Journal of Chromatography, 391 (1987) 452–456
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 354

Note

Interaction of mannose-binding proteins with different types of immobilized affinity ligands

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(Received December 16th, 1986)

Immobilized glycoproteins or carbohydrates have been widely employed for the purification of a variety of plant lectins which are versatile tools for the structural investigation and localization of glycoconjugates. Besides their well known presence in plants, endogenous carbohydrate-binding proteins without enzymatic activity (lectins) are now being detected in a steadily increasing number of organisms and cell types and have also been detected in vertebrates[1,2]. Here they appear to be involved in a variety of physiologically significant recognitive interactions[3,4]. Since they are also present in tumours, forming a new class of tumour markers and targets for therapy[5] and participating in the pathogenesis and spread of cancer at different stages[6], their chromatographic purification from sources of limited quantity like solid tumours requires thorough standardization for the best possible yields.

By using a series of immobilized ligands for the Ca^{2+}-dependent mannose-binding protein, consisting of natural glycoproteins, synthetic neoglycoproteins and immobilized D-mannose, we here demonstrate significant quantitative differences in the yield of this type of lectin from three different sources.

EXPERIMENTAL

Materials

Sepharose 4B and concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals (Freiburg, F.R.G.), *p*-nitrophenyl-α-D-mannopyranoside, RNase B, mannan, invertase and D-mannose were from Sigma (Munich, F.R.G.) and cyanogen bromide and divinyl sulphone were from Merck (Darmstadt, F.R.G.). Human liver and a fibroadenoma from rat mammary gland were kindly provided by the department of pathology in Göttingen and the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.), respectively. Livers from roe-deers were donated by R. Engelhardt. The commercially available glycoproteins were purified by affinity chromatography on concanavalin A-Sepharose 4B prior to the coupling procedure.

Preparation of neoglycoproteins

Neoglycoproteins containing α-mannopyranoside residues were synthesized by diazo coupling of *p*-aminophenyl glycosides, prepared from *p*-nitrophenyl glycosides

0021-9673/87/$03.50

by catalytic reduction in the presence of palladium on charcoal, or coupling of *p*-isothiocyanate glycosides, prepared from *p*-aminophenyl glycosides by reaction with thiophosgene, to bovine serum albumin[7]. After purification of the conjugates by gel filtration and extensive dialysis against distilled water, the mannose content was determined by the resorcinol–sulphuric acid method[8].

Preparation of affinity column supports

Glycoproteins were coupled to Sepharose 4B which had been activated by cyanogen bromide[9]. To exclude any non-specific binding during preparation of the lectin to the immobilized protein, chemically deglycosylated glycoproteins were also coupled to Sepharose 4B. D-Mannose was coupled to the resin after activation of the resin by divinyl sulphone[10], using 12 ml of divinyl sulphone per 100 ml of packed gel. To demonstrate coupling, the plant lectin concanavalin A was passed through a small column and was specifically eluted by D-mannose. Protein coupled to the gel was estimated in the commonly employed manner by determining the amount of protein in the supernatant and washes after the coupling reaction, but before blocking with ethanolamine and further washing, additionally ascertained by the use of iodinated mannan.

Preparation of mannose-binding proteins by affinity chromatography

Frozen and thawed tissues (30 g), trimmed of connective tissue, necrotic parts and fat, were blended in a Waring blender with 250 ml of cold acetone for 1 min and processed as described[11,12]. Briefly, the resulting acetone powder was subsequently extracted with salt medium (20 m*M* Tris–HCl, pH 7.8, containing 0.2 *M* sodium chloride, 1 m*M* dithiothreitol and 0.1 m*M* phenylmethanesulphonyl fluoride) and detergent medium (20 m*M* Tris–HCl, pH 7.8, containing 2% peroxide-free Triton X-100, 0.4 *M* potassium chloride, 1 m*M* dithiothreitol and 0.1 m*M* phenylmethanesulphonyl fluoride). After centrifugation the supernatants from both extractions were pooled and brought to a final concentration of 1.25 *M* and 25 m*M* calcium chloride. This solution was passed over a precolumn of Sepharose 4B to avoid further binding to the unsubstituted column material and then aliquots were recirculated over 18-ml capacity columns of each affinity medium for 10 h. The Ca^{2+}-dependent mannose-binding proteins were eluted by EDTA. The analytical procedures and ascertainment of carbohydrate specificity with fluoresceinylated (*i.e.* fluorescein isothiocyanate labelled) neoglycoproteins have been described in detail[11–13]. Protein was determined by the dye-binding assay adapted for microtitre plates[14] and the yield is given as the mean of two independent preparations.

