PREPARATION OF BOVINE MERCAPTALBUMIN BY MEANS OF COVALENT CHROMATOGRAPHY

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1. Introduction

Bovine serum albumin is a mixture of mercaptalbumin containing one thiol group/mole, and nonmercaptalbumin in which the thiol group is masked by cysteine or glutathione [1,2]. Commercial albumin preparations therefore show a fractional titer of 0.6–0.7 thiol group/mole. Mercaptalbumin has previously been prepared using the affinity of the thiol group for mercury [3,4]. Methods based on ion exchange chromatography have also been used [5,6]. The latter methods, however, do not resolve mercaptalbumin and nonmercaptalbumin completely.

We now report the preparation of mercaptalbumin by covalent chromatography using a Sepharose-(glutathione-2-pyridyl-disulfide) conjugate. This method, which is based on a thiol–disulfide interchange reaction between protein thiol groups and the mentioned conjugate, has recently been used for the preparation of fully active papain from dried papaya latex [7].

By this technique a bovine serum mercaptalbumin (1.00–1.02 thiol groups/mole) and purified nonmercaptalbumin containing not more than 0.02 thiol group/mole were obtained.

2. Materials and methods

Sephadex G25, G200 and Sepharose 2B were purchased from Pharmacia Fine Chemicals Co., Uppsala, Sweden. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo., USA. Lipid impurities were removed by charcoal defatting [8,9]. Monomeric serum albumin containing 0.59 of thiol/mole was prepared by gel filtration on Sephadex G200. The thiol contents of serum albumin and Sepharose-conjugate were determined spectrophotometrically by titration with 2,2′-dipyridyldisulfide (Aldrich-Europe, Beerse, Belgium) [10]. For calculations $A^{1\%} = 6.7$ at 278 nm and a molecular weight of 66 000 were used for bovine serum albumin. The same values were assumed for mercaptalbumin and nonmercaptalbumin.

Preparation of the Sepharose-(glutathione-2-pyridyldisulfide) was carried out as described by Brocklehurst et al. [7]. The derivative contained 40 μmoles mixed disulfide residues per g dried conjugate as determined by measuring the 2-thiopyridone released in reaction of the reduced Sepharose-(glutathione) gel with 2,2′-dipyridyldisulfide. After use the Sepharose derivative was reactivated by reduction with 20 mM dithiothreitol followed by reaction with 2,2′-dipyridyldisulfide [7].

Electrophoresis of the albumin fractions was performed in Gradipore gradient gel plates (4–24%) in a Gradipore® 342 000 apparatus. 5–10 μg of protein was applied on top of the plate and the electrophoresis was run for 24 hr in 0.089 M Tris–borate buffer pH 8.28, containing 2.5 mM EDTA. The plate was stained with Amidoblack and destained with acetic acid (7%).

Isoelectric focusing in polyacrylamide plates was carried out in an LKB 2717 Multiphore apparatus (LKB, Bromma. Sweden) as outlined in the instruction sheet, using a pH 3.5–10 gradient.

Gel electrophoresis of mercaptalbumin isomers was done essentially as described by Stroupe and Foster [11]

3. Results and discussion

The chromatographic procedure involves two consecutive thioldisulfide interchange reactions. The thiol-containing protein is fixed to the column material and subsequently removed by a low molecular weight thiol compound (see fig. 1).

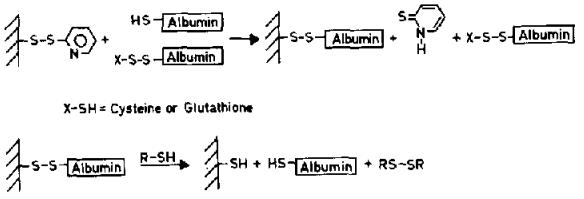

Fig. 1. Reaction scheme for covalent chromatography of bovine serum albumin.

Defatted monomeric serum albumin (385 mg), containing 0.59 mole of thiol per mole of protein, in 77 ml of 0.1 M Tris–HCl buffer pH 7.5 containing 0.3 M KCl and 1 mM EDTA was applied to a column containing Sepharose 2B-(glutathione-2-pyridyldisulfide) gel. The column (3.2 × 18 cm, bed volume 145 ml) was eluted with the same buffer at a flow-rate of 11 ml/hr. Ultraviolet absorption at 278 and 343 nm was determined in fractions of the effluent. 2-thiopyridone absorbing at 343 nm was detected in the effluent, indicating that thiol-containing material had reacted with the gel. The absorption due to protein was calculated by subtracting the contribution of 2-thiopyridone at 278 nm from A_{278}. This contribution was calculated from the ultraviolet spectrum of 2-thiopyridone as $1.25 \times A_{343}$ [10]. Elution was continued until A_{278} was less than 0.03.

Covalently bound material was removed from the column with 25 mM cysteine dissolved in the elution buffer. Fig. 2 shows the chromatogram obtained. Material from the two peaks was gel filtrated on Sephadex G25 to remove 2-thiopyridone and cysteine. The buffer used was 0.1 M sodium–acetate buffer (pH 5.4), containing 0.3 M KCl and 1 mM EDTA. The thiol content in the first peak was 0.00–0.02 and in the second 1.00–1.02 thiol groups/mole.

