Glycoconjugate Journal (1995) **12**:51–54

Water-soluble poly(acrylamide-allylamine) derivatives of saccharides for protein-saccharide binding studies

J. KLEIN[1], M. KRAUS[1], M. TICHÁ[1], B. ŽELEZNÁ[2], V. JONÁKOVÁ[2] and J. KOCOUREK[1]*‡

[1] *Department of Biochemistry, Charles University, Albertov 2030, 128 40 Praha 2, Czech Republic*

[2] *Institute of Molecular Genetics, The Academy of Sciences of the Czech Republic, Flemingovo nám. 2. 166 37 Praha 6, Czech Republic*

Received 13 May 1994, revised 8 August 1994

Water-soluble poly(acrylamide-allylamine) copolymers containing covalently bound amino groups, prepared by copolymerization of acrylamide and allylamine, can be used as general carriers for coupling of different types of saccharides or saccharide derivatives. The water-soluble macromolecular carbohydrate derivatives can be easily labelled and used in various solid-phase techniques to study protein-saccharide interaction. Two types of coupling reaction were used to prepare polyacrylamide derivatives of saccharides: reductive amination was applied to couple the reducing disaccharides and a carbodiimide reaction was used to couple heparin via its carboxyl groups to the amino groups of the poly(acrylamide-allylamine) derivative. Peroxidase labelled or biotinylated derivatives were shown to be useful in studies on the binding properties of lectins and proteins from boar seminal plasma.

Keywords: Water soluble polyacrylamide derivatives of saccharides, protein-saccharide binding studies, heparin coupled to polyacrylamide

Introduction

Polyacrylamide based affinity carriers have been shown to be useful in affinity chromatography of various proteins. Their easy preparation makes it possible to obtain matrices with an eligible ligand and with the desired degree of substitution. They can be prepared in water-insoluble form as matrices for affinity chromatography [1, 2] or in water-soluble form as macromolecular derivatives of a ligand for the study of protein-ligand interactions [3–6]. For studies of lectin-binding properties, *O*-glycosyl polyacrylamide derivatives either unlabelled [3, 4] or labelled were successfully used [5, 6]. For preparation of these substances it was necessary to prepare the corresponding allyl glycoside.

The present paper describes a more general method for coupling of saccharide ligands to polyacrylamide derivatives.

Material and methods

Materials Boar seminal plasma was obtained after centrifugation of ejaculated boar spermatozoa at 600 *g* for 20 min at 5°C. A fraction containing heparin-binding proteins was obtained from boar seminal plasma by means of affinity chromatography on immobilized heparin [2]. Another protein fraction (a mixture of spermadhesins) was obtained from boar seminal plasma after HPLC on a Vydac C_{18}RPC column as described by Jonáková *et al.* [7].

Concanavalin A and lectins from *Arachis hypogaea*, *Lens culinaris* and *Ricinus communis* were isolated on corresponding *O*-D-glycosyl derivatives of Spheron [8].

Analytical methods The amount of coupled heparin was determined by the method described by Smith *et al.* [9]. Neutral sugar content was determined by the phenol-sulfuric acid method [10] using the corresponding free sugar as a standard. The amount of coupled horseradish peroxidase in polyacrylamide copolymers was determined by measuring absorbance at 403 nm using oxidized enzyme as reference substance. The amount of coupled biotin was determined as described by Avellana-Adalid *et al.* [11].

Preparation of poly(acrylamide-allylamine) copolymers containing amino groups Acrylamide (400 mg) was mixed with a solution containing allyl amine (100–300 μl) in 0.2 M phosphate buffer pH 7.2 (4 ml); after dissolution, the pH of the solution was adjusted to 7.0. The volume was adjusted to 8 ml and $(NH_4)_2S_2O_8$ solution (200 μl of 10% solution, freshly prepared) and *N,N,N,N*-tetramethyl-ethylene-diamine (TEMED, 5 μl) were added). The solution was heated on a

* This is paper No. LXXXIV of the series 'Studies on Lectins' by J.K.

‡ To whom correspondence should be addressed.

water bath so that the temperature rose from 20 to 100°C within 3–5 min and then heating at 95°C was continued for 5 min. After being cooled the solution was diluted with the same volume of distilled water, exhaustively dialysed against distilled water and then lyophilized.