RESULTS AND DISCUSSION

The influence of the type of affinity ligand, immobilized on Sepharose, on the qualitative and quantitative aspects of purification of mammalian Ca^{2+}-dependent mannose-binding proteins was investigated under identical conditions. To allow a more general answer to the problem of optimal affinity-ligand selection, we have used different types of mammalian species and tissue as starting material. A series of affinity columns was prepared by coupling mannosylated bovine serum albumin (BSA), containing 4 and 18 mannose residues per BSA molecule, respectively, RNase

B, which bears one high-mannose type oligosaccharide[15], the high-mannose type glycoproteins invertase[16] and mannan[17] with high degrees of glycosylation and differences in branching and the sugar D-mannose itself to the column support. For comparative purposes, the protein concentration on the gels was adjusted to 2 ± 0.2 mg/ml. This amount of coupling also reduced the occurrence of leakage, notable at higher protein densities especially for mannosylated BSA with high mannose content, coupled to the amino groups of the protein, and assured constant conditions during the analysis. Interference from binding to the column support of proteins non-specifically or even specifically, known for serum amyloid P component, a lectin with specificity for the cyclic 4,6-pyruvate acetal of galactose in Sepharose[18], was excluded by the use of a precolumn. This precolumn was also indispensable to prevent galactose-dependent binding to invertase.

The material, purified by affinity chromatography, gave an identical electrophoretic pattern for all types of affinity ligands (Fig. 1). Human liver contained a protein having an apparent molecular weight of 30 000, rat fibroadenoma gave a protein band at a similar molecular weight, whereas two protein bands having apparent molecular weights of 58 000 and 22 000 were present in preparations from roe-deer liver. The yields of these proteins for each type of affinity ligand, summarized

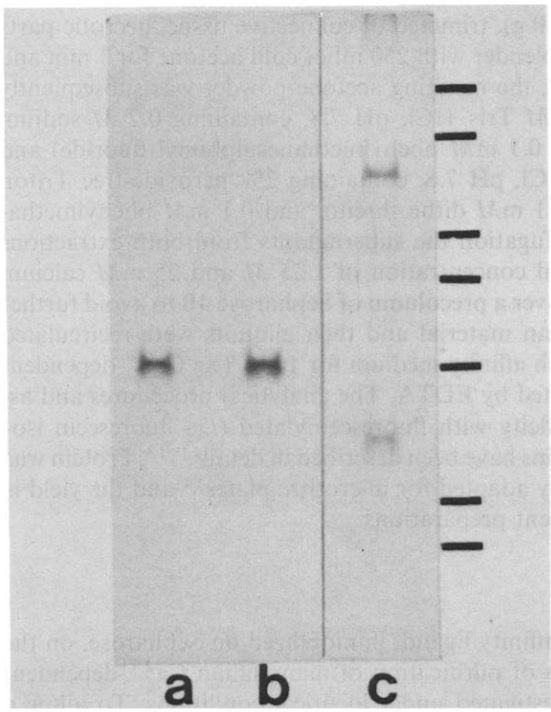

Fig. 1. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate of mannose-binding proteins from human liver (a), rat fibroadenoma (b) and roe-deer liver (c). Standards for molecular weight designations, indicated by bars, are: phosphorylase b (97 000); bovine serum albumin (66 000); egg albumin (44 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); β-lactoglobin (18 400) and lysozyme (14 300).

TABLE I

EFFECT OF THE TYPE OF IMMOBILIZED LIGAND ON THE YIELD OF Ca^{2+}-DEPENDENT MANNOSE-BINDING PROTEINS

Yields are given in μg per 5 g liver or tumour.

Tissue	*Ligand*					
	*BSA_4**	*BSA_{18}***	*RNase B*	*Mannan*	*Invertase*	D-*Mannose*
Human liver	30	46	63	86	68	89
Rat fibroadenoma	12	17	19	28	31	202
Roe-deer liver	34	82	450	680	585	1250

* Mannosylated bovine serum albumin with 4 sugar residues.
** Mannosylated bovine serum albumin with 18 sugar residues.

in Table I, demonstrated clear differences, revealing that immobilized D-mannose was most efficient. A comparison between the various immobilized glycoproteins showed that the results for mannan were very favourable, with decreased yields from immobilized invertase, RNase B and the two neoglycoproteins.

