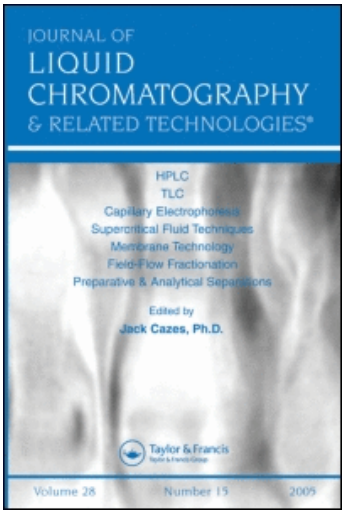

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Affinity Chromatography of Glycoenzymes and Glycoproteins on Concanavalin A-Bead Cellulose

D. Mislovičová[a]; E. Stratilová[a]; N. Kolarova[a]
[a] Institute of Chemistry Slovak Academy of Sciences Dúbravská cesta, Bratislava, Slovak Republic

To cite this Article Mislovičová, D. , Stratilová, E. and Kolarova, N.(1997) 'Affinity Chromatography of Glycoenzymes and Glycoproteins on Concanavalin A-Bead Cellulose', Journal of Liquid Chromatography & Related Technologies, 20: 9, 1367 — 1379

To link to this Article: DOI: 10.1080/10826079708010981

URL: http://dx.doi.org/10.1080/10826079708010981

J. LIQ. CHROM. & REL. TECHNOL., 20(9), 1367–1379 (1997)

AFFINITY CHROMATOGRAPHY OF GLYCOENZYMES AND GLYCOPROTEINS ON CONCANAVALIN A–BEAD CELLULOSE

D. Mislovičová, E. Stratilová, N. Kolarova

Institute of Chemistry
Slovak Academy of Sciences
Dúbravská cesta 9
842 38 Bratislava, Slovak Republic

ABSTRACT

Concanavalin A immobilized on chlorotriazine bead cellulose was applied to affinity purification of glycoenzymes and glycoproteins. Enzymes such as invertase from baker's yeast, endopolygalacturonase (Rohament P) and exopolygalacturonase from carrot roots, as well as extracellular mannoproteins from the yeast *Cryptococcus laurentii* were examined. Chromatography was performed on minicolumns filled with Concanavalin A-triazine bead cellulose gel with the content of immobilized Concanavalin A within the range 1.2 – 8.2 mg per mL of gel. The specifically bound glycoenzymes or glycoproteins were eluted with a solution of the corresponding counter-ligand α-methyl mannopyranoside. Individual degrees of purification, estimated from the measurements of specific activity of crude and purified glycoenzymes, were 14.5-fold for invertase, 93-fold for polygalacturonase and 3.9-fold for exopolygalacturonase. The yeast mannoprotein was isolated from the heteroglycoprotein fraction. The purified mannoprotein contained mainly mannose, with traces of glucose. The purification effect was verified by FPL-chromatography.

INTRODUCTION

Lectins are proteins (glycoproteins) that specifically bind particular carbohydrate structures and have been shown to be widely distributed in plants, animals and bacteria.[1,2] The ability of lectins to interact with glycoconjugates has been evaluated for isolation and fractionation of glycoproteins or oligosaccharides by affinity chromatography. Affinity chromatography, with immobilized lectins, seems to be quite an effective technique, because it can achieve not only fractionation of the glycoproteins or oligosaccharides, but also their structural assessment on the basis of the elution profile from an immobilized lectin column. Most of the well-characterized lectins and their immobilized derivatives on agarose are commercially available. Sepharose carrier with immobilized Concanavalin A (Con A) is widely used in affinity chromatography of many saccharide compounds from the biological sources.[3] Con A is a lectin with an ability to bind, specifically, the nonreducing terminal mannosyl or glucosyl residue of saccharide chains. Con A-Sepharose was evaluated in the purification procedure of glycoenzymes (glucanases, glucosidase, mannosidase or cellobiase),[4-7] glycoproteins from human sera and from cellular membranes,[8,9] antibodies and antigens,[10,11] lysozymes,[12] heteropolysaccharides from plants,[13] and many other compounds.[14]

