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Note

Possible means of improving yields in protein chromatography

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The majority of procedures for isolating enzymes and other proteins involve column chromatography in one form or another, often with a hydrophilic gel or substituted cellulose as the solid medium. Recoveries of protein from such columns are usually good but they are rarely so good as to leave no room for improvement. Although packed columns may be used many times with small, clean samples without much deterioration in recoveries or performance, as in analytical gel filtration[1], separations involving relatively large amounts of protein or crude material soon contaminate chromatographic media and low recoveries and poor separations usually result. Problems of this sort became evident in both gel filtration with Sepharose 6B and in affinity chromatography with Sepharose–α-lactalbumin when a procedure for isolating lactose synthetase A protein from human milk[2] was modified for larger scale preparations. However, the ease with which they were overcome, by washing the columns with a strong urea solution, suggests that this device has wider application. Barker *et al.*[3] have also used urea solutions to wash affinity columns between experiments but without indicating to what extent the treatment affected the results.

EXPERIMENTAL AND RESULTS

Gel filtration in Sepharose 6B

The first stage in the purification procedure for lactose synthetase A protein involved gel filtration on Sepharose 6B of human milk, which had been centrifuged at 35,000 *g* for 60 min to remove as much fat as possible. Samples of the centrifuged milk (250 ml) were applied to a column (9.5 cm × 42 cm, containing about 3 l of gel) of Sepharose 6B [obtained from Pharmacia (Great Britain), London, Great Britain] equilibrated at 8° with 10 m*M* Tris–HCl buffer, pH 7.5, containing 0.2 *M* KCl and eluted with the same solution. The enzyme emerged in a narrow band, partly overlapping and followed by the α-lactalbumin band[2]. After several runs, between which the column was well washed with the eluting solution, the enzyme emerged in a broader band and the overlap with α-lactalbumin was much increased. Although recovery of enzymic activity was about 90% in the first few runs with new gel, it decreased to 70–80% as the separation efficiency declined then suddenly dropped to almost nil.

When the gel from the column at this stage was suspended in a large volume of water and allowed to settle, the supernatant was turbid. When the gel was washed by

decantation and repacked into the column it at first gave the original yield and separation of enzyme but the performance soon deteriorated and similar attempts to clean it again were progressively less effective.

A much more successful way of cleaning the gel column was to wash it with 1 *M* NaCl containing 5 *M* urea (2 l). After equilibration again with the Tris–KCl buffer the column fractionated centrifuged milk as well as did the column of new gel and the recovery of enzyme activity was 95–100%. When further contamination was indicated by a drop in the recovery of enzyme a wash with the urea solution consistently restored the original performance.

Affinity chromatography on Sepharose–α-lactalbumin

When the eluting buffer contains N-acetylglucosamine, complex formation between lactose synthetase A protein, N-acetylglucosamine and α-lactalbumin results in a retardation of the A protein in its passage through a column of Sepharose gel to which α-lactalbumin has been covalently attached. The extent of retardation depends on the N-acetylglucosamine concentration[4] and when the concentration is high enough the method is a valuable way of purifying the A protein which is accumulated on the column whilst other protein is washed out, and is then released from the column by omitting the amino sugar from the eluting buffer[2].

Sepharose–α-lactalbumin was prepared in 0.1 *M* $NaHCO_3$ by coupling bovine α-lactalbumin to Sepharose 6B which had been activated with cyanogen bromide[5] and was thoroughly washed with 0.1 *M* $NaHCO_3$ containing 1 *M* NaCl before use. It contained approximately 2.5 mg of α-lactalbumin per ml of gel. In experiments conducted at 8° with a 1 × 5 cm column of the substituted gel equilibrated with 10 m*M* Tris–HCl buffer, pH 7.5, containing 40 m*M* KCl, a buffer flow-rate of 30 ml/h and 50–100-μg portions of purified A protein, the recovery of enzyme activity from the column was about 70% whether or not the buffer contained N-acetylglucosamine. In preparative experiments with a 1 × 48 cm column of the same gel equilibrated with buffer containing 20 m*M* N-acetylglucosamine to retain the enzyme on the column, and samples containing milligram amounts of A protein plus several hundred milligrams of other protein, recoveries of enzyme were 70% at first but after several runs dropped to 40–50%.

A prolonged wash with 1 *M* NaCl effected no improvement in the performance of either column although it did elute some adsorbed protein (as shown by extinction measurements). However, the recovery of enzyme was much improved after the columns had been washed with three volumes of 1 *M* NaCl containing 5 *M* urea, a process which removed more adsorbed protein, and equilibrated again with buffer. The A protein (0.6 mg), retained on the long column in the presence of 20 m*M* N-acetylglucosamine, was then recovered in 95% yield. Similar recoveries were consistently obtained when the samples applied to the column contained relatively large amounts of other proteins, provided the wash with urea was carried out between each run. No decrease in the capacity of the substituted gel to retain A protein, as a result of urea treatment, was detected.

CONCLUSIONS

Adsorption of proteins to chromatographic media is well known, although the

extent to which it occurs undoubtedly varies with different proteins and different media[6,7]. In general, the adsorption seems to be a continuing process, possibly accelerated by protein already adsorbed or by the presence of lipids or other materials. A high concentration of urea seems to be required to remove much of the adsorbed protein since, in the examples described, the lower concentration (2 *M*) used in some cases by Barker *et al.*[3] to wash affinity columns was less effective in removing protein and was ineffective in restoring the original performance of the columns. Whatever the extent to which the high urea concentration removed adsorbed material, however, the main point is that it left the chromatographic media in a state which showed negligible adsorption of the protein of interest. Experience suggests that lactose synthetase A protein is particularly susceptible to adsorptive losses in chromatography so that the high recoveries obtained after washing columns with urea, which nearly doubled the overall recovery of enzyme during purification, are especially notable. In some cases, of course, the use of new chromatographic medium will be preferred to the regeneration of used material but against this must be put the question of cost. This is well illustrated by the first example given above, since frequent replacement of the gel was impracticable on these grounds alone. The urea treatment seems worth trying on any suitable stable medium for protein chromatography when adsorption of product becomes a problem.

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