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Note

Salting-out chromatography on unsubstituted Sepharose CL-6B as a convenient method for purifying proteins from dilute crude extracts

Application to horseradish peroxidase

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The purification of proteins from dilute crude extracts is often a difficult task in large-scale purification procedures. Ammonium sulphate precipitation is not convenient as it necessitates high-speed centrifugation of large volumes of sample. Ultrafiltration is relatively fast and does not concentrate salts and other low-molecular-weight substances, but expensive equipment is needed. For these reasons the absorption of the proteins on ion exchangers or on affinity materials, batchwise or in a column procedure, is the method used in many situations. However, this is not always possible because of the unavailability of specific affinity materials, their high cost and leakage of ligand and also because of the presence in the crude extract of large amounts of salt.

A technique that can be used irrespective of the protein and the composition of the extract is hydrophobic chromatography. In many instances, however, the asbsorption is irreversible or the elution requires severe conditions (high concentration of urea, chaotropic salts), which inactivate the enzymes. We realized that salting-out chromatography on unsubstituted Sepharose[1,2] can be the method of choice. As the interactions are weaker, elution is effected simply by decreasing the ionic strength. Sepharose is inexpensive and ready available.

We report here studies on the purification of horseradish peroxidase as a model enzyme. Despite a negative report[3], it was found that this enzyme bound to Sepharose CL-6B. Stepwise elution yielded the enzyme with a purity of 30–40% in a volume 100-fold smaller that that of the extract in only one step.

EXPERIMENTAL

Sepharose 6B, Sephadex G-50 Fine and Sephadex G-25 Medium were purchased from Pharmacia (Uppsala, Sweden). All chemicals were of analytical-reagent grade. Sepharose 6B was cross-linked as described in ref. 4, omitting sodium borohydride from the reaction mixture.

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The peroxidase assay

The reaction medium contained, in a final volume of 1.0 ml, 20 m*M* sodium phosphate buffer (pH 6.0), 0.4 m*M* *o*-dianisidine and 1.0 m*M* hydrogen peroxide at 37°C. The increase in absorption at 436 nm was recorded using an Eppendorf Spectralline photometer. The purity of the enzyme was also monitored after gel filtration on Sephadex G-50 Fine by calculating the ratio of the absorbance at 405 nm and 280 nm (the *RZ* value). The pure enzyme has an *RZ* value of about 3.0 (ref. 5).

Preparation of the crude extract

The horseradish was obtained from the local market. The extract (about 1 l from every kilogram of horseradish), obtained as described[5], was brought to 55% ammonium sulphate saturation. After 24 h the clear solution was carefully siphoned and filtered through filter-paper. The enzyme activity was stable for at least 1 month at room temperature, but a considerable amount of polymeric brown pigment developed, which interfered with the desalting by gel filtration and partially bound to Sepharose CL-6B.

Purification procedure using the column experiment

A 1700-ml volume of extract containing ammonium sulphate at 55% saturation was pumped at a flow-rate of 60–100 ml/h through a Sepharose CL-6B column (7.0 × 2.2 cm), equilibrated with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 60% saturation. The column was washed with the starting buffer. The enzyme was eluted with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 30% saturation, with a flow-rate of 30 ml/h.

Purification procedure using the batchwise experiment

A 1700-ml volume of extract containing ammonium sulphate at 55% saturation was efficiently stirred with 40 ml of Sepharose CL-6B. After 30 min the concentration of ammonium sulphate was gradually increased to 65% saturation during 30 min by the addition of solid salt. The enzyme activity was more than 97% absorbed. The gel was poured into a chromatographic column and washed with 0.1 *M* phosphate buffer containing ammonium sulphate at 65% saturation. The enzyme was eluted with phosphate buffer containing ammonium sulphate at 30% saturation.

Further enzyme purification

The fractions containing the enzyme, obtained by either procedure, were pooled and the peroxidase was precipitated by dialysis against a saturated solution of ammonium sulphate. After centrifugation the pellet was disolved in 0.1 *M* phosphate buffer (pH 7.0) and desalted by gel filtration. The enzyme at this stage had an *RZ* value of 1.4–1.6. The enzyme solution was brought to 50% ammonium sulphate saturation by adding solid salt. The slight precipitate was removed by centrifugation. The enzyme was absorbed on an 8.0 × 2.2 cm column of Sepharose CL-6B equilibrated with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 60% saturation and eluted with a linear gradient generated by mixing ammonium sulphate solutions of 60% and 30% saturation in 0.1 *M* phosphate buffer (pH 7.0). The volume of the gradient was about 400 ml and the flow-rate about 30 ml/min.

