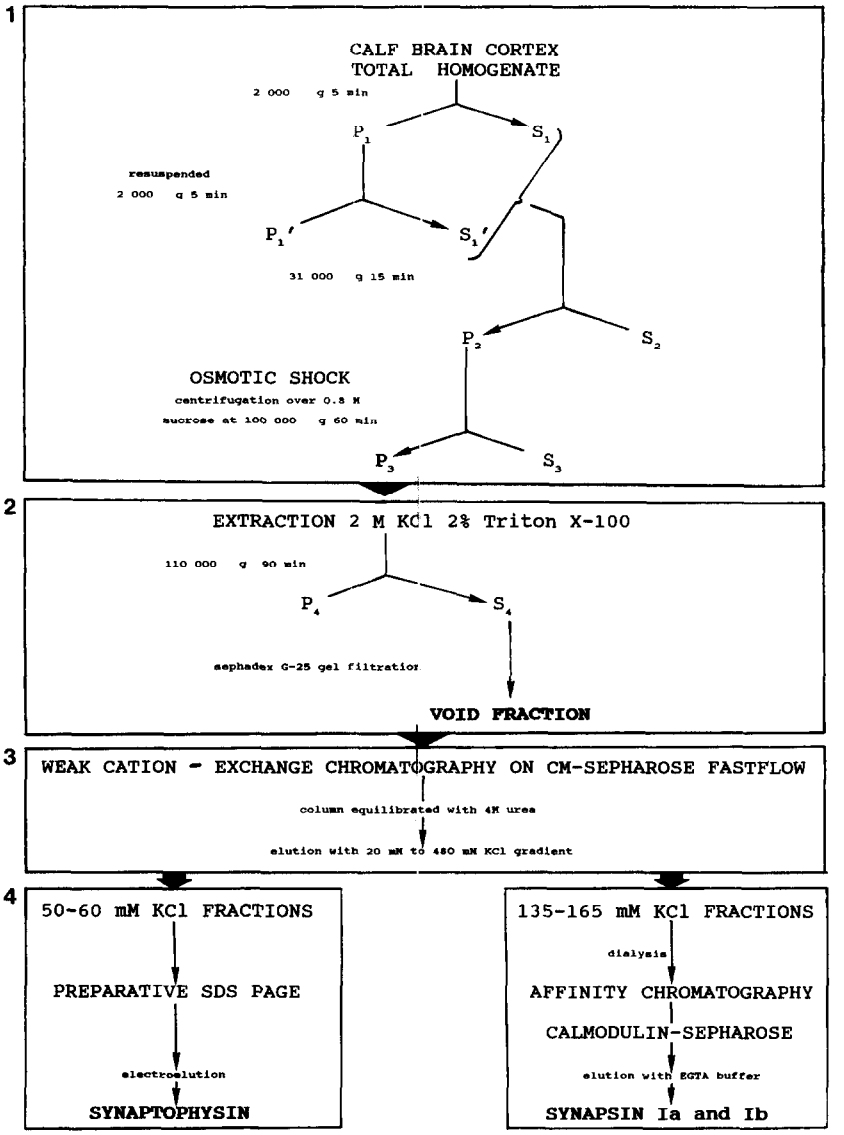

Fig. 1. Scheme of the isolation procedure for synapsin I and synaptophysin.

were performed at 4°C. The homogenate was diluted 1:8 (w/v) and centrifuged at 2000 *g* for 5 min. The pellet (P_1) was resuspended and centrifuged again. The combined supernatants (S_1 + S_1') were pelleted at 31 000 *g* for 15 min. The synaptosomal pellet (P_2) obtained was subjected to hyposmotic lysis by resuspension in buffer B [5 m*M* NaH_2PO_4 (pH 7.0)–1 m*M* EGTA–0.3 m*M* PMSF]. Subsequently, the suspension was centrifuged over a cushion of 0.8 *M* sucrose in buffer B at 100 000 *g* for 60 min. The pellet (P_3) was extracted with buffer C [5 m*M* NaH_2PO_4 (pH 7.2)–2 *M* KCl–2% (v/v) Triton X-100–0.3 m*M* PMSF] for 30 min at 37°C and centrifuged at 110 000 *g* for 90 min. The supernatant (S_4) was collected and subjected to chromatography on a Sephadex G-25 column (60 × 3 cm I.D.) equilibrated with buffer D [5 m*M* NaH_2PO_4 (pH 8.0)–20 m*M* KCl–0.1% Tween 20]. The void volume fraction was collected, diluted with one volume of 8 *M* urea and applied to a carboxymethyl-Sepharose Fast Flow column (16 × 1.6 cm I.D.), previously equilibrated with buffer D plus 4 *M* urea. After washing the unbound material with buffer D, bound proteins were eluted with a gradient from 20 to 480 m*M* KCl in buffer D.

