PREPARATION AND PROPERTIES OF GLYCOSYLATED CYTOCHEMICAL MARKERS

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1. Introduction

Lectins which are known to bind selectively carbohydrate moieties [1] have been used to visualize cellular carbohydrate components by electron microscopy [2]. Because lectins have at least two binding sites, they are able to bind a cellular sugar on one hand and a glycosylated cytochemical marker on the other hand. However, the main cytochemical markers either are not glycosylated (ferritin) or are glycosylated (horseradish peroxidase [3], hemocyanin [4], dextran–iron complex [5] or mannan–iron [6]) but bind only glucose- or mannose-binding lectins. In oder to detect various lectins by such a sequential method, we proposed using cytochemical markers covalently substituted with oligosaccharides [7,8]. In this paper, we describe the preparation and the properties of various glycosylated horseradish peroxidases and glycosylated ferritins.

2. Materials and methods

2.1. *Materials*

Horseradish peroxidase (HRP), agglutinins of *Canavalia ensiformis* (Con A), of *Triticum vulgare* (WGA) [9], of *Phaseolus vulgaris* (PHA-Els) and porcine thyroglobulin were obtained from Industrie Biologique Française, Clichy (France). Lectins of *Solanum tuberosum* (StA) and *Limulus polyphemus* (Limulin) were prepared according to Delmotte et al. [10] and Roche et al. [11], respectively. Lectins of *Arachis hypogea* (PNA) and *Glycine max* (SBA) were generous gifts from Dr R. Lotan and Dr N. Sharon, Weizmann Institute, Rehovot (Israel). Lectin of *Ricinus communis* (RcA) was a gift of Dr Olsnes, Oslo (Norway). Pronase was obtained from Calbiochem, *p*-aminophenyl-β-lacto-side was purchased from Cyclo Chemical, Los Angeles (USA), *p*-nitrophenyl-β-lactoside from Koch-Light, Colnbrook (England). *p*-Nitrophenyl-β-di-*N*-acetyl chitobioside was prepared according to Zurabian et al. [27], ferritin, 2 × cryst. was from Miles Laboratories Inc., Kankakee, Ill. (USA).

2.2. *Preparation of p-aminophenyl-glycopeptides*

2.2.1. Isolation of glycopeptides from porcine thyroglobulin (PT-glycopeptides)

The protein moiety of porcine thyroglobulin (3 g) was digested by pronase according to Monsigny et al. [12]. After the fourth digestion, glycopeptides were purified by gel-filtration on a Sephadex G-50 column (2 × 90 cm) [13].

2.2.2. Preparation of *p*-aminophenyl derivatives of PT-glycopeptides

(i) *p*-Nitrobenzoyl chloride (50 mg) dissolved in 5 ml pyridine and PT-glycopeptides (5 mg) dissolved in 5 ml pyridine/water mixture (4:1, v/v) were mixed. The solution was stirred at 25°C. After 24 h, specific reaction for amino group [14] was negative.

(ii) An alternative procedure was tested, using the hydroxysuccinimide ester of *p*-nitrobenzoic acid. *p*-Nitrobenzoic acid (2.25 g, 12 mmol) and *N*-hydroxysuccinimide (1.75 g, 17 mmol) were dissolved in di-*N*-methylformamide (7.5 ml) and 20 ml dichloromethane was added. The solution was cooled, and dicyclohexyl carbodiimide (3.1 g) in dichloromethane

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(3 ml) was added; the solution was kept for 6 h at 0°C. Dicyclohexylurea was removed by filtration. The filtrate was concentrated to dryness under vacuum. The solid residue was dissolved in boiling isopropanol (100 ml). Upon cooling *p*-nitrobenzoyl *N*-hydroxysuccinimide ester (1.5 g) crystallized.

The PT-glycopeptide (100 mg) dissolved in 5 ml pyridine/water mixture (4:1, v/v) and *p*-nitrobenzoyl *N*-hydroxysuccinimide ester (100 mg) dissolved in pyridine was mixed and stirred for 20 h at 25°C. The *p*-nitrobenzoyl-PT-glycopeptide was purified by gel-filtration on a Sephadex G-25 column (2 × 90 cm) and freeze-dried. No free amino groups were left.

(iii) The *p*-nitrobenzoyl-PT-glycopeptides (10 mg) were dissolved in 5 ml of 0.1 M sodium hydrogen carbonate buffer, containing 0.1 M sodium dithionite. The solution was stirred for 3 h at 25°C. The *p*-aminobenzoyl-PT-glycopeptide was isolated by gel-filtration on a Sephadex G-25 column (2 × 90 cm). The fractions containing the glycopeptide were cooled and freeze-dried (yield 20 mg).

2.3. *Preparation of p-aminophenyl-glycosides*

The reduction of *p*-nitrophenyl-glycosides (0.1 mmol) (*p*-nitrophenyl-di-*N*-acetylchitobioside, *p*-nitrophenyl-β-lactoside) were carried out in methanol (10 ml) by catalytic hydrogenation at atmospheric pressure (25°C, 6 h) in presence of 5% palladium on charcoal (20 mg) and of hydrochloric acid (0.1 mmol). The charcoal was filtered off and the solution was evaporated to dryness, the residue was then dissolved in 5 ml water and freeze-dried (yield 95%).

