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Procedure for the purification of streptavidin by hydrophobic interaction chromatography

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ABSTRACT

A procedure is described for the purification of hydrophobic microbial proteins such as streptavidin from *Streptomyces avidinii*, using Benzyl-DC bead cellulose as the column material. The separation is rapid with a high loading capacity and sufficient resolution for preparative uses. Advantages are discussed especially for industrial purposes.

INTRODUCTION

Streptavidin, an extracellular fermentation product from a culture of *Streptomyces avidinii*, shows a high affinity for the vitamin biotin and for biotin-coupled compounds. Its association constant with biotin is 10^{15} l/mol. It is becoming increasingly important as a tool in immunology and other affinity techniques in medicine, biochemical analysis and genetic engineering.

Some applications [1,2] are in the isolation and purification of biotinylated substances by affinity chromatography, as an affinity marker for identification studies by fluorescence or electron microscope methods, as an inhibitor of biotinylated bacteriophages similarly to an antibody–antigen complex in studies of molecular interactions with components of cellular surfaces, for substitution of radioimmunoassays by non-radioactive enzyme-linked immunosorption assays (ELISA) using biotinylated peroxidase or alkaline phosphatase, and in non-radioactive DNA/RNA probes.

Previously the purification of this protein was accomplished with affinity chromatography as the most important step [3,4]. This technique is very effective for the preparation of small amounts but there are some disadvantages with reagard to an

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industrial process, *e.g.*, high cost of the column material and a complicated regeneration process of the matrix.

A spherical bead cellulose named Divicell (DC) is produced by VEB Leipziger Arzneimittelwerk (Leipzig, G.D.R.). Well-known modified bead celluloses include DEAE-DC and CM-DC for ion-exchange chromatography and Concanavalin A-DC and Cibacron Blue F-3-GA-DC for affinity chromatography. In this work we used the recently introduced Benzyl-DC bead cellulose.

EXPERIMENTAL

Streptomyces avidinii ATCC 27419 was grown in a batch culture using a 30-l fermenter system (Mytron, Heiligenstadt, G.D.R.) with a working volume of 12 l. The strain was precultured on a medium consisting of 1 l of aqueous culture medium containing 4 g of meat extract, 4 g of peptone, 5 g of NaCl, 1 g of yeast extract and 5% glucose as a carbon source, adjusted to pH 6.8. The same composition was used as a medium for the production of streptavidin. During the growth the pH was periodically corrected to the original value. The temperature, the stirrer speed (300 rpm) and the amount of dissolved oxygen were kept constant (aeration 144 l/h at the beginning and 288 l/h after 13 h).

Cells were separated from the culture broth by centrifugation after a cultivation time of 72 h. The protein in the supernatant was concentrated by precipitation with acetone to a final concentration of 75% (v/v). After dissolving the filtered precipitate in the minimum volume of phosphate buffer (0.075 *M* potassium sodium hydrogenphosphate + 0.188 *M* ammonium sulphate, pH 6,8) it was dialysed against the same buffer overnight.

The filtered dialysed crude protein solution was subjected to size-exclusion chromatography on a Spherogel TSK 3000 SW column (300 × 7.5 mm I.D.) (Beckmann) under isocratic conditions using the above-mentioned phosphate buffer. A substantial part of inactive protein and brownish associated fermentation products were removed by this procedure. One-minute fractions were collected and the biologically active fractions were pooled and concentrated by acetone precipitation as described above. Thereafter the precipitate was redissolved in 0.75 *M* ammonium sulphate solution (pH 6.8) (buffer A) and dialysed against the same buffer.

A stainless-steel column (250 × 4 mm I.D.) was filled with buffer-slurried Benzyl-DC using a commercial filling device combined with a high-performance liquid chromatographic pump (Shimadzu LC-6A). The filling was done at a constant pressure of 2 MPa. For separation runs the column was used in the opposite direction to that for filling. The separation conditions and the time programme for buffer gradients are given in the figure legends.

The loading capacity was measured by runs with increasing sample volumes of purified streptavidin solution. Saturation was reached if a biologically active component was measured in the breakthrough fractions under constant application conditions.