Synaptophysin was highly enriched in the fractions eluting between 50 and 60 m*M* KCl. Final purification was achieved by subjecting these fractions to preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

The fractions eluting between 135 and 165 m*M* KCl were pooled and dialysed extensively against buffer E [5 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.2)–0.1 m*M* EGTA–0.1 m*M* dithiothreitol (DTT)–100 m*M* KCl]. $CaCl_2$ was added to the dialysed material to give a final concentration of 1 m*M*. Subsequently, it was applied to a calmodulin-Sepharose column (10 × 0.9 cm I.D.) equilibrated with buffer F [5 m*M* HEPES (pH 7.2)–1 m*M* $CaCl_2$–0.1 m*M* DTT–100 m*M* KCl]. The column was washed with six to seven bed volumes of buffer F, followed by ten bed volumes of buffer F but containing 500 m*M* KCl. Finally, synapsin I was eluted with buffer F containing 5 m*M* EGTA instead of 1 m*M* $CaCl_2$.

Quantification of synapsin I and synaptophysin in the different steps of purification was achieved by quantitative immunoblotting. Samples were separated by SDS-PAGE and proteins transferred to nitrocellulose membranes. The latter were incubated overnight with synapsin I and synaptophysin antibodies. The immunocomplexes were detected using [^{125}I]protein A (0.1 μCi/ml). After autoradiography, the labelled bands were cut and counted in a gamma scintillation counter (Packard, Cobra 5005). Curved relating the radioactivity bound to synapsin I and synaptophysin with the amount of protein loaded were constructed. From the slopes of the linear graphs the relative concentration of synapsin I and synaptophysin per milligram of protein was calculated for each sample.

Miscellaneous procedures

Proteins were measured by the method of Bradford [22]. With detergent-containing samples, the bicinchoninic acid (BCA) kit from Pierce (Rockford, IL, USA) was used. Bovine serum albumin was used as a standard.

Protein samples from each step were separated by SDS-PAGE using 12.5% gels according to the procedure of Laemmli [23]. Gels were stained with Coomassie Brilliant Blue (CBB) (Serva, Heidelberg, Germany). In preparative SDS-PAGE, protein bands were made visible by copper staining according to Lee *et al.* [24]. The stained protein band was cut out and the piece of gel destained by incubation in 0.25 *M* Tris (pH 9)–0.25 *M* EDTA. Synaptophysin was electroeluted using a Centrilutor from Amicon (Lexington, MA, USA). Immunoblotting was performed according to established procedures [25]. Prior to the incubation with the antibodies, the nitrocellulose membranes were stained reversibly with Ponceau S [26]. After destaining, the membranes were incubated with antibodies overnight. Alkaline phosphatase-conjugated second antibodies were used for immunodetection.

RESULTS AND DISCUSSION

The expression of synapsin I and synaptophysin has been reported to correlate with the period of synapse formation [27–30]. Therefore, calf brain cortex was chosen as a starting material for the purification procedure. It is known that both synapsin I and synaptophysin are highly sensitive to degradation by calcium-dependent proteases [19]. For this reason, EGTA and PMSF were included in all buffers to control proteolytic degradation.

The isolation procedure consists of four main steps (Fig. 1): (1) preparation of a demyelinated membrane faction (P_3); (2) extraction of proteins with 2 *M* KCl and 2% Triton X-100; (3) separation of the extracted proteins by weak cation-exchange chromatography on carboxymethyl-Sepharose (CM-Sepharose FF); and (4) final purification of each protein.

The first step allows the removal of myelin-enriched membranes, which float as a white dense layer on the 0.8 *M* sucrose cushion. Myelin basic proteins are major contaminants in synapsin I preparations [19].

Second, the demyelinated membranes were extracted with a combination of high ionic strength (2 *M* KCl) and a non-ionic detergent (2% Triton X-100) containing buffer. Because synapsin I is a peripheral membrane protein and synaptophysin is an integral membrane protein of SSV, both proteins can be extracted simultaneously using this buffer. The extracted material (S_4) was immediately desalted by gel filtration on Sephadex G-25. We chose this procedure to avoid degradation of the proteins by a long period of dialysis.