In the present work, a conjugate of Con A with macroporous bead cellulose was prepared and investigated as an affinity chromatography matrix for glycoenzymes and extracellular glycoproteins. Mild conditions of lectin immobilization were chosen to avoid impairment of its mannose/glucose binding activity. The prepared Con A-triazine bead cellulose was used in affinity purification of the glycoenzymes containing mannosyl units, such as invertase, polygalacturonase and exopolygalacturonase, as well as extracellular heteromannoproteins from yeast.

EXPERIMENTAL

Materials

Concanavalin A-triazine bead cellulose (Con A-TBC) was prepared according to the procedure described in our previous work.[15] Con A was provided by Lectinola (Charles University, Prague, Czech Republic), Perlose MT 100 (bead cellulose) with the size of particles 0.100-0.250 mm was supplied by Lovochemie (Lovosice, Czech Republic), 2,4,6-trichloro-1,3,5-triazine and invertase (INV) (EC.3.2.1.26, β-D-fructofuranosidase) grade V: practical from baker's yeast (2.3 U/mg) were obtained from Sigma (St.Louis,

MO., USA), α-methyl-D-mannopyranoside (α-MMP) from Fluka (Buchs, Switzerland) and polygalacturonase Rohament P (PG) lyophilizate (1.86 U/mg) was from Rohm GmbH (Darmstadt, Germany). Exopolygalacturonase (exoPG) lyophilizate (0.085 U/mg) was prepared from carrot roots[16] and extracellular glycoproteins (GPCL) produced by *Cryptococcus laurentii* were isolated by a procedure described by Masler et al.[17]

Methods

Affinity chromatography on Con A-TBC

Minicolumn filling of 1g Con A-TBC (2.0 x 1.0 cm), containing 1.2 - 8.2 mg Con A/mL of gel, was equilibrated with 50 mM acetate buffer pH 4.7 or 5.8 containing 100 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. 4 H_20. After loading of solution of one of the above mentioned glycoenzymes or glycoproteins in equilibration buffer (1 mL), the non-bound proteins were washed out with the some buffer but without $CaCl_2$ and $MnCl_2$. $4H_20$. For elution of specifically bound glycoenzymes/glycoproteins, a solution of α-MMP (as counter-ligand) was used with the concentration dependent upon the strength of Con A–glycoenzyme/glycoprotein interaction. Flow rate was within the range of 12-18 mL/hod. In the collected fractions (1-3 mL), protein content[18] and enzyme activity of INV[19] or PG, exoPG[20] or saccharide content[21] were determined.

Fast protein liquid chromatography (FPLC)

Purity of all glycoenzymes/glycoproteins was checked by size-exclusion chromatography. FPLC equipment from Pharmacia (Uppsala, Sweden) with standard prepacked columns of Superose 12^{TM} or 6^{TM}, both HR 10/10 were used. The elution buffer was 50 mM phosphate buffer (pH 7) with 150 mM NaCl, flow rate was 0.5 mL/min or 0.3 mL/min. UV absorbance of effluent was monitored at 280 nm. The fractions of volume 0.5 or 0.3 mL were tested also for enzyme activity or saccharide content.

Gas chromatography (GC)

Glycoproteins were hydrolysed with 1 M HCl for 8 hours, then reduced with $NaBH_4$ and acetylated in pyridine-acetic anhydride (1:1) 16 hours at room temperature. The resulting alditol acetates were analysed by GC using a Hewlett-Packard Model 5890. Temperature was programmed to hold at 125°C for 2 min, then to increase to 165°C for 20 min; a column. PAS 1701 (250 cm x 0.32 cm), was used. The carrier gas was H_2 (15 cm^3 . min^{-1}).

