PURIFICATION OF SOYBEAN AGGLUTININ BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE-*N*-ε-AMINOCAPROYL-β-D-GALACTOPYRANOSYLAMINE

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1. Introduction

Affinity chromatography has been successfully used for the purification of proteins with specific binding sites, such as enzymes, antibodies and lectins [1–3]. Soybean agglutinin (SBA), a lectin isolated from soybean oil meal [4, 5] has been shown to bind specifically *N*-acetyl-D-galactosamine and D-galactose [6]. This property has now been utlized by us for the purification of SBA on a column made of a conjugate of Sepharose and *N*-ε-aminocaproyl-β-D-galactopyranosylamine (SAG).

The method used for the preparation of SAG was essentially the same as that developed very recently by Blumberg et al. [7] for the binding of β-L-fucopyranosylamine to Sepharose. *N*-ε-aminocaproyl-β-D-galactopyranosylamine was prepared by a reaction of β-D-galactopyranosylamine (1-β-amino-1-deoxy-D-galactopyranoside) and *N*-benzyloxycarbonyl-ε-aminocaproic acid [8], and removal of the benzyloxycarbonyl group by hydrogenolysis. The product was coupled to Sepharose by the cyanogen bromide method of Axén et al. [9] as described by Blumberg et al. [10]. SBA was adsorbed from a partially purified extract of soybean oil meal to a column made of SAG, and the active material was eluted from the column by a solution of D-galactose. Separation of SBA from minor agglutinating components present in the soybean oil meal [11] was achieved by chromatography on DEAE cellulose. From 50 g of soybean oil meal, 80 mg of purified SBA, with a specific activity of 7–8000 hemagglutinating units/ml, was thus obtained.

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2. Materials and methods

Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), D-galactose from Pfanstiehl Lab. Inc. (Waukegan, Ill.), cyanogen bromide from Fluka A.G. (Buchs, Switzerland) and DEAE-cellulose from Biorad (Richmond, Calif.). All other reagents were commercial preparations of the highest purity available. Protein was determined according to Lowry et al. [12] and hemagglutinating activity by the spectrophotometric method of Liener [13]. Soybean oil meal was kindly supplied by Etz Hazait factory, Petah Tikva, Israel. The meal was taken out from the processing line after extraction of the oil with *n*-hexane. Removal of the solvent was carried out in the laboratory by drying in a hood at room temp.

2.1. *Preparation of β-D-galactopyranosylamine (1-β-amino-1-deoxy-D-galactopyranoside)*

This compound was prepared essentially as described [14, 15]. D-Galactose (27 g, 150 mmoles) was dissolved in 200 ml of liquid ammonia in methanol (30%, v/v). After standing in a closed vessel at room temp. for 1 week, the precipitate formed, which consisted of α-D-galactopyranosylamine, was discarded. The supernatant was kept in a closed vessel in a hood at room temp. for another 3–4 days; during this period the vessel was opened every day for 1–2 hr to permit the evaporation of excess ammonia. Under these conditions, the desired β-isomer of D-galactopyranosylamine crystallized. It

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was collected by filtration, washed with absolute methanol and dried in a vacuum dessicator over NaOH. Yield ca: 25%; m.p. 136–137°. Found: C, 40.04; H, 7.12; N, 7.64. $C_6H_{13}O_5N$ requires C, 40.22; H, 7.31; N, 7.82.

2.2. *Preparation of N-(N-benzyloxycarbonyl-ε-aminocaproyl)-β-D-galactopyranosylamine*

Isobutylchloroformate (30 mmoles) and triethylamine (30 mmoles) were added to a solution of *N*-benzyloxycarbonyl-ε-aminocaproic acid [8] (30 mmoles) in dimethylformamide (50 ml), kept at −5°. The mixture was stirred for 20 min at −5° and then filtered. The filtrate was immediately added to a suspension of β-D-galactopyranosylamine (25 mmoles) in dimethylformamide (50 ml) and the mixture stirred at room temp. until the solution was clear (1 hr). The solution was allowed to stand at room temp. for 16 hr after which the solvent was removed in vacuo. The residue was crystallized twice from ethanol. Yield 60%; m.p. 159–160°. Found: C, 54.33; H, 7.06; N, 6.25; N, 6.25. $C_{20}H_{30}O_8N_2.H_2O$ requires C, 54.04; H, 7.26; N, 6.30.

