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A novel method for glycoconjugate synthesis

M. TICHÁ[1], M. ČERNÝ[2] and T. TRNKA[2]

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

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2-Chloroethyl 1-thio-β-D-galactopyranoside and the corresponding 1-thio-β-D-glucopyranoside have been found to be suitable glycosylating agents for the preparation of different types of glycoconjugates. Glycosylation of bovine serum albumin and chymotrypsin were chosen as examples of an application of the described compounds. The glycosylating agents can modify not only amino groups, but also alkyl hydroxyl and aryl hydroxyl groups, as was shown in experiments with model water soluble and water insoluble polyacrylamide copolymers.

Keywords: glycosylation, 1-thio-β-D-glycosides, glycosylation of proteins, water soluble polyacrylamide copolymers

Introduction

The importance of saccharide moiety for the biological function, distribution and stability of glycoproteins has been recognized [1–2]. Saccharides of cell surface glycoproteins have been shown to be responsible for cell-cell recognition and adhesion in several biological processes [3–6]. On the other hand, an attachment of a defined saccharide to a macromolecule can result in significant changes in its biological properties, as well as its distribution in living systems. The protective role of saccharides is also important, as previously shown [7].

With increasing interest in the study of the role of saccharide parts of natural glycoconjugates in biological processes, the preparation of glycoconjugates with defined site of their attachment attracts still more attention.

The present communication describes a novel type of glycosylating agent for glycoconjugate synthesis.

Materials and methods

Materials

Sweet almond β-glucosidase (E.C. 3.2.1.21), β-galactosidase (E.C. 3.2.1.23) from *Escherichia coli* and *N*-succinyl-phenylalanine-p-nitroanilide were purchased from Sigma Chemical Co., St Louis, USA, bovine serum albumin and chymotrypsin from Léčiva, Praha, Czech Republic, allyl alcohol, allyl amine and 2-allyl phenol from Aldrich, Steinheim, FRG. Concanavalin A was isolated as described by Filka *et al.* [8].

For the preparation of 2-chloroethyl 1-thio-β-D-galactopyranoside (CET-Gal), the modification of the procedure described by Frgala *et al.* [9] was used [10]. Similarly, 2-chloroethyl 1-thio-β-D-glucopyranoside (CET-Glc) was obtained [10]. Both thioglycosides can be stored in the presence of P_2O_5 at a temperature below 10 °C for several months; they slowly decompose in aqueous solutions.

Preparation of polyacrylamide copolymers

Water soluble polyacrylamide copolymers containing covalently bound amino groups (poly(acrylamide-allyl amine) copolymers) were prepared by copolymerization of acrylamide and allyl amine as described previously [11, 12]. 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 pH 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′*-tetramethylethylenediamine (TEMED, 5 µl) were added. The solution was heated in a water bath so that the temperature rose from 20 to 100 °C within 3–5 min followed by heating at 95 °C for a further 5 min. After being cooled the solution was diluted with the same volume of distilled water, exhaustively dialysed against distilled water and then lyophilized.

An analogous procedure was used for the preparation

*To whom correspondence should be addressed.

of water soluble poly(acrylamide-allyl alcohol) copolymer and poly(acrylamide-2-allyl phenol) copolymer. In the copolymerization mixture, allyl alcohol or 2-allyl phenol were used instead of allyl amine. Lyophilized preparations of polyacrylamide copolymers were used for further experiments.

Water insoluble poly(acrylamide-allyl alcohol) copolymer was prepared by copolymerization of acrylamide (3 g), allyl alcohol (3 ml), bis-methylene-acrylamide (0.3 g) in 60 ml distilled water in the presence of $(NH_4)_2S_2O_8$ (160 mg) and TEMED (50 µl). The solution was heated in a water bath to 95 °C and then kept at 95 °C for 30 min. After being cooled, the gel formed was disintegrated by pushing it through a nylon net and exhaustively washed with distilled water.

Analytical methods

The content of saccharides in glycosylated products was determined by the Dubois procedure [13] using D-galactose or D-glucose as a standard.