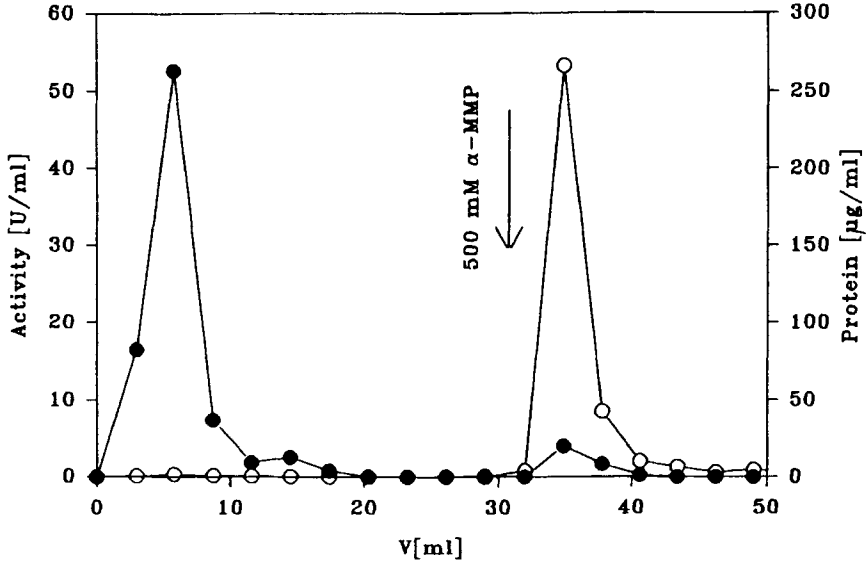

Figure 1. Affinity elution of invertase. Equilibration buffer - pH 4.7, loading: 1mL solution of INV (80mg of lyophilizate); ● - protein, ○ - activity.

RESULTS AND DISCUSSION

In our previous work[15] we described ability of Con A-TBC matrix to bind baker's yeast invertase and quantitative parameters (binding capacity and dissociation constants) of this interaction were determined. Because of a very strong interaction between invertase and Con A, the elution reagent of high concentration (500 mM α-MMP) have left to act at least 2 h (incubation time) in order to obtain a substantial improvement of the elution characteristics.

The purpose of this work is to investigate the possibility of using of this sorbent for purification of glycoenzymes and separation of glycoproteins containing mannosyl units. The sorbent was prepared by a described procedure[15] where Con A was bound to chlorotriazine bead cellulose (CHTBC). The content of immobilized Con A was within the range of 1.2 – 8.2 mg/mL of gel. The minicolumns containing of 1g of wet Con A-TBC were used for affinity purification of the glycoenzymes (INV, PG, exoPG) and isolation of mannoproteins from the glycoproteins (GPLC).