2.4. *Preparation of diazophenyl derivatives*

The *p*-aminophenyl-glycosides or the *p*-aminobenzoyl-PT-glycopeptide (0.10 mmol) were dissolved in 3 ml 80 mM hydrochloric acid and 8 mM potassium bromide in water at 0°C. Sodium nitrite (10 mg, 0.15 mmol) in distilled water (1 ml) was added slowly over 20 min. The solution was stirred for 1 h at 0°C. Urea 12 mg (0.2 mmol) was added. After an additional stirring for 30 min, distilled (6 ml) was added.

2.5. *Horseradish peroxidase and ferritin glycosylation*

2.5.1. Preparation of glycosylated horseradish peroxidase

Horseradish peroxidase (HRP) (75 mg, 2 mmol) was dissolved in 0.01 M borate buffer, pH 9.3 (7.5 ml) at 0°C. The *p*-diazophenyl derivative (10 mmol) in 10 ml solution was added slowly over 30 min. The pH was maintained at 9.3 ± 0.2 by adding 0.5 M sodium hydroxide. The disappearance of diazonium was monitored using the resorcinol method according to Koltun [15]. The solution was passed through a Sephadex G-50 column (2 × 90 cm) equilibrated with 0.10 M NaCl, 0.05 M Tris–HCl buffer, pH 7.5. The fractions containing the glycosylated horseradish peroxidase were dialyzed against distilled water and then freeze-dried (yield 70 mg).

2.5.2. Preparation of glycosylated ferritin

Ferritin was substituted in conditions described for horseradish peroxidase except that the glycosylated ferritin was not freeze-dried.

2.6. *Analytical assays*

2.6.1. Peroxidase activity

The activity of horseradish peroxidase and of their glycosylated derivatives were assayed using the *o*-dianisidine method according to Shannon et al. [16].

2.6.2. Double-diffusion precipitation

The capacity of the glycosylated cytomarkers (2 mg/ml) to bind lectins (2 mg/ml) was assayed by the double-diffusion technique [17]. Gels (1%) were prepared using Agar Noble (Difco) and veronal buffer, Γ/2 0.1, pH 8.2.

The plates were stained with Coomassie Blue or using the specific reaction for peroxidase activity with 3,3′-diamino-benzidine tetrahydrochloride (Sigma) and hydrogen peroxidase [18].

2.6.3. Inhibition of agglutination

Specificity of the glycosylated cytomarkers was tested by inhibition assays using a 3% erythrocyte-suspension in the condition previously described [19].

2.6.4. Ultracentrifugation analysis

Ultracentrifugation experiments were carried out in 0.1 M NaCl, 0.05 M Tris–HCl buffer, pH 7.5. Measurements of the sedimentation velocity were made at 60 000 rev./min on a Spinco Beckman model E ultracentrifuge equipped with an ultraviolet optical system at 280 nm.

3. Results and discussion

In order to define a general method of visualizing specifically various cell-surface carbohydrates, substitution of horseradish peroxidase and of ferritin with diazophenyl-carbohydrates was selected, for the following reasons:

(i) Most *p*-nitrophenyl-monosaccharides are commercially available.

(ii) *N-p*-Nitrobenzoyl-glycopeptide can be easily prepared with an almost quantitative yield.

(iii) Substitution of the amino group was thought better than activation of the carboxylic group [20] because with the latter both the carboxylic groups of the glycoamino-acid or glycopeptide and of sialic acid would be involved.

(iv) Reduction of the *p*-nitrophenyl group into an amino-phenyl-group and diazotization of the amino-phenyl derivative are almost quantitative.

(v) The reactions of diazophenyl compounds with protein do not induce oligomerization of, or charge changes in, the protein.

(vi) Finally, the azo-derivatives are stable enough to be kept for months or years, at low temperature.

3.1. *Size of the cytochemical markers*

Sedimentation constants of glycosylated horseradish peroxidase and of ferritin are slightly higher than those of the native proteins (table 1). The increase of molecular weight, estimated from the equation : $M_1 = M_2(S_1/S_2)^{3/2}$, is about 15% for L-ferritin, CB-ferritin and PTG-HRP and only about 5% for L-HRP and CB-HRP. Furthermore, substitutions by diazophenyl-sugars did not dissociate ferritin into smaller oligomers and glycosylated ferritin can be detected by electron microscopy as well as the native protein [8]. Glycosylated horseradish peroxidase was freeze-dried and kept for months at 10°C without any oligomerization or aggregate formation.

Table 1
Sedimentation constants $s^{0.1\%}_{20,w}$ of cytochemical markers and of their glycosylated derivatives[a]

Native HRP	1.59
L-HRP	1.64
CB-HRP	1.65
PTG-HRP	1.75
Native ferritin	27
L-Ferritin	29.6
CB-Ferritin	29.7

[a] *Abbreviations:* HRP horseradish peroxidase, CB di-*N*-acetyl chitobiose, L lactose, PTG porcine thyroglobulin glycopeptide

3.2. *Binding properties of glycosylated markers*

Porcine thyroglobulin was selected