Recycling of the Sepharose CL-6B

The gel (yellow to brown in colour) was suspended in distilled water and sodium hydroxide was added to a final concentration of 0.1 *M*. After 1–2 days at room temperature it was washed with a large volume of distilled water and finally with the equilibration buffer.

RESULTS AND DISCUSSION

By using either the column or the batchwise procedure, the horseradish peroxidase could be recovered in a small volume, typically 100 times smaller than the volume of the extract. In both instances the *RZ* value was 1.3–1.5, *i.e.*, the purity of the enzyme was about 30–40%. The enzyme obtained by precipitation with ammonium sulphate between 55 and 80% saturation had an *RZ* value of only 0.6–0.7. Using the column procedure the yield was lower (50–55%) because as much as 25% of the enzyme activity was not bound to the column. The batchwise procedure was noteworthy in that the yield was over 80% and the experiment could be completed in only 4 h. For further purification, the enzyme was eluted with a decreasing gradient of ammonium sulphate from a Sepharose CL-6B column (Fig. 1); 75% of the enzyme activity applied to the column was recovered in the peak fractions having an *RZ* value of 2.35–2.70. The distribution of the various isozymes and the carbohydrate content were not investigated. The contaminating proteins are relatively uniformly

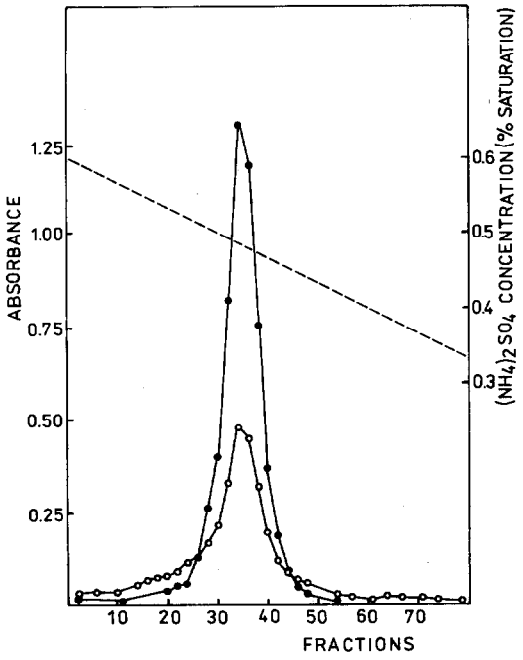

Fig. 1. Final purification of peroxidase by salting-out chromatography on Sepharose CL-6B. For details see Experimental. (○) Absorbance at 280 nm; (●) absorbance at 405 nm; (--------) slope of the ammonium sulphate gradient.

distributed so methods employing different physical properties of the enzyme are needed in order to obtain highly purified enzyme, *i.e.*, gel filtration on Sephadex G-75.

In a recent paper, Chavez and Flurkey[3] found no interaction between horseradish peroxidase and unsubstituted agarose. They used very low concentrations of enzyme and lower concentrations of ammonium sulphate that we did. Moreover, we used cross-linked Sepharose, which is probably slightly more hydrophobic than the non-cross-linked Sepharose.

The results demonstrate the usefulness of salting-out chromatography in concentrating and purifying proteins starting from crude extracts, particularly in batchwise procedures. Although the binding of proteins to unsubstituted Sepharose was noted many years ago, there have been few preparative applications[1,2]. Probably every protein binds to Sepharose in the presence of ammonium sulphate at a concentration slightly less than needed for precipitation. The proteins of rabbit muscle extract were totally absorbed on Sepharose CL-6B as the concentration of ammonium sulphate was gradually increased to 80% saturation (unpublished results). This seems to be the first report describing the use of salting-out chromatography for primary purification, *i.e.*, directly from the dilute crude extract. The advantages of this method are speed, high capacity and low cost of Sepharose, high yields and no need for costly equipment even if the purification is carried out on a fairly large scale. Potential applications of this method are the purification of plant proteins, monoclonal antibodies and other proteins from mammalian cell culture filtrates and proteins from urine.

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