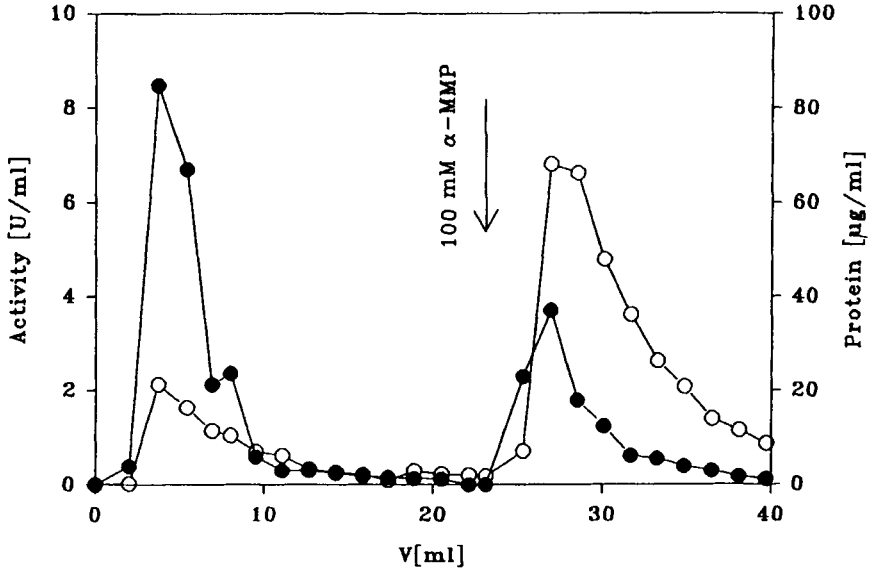

Figure 2. Affinity elution of polygalacturonase. Equilibration buffer - pH 4.7, loading: 1 mL solution of PG (1mg of lyophilizate); ● - protein ○ -activity.

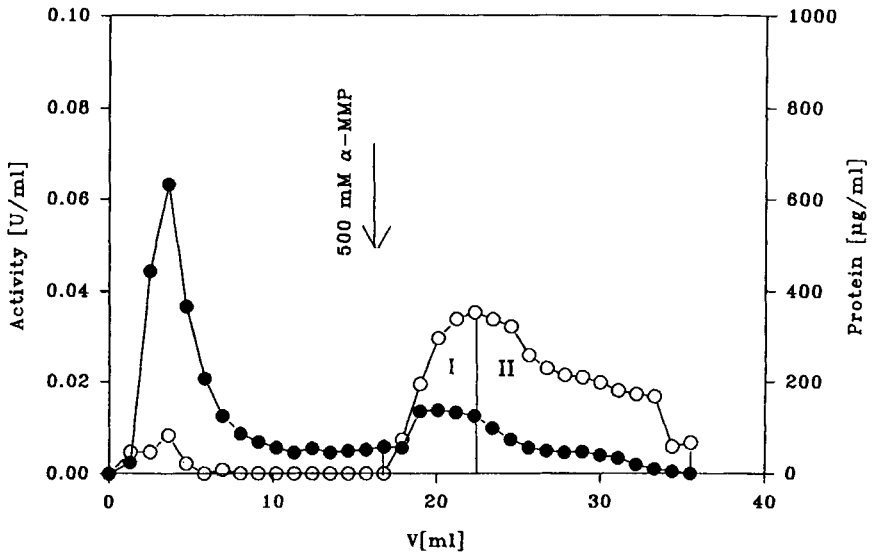

Figure 3. Affinity elution of exopolygalacturonase. Equilibration buffer - pH 4.7, loading: 1 mL solution of exoPG (10 mg of lyophilizate); Collected fractions : I - *first* fraction , II - second fraction, ● - protein, ○ - activity.