The amount of bound amino groups in polyacrylamide copolymers was determined as described previously [11] and the amount of phenolic groups from the measurement of absorbance at 272 nm. In the course of gel chromatography on Sephadex G-25, the protein content was determined by measurement of the absorbance at 280 nm.

The chymotrypsin activity was determined using *N*-succinyl-phenylalanine-p-nitroanilide as a substrate [14]. Paper chromatography (Whatman No. 3), the eluent butyl alcohol-acetic acid = 10:1:3 and the detection with $AgNO_3$ solution were used to follow the enzymic hydrolysis of prepared glycoconjugates.

Double diffusion in agarose gel

A 2% solution of modified bovine serum albumin or glycosylated polyacrylamide derivative in 0.1 M barbiturate buffer pH 8.0, and 2% solution of concanavalin A in the same buffer were applied to wells in 3% agarose gel in 0.1 M barbiturate buffer pH 8.0. The following procedure was the same as described in the LKB manual. Gels were stained for protein using 0.5% Amido Black 10B in 7% acetic acid. For inhibition studies, D-glucose was added to the suspension for the preparation of agarose gel to give the final monosaccharide concentration of 2%.

Affinity chromatography of concanavalin A

The solution of concanavalin A (2 mg per 0.5 ml) in phosphate buffered saline (PBS) was applied to a column (1.5 × 8 cm) of water insoluble D-glucosylated poly(acrylamide-allyl alcohol) copolymer washed with PBS. Material that was not adsorbed was eluted with PBS (1 ml fractions were collected at a flow rate of 8 ml h^{-1}). The adsorbed lectin was eluted with 0.2 M D-glucose.

Glycosylation of bovine serum albumin

To the solution of bovine serum albumin (25 mg) in 0.1 M $NaHCO_3$ (3 ml), CET-Gal or CET-Glc (100 mg) were added. The solution was incubated at 37 °C for 20 h, then exhaustively dialysed against distilled water and finally lyophilized. In alternative experiments 0.1 M phosphate buffers pH 5, 6, 7 and 8 were used instead of 0.1 M $NaHCO_3$.

Glycosylation of chymotrypsin

The solution of chymotrypsin (20 mg) in 0.1 M phosphate buffer pH 6.0 (2 ml) was mixed with an aqueous solution of freshly prepared CET-Gal (100 mg per 2 ml) and then incubated for 8 h at 37 °C. Modified chymotrypsin was separated using gel chromatography on Sephadex G-25 (65 × 1.1 cm I.D.) equilibrated with 3% acetic acid. The chromatography was performed in 3% acetic acid at the flow-rate of 20 ml h^{-1}. The absorbance at 280 nm, the chymotrypsin activity [14] and the content of neutral sugar [13] were determined in the elution fractions obtained. Fractions containing modified enzyme were dialysed against 0.1 M NH_4HCO_3 and lyophilized. The same conditions of chromatography were applied to unmodified enzyme.

Glycosylation of polyacrylamide copolymers containing covalently bound amino, alkyl hydroxyl and aryl hydroxyl groups

To the solution of water-soluble polyacrylamide copolymer (25 mg) in 0.1 M $NaHCO_3$ (3 ml), CET-Gal (100 mg) was added. The solution was incubated at 37 °C for 20 h, then exhaustively dialysed against distilled water and lyophilized.

A similar procedure was used for the glycosylation of water-insoluble poly(acrylamide-allyl alcohol) copolymer. To the suspension of polyacrylamide copolymer gel (20 ml in 20 ml 0.05 M $NaHCO_3$), CET-Glc (300 mg) was added. The suspension was shaken slowly for 4 h and incubated at 37 °C for 20 h, and then exhaustively washed with distilled water and then with PBS.