Owing to its high isoelectric point (10.5), synapsin I can be bound to the weak cation-exchange column (CM-Sepharose FF) at pH 8.0, whereas most of the contaminating proteins remain unbound at the given pH. Synaptophysin also binds to the column but the nature of the interaction with the gel matrix is not clear, as the isoelectric point of synaptophysin is 4.8 [13]. Perhaps urea denatures the protein, exposing highly positive groups, which allows the binding to CM-Sepharose FF. Fig. 2A

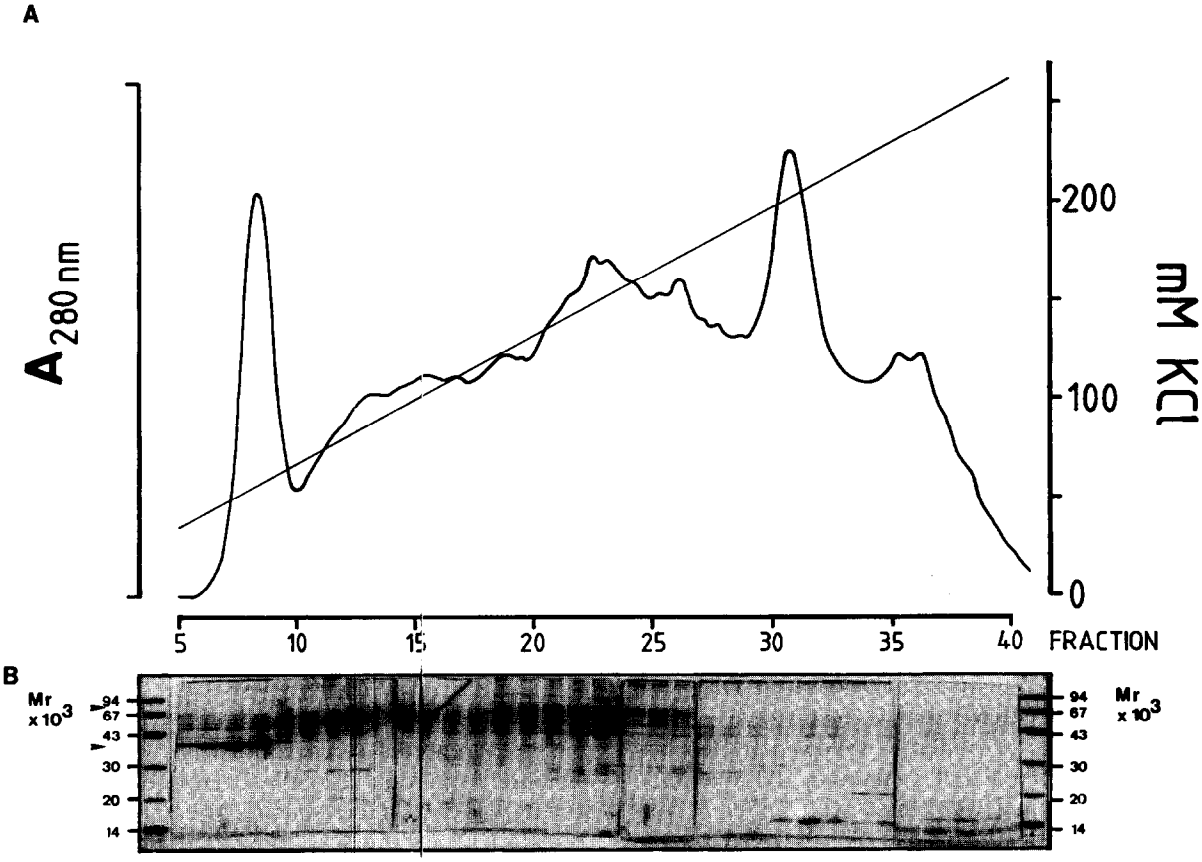

Fig. 2. Weak cation-exchange chromatography. (A) Elution profile (absorbance at 280 nm) of the CM-Sepharose FF column. The bound material was eluted with a gradient from 20 to 480 m*M* KCl. (B) Corresponding SDS-PAGE of the different fractions eluted from the column. Arrowheads point to the positions of synapsin I (M_r = 80 000–85 000) and synaptophysin (M_r = 38 000).