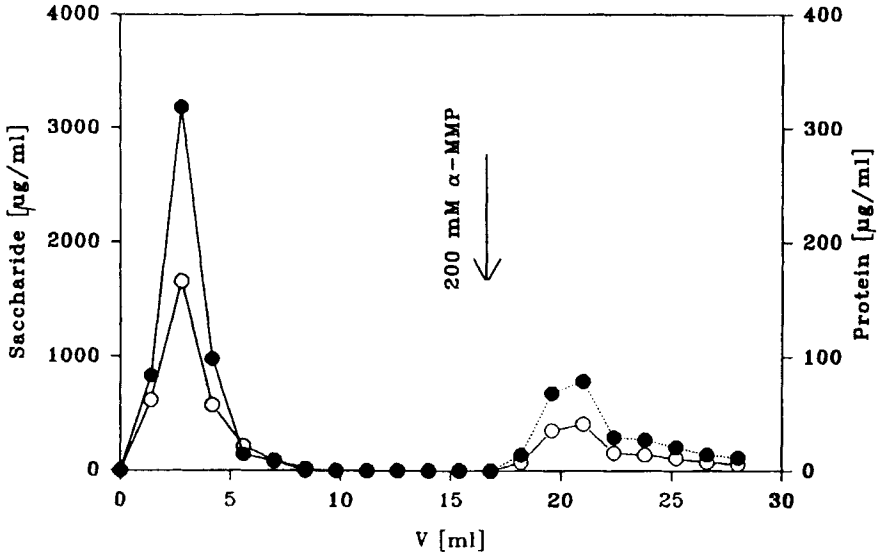

Figure 4. Affinity elution of extracellular glycoproteins. Equilibration buffer - pH 5.8, loading: 1 mL solution of GPLC (10 mg of lyophilizate); ● - saccharide, ○ - protein.

The elution course of the INV is shown in the Figure 1. Sorbent containing 1.2 mg of Con A per mL of gel was used. For more effective elution of the sorbed enzyme, prolonged treatment with α-MMP solution (20 h) was used. The recovery of purified invertase was 71.4 %.

The purification courses of polygalacturonase and exopolygalacturonase on Con A-TBC matrix are presented in Figures 2 and 3. Due to the lower interaction of the polygalacturonases with Con A-TBC, sorbent with 5 mg per mL of gel of immobilized Con A was used. The satisfactory elution of purified PG was reached already with 100 mM solution of α-MMP.

Exopolygalacturonase with very low specific activity (0.142 U/mg) was loaded on the column with the same content of immobilized Con A (5 mg/mL of gel) as polygalacturonase. Interaction with the immobilized Con A was observed, but the enzyme specifically eluted with 500 mM α-MMP was not so successfully purified as INV or PG.

Table 1

Purification of Glycoenzymes on Con A/TBC Minicolumns

Glycoenzyme	Content of Con A (mg/mL Gel)	Total Activity (U)	Specific Activity of Crude Enz. (U/mg)	Recovery (%)	Specific Activity of Purif. Enz. (U/mg)	Purification Factor
INV	1.2	281.0	147. 90	71.4	2154.0	14.5
PGA	5.0	1.86	2.88	68.6	268.1	93.1
e-PGA	5.0	0.85	0.14	I.fr.* 19.6	0.267	1.9
				II.fr. 27.5	0.554	3.9

*The fractions collected after elution with α-MMP were pooled in two main parts (Fract. I & II).

Table 2

Molar Content of Monosaccharides (Man = 1) in Heteroglycoprotein (A) and Purified Mannoprotein (B)

Saccharide	Molar Ratio X_R* A	B
Arabinose	0.07	---
Glucose	0.10	0.06
Galactose	0.13	---
Mannose	1.00	1.00
Xylose	0.02	---

*$X_R = X_i / X_{(mann)}$; X_i = molar content of monosaccharide; $X_{(mann)}$ = molar content of mannose.

Figure 4 shows affinity chromatography of the extracellular heteroglycoproteins on Con A-TBC with content of immobilized Con A of 8.2 mg/mL of gel. In this case, the fraction of bound glycoprotein, obtained with 200 mM α-MMP elution, contained the same saccharide : protein ratio as the original sample of GPLC (95:5).

The results of glycoenzymes purification on Con A - affinity column are shown in Table 1. A significant increase of invertase and polygalacturonase specific activities was observed. In the case of polygalacturonase, the high purification effect can be caused, also, by removal of the inhibitors present in the original sample.