2.3. *Preparation of N-ε-aminocaproyl-β-D-galactopyranosylamine*

N-(*N*-benzyloxycarbonyl-ε-aminocaproyl)-β-D-galactopyranosylamine (10 mmoles) in 80% aqueous methanol (100 ml) was hydrogenated over palladium on charcoal (10%, 100 mg) at atmospheric pressure for 8 hr. The mixture was filtered and the filtrate concentrated in vacuo. The residue was crystallized from methanol–ether. Yield 75%; m.p. 206–208°. Found: C, 49.22; H, 8.42; N, 9.64. $C_{12}H_{24}O_6N_2$ requires C, 49.30; H, 8.28; N, 9.58.

2.4. *Preparation of the conjugate of Sepharose and N-ε-aminocaproyl-β-D-galactopyranosylamine (SAG)*

Cyanogen bromide (10 g) was stirred with water (100 ml) for 10 min, when most of it dissolved. A slurry of Sepharose 4B (100 ml), washed with water, was then added to the cyanogen bromide–water mixture. The reaction mixture was adjusted to pH 11 and maintained at this pH for 6 min by adding 4 N NaOH. The temperature of the reaction mixture was kept below 26° by the addition of crushed ice. The activated Sepharose was washed rapidly with 15 vol of cold water on a Buchner funnel. The wet Sepharose was then added quickly to a solution of *N*-ε-aminocaproyl-β-D-galactopyranosylamine (1.6 mmoles in 40 ml of 0.5 M $NaHCO_3$) and the mixture was stirred gently for 16 hr at 4°. The conjugate (SAG) was filtered and washed thoroughly with 0.1 M $NaHCO_3$ and water. The amount of D-galactose derivative covalently bound to the Sepharose was determined by hydrolysis of SAG in 6 N HCl (22 hr, 110°) and estimation of the liberated ε-aminocaproic acid on a Beckman-Spinco amino acid analyser. SAG was found to contain 2 μmoles of covalently bound *N*-ε-aminocaproyl-β-D-galactopyranosylamine per ml.

2.5. *Purification of SBA*

Untoasted defatted soybean oil meal (50 g) was extracted with saline (250 ml) for 1 hr at room temp. with constant stirring. The insoluble residue was removed by centrifugation (10 min at 6,000 rpm in a Sorvall RC 2 centrifuge), and the supernatant was cooled to 4°. The following steps were all carried out in the cold room. To the supernatant was added $(NH_4)_2SO_4$ (30 g/100 ml), the precipitate was removed by centrifugation (10 min, 6,000 rpm) and discarded. An additional amount of $(NH_4)_2SO_4$ was added to the supernatant (25 g/100 ml) and the precipitate was collected as above. It was dissolved in a minimal volume of water and dialyzed extensively, first against distilled water and finally against saline. The dialyzed solution was centrifuged to remove any precipitate formed and was applied to a column (2.4 × 30 cm) of SAG, which had previously been washed with 1 l of saline. The column was washed with saline (approx. 500 ml) until no significant amount of material absorbing at 280 nm was detected in the effluent (absorbancy < 0.1). Elution of the agglutinin was carried out with 200 ml of a solution of D-galactose in saline (5 mg/ml). Fractions of 10 ml were collected at a rate of 100 ml/hr and monitored at 280 nm. The fractions containing U.V. absorbing material were pooled, dialyzed against distilled water and lyophilized.

3. Results and discussion

The purification procedure described above involves only three steps: extraction of the soybean oil