shows the elution profile for the column using a salt gradient from 20 to 480 m*M* KCl. The SDS-PAGE pattern from the different fractions is shown in Fig. 2B. Using polyclonal anti-synapsin I and monoclonal anti-synaptophysin antibodies, the two proteins could be identified. The corresponding immunoblots are shown in Fig. 3A and B, respectively. The binding of synaptophysin to the CM-Sepharose FF is weak because synaptophysin was eluted from the column at low ionic strength (between 50 and 60 m*M* KCl). As reflected by SDS-PAGE, synaptophysin is almost pure after CM-Sepharose FF chromatography (Fig. 2B, fractions 5–9). The final purification was achieved by preparative SDS-PAGE, followed by electroelution of the protein. The protein band at M_r 38 000 was excised and used for raising polyclonal antibodies in rabbits.

On the other hand, synapsin I-containing fractions (Fig. 2B, fractions 25–30) are still contaminated by several proteins. In contrast, Krebs *et al.* [21] purified synapsin I using a similar gel (CM-52 from Whatman). In addition to this gel type we also tested CM-Sephadex. At least in our hands, both gel types gave unsatisfactory results. Therefore, an additional purification step had to be developed. Synapsin I was identified as a Ca^{2+}-dependent calmodulin-binding protein by Okabe and Sobue [20]. Taking advantage of this property of synapsin I, we achieved the final purification of the protein using calmodulin affinity chromatography. Fig. 4A shows the elution profile of the calmodulin-Sepharose column. The first peak corresponds to the Ca^{2+}-independent bound proteins that were eluted at high ionic strength. Synapsin I was eluted by chelating Ca^{2+} ions with EGTA. As shown in Fig. 4B, the CBB pattern of pure synapsin I resembles the corresponding immunoblot fairly well, but there are some differences (see below).

Table I summarizes the protein recovery and the enrichment factor for the different fractions obtained during the purification procedure. Synaptophysin was enriched 386 times over total homogenate in the peak fraction eluted from the CM-Sepharose FF. A similar value has been reported by Navone *et al.* [18] using affinity chromatography with an anti-synaptophysin monoclonal antibody. The recovery was *ca.* 40 μg of synaptophysin per gram of brain. On the other hand, synapsin I was