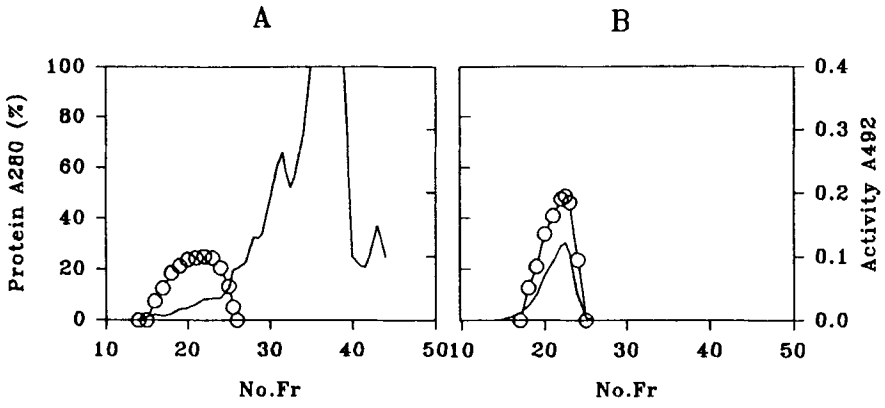

Figure 5. FPLC of invertase on Superose 6™. A - original sample, B - sample after purification; —— protein, ❍ - activity.

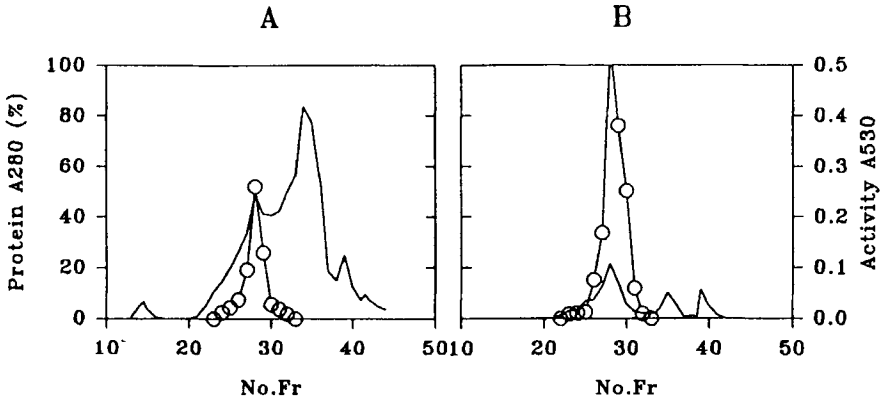

Figure 6. FPLC of polygalacturonase on Superose 12™. A. original sample, B. sample after purification: —— protein, ❍ - activity.

By purification of exopolygalacturonase, the eluted enzyme was divided into two fractions with different specific activities. The efficiency of purification was very low in both fractions, as was the recovery of bound enzyme. This could have been caused by deactivation of enzyme during the purification procedure.

