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IMPROVED CONCANAVALIN A-SEPHAROSE ELUTION BY SPECIFIC READSORPTION OF GLYCOPROTEINS

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SUMMARY

Elution of bound glycoproteins from concanavalin A-Sepharose can be made more efficient by their readsorption to a Blue A agarose column (specific) and Green A agarose column (less-specific) during recycling of the elution buffer. Three lysosomal enzymes were eluted in this way with marked improvement in their specific activities, time and handling and amount of eluting ligand used.

INTRODUCTION

Elution of bound glycoproteins from concanavalin A-Sepharose columns is usually performed at concentrations of the ligand α-methylmannoside between 5 and 15% in high ionic strength buffer. Since the affinity of the concanavalin A matrix for this monosaccharide is much lower than for α-mannose containing type glycoproteins[1,2] (the proteins being desorbed), elution takes place because of mass action in the presence of a large excess of ligand and requires a long period of time to allow the slow dissociation of the bound glycoprotein to occur.

This process is not only time consuming but can be expensive if large amounts of α-methylmannoside are required. The amount of ligand can be reduced by recycling the eluent back over the column, however, this returns the desorbed proteins to the column and elution comes to equilibrium rather than to completion. If the concentration of glycoprotein increases in the recycled buffer the equilibrium may shift toward readsorption of the glycoprotein, particularly if the total volume is kept low. Increasing the volume requires more α-methylmannoside and increases the problems of concentrating and recovering the protein from the eluent and of removing the salts and monosaccharide from the recovered glycoprotein. This may require large dialysis volumes, desalting columns, ammonium sulphate precipitation or some

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semipermeable concentration procedure all of which can result in losses of the glycoprotein.

During the course of purification of three α-mannose containing, lysosomal enzymes, α-L-iduronidase (E.C. 3.2.1.76), sulphamate sulphohydrolase (E.C. 3.10.11) and sulphoiduronate sulphohydrolase (E.C. 3.1.6.-) it was determined empirically that adsorption of two of these enzymes to Blue A agarose in the concanavalin A-Sepharose elution buffer allowed more efficient elution, concentration and exchange of buffer. This afforded a useful degree of purification of all three enzymes all in the one operation and in the same time taken to perform the concanavalin A-Sepharose elution procedure alone.

MATERIALS AND METHODS

Concanavalin A-Sepharose and α-methyl-D-mannopyranoside (α-methylmannoside) were from Sigma (St. Louis, MO, U.S.A.) and Matrex Dyes, Blue A agarose and Green A agarose were from Amicon (Danvers, MA, U.S.A.).

Enzyme assays

α-L-Iduronidase activity was measured using the fluorogenic substrate 4-methylumbelliferyl-α-L-iduronide as described[3] except that bovine serum albumin (7 mg/ml) was added to the assay mixture. Relative fluorescence measurements were converted to pmol/min · ml.

Sulphamate sulphohydrolase activity was assayed using the labelled disaccharide O-(α-2-sulphamino-2-deoxy-D-glucopyranosyl)-(1 → 3)-L-[6-^{3}H]idonic acid as the substrate[4]. Activities are reported as % breakdown of this substrate in 16 h.

Sulphoiduronate sulphohydrolase activity was assayed using the labelled disaccharide O-(α-L-idopyranosyluronic acid 2-sulphate)-(1 → 4)-[1-^{3}H]2,5-anhydro-D-mannitol-6-sulphate as the substrate[5]. Percent breakdown of this substrate was converted to pmol/h · ml.

Application of samples to concanavalin A-Sepharose

Homogenates of human liver were delipidated by a regime described elsewhere[6] before application to a 300-ml concanavalin A-Sepharose column in 0.015 *M* dimethyl glutarate buffer pH 6.0 containing 0.5 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ and 0.1 m*M* dithiothreitol (buffer A) at 4°C. The column was washed with a further two column volumes of buffer A before beginning the recycling procedure.

Recycling procedure for elution of enzymes from concanavalin A-Sepharose

As shown in Fig. 1 a Blue A agarose column (300 ml) was connected to the effluent of the concanavalin A-Sepharose column and a Green A agarose column (100 ml) was connected to the effluent of the Blue A agarose column and 650 ml of elution solution containing 10% (w/v) α-methylmannoside in buffer A were allowed to run into the three columns at 4°C. The first 600 ml were discarded and the solution containing α-methylmannoside emerging from the Green A agarose column was passed into a further 100 ml of the same buffer, stirred and pumped back to the top of the concanavalin A-Sepharose column.