Coupling of heparin to the poly(acrylamide-allylamine) copolymer containing free amino groups To the solution of polyacrylamide copolymer (100 mg in 2 ml of distilled water), a solution of heparin (11.5 mg) in water (2 ml) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (10 mg) was added. The solution was shaken for 24 h at laboratory temperature and then dialysed against 0.05 M NH_4HCO_3. Copolymer bound heparin was separated from free heparin by means of gel chromatography on a Sephadex G 100 column (1.2 × 75 cm) in 0.05 M NH_4HCO_3. Fractions containing copolymer bound heparin were collected and lyophilized.

Coupling of disaccharides to poly(acrylamide-allylamine) copolymers by reductive amination For this purpose, the modified method described by Roy *et al.* [12] was used. To the solution of polyacrylamide copolymer containing covalently bound amino groups (100 mg) in phosphate-buffered saline (PBS) pH 7.5 (2 ml), a disaccharide (lactose or maltose 400 mg) and sodium cyanoborohydride (10 mg) were added. The solution was incubated at 37°C for 72 h, then acidified to pH 2.5 with 50% acetic acid, exhaustively dialysed against distilled water and finally lyophilized.

Coupling of horseradish peroxidase to saccharide poly(acrylamide-allylamine) copolymers For the preparation of peroxidase labelled polyacrylamide derivatives of saccharides, the enzyme was oxidized in the presence of 35 mM $NaIO_4$ for 1 h. The solution of oxidized peroxidase (50 mg in 12.5 ml), dialysed against water, was mixed with a solution of polyacrylamide copolymer containing coupled saccharide (50 mg in 20 ml of 0.2 M sodium carbonate buffer pH 9.4) and incubated for 3 h at 20°C. The reaction was stopped by the addition of NaBH4 solution (4 mg in 1 ml of water). After 2 h standing at 4°C the solution was dialysed against 0.05 M NH_4HCO_3. The peroxidase labelled polyacrylamide derivative of a saccharide was separated by means of gel chromatography on Sephadex G-100 in 0.05 M NH_4HCO_3. The eluate was monitored at 403 nm; fractions corresponding to the first peak were collected, dialysed against water and lyophilized.

Biotinylation of saccharide poly(acrylamide-allylamine) copolymers The saccharide substituted polyacrylamide was labelled by means of *N*-hydroxysuccinimido-biotin (Sigma) [11]. *N*-hydroxysuccinimido-biotin (10 mg) dissolved in dimethyl- formamide (25 μl) was added to the solution of the poly(acrylamide-allylamine) derivative of a saccharide (20 mg) in 0.1 M borate buffer, pH 8.5. The mixture was stirred for 30 min at laboratory temperature and then 0.2 M NH_4Cl was added to adjust the pH to 6.0. After dialysis against distilled water, the solution was lyophilized.

Enzyme-linked binding assay (ELBA) Microtitre plates were coated with 50 μl of protein solution at various concentrations (0–100 μg ml^{-1}) in 0.1 M TRIS-HCl buffer, pH 8.0, containing 1% glutaraldehyde. Wells were washed three times with PBS containing 0.02% Tween and then 3% bovine serum albumin (BSA) solution was added to each well (250 μl) and incubated at 37°C for 2 h. After three washings with PBS containing 0.02% Tween, the solution (50 μl) of labelled poly(acrylamide-allylamine) derivative of a saccharide was applied (100 μg ml^{-1} in TRIS-HCl buffer, pH 7.2, containing 1% BSA), incubated at 37°C for 2 h and then washed three times with PBS. In the case of biotinylated derivatives, a solution (50 μl) of Avidin-Peroxidase (Sigma) (0.25 μg ml^{-1} in a phosphate buffer, pH 7.2, containing 1% BSA) was added to each well and incubated for 1 h. After three washings with PBS, the substrate solution (250 μl) was added containing ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma) (1 mg ml^{-1}) in 0.05 M phosphate-citrate buffer, pH 5.0, and 0.012% sodium perborate. After 30 min incubation at 37°C, the reaction was stopped by the addition of 1% sodium dodecyl sulfate solution (50 μl). The content of each well was diluted four times with water and read at 405 nm.