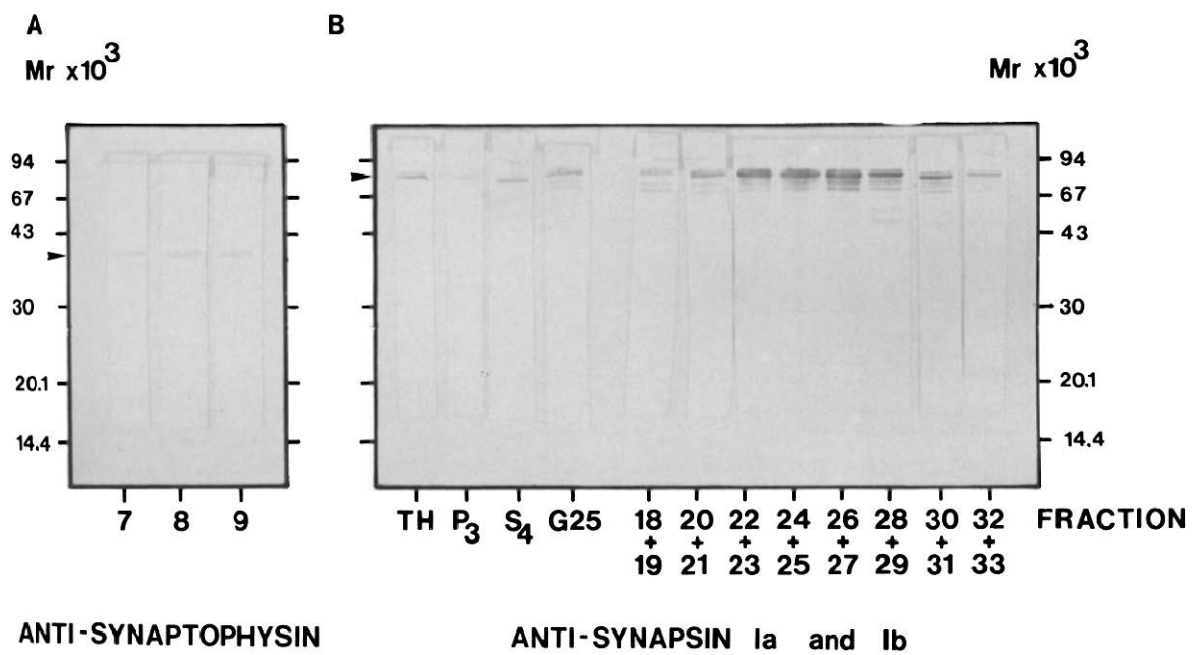

Fig. 3. Immunoblot of the eluted fractions from the CM-Sepharose FF column. (A) Synaptophysin-containing fractions (fractions 7–9); (B) synapsin I-containing fractions (fractions 20–30).