Similar mannose-binding proteins have been reported from rat liver with yields of 3.2[19] and 4.6 μg/g liver[20], using mannan-Sepharose, of 140 and 10 μg/g liver[21] using RNase B or mannose and of 1.5 and 2 μg/g liver[22] using invertase or mannose respectively. Since the protein densities on the column and the conditions differed, no clear answer as to the selection of the affinity ligand could be drawn.

Whereas a similar protein has also recently been described for rat mammary adenocarcinoma[23], the different molecular weights in the preparations from roe-deer liver underscore that variations in the molecular weights of related proteins can occur in different species, as noted for fucose-binding lectins from rat, mouse and human liver[24]. Differences in the subunit-composition, isolated by use of different affinity ligands, as reported for the rat liver mannose-specific lectin[21], could not be detected. In conclusions, these studies reveal that under controlled conditions the yield of a mannose-specific lectin is consistently dependent on the type of immobilized affinity ligand employed. Careful selection among possible choices can therefore lead to significant quantitative increases in the protein, enabling structural studies with less starting material. This result may also be of relevance to other types of lectins with different specificities.

ACKNOWLEDGEMENTS

We gratefully acknowledge the excellent secretarial assistance of U. Rust. This work was supported by a grant from Dr.-Mildred-Scheel-Stiftung für Krebsforschung.

REFERENCES

1 S. H. Barondes, *Annu. Rev. Biochem.*, 50 (1981) 207.
2 H. Lis and N. Sharon, *Ann. Rev. Biochem.*, 55 (1986) 35.

3 G. Ashwell and J. Harford, *Ann. Rev. Biochem.*, 51 (1982) 531.
4 H.-J. Gabius, R. Engelhardt and F. Cramer, *Anticancer Res.*, 6 (1986) 573.
5 H.-J. Gabius, R. Engelhardt and F. Cramer, *Med. Hypotheses*, 18 (1985) 47.
6 H.-J. Gabius, *Cancer Investigation*, 1 (1987) in press.
7 C. R. McBroom, C. H. Samanen and I. J. Goldstein, *Methods Enzymol.*, 28 (1972) 212.
8 A. C. Roche, M. Barzilay, P. Midoux, S. Junqua, N. Sharon and M. Monsigny, *J. Cell. Biochem.*, 22 (1983) 131.
9 J. Kohn and M. Wilchek, *Biochem. Biophys. Res. Commun.*, 107 (1982) 878.
10 N. Fornstedt and J. Porath, *FEBS Lett.*, 57 (1975) 187.
11 H.-J. Gabius, R. Engelhardt, S. Rehm and F. Cramer, *J. Natl. Cancer Inst.*, 73 (1984) 1349.
12 H.-J. Gabius, K. Vehmeyer, R. Engelhardt, G. A. Nagel and F. Cramer, *Cell Tissue Res.*, 246 (1986) 515.
13 H.-J. Gabius, R. Engelhardt, J. Casper, D. Reile, S. Schumacher, H.-J. Schmoll, G. Graupner and F. Cramer, *Tumour Biol.*, 6 (1985) 145.
14 M. G. Redinbaugh and W. H. Campbell, *Anal. Biochem.*, 147 (1985) 144.
15 C.-J. Liang, K. Yamashita and A. Kobata, *J. Biochem. (Tokyo)*, 88 (1980) 51.
16 N. P. Neumann and J. O. Lampen, *Biochemistry*, 6 (1967) 468.
17 T. Nakajima and C. E. Ballou, *J. Biol. Chem.*, 249 (1974) 7685.
18 C. R. K. Hind, P. M. Collins, M. L. Baltz and M. B. Pepys, *Biochem. J.*, 225 (1985) 107.
19 Y. Mizuno, Y. Kozutsumi, T. Kawasaki and I. Yamashina, *J. Biol. Chem.*, 256 (1981) 4247.
20 Y. Maynard and J. U. Baenzinger, *J. Biol. Chem.*, 257 (1982) 3788.
21 J. Wild, D. Robinson and B. Winchester, *Biochem. J.*, 210 (1983) 167.
22 R. Townsend and P. Stahl, *Biochem. J.*, 194 (1981) 209.
23 H.-J. Gabius, R. Engelhardt, S. Rehm, F. Deerberg and F. Cramer, *Tumour Biol.*, 7 (1986) 71.
24 R. S. Haltiwanger, M. A. Lehrman, A. E. Eckhardt and R. L. Hill, *J. Biol. Chem.*, 261 (1986) 7433.