The principle of manufacture of the matrix Divicell involves the decomposition (regeneration) of drop-shaped distributed soluble derivatives of cellulose (especially esters and xanthates). The production process is started by viscose, which is dispersed in chlorobenzene to coagulate and regenerate. It is possible to adjust the size of the

particles in the range 20–1000 μm and the porosity in the range 70–90%. The resulting pore-size distribution from 1 to 50 nm guarantees a size-exclusion limit of $7 \cdot 10^6$ daltons. The activation and coupling of the ligand were achieved according to Büttner *et al.* [5].

ELISA with a biotinylated horseradish peroxidase-catalysed dye formation as the detection system was performed according to Kohen *et al.* [6]. The protein content in the fractions was measured by a modified Bradford method [7]. The purity of the protein was proved by sodium dodecyl sulphate (SDS) electrophoresis with 12.5% cross-linked polyacrylamide vertical gel plates according to Neville [8].

RESULTS AND DISCUSSION

The proposed purification process for streptavidin was developed as a model for the separation of proteins with a high degree of hydrophobic domains. The following main demands must be accomplished by such a process:

(1) The resolution and capacity of the column material must be increased by activation of the unmodified bead cellulose by reaction with a chloroformic acid ester [5] followed by benzylation with benzylamine [9]. The capacity was adjusted by applying a reaction ratio of the compounds to give the same results as those with phenylated Sepharose from Pharmacia. The resolution was compared with Spherogel TSK Phenyl-5PW (Beckman).

(2) It must be possible to scale up the process easily and economically. The production costs for Benzyl-DC are considerably lower than those for other column materials.

(3) The separation process should be achieved rapidly with high flow-rates. Therefore, it was necessary to test the stability of the column material under the influence of increased pressure.

The properties of the Divicell bead celluloses are as follows. The material is of high rigidity and mechanical stability owing to the spherical shape and a density of about 1. Chemical modifications and small particle sizes increase these properties. The physical structure of the beads is characterized by a variable pore size in the outer surface and a large inner volume which takes part in the exchange reactions. The chemical structure of the support and surface of the beads is highly inert and therefore the biomolecules remain uncharged. This is why the material is favoured for use in the chemical modification and immobilization of biologically active compounds [10].

Separation of streptavidin by hydrophobic interaction chromatography on a Spherogel TSK Phenyl-5PW column is shown by Fig. 1. Peaks 1, 2 and 3 are unidentified contaminants in the fractions with retention time up to 4.8 min. Peaks 4 and 5 were identified as active streptavidin. They were eluted with different salt concentrations in a negative non-linear gradient. Both fractions had the same biological activity.

Fig. 2. shows the chromatogram of a run on a column filled with Benzyl-DC. The same buffer A was used as above. The ionic strength of eluent B was lowered. The substances corresponding to peaks 1–3 were not separated under these conditions. The retention time is very short owing to the relatively large particle size, in the range 80–100 μm. A biotin binding activity was measured in the following peak. It was