Enzymic hydrolysis of prepared glycoconjugates

D-glucosylated and D-galactosylated preparation of bovine serum albumin or both types of glycosylated polyacrylamide copolymers (2.5 mg or 0.5 ml of the gel) dissolved or suspended in 0.05 M phosphate buffer pH 7.5 (1 ml) was mixed with sweet almond β-glucosidase (Sigma) solution (1 mg in 0.1 ml of the same buffer) and β-galactosidase suspension (from *Escherichia coli*, Sigma) (10 µl) and incubated for 3 h at 37 °C. The enzymic reactions were stopped by heating in a boiling water bath for 2 min.

In the case of water soluble derivatives, the suspensions obtained were centrifuged and supernatants were

evaporated to dryness. After dissolution in distilled water (0.1 ml), their saccharide composition was examined using paper chromatography with corresponding standards. In the case of D-glucosylated derivatives, the presence of liberated D-glucose was proved using the glucose oxidase test [15]: to aliquots of obtained supernatants, a solution (1 ml) containing glucose oxidase, peroxidase, 3-methylphenol and 4-aminophenazone (Bio-La-Test, Oxochrom GLUKOSA, Lachema, Brno, Czech Republic) was added and the absorbance was read at 492 nm.

In the case of water insoluble D-glucosylated polyacrylamide copolymer, the gel after β-glucosidase treatment was exhaustively washed with PBS and distilled water. The content of bound saccharides was determined both in swollen gel and the dried samples using the Duboise procedure [13].

For control experiments, 2-hydroxyethyl β-D-thiogalactoside (1 mg in 0.5 ml of 0.05 M phosphate buffer, pH 7.5) was incubated in the presence of β-galactosidase (10 µl of suspension). A composition of the incubation mixture was examined by paper chromatography as described above.

Results and discussion

A novel type of glycosylating agent (2-chloroethyl 1-thioglycosides) was used for the modification of two types of proteins: bovine serum albumin and chymotrypsin.

In the case of bovine serum albumin the glycosylated product obtained in 0.1 M $NaHCO_3$ contained from 30 to 33 mol of covalently bound saccharide residues per mol of the protein (Table 1).

The presence of bound D-glucosyl residues in bovine serum albumin preparation modified using CET-Glc was proved by means of double diffusion in agarose gel: the obtained modified protein yielded with concanavalin A precipitation lines; no precipitation was observed in the presence of D-glucose in the agarose gel.

Table 1. Content of saccharides in prepared glycoconjugates.

Glycosylated	*Content of saccharides*			
	Gal[a] (%)	*Glc*[b] (%)	*Gal*[a] (*mol per mol*)	*Glc*[b]
BSA	8.5	9.5	30	33
chymotrypsin	6.5	–	9	–
COP-NH_2[a]	4.2	4.3	–	–
COP-OH[a]	4.8	4.5	–	–
COP-Phenol[a]	1.5	–	–	–

BSA, bovine serum albumin; COP, polyacrylamide copolymer; [a]CET-Gal; [b]CET-Glc.

The modification degree is not significantly dependent on the pH of the reaction mixture, as was proved using buffers of different pH in the range of pH 5–8. This fact is advantageous for the further application of the described glycosylating agent: the pH of the reaction mixture can be chosen to be suitable for the particular protein or peptide.

Modification of the chymotrypsin was carried out at pH 6.0. An excess of the glycosylating agent and low-molecular weight products were not removed by dialysis (as in the case of bovine serum albumin) but by using gel chromatography on Sephadex G-25 in 3% acetic acid. The course of separation of modified protein in comparison with that of unmodified enzyme is shown