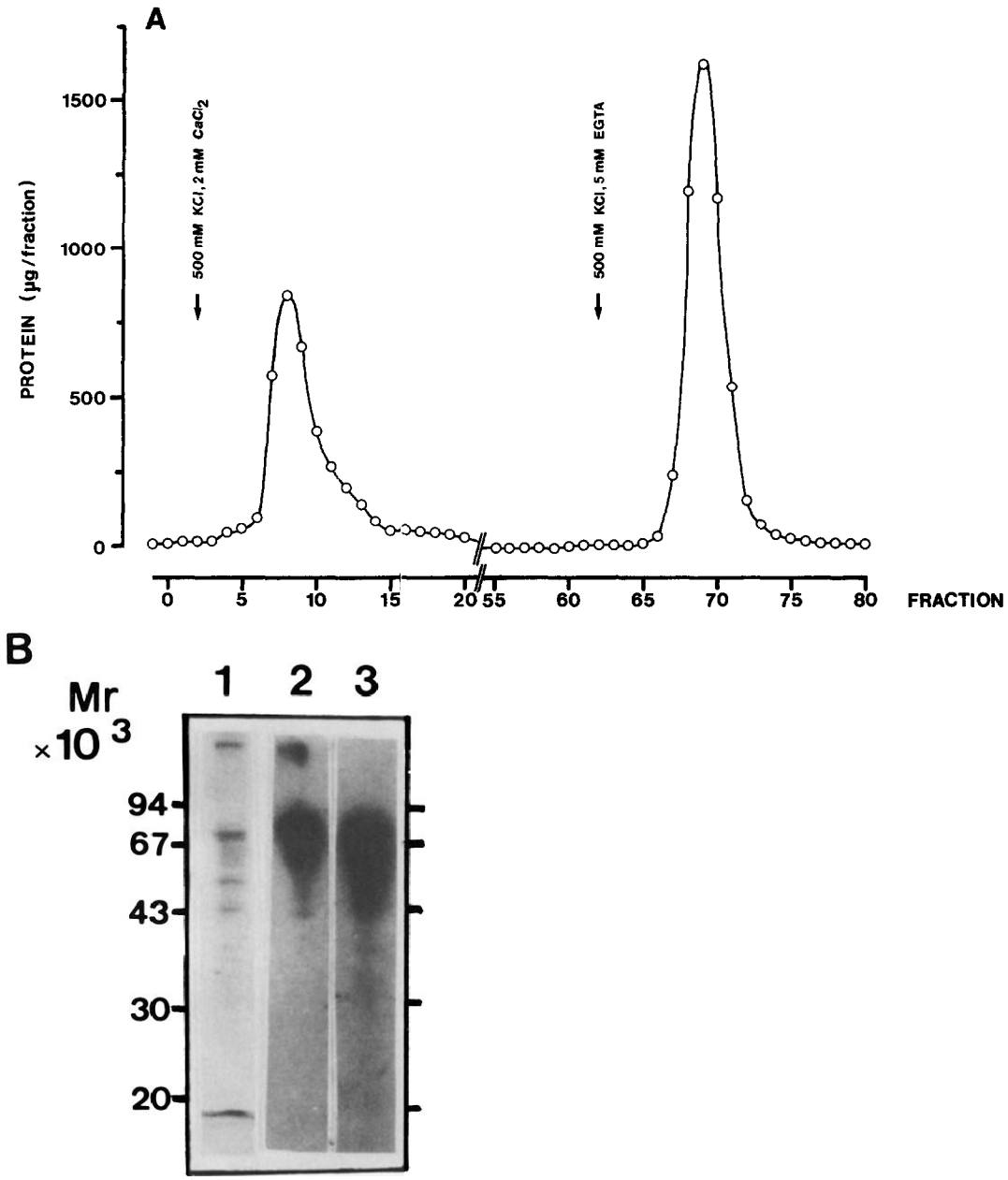

Fig. 4. Calmodulin affinity chromatography. (A) Protein elution profile of the column. Non-specific bound material was washed with buffer F plus 500 m*M* KCl. Synapsin I was eluted with buffer F plus 1 m*M* EGTA. (B) SDS-PAGE and corresponding immunoblot using [^{125}I]protein A for immunodetection. Lanes: 1 = CBB staining; 2 = 6 h exposure; 3 = 18 h exposure (5 µg of protein loaded per lane).

enriched only 87 times over total homogenate in the peak fraction eluting from the calmodulin column. The recovery was 25 µg of synapsin I per gram of brain. The original method of Ueda and Greengard [31] for the purification of synapsin I involved several chromatographic steps resulting in a low recov-

TABLE I

PROTEIN RECOVERY AND RELATIVE ENRICHMENT OF SYNAPSIN I AND SYNAPTOPHYSIN IN THE VARIOUS FRACTIONS OBTAINED IN THE COURSE OF THE PURIFICTION PROCEDURE

	Protein (%)	Enrichment factor[a]	
		Synapsin I	Synaptophysin
Total homogenate	100	1.00	1.00
S_1	57.5	0.91	0.90
P_2	27.2	0.83	1.42
P_3	19.1	1.62	1.31
Sephadex G-25	5.8	2.73	1.38
CM-Sepharose FF total bound	0.37	N.D.[d]	N.D.
CM-Sepharose FF fraction:			
7	0.027[b]	N.D.	84.7
8		N.D.	355.3
9		N.D.	386.2
10		N.D.	138.7
26	0.056[c]	25.6	N.D.
27		26.3	N.D.
28		40.0	N.D.
29		32.8	N.D.
30		29.8	N.D.
Calmodulin-Sepharose peak fraction	0.031	87.0	N.D.

[a] Enrichment factors for synapsin I and synaptophysin were calculated as described under Experimental. The values correspond to a typical experiment that was repeated at least four times with similar results.
[b] Percentage of protein recovered in fractions 7–10.
[c] Percentage of protein recovered in fractions 26–30.
[d] N.D. = Not determined.

ery (0.4 μg/g brain). Later, the method was modified by Schiebler *et al.* [32] and Bähler and Greengard [14], giving ten times higher recoveries (ca. 4 μg/g brain). A similar approach was used by Baines and Bennett [33] but with recoveries 5–6 times higher (20–23 μg/g brain). In 1986, Krebs *et al.* [21] published a rapid and simple method for the purification of synapsin I. Their recovery (80 μg/g brain) was much higher than any of the previous published methods. However, we were not able to reproduce the method with similar recoveries. If we assume that synapsin I comprises 0.5% of the total brain membrane protein [19], we recovered *ca.* 14% of the initial synapsin I. This low recovery can be atributed to several factors. First, synapsin I is a very "sticky" protein and adsorbs on tubes and surfaces, giving underestimates of protein measurements. Second, the presence of Ca^{2+} ions faciliates degradation of purified synapsin I [34]. As we eluted the calmodulin column with 5 m*M* $CaCl_2$, this degradation is inevitable. Breakdown products can be recognized in the immunoblots after longer incubation periods. Probably this also explains why the CBB staining does not match completely with the corresponding immunoblot (Fig. 4B). Some of the degradation products probably are not recognized by the polyclonal antibody. Degradation of synapsin I has also been reported *in vivo* in nerve terminals originating from sciatic nerve [35]. Moreover, recently it has been shown *in vitro* that synapsin I phosphorylated by Ca^{2+},calmodulin-dependent protein kinase II is able to self-degrade [36].

With the present method of purification, the recovery (25 μg of synapsin I per gram of brain) is similar to that reported by Bennett *et al.* [19] (20–23 μg/g brain). The additional advantage of this method is the simultaneous purification of synaptophysin with a high recovery (40 μg of synaptophysin per gram of brain). Until now, the methods for the purification of synapsin I consisted in the extraction of the protein starting from demyelinated membranes, followed by chromatography on carboxymethylcellulose [19]; a major contaminant protein has M_r = 38 000. This protein is also present in our chromatography on the CM-Sepharose FF column. However, we were able to identify this protein as synaptophysin. This positive identification of synaptophysin enables us to purify both the proteins synapsin I and synaptophysin simultaneously with a high recovery in a shorter time using a fairly simple method.