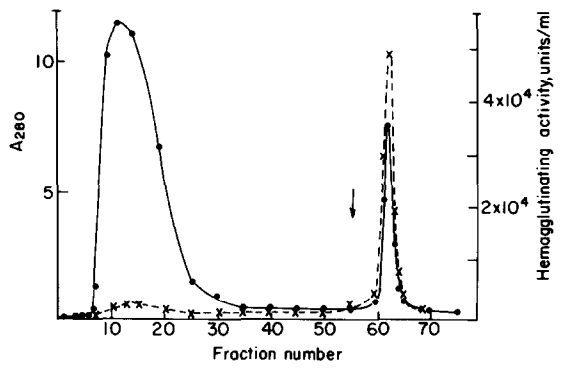

Fig. 1. Chromatography of partially purified soybean agglutinin (SBA) on a column of Sepharose-*N*-ε-aminocaproyl-β-D-galactopyranosylamine (SAG). One hundred ml of the 30–55% ammonium sulfate fraction, after dialysis, was applied to a 2.4 × 30 cm column, containing 100 ml of SAG equilibrated with saline. The column was washed with saline (about 500 ml) and SBA eluted with D-galactose (0.5% in saline). Chromatography was performed at 4°. Fractions of 10 ml were collected at a rate of 100 ml/hr. Arrow denotes application of D-galactose. Absorbance at 280 nm (•—•—•); hemagglutinating activity (x—x—x).

meal with saline, fractionation with ammonium sulfate and affinity chromatography on a column made of a conjugate of Sepharose and *N*-ε-aminocaproyl-β-D-galactopyranosylamine. Ammonium sulfate precipitation resulted in a 5-fold purification of SBA with 30% yield. The bulk of the proteins present in the active ammonium sulfate fraction was not absorbed to the column (fig. 1), but these proteins exhibited only little hemagglutinating activity. The agglutinin was quantitatively (> 90%) eluted from the column with a dilute (0.5%) solution of D-galactose, affording 10-fold purification. The yield was 80 mg/50 g soybean oil meal with a specific activity of 7000–8000 units/mg. This yield is from 4 to 6 times higher than that obtained by the method previously described for the isolation of SBA [5]; the specific activity is also higher. The increase in specific activity may be due to the fact that in the affinity chromatography procedure, we have eliminated two steps – acid precipitation and dialysis against alcohol – which may have caused partial inactivation of SBA. In this connection, it is of interest to note that denaturation of soybean proteins by mildly acid pH's have been recently reported [16].

The protein fraction eluted from the SAG column with D-galactose was comprised mainly of the major hemagglutinin (SBA) present in soybean oil meal, but also contained trace amounts of the minor hemagglutinins known to occur in the meal [11]. These could be removed from SBA by chromatography on DEAE-cellulose under the conditions previously described [11]. The major peak obtained at 90% yield was pooled, dialyzed and lyophilized, giving a final product with a specific activity of 7000–8000 units/mg. It was homogeneous on disc electrophoresis at pH 4.5 (7.5% gel, 1.5 mA per column, staining with Coomassie Blue) and migrated at the same rate as SBA prepared by the previous procedure [5]. Its amino acid composition and carbohydrate content were also in excellent agreement with those of the earlier SBA preparations.

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References

[1] P. Cuatrecasas, M. Wilchek and C.B. Anfinsen, Proc. Natl. Acad. Sci. U.S. 61 (1968) 636.
[2] P. Cuatrecasas and C.B. Anfinsen, Ann. Rev. Biochemistry 41 (1971) 253.
[3] N. Sharon and H. Lis, Science, in press.
[4] I.E. Liener and M.J. Pallansch, J. Biol. Chem. 197 (1952) 29.
[5] H. Lis, N. Sharon and E. Katchalski, J. Biol. Chem. 241 (1966) 684.
[6] H. Lis, B.A. Sela, L. Sachs and N. Sharon, Biochim. Biophys. Acta 211 (1970) 582.
[7] S. Blumberg, J. Hildesheim, J. Yariv and K.J. Wilson, Biochim. Bioophys. Acta 264 (1972) 171.
[8] R. Schwyzer, B.M. Iselin and M. Feurer, Chem. Abstr. 56 (1962) 4864.
[9] R. Axén, J. Porath and S. Ernback, Nature 214 (1967) 1302.
[10] S. Blumberg, I. Schechter and A. Berger, European J. Biochem. 15 (1970) 97.
[11] H. Lis, C. Fridman, N. Sharon and E. Katchalski, Arch. Biochem. Biophys. 117 (1966) 301.

[12] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 188 (1951) 265.
[13] I.E. Liener, Arch. Biochem. Biophys. 54 (1955) 223.
[14] C.A. Lobry de Bruyn and F.H. van Leent, Rec. Trav. Chim. Pays-Bas 14 (1895) 134.
[15] H.S. Isbell and H.L. Frush, J. Org. Chem. 23 (1958) 1309.
[16] A.M. Nash, W.K. Kwolek and W.J. Wolf, Cereal Chem. 48 (1971) 360.