The thiol-containing protein was analyzed by electrophoresis in polyacrylamide gradient gel. Fig. 3 shows that the electrophoretic pattern was identical

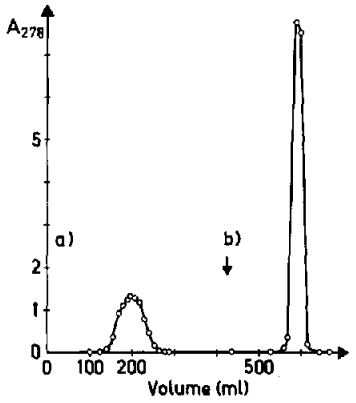

Fig. 2. Covalent chromatography of defatted monomeric serum albumin on Sepharose 2B-(glutathione-2-pyridyldisulfide) gel. Conditions were: 3.2 × 18 cm (145 ml) gel bed, sample volume 77 ml (385 mg albumin), elution system: a) 0.1 M Tris–HCl buffer pH 7.5 containing 0.3 M KCl and 1 mM EDTA, flow rate 11 ml/hr, b) 0.1 M Tris–HCl buffer pH 7.5 containing 0.3 M KCl, 1 mM EDTA and 25 mM cysteine, flow rate 35 ml/hr. A_{278} in the 589 and 600 ml fractions was determined after gelfiltration of the fractions on Sephadex G25, because of the high concentration of 2-thiopyridone in the peak.

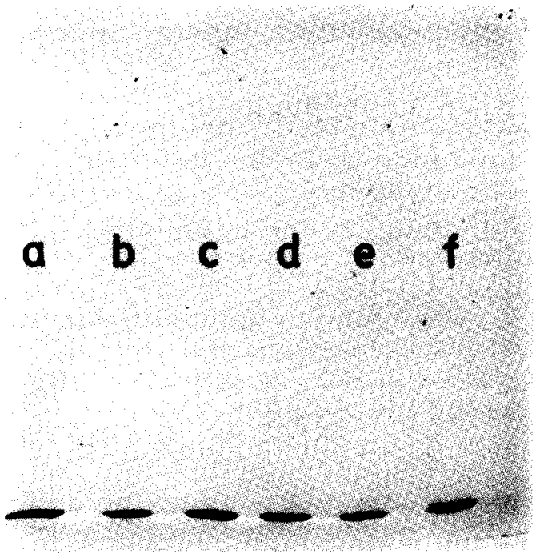

Fig. 3. Gradipore electrophoresis of serum albumin in 0.089 M Tris–borate buffer pH 8.28, containing 2.5 mM EDTA. Samples a,b) 10 and 5 μg defatted monomeric serum albumin, c,d) 10 and 5 μg mercaptalbumin (peak b in fig. 2), e,f) 10 and 5 μg nonmercaptalbumin (peak a in fig. 2).

with that of monomeric serum albumin and the fraction containing no thiol.

The possibility of introducing artifacts during the preparation procedure was further checked by isoelectric focusing. Focusing in a pH-gradient 3.5–10 gave similar patterns for the starting material and both fractions of albumin: 2–3 bands in the pH-range 4.8–5.0. The bands could not be ascribed to differences in thiol content. This is in agreement with the resuls reported by Salaman and Williamson [12] who examined the isoelectric behaviour of reduced and alkylated bovine serum albumin.

Above pH 7.5 and at low ionic strength, bovine mercaptalbumin isomerizes to give a compound with higher electrophoretic mobility at pH 4.2 [11]. Thiol compounds such as cysteine catalyze this isomerization. When mercaptalbumin prepared by covalent chromatography was analyzed by polyacrylamide gel electrophoresis at pH 4.2, one band was obtained as shown in fig. 4. Mercaptalbumin stored at pH 9.1 in

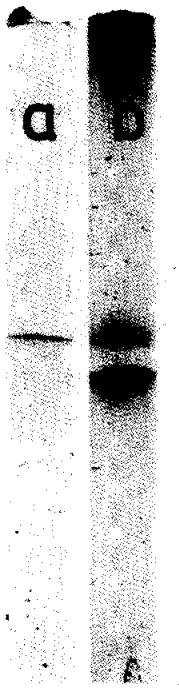

Fig. 4. Cel electrophoresis of bovine mercaptalbumin in polyacrylamide (T = 15%, C = 4%). Electrophoresis was run in 0.06 M sodium citrate pH 4.2 (23°C) for 6 hr at 40 V. Samples a) 20 μg mercaptalbumin prepared by covalent chromatography, b) 20 μg mercaptalbumin prepared as in a) but stored at pH 9.1 for 5 hr.

0.005 M Tris–Cl buffer for 5 hr was used as a reference. Mercaptalbumin coincided with the slower component in the reference sample, which is the native form. The electrophoretic mobilities agree well with those obtained by Stroupe and Foster [11]. Evidently, the mercaptalbumin did not isomerize in the purification procedure.

The capacity of the chromatographic material to bind mercaptalbumin was determined in a separate experiment. Mercaptalbumin prepared by covalent chromatography was passed through a column containing the Sepharose-glutathione-2TP-derivative until protein appeared in the eluate. The gel conjugate reacted with 2.4 mg mercaptalbumin per ml of swollen gel (1.8 μmoles/g dry gel derivative). This value is less than the theoretical capacity (40 μmoles/g) indicating that some active groups are inaccessible for reaction. This was also observed in the preparation of papain by covalent chromatography [7].

The intermediate conjugate of bovine mercaptalbumin and Sepharose-glutathione-2-pyridyl-disulfide could be stored for at least two weeks at +4°C. After removal of mercaptalbumin the gel derivative was easily regenerated. The same glutathione-Sepharose derivative was used and reactivated four times during a period of four months.

The immobilized mercaptalbumin might also be useful in binding studies, affinity chromatography and for specific modification reactions.

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