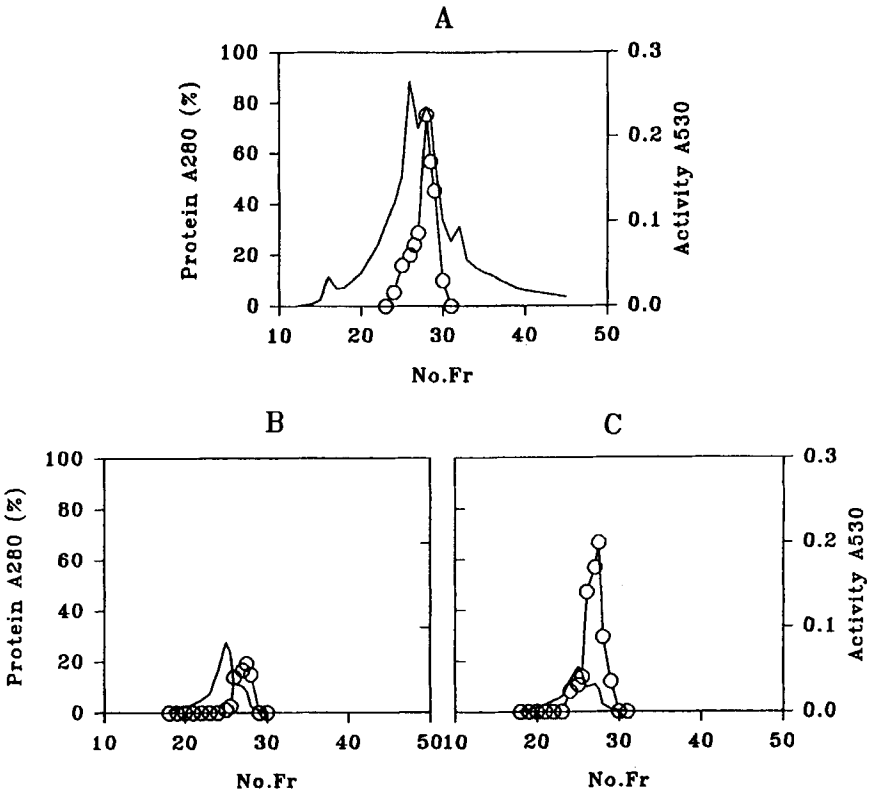

Figure 7. FPLC of exopolygalacturonase on Superose 12™. A. original sample, B. sample after purification (I), C. sample after purification (II); —— protein, ○ - activity.

The purification of the studied glycoenzymes was verified by FPLC chromatography. Superose 6™ or Superose 12™ column was used to determine purity and molecular distribution of the samples separated by affinity chromatography on Con A-TBC. Samples before and after purification were analysed, whereby the sensitivity of protein detection was usually at purified sample higher than at original one.

The significant invertase purification is demonstrated in Figure 5B. The position of the peak in FPLC corresponds to a molecular mass of 26–27 x 10^4. Interestingly, the invertase as protein was not identified in FPLC of the crude sample (Figure 5A), but instead the activity in the fractions was measured.

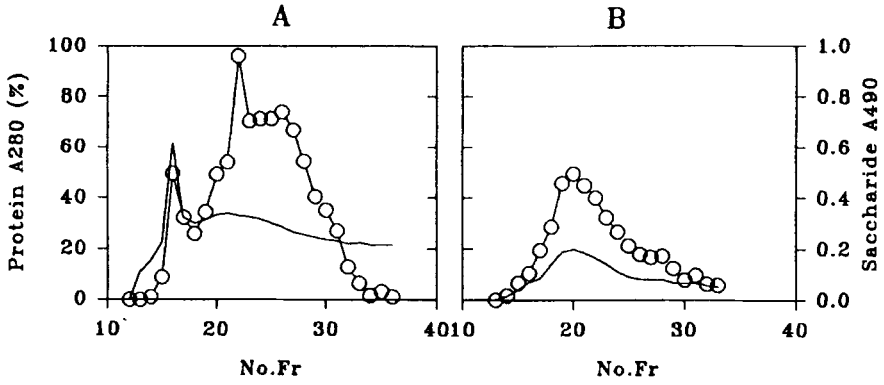

Figure 8. FPLC of extracellular glycoproteins on Superose 12™. A. original sample B. sample after purification; —— protein, ○ - saccharide.

Using affinity chromatography accompanying proteins have been almost completely removed and, in Figure 5B, one can observe, mainly, the peak of the invertase proteins. The results of FPLC chromatography of polygalacturonase (Fig.6 A,B) showed the purification effect of Con A-TBC on this glycoenzyme.

Similarly, as with invertase, a large amount of accompanying proteins was removed and the purity of the eluted enzyme with molecular mass about 35,000 was enriched 93.1-fold.

The lyophilizate from carrot root extract contained exopolygalacturonase of very low specific activity. Some of the accompanying glycoproteins can also interact with Con A immobilized on bead cellulose. Removal of these proteins by Con A-affinity chromatography was, therefore, more complicated than in the previously described experiments. The fractions collected after elution with α-MMP (Fig. 3) were pooled into two main parts (fraction I and II) with different specific activities. Purity of the obtained fractions was examined by FPLC and compared with that of the original sample (Figure 7A, B, C).