Recycling was continued until enzyme activity was determined to be at the

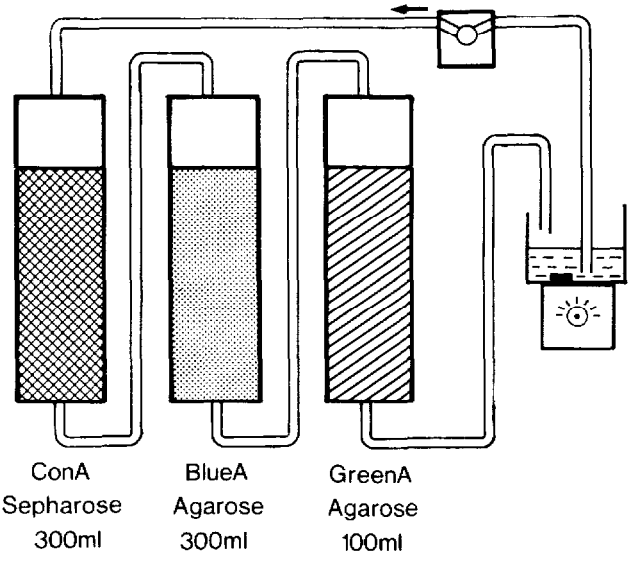

Fig. 1. Arrangement of the concanavalin A (Con A)-Sepharose and dye affinity columns for the recycling procedure as described under Materials and methods.

same low level between the concanavalin A and Blue A columns as between the Blue A and Green A columns.

Elution of enzymes from Blue A agarose

After recycling, interconnections between columns were broken and recycling buffer was replaced with one column volume of buffer A. This was in turn replaced with 0.015 *M* dimethyl glutarate buffer pH 6.0 containing 10% glycerol and 0.1 m*M* dithiothreitol (three column volumes) and finally with a 2-l wash with 0.01 *M* Tris–HCl pH 7.6 containing 10% glycerol and 0.1 m*M* dithiothreitol. An insignificant level of absorbance at 280 nm was ascertained before a 2 × 1.5 l gradient of 0–1 *M* NaCl was applied to the column in the same Tris buffer. Fractions were collected and enzyme activities were determined.

Elution of concanavalin A-Sepharose-bound enzymes without dye readsorption

Enzymes were eluted from a 3-ml concanavalin A-Sepharose column by recycling 25 ml 15% (w/v) α-methylmannoside in buffer A at 4°C until the enzyme activity in the recycling buffer was at a maximum (2–3 days). The enzymes were washed from the column with one volume of buffer A containing 10% (w/v) α-methylmannoside and total protein and enzyme activity determined.

RESULTS

α-L-Iduronidase eluted from concanavalin A-Sepharose underwent total rebinding to the Blue A agarose column during the recycling procedure which was essentially complete in 4 days as shown in Fig. 2. Application of a salt gradient to the Blue A agarose column in buffer at pH 7.6 afforded α-L-iduronidase and sul-

TABLE I

COMPARISON OF DATA FOR THREE ENZYMES ELUTED FROM CONCANAVALIN A-SEPHAROSE WITH OR WITHOUT AFFINITY DYE REBINDING

Experiment No.	*Amount of tissue (g)*	*Treatment*	*Total activity (units*)*	*Total protein (mg)*	*Recovery of activity (%)*	*Specific activity (units/mg)*	*Purification (fold)*	*Amount of α-methyl mannoside (g)*
α-L-Iduronidase								
1	10	Homogenate	982,350	1688	100	582	1	
		Concanavalin A eluate	613,053	28	62	21,894	38	3.75
2	1000	Homogenate	17.5×10^6	84,175	100	208	1	
		Blue A eluate	9.9×10^6	219	57	45,063	207	75
Sulphamate sulphohydrolase								
1	10	Homogenate**	4496	1688	100	2.7	1	
		Concanavalin A eluate	1736	28	38	62	23	3.75
2	1000	Homogenate	287,000	84,175	100	3.4	1	
		Blue A eluate	256,000	193	89	1327	390	75
Sulphoiduronate sulphohydrolase								
1	10	Homogenate**	544	1688	100	13	1	
		Concanavalin A eluate	504	28	92	504	39	3.75
2	1000	Homogenate	189,394	84,175	100	2.3	1	
		Recycle buffer + Blue A eluate	298,398	395	158	755	335	75

* See Materials and methods for details.