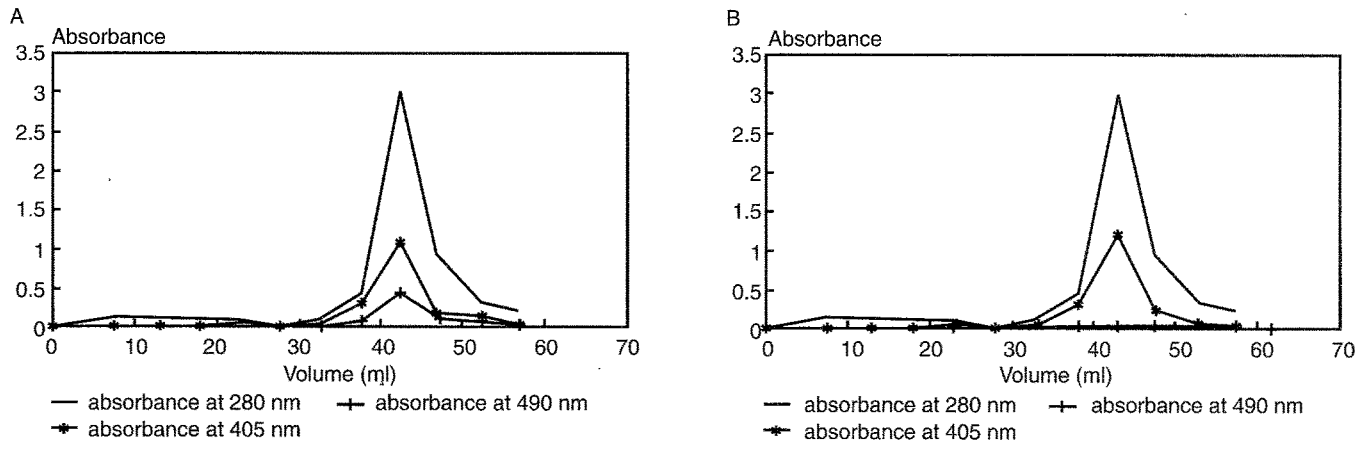


Figure 1. Separation of galactosylated (A) and not modified chymotrypsin (B) on Sephadex G-25 in 3% acetic acid.
Absorbance at 280 nm – content of proteins;
Absorbance at 490 nm – content of neutral saccharide [13];
Absorbance at 405 nm – the chymotrypsin activity [14].

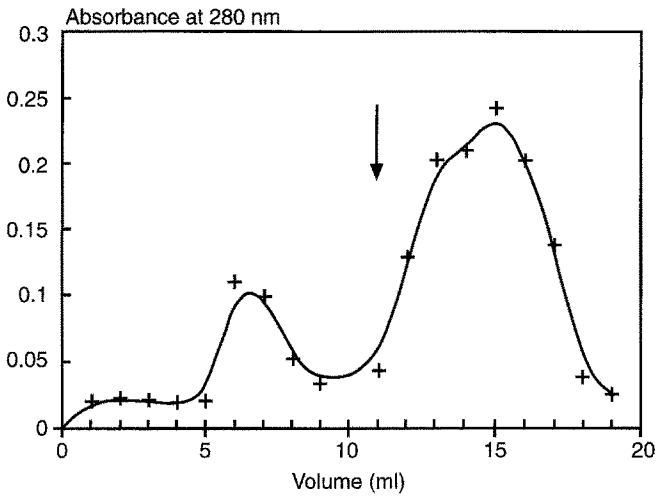


Figure 2. Affinity chromatography of concanavalin A on D-glucosylated poly(acrylamide-allyl alcohol) copolymer. An arrow denotes the start of elution with 0.2 M D-glucose solution.

in Fig. 1, and the content of bound saccharides in Table 1. The enzymic activity of modified chymotrypsin decreased by 8%.

To exploit the possible sites of the protein modification, model substances were used. For this purpose the following water soluble polyacrylamide derivatives were used: poly(acrylamide-allyl amine), poly(acrylamide-allyl alcohol) and poly(acrylamide-2-allyl phenol) copolymers. In an alternative experiment, water insoluble poly(acrylamide-allyl alcohol) copolymer was used. In all cases, glycosylated polyacrylamide copolymers were obtained; the content of bound saccharides in water-soluble preparations is given in Table 1. Modified polyacrylamide copolymers with amino or alkyl hydroxyl groups contained comparable content of the linked saccharide (Table 1). The amount of bound saccharides to aryl hydroxyl groups of the third polyacrylamide copolymer was lower. In the case of polyacrylamide copolymer containing covalently linked amino groups, the degree of modification using CET-Gal was similar to that obtained after reductive amination of disaccharides described previously [12].