Fraction I, with molecular mass of the main peak of inactive proteins Mw ≅ 68,000, has lower specific activity than fraction II with molecular mass of the main enzyme peak Mw ≅ 48,000. Molecular distribution of the extracellular glycoproteins, before and after Con A-affinity chromatography, revealed by FPLC, is presented in Figure 8 A, B.

The separation of a fraction with broad distribution of molecular mass was observed. Changes in the composition of saccharides in glycoproteins were determined by GC (after hydrolysis, reduction and acetylation) (Table 2).

CONCLUSION

Con A-bead cellulose was successfully used as affinity sorbent for purification of two glycoenzymes: invertase and polygalacturonase. One-step chromatography procedure provided high purification factors estimated from the measurements of specific activity of the crude and purified glycoenzymes: 14.5-fold for invertase and 93.1-fold for polygalacturonase. The good increase of the specific activity of described enzymes was achieved. The results of the enzyme recovery were satisfactory as well. By FPLC monitoring of their purities, it was found that the final products of Con A-affinity chromatography were practically homogeneous in molecular weight. Purification of exopolygalacturonase was less successful because it contained accompanying glycoproteins with affinity to Con A-TBC. Good results were obtained in the separation of mannoproteins from heteroglycoproteins. The purified fraction contained mainly mannose with traces of glucose without contaminating saccharides such as arabinose, galactose and xylose.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Grant Agency for Science (Grant No. 2/1238/95).

REFERENCES

1. H. Lis, N. Sharon, Ann. Rev. Biochem., **56**, 35-67 (1986).

2. I. J. Goldstein, T. Tsuji, Adv. Carbohydr. Chem. Biochem., **35**, 127-340 (1978).

3. T. Osawa, T. Tsuji, Ann. Rev. Biochem., **56**, 21-24 (1987).

4. C. S. Gong, L. F. Chen, G. T. Tsao, Biotechnol. Bioeng., **21**, 167-171 (1979).

5. J. Woodward, H. J. Maquess, C. S. Picker, Prep. Biochem., **16**, 337-352 (1986).

6. N. C. Phillips, B. G. Winchester, R. D. Jolly, Biochem. J., **163**, 269-277 (1977).

7. M. Kmínková, J. Kučera, J. Chromatogr., **244**, 166-168 (1982).

8. I. Nicollet, J. P. Lebreton, M. Fontaine, M. Hiron, Biochem. Biophys. Acta, **668**, 235-245 (1981).

9. L. Poliquin, G. C.Shore, Anal. Biochem., **109**, 460-465 (1980).

10. J. Arens, Methods Enzymol., **73**, 166-175 (1981).

11. M. M. Chua, S. T. Fan, F. Karush, Biochem. Biophys. Acta, **800**, 4677-4681 (1984).

12. T. A. Springer, J. Biol. Chem., **256**, 3833-3839 (1981).

13. T. Usui, Y. Iwasaki, T. Mizuno, Carbohydr. Res., **92**, 103-114 (1981).

14. J. Turková, **Bioaffinity Chromatography**, Journal of Chromatography Library, Elsevier, Amsterdam, London, New York, Tokyo, 1993, pp. 372-551.

15. D. Mislovičová, M. Chudinová, P. Gemeiner, P. Dočolomanský, J. Chromatogr., B, **664**, 145-153 (1995).

16. K. Heinrichová, Collect. Czech. Chem. Commun., **42**, 3214-3221 (1977).

17. L. Masler, D. Šikl, Š. Bauer, Collect. Czech. Chem. Commun., **33**, 942-950 (1968).

18. O. H. Lowry, N. Y. Rosebrough, A. L. Farr, R. J. Randall, J. Biol. Chem., **193**, 265-275 (1951).

19. P. Trinder, Ann. Clin. Biochem., **6**, 24-27 (1969).

20. M. Somogyi, J. Biol. Chem., **195**, 19-21 (1952).

21. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, Anal. Chem., **28**, 350-356 (1956).

Received April 8, 1996
Accepted September 30, 1996
Manuscript 4134