** Dialysed to remove small-molecular-weight inhibitors.

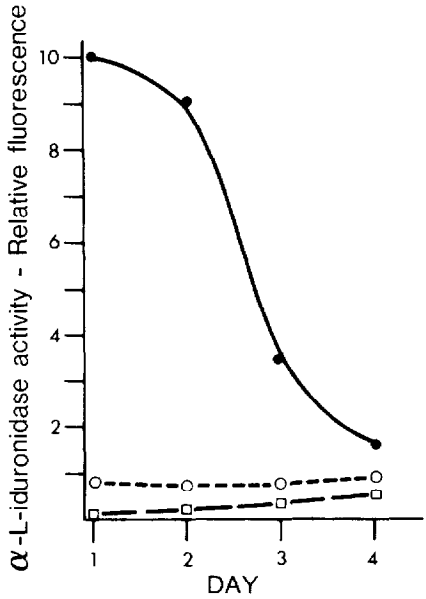

Fig. 2. Activity of α-L-iduronidase during the recycling procedure. Aliquots (5 μl) were taken for assay on the days shown, day 1 being 24 h after recycling was commenced. ●, Concanavalin A–Blue A; ○, Blue A–Green A; □, Green A–concanavalin A.

phamate sulphohydrolase activity peaks well separated from each other while the protein remained at a low level throughout the elution.

It should be noted that an important part of the purification procedure of these two enzymes was the change in pH from 6.0 to 7.6 and the exhaustive washing of the Blue A agarose column. At pH 6 the Blue A agarose column bound 80% of the glycoprotein in the concanavalin A-Sepharose eluent, even in the presence of 0.5 *M* NaCl. At pH 7.6, on the other hand, only 30% of the glycoproteins were bound even with no NaCl present.

A total of 60% of the activity of the third enzyme, sulphoiduronate sulphohydrolase was bound to both dye columns while about 40% of the activity remained in solution. Inclusion of the Green A agarose column in the recycling procedure as shown in Fig. 1 allowed a substantial purification of this enzyme as 90% (18% of total) of the protein eluting from the Blue A agarose column bound to the Green A agarose column. This buffer returned to the concanavalin A-Sepharose column was substantially reduced in glycoprotein content and was totally free of α-L-iduronidase and sulphamate sulphohydrolase activities.

Purification data are shown in Table I where the three enzymes taken in order down the table underwent five-, four- and nine-fold increases respectively in purification for a five-fold decrease in the amount of α-methyl-mannoside.

The values obtained for recovery of activity in both of the sulphohydrolase enzyme cases are better in the recycling experiment than in the "concanavalin A only" experiment. This is probably due to the presence of unidentified protein inhibitors[7] which are present in the homogenate and concanavalin A eluate but removed by the dye column chromatography step. The homogenate samples were also dialysed against 0.2 *M* sodium acetate buffer pH 5.0 to remove small-molecular-weight inhibitors such as phosphate and sulphate ions.

The data in Table I are taken from two different experiments, one for the recycling procedure and one for the "concanavalin A only" procedure which was scaled down 1/100-fold to use 10 g liver homogenised with 30 ml buffer and passaged through a 3-ml concanavalin A-Sepharose column and eluted in a volume of 25 ml.

DISCUSSION

Combination of two purification steps into one recycling procedure using three affinity columns, concanavalin A-Sepharose, Blue A agarose and Green A agarose, afforded a marked improvement in specific activity of three enzymes with a large savings in time, expensive eluting ligand and handling losses. Yield was also improved for two of the three enzymes. The recycling time, although not reduced in this procedure compared with the "concanavalin A only" procedure included the extra column purification step which results in a substantial saving in time and increased yield for the combined procedures. The adsorption of the enzymes eluted from the concanavalin A-Sepharose column by the dye column thus allowed significant reduction in handling, easy removal of salt and α-methylmannoside and exchange of buffers. The removal of the glycoproteins from the concanavalin A-Sepharose column eluate allowed the return of a substantially glycoprotein-free α-methylmannoside solution to the concanavalin A-Sepharose column thus increasing the ligand to glycoprotein ratio in the solution and changing the equilibrium of the elution to one favouring α-methylmannoside displacement of any remaining bound glycoprotein.

The empirical procedures worked out for three enzymes α-L-iduronidase, sulphamate and sulphoiduronate sulphohydrolases can be adapted by experimentation to suit other enzymes. Other dye affinity columns (or other adsorbent) can be used in place of Blue A to remove eluted glycoprotein from the concanavalin A column eluent solution, however, Blue A agarose alone at pH 6 bound $\approx 80\%$ of the eluted protein and Green A agarose an additional $\approx 18\%$, thus this system could be used for most liver glycoproteins eluted from concanavalin A-Sepharose. Those unbound by either dye column would also be substantially purified by the procedure as shown by the sulphoiduronate sulphohydrolase which remained in the recycling buffer.

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