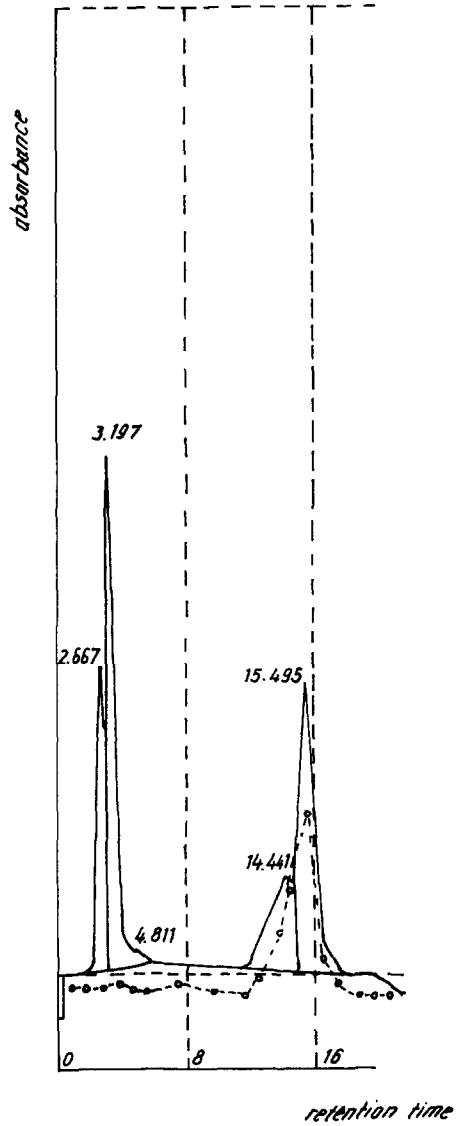

Fig. 1. Chromatogram of a previously designed final step to purify streptavidin in an analytical run. Column: Spherogel TSK Phenyl-5PW (75 × 7.5 mm I.D.), particle size 10 μm, pore diameter 100 nm. Eluents: (A) 0.75 *M* $(NH_4)_2SO_4$ (pH 6.8); (B) 0.066 *M* $KNaHPO_4$ (pH 6.8). Sample: prepurified streptavidin (see Experimental), 10 μl (1.95 mg/ml). Temperature, 35°C; pressure, 5.4 MPa; flow-rate, 1 ml/min; detection wavelength, 280 nm. o--o, Biotin binding activity of the fractions (relative). The time programme was as follows (times in minutes): 0, 100% A; 0.01, inject; 7.99, 100% A; 8.00 to 15.99, gradient function, A (%) = 100 $[1 - 0.125 (\text{time} - 8.00)]^5$; 16.00, 100% B; 20.00, end. Retention times in min.

eluted by eluent B. This demonstrates the good aptitude of the material for the preparative purification of streptavidin from prepurified extracts. The product finally obtained showed, in accordance with the purified product of Fig. 1, a biological

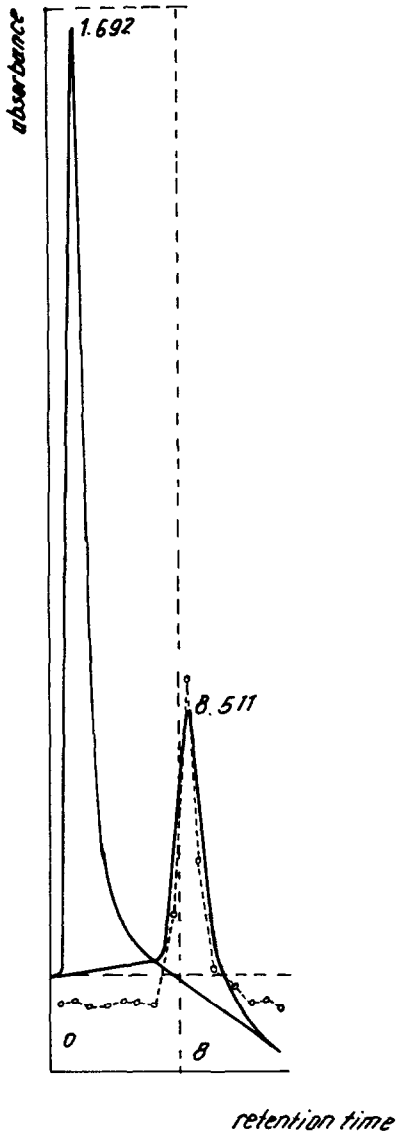

Fig. 2. Chromatogram of the proposed final step to purify streptavidin by hydrophobic interaction chromatography. The pooled fractions of the second peak showed a specific activity of 12–14 U/mg depending on the protein content. Column: Benzyl-DC (250 × 4.0 mm I.D.), particle size 80–100 μm, pore diameter 1–50 nm. Eluents: (A) 0.75 *M* $(NH_4)_2SO_4$ (pH 6.8); (B) water, enriched by phosphate (< 5 μM) (pH 6.8). Sample: prepurified streptavidin as in Fig. 1; 20 μl (2.68 mg/ml). Temperature, 25°C; pressure, 0.8–1 MPa; Flow-rate, 3.5 ml/min; detection wavelength, 280 nm. o--o, Biotin binding activity of the fractions (relative). The time programme was as follows (times in minutes): 0, 100% A; 0.01, inject; 5.99, 100% A; 6.00, 100% B; 12.99, 100% B; 13.00, 100% A; 15.00, end. Retention times in min.