The presence of D-glucosyl residues in D-glucosylated polyacrylamide copolymers bound to amino or alkyl hydroxyl groups was proved by two methods.

a) Double diffusion experiment in agarose gel: both types of polyacrylamide derivatives of D-glucose yielded precipitation lines with concanavalin A, as in the case of modified bovine serum albumin. The presence of free D-glucose in agarose gel inhibited the formation of precipitate.

b) Using water-insoluble D-glucosylated poly(acrylamide-allyl alcohol) copolymer as an affinity carrier; the prepared modified matrix specifically adsorbed concanavalin A, which could be eluted with the D-glucose solution (Fig. 2).

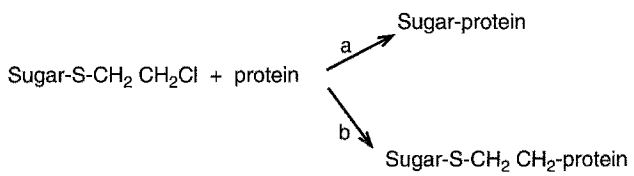
Enzymic hydrolysis with β-glucosidase and β-galactosidase was used to characterize the linkage of saccharide residues in the prepared glycoconjugates. Control experiment showed that 2-hydroxyethyl β-D-thiogalactoside was not hydrolysed by β-galactosidase, as was proved using paper chromatography. After enzymic hydrolysis of D-glucosylated derivative of bovine serum albumin or poly(acrylamide-allyl alcohol) copolymer, both the water-soluble and the water-insoluble one, traces of D-glucose in the incubation mixture containing β-glucosidase was detected; paper chromatography or glucose oxidase test were used. Similarly, β-galactosidase released traces of D-galactose from D-galactosylated glycoconjugates. In the case of D-glucosylated water-insoluble poly(acrylamide-allyl alcohol) copolymer, β-glucosidase released approximately 10–15% of linked saccharides; the second part of the saccharides remained bound to polyacrylamide copolymer after the enzymic hydrolysis, as was proven by the determination of neutral sugar in the gel after β-glucosidase treatment (Table 2).

As far as the course of glycosylation of proteins is concerned, two alternative explanations can be suggested: a) an attack of the protein molecule by a glycopyranosyl cation originating from chloroethyl thioglycosides [10] in the reaction medium; b) substitution of chlorine in chloroethylthioglycoside by a nucleophilic group of the protein molecule (see Scheme 1).

As the enzyme hydrolysis of modified glycoconjugates with β-glucosidase or β-galactosidase yielded only a small portion of free D-galactose or D-glucose, respectively, mechanism b) probably predominates. This suggestion is supported by the fact that thioglycosides are not

Table 2. Content of D-glucose in modified water-insoluble poly (acrylamide-allyl alcohol) copolymer.

	Saccharide content	
	(μg *per ml of gel*)	(%)
D-Glc-COP	60	3.0
β-glucosidase treated D-Clc-COP	52	2.6



Scheme 1.

hydrolysed with exoglycosidases [16] and also by our findings: 2-hydroxyethyl β-D-thiogalactoside was not hydrolysed with β-galactosidase.

Water-soluble polyacrylamide copolymers containing linked amino, alkyl hydroxyl or aryl hydroxyl groups have been found to be very suitable for studying the site of modification of proteins. In our previous communication [12], we have shown that poly(acrylamide-allylamine) copolymers can be used for binding saccharides either by reductive amination or using carbodiimide reaction. New types of water soluble polyacrylamide copolymers containing bound alkyl or aryl hydroxyl groups described in the present communication broadened the possibility of preparation of different types of glycoconjugates and their application in different binding studies.

It is possible to prepare analogous water-insoluble polyacrylamide copolymers modified by the same way which could be used as carriers for affinity chromatography. On the basis of results of binding studies with water soluble derivatives, the most suitable affinity carrier can be prepared, as was shown on an example of heparin derivative of poly(acrylamide-allylamine) copolymer [12, 17] and D-glucosylated poly(acrylamide-allyl alcohol) copolymer (this paper).

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