Isoelectric focusing and electrophoretic transfer of resolved proteins to nitrocellulose membrane Isoelectric focusing (IEF) was performed with the Pharmacia apparatus (Pharmacia, Uppsala, Sweden) using Pre-Nets (SERVA, Heidelberg, FRG) pH 3–10 and a pI calibration kit (pH range covered 3–10).

Proteins resolved by isoelectric focusing were transferred immediately to a nitrocellulose membrane in a semidry electroblotter (OMNI-TRANS, Omni-Bio, Czech Republic). Electrotransfer was conducted at a current density of 0.8 A cm^{-2} in 0.05 M citrate-phosphate buffer, pH 4.6, or in 0.05 M TRIS-HCl buffer, pH 8.5, for 90 min.

Dotting assay and analysis of nitrocellulose membranes Protein solutions (50–500 μg ml^{-1} in 0.1 M phosphate buffer, pH 7.2) were spotted (2 μl) on to nitrocellulose strips.

Nitrocellulose membranes after either electroblotting or after dotting assay were specifically detected using biotinylated or peroxidase labelled poly(acrylamide-allylamine) derivatives of saccharides. Nitrocellulose membranes were incubated in 3% BSA in 0.1 M phosphate buffer, pH 7.2, for 2 h. After being washed with PBS, membranes were incubated in the solution of labelled polyacrylamide derivative (100 μg ml^{-1} in 0.1 M phosphate buffer, pH 7.2) for 2 h; they were then washed (3 × 5 min) with the same phosphate buffer. With biotinylated substances, membranes were incubated in a solution of Avidin-Peroxidase (Sigma) (0.25 μg ml^{-1} in the phosphate buffer containing 1% BSA) and washed with the same buffer. The bound peroxidase was developed by incubation of membranes in ABTS substrate solution as described above for the ELBA experiments.

Results

Preparation of water-soluble poly(acrylamide-allylamine) derivatives of saccharides

Water-soluble poly(acrylamide-allylamine) copolymers containing covalently bound amino groups have been used as a matrix for coupling of saccharides, prepared by copolymerization of acrylamide and allylamine as described in the Materials and methods section. The amount of bound amino groups in copolymers ($4.0–6.0 \times 10^{-2}$ moles per 100 g of copolymer) corresponded to that described previously [5] as did their molecular masses (380 000–400 000) [5]. Reducing disaccharides and heparin were chosen as examples of two different types of coupled carbohydrates.

Carbodiimide reaction was used to couple heparin via its carboxyl groups to amino groups of the polyacrylamide copolymer. Reducing disaccharides were coupled to polacrylamide copolymers by reductive amination. The amount of bound saccharides is given in Table 1.

Labelling of poly(acrylamide-allylamine) derivatives of saccharides Non-substituted residual free amino groups of the poly(acrylamide-allylamine) copolymers were used for coupling of horseradish peroxidase or a biotin derivative. Horseradish peroxidase was coupled after periodate oxidation of the sugar moiety of the enzyme. The periodate-oxidized enzyme retained a sufficient degree of enzyme activity to allow detection and, at the same time, lost its ability to interact with some lectins through its sugar moiety [17]. The amount of coupled labels of both types (biotin or peroxidase) was in the range $0.7 - 1.0 \times 10^{-3}$ mol per 100 g of poly(acrylamide-allylamine) derivative.

Binding of poly(acrylamide-allylamine) derivatives of saccharides to proteins and lectins The poly(acrylamide-allylamine) derivatives of saccharides labelled either with biotin or horseradish peroxidase were shown to be useful for detection or semi-quantitative determination of saccharide-binding proteins. The following techniques were used.

(a) Peroxidase labelled heparin poly(acrylamide-allylamine) copolymer derivative was used to detect heparin binding proteins from boar seminal plasma after isoelectric focusing and electroblotting on to a nitrocellulose membrane. Both the protein fraction prepared by affinity chromatography on immobilized heparin [2] and the mixture of spermadhesins prepared by HPLC on a Vydac C_{18} reversed phase chromatographic column [7] were stained by the peroxidase labelled heparin poly(acrylamide-allylamine) copolymer (data not shown).