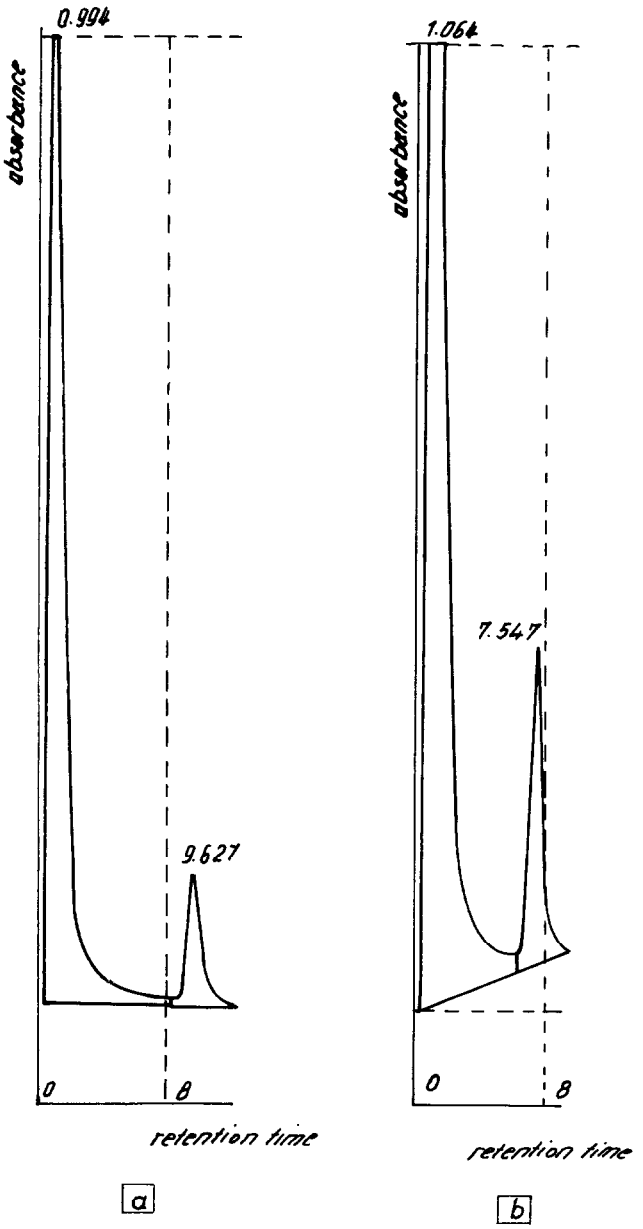

Fig. 3. Scale-up of preparative streptavidin purification. The conditions were the same as in Fig. 2, but the time programme was modified (eluent B was started at 5.00 min). In these experiments a different preparation according to Fig. 2 was used. Between the two runs shown there was a stop period of 21 days. (a) Sample volume, 100 μl (for concentration, see Fig. 2); (b) sample volume, 200 μl (2.95 mg/ml). The biological activity of 12–14 U/mg and the 10.4–10.8% purified streptavidin (peak 2) were unchanged in both runs. Retention times in min.

activity of 14 U/mg (1 unit binds 1 μg of biotin). The protein was homogeneous in SDS electrophoresis [8].

The advantages of the proposed hydrophobic interaction chromatographic (HIC) method relate especially to preparative industrial applications. The material shows a surprisingly high rigidity without changes in its properties during loading with pressures up to 2 MPa. The run time is reduced in comparison with the original separation in Fig. 1 because of the shorter retention time of the contaminant fractions. After the described procedure for filling the column, flow-rates of more than 30 ml/cm^2 can be reached with a pressure of 1–1.5 MPa with a slight dependence on the buffer ion strength. The loading capacity is slightly higher than those of other commercial HIC supports (tested for streptavidin: 112% with respect to Phenyl-Sepharose with aproximately the same degree of modification; 108% with respect to Spherogel TSK Phenyl-5PW). The reproducibility after 22 runs without regeneration of the column is excellent, and even after a longer period it remained constant (*cf.* Fig. 3). The application of this method is not limited to streptavidin. For instance, lipase and alkaline phosphatase (AP) were purified successfully. With AP it was possible to separate from a commercial high-purity product [11] about 20% of non-active protein. There was no significant loss of biological activity in any of the applications tested.

This research provides a basis for scale-up and industrial application of the process for the purification of proteins with hydrophobic structure domains.

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