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REFERENCES

1 E. Floor, S. F. Schaeffer, B. E. Feist and S. E. Leeman, *J. Neurochem.*, 50 (1988) 1588.
2 V. P. Whittaker, *Ann. N.Y. Acad. Sci.*, 493 (1987) 77.
3 E. Floor and S. E. Leeman, *J. Neurochem.*, 50 (1988) 1597.
4 M. Baumert, P. R. Maycox, F. Navone, P. De Camilli and R. Jahn, *EMBO J.*, 8 (1989) 379.
5 M. Baumert, K. Takei, J. Hartinger, P. M. Burger, G. Fisher von Mollard, P. R. Maycox, P. De Camilli and R. Jahn, *J. Cell Biol.*, 110 (1990) 1285.
6 S. Fournier and J. M. Trifaró, *J. Neurochem.*, 50 (1988) 27.
7 M. Bähler, R. L. Klein, J. K. T. Wang, F. Benfenati and P. Greengard, *J. Neurochem.*, 57 (1991) 423.
8 T. C. Südhof and R. Jahn, *Neuron*, 6 (1991) 665.
9 P. De Camilli and R. Jahn, *Annu. Rev. Physiol.*, 52 (1990) 625.
10 E. M. Johnson, T. Ueda, H. Maeno and P. Greengard, *J. Biol. Chem.*, 247 (1972) 5650.
11 E. J. Nestler and P. Greengard, *Prog. Brain Res.*, 69 (1986) 323.
12 T. C. Südhof, A. J. Czernik, H. Kao, K. Takei, P. A. Johnston, A. Horiuchi, S. D. Kanazir, M. A. Wagner, M. S. Perin, P. De Camilli and P. Greengard, *Science (Washington, D.C.)*, 245 (1989) 1474.
13 N. Hirokawa, K. Sobue, A. Harada and H. Yorifuji, *J. Cell Biol.*, 108 (1989) 111.
14 M. Bähler and P. Greengard, *Nature (London)*, 326 (1987) 704.
15 B. Wiedenmann and W. Franke, *Cell*, 41 (1985) 1017.
16 R. Jahn, W. Schiebler, C. Ouimet and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 4137.
17 H. Rehm, B. Wiedenmann and H. Betz, *EMBO J.*, 5 (1986) 535.
18 F. Navone, R. Jahn, G. Di Gioia, H. Stukenbrok, P. Greengard and P. De Camilli, *J. Cell Biol.*, 103 (1986) 2511.
19 V. Bennett, A. J. Baines and J. Davis, *Methods Enzymol.*, 134 (1986) 55.
20 T. Okabe and K. Sobue, *FEBS Lett.*, 213 (1987) 184.
21 K. E. Krebs, I. S. Zagon and S. R. Goodman, *Brain Res. Bull.*, 17 (1986) 237.
22 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
23 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
24 C. Lee, A. Levin and D. Branton, *Anal. Biochem.*, 166 (1987) 308.
25 H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350.
26 R. H. Aebersold, J. Laevitt, R. A. Saavedra, L. E. Hood and S. B. H. Kent, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 6970.
27 S. M. Lohmann, T. Ueda and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 4037.
28 P. Knaus, H. Betz and H. Rehm, *J. Neurochem.*, 47 (1986) 1302.
29 C. A. Haas and L. J. De Gennaro, *J. Cell Biol.*, 106 (1988) 195.
30 N. Leclerc, P. W. Beesley, I. Brown, M. Colonnier, J. W. Gurd, T. Paladino and R. Hawkes, *J. Comp. Neurol.*, 280 (1989) 197.
31 T. Ueda and P. Greengard, *J. Biol. Chem.*, 252 (1977) 5155.
32 W. Schiebler, R. Jahn, J. P. Doucet, J. Rothlein and P. Greengard, *J. Biol. Chem.*, 261 (1986) 8383.
33 A. Baines and V. Bennett, *Nature (London)*, 315 (1985) 410.
34 R. Jahn, personal communication.
35 S. Bööj, P.-A. Larsson, A.-G. Dahllöf and A. Dahlström, *Acta Physiol. Scand.*, 128 (1986) 155.
36 A. N. Gubin and S. E. Severin, *FEBS Lett.*, 289 (1991) 11.