(b) Biotinylated or peroxidase labelled saccharide poly(acrylamide-allylamine) copolymer derivatives were used to detect plant lectins (0.2–1 μg per spot) immobilized by spotting on nitrocellulose membranes (dotting assay). Concanavalin A was stained with a peroxidase labelled derivative containing bound maltose and *Ricinus communis* agglutinin (RCA_{120}) was detected with a biotinylated derivative containing bound lactose (data not shown).

(c) Biotinylated and peroxidase-labelled polyacrylamide derivatives containing bound maltose and bound heparin were used to detect, respectively, Concanavalin A and boar seminal plasma proteins immobilized on plastic microtitre wells by the enzyme linked binding assay (ELBA) (Fig. 1).

In similar experiments, it was shown that *Lens culinaris* lectin as well as Concanavalin A interacted with maltose bound to polyacrylamide copolymers and *Arachis hypogaea* lectin as well as *Ricinus communis* agglutinin interacted with lactose bound to polyacrylamide copolymers. Non-specific interactions were not observed.

No significant differences were found between peroxidase labelled and biotinylated glycoconjugates. An advantage of the biotinylated material is its facile preparation and its extremely long shelf life.

Table 1. The content of saccharides in the water soluble poly(acrylamide-allylamine) derivatives.

	Neutral sugar content (mol 100 g^{-1})	Heparin content (g 100 g^{-1})
Lac-PAA	3.8×10^{-2}	
Mal-PAA	2.6×10^{-2}	
Heparin-PAA		6.1

PAA, water soluble poly(acrylamide-allylamine) derivative; Lac, lactose; Mal, maltose.

Discussion

Natural, semi-synthetic and synthetic glycoconjugates have all been used for the detection of endogenous lectins as well as for studies of their properties [4, 5, 13–20]. Polyacrylamide based synthetic macromolecular derivatives of saccharides have been prepared by copolymerization of acrylamide derivatives of saccharides [4, 5, 17, 18]. Another approach was used by Bovin *et al.* [20] who prepared polyacrylamide derivatives by reaction of activated polyacrylamide polymer with aminoalkylglycosides. In both cases it was necessary to synthesize a suitable saccharide derivative. The method described in this report for the synthesis of polyacrylamide based glycoconjugates does not require the use of saccharide derivatives.

The polyacrylamide copolymer containing covalently bound amino groups represents a new type of general carrier which can be used for the coupling of ligands carrying not only carboxyl groups or reducing disaccharides but also other substances such as aldehydes. Aldehydes can be used to immobilize ligands containing saccharide moieties after periodate oxidation.

Another advantage of the method described in this report is the possibility of preparing analogous water-insoluble polyacrylamide copolymers of coupled ligands that can be used for

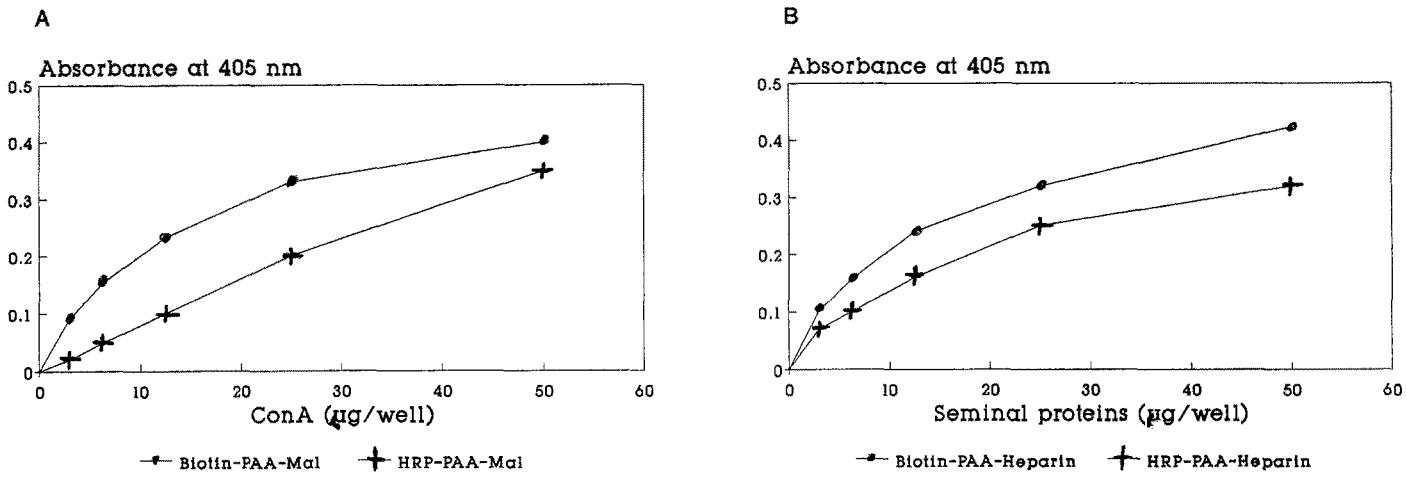

Figure 1. The enzyme linked binding assay (ELBA). Binding of biotinylated (- ○ -) and peroxidase labelled (- + -) polyacrylamide derivatives containing bound maltose to Concanavalin A (A) and containing bound heparin to boar seminal plasma proteins (B) immobilized on plastic microtitre wells.

affinity chromatography. Preliminary experiments can be carried out with water soluble derivatives to determine the most suitable ligands for subsequent use in affinity chromatography. The poly(acrylamide-allylamine) derivative of heparin can serve as an example: e.g. for coupling of heparin to the polyacrylamide derivative containing free amino groups, free carboxyl groups of the polysaccharide were used. The same type of reaction was successfully used for preparation of an affinity carrier containing immobilized heparin [2].

The degree of substitution with ligand depends on the amount of covalently bound amino groups of the polyacrylamide derivative. The amount of amino groups bound can be changed by an increase or a decrease of the allylamine concentration in the copolymerization mixture. The yield of coupling of the disaccharide to the copolymer was similar to that described for the binding of disaccharides to bovine serum albumin by the same reaction (reductive amination) [12].

References

1. Kocourek J (1982) *Acta Histochem* **71**: 57–66.
2. Tichá M, Železná B, Jonáková V, Filka K (1994) *J Chromatogr* **656**: 423–26.
3. Hořejší V, Smolek P, Kocourek J (1978) *Biochim Biophys Acta* **538**: 293–98.
4. Hořejší V, Tichá M (1986) *J Chromatogr* **376**: 49–67.
5. Tichá M, Kocourek J (1991) *Carbohyd Res* **213**: 339–43.
6. Chadli A, Caron M, Tichá M, Jourbet R, Bladier D, Kocourek J (1992) *Anal Biochem* **204**: 198–203.
7. Jonáková V, Čechová D, Töpfer-Petersen E, Calvete JJ, Veselský L (1991) *Biomed Biochim Acta* **50**: 691–95.
8. Filka K, Čoupek J, Kocourek J (1978) *Biochim Biophys Acta* **539**: 518–28.
9. Smith PK, Malia AK, Hermanson GT (1980) *Anal Biochem* **109**: 466–73.
10. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Chem* **18**: 350–56.
11. Avellana-Adalid V, Jourbet R, Bladier D, Caron M (1990) *Anal Biochem* **190**: 26–31.
12. Roy R, Katzellenbogen E, Jennings HJ (1984) *Can J Biochem Cell Biol* **62**: 270–75.
13. Lesniak AP, Lin EH (1984) *Anal Biochem* **142**: 140–47.
14. Gabius HJ (1991) *Biochim Biophys Acta* **1071**: 1–8.
15. Stowell CP, Lee YC (1980) *Adv Carbohydr Chem Biochem* **37**: 225–281.
16. Kieda C, Delmotte F, Monsigny M (1977) *FEBS Lett* **76**: 257–61.
17. Tichá M, Machoň O, Kocourek J (1991) *Interlec 13, Abstr. Vol.* P58, Berlin.
18. Chernyak AY, Weintraub A, Norberg T, Kallin E (1990) *Glycoconjugate J* **7**: 111–20.
19. Schnaar RL, Weigel PH, Kuhlenschmidt MS, Lee YC, Roseman S (1978) *J Biol Chem* **253**, 7940–51.
20. Bovin, NV, Korchagina EY, Zemlyakhina TV, Byramova NE, Galanina OE, Zemlyakov AE, Ivanov AE, Zubov VP, Mochalova LV (1993) *Glycoconjugate J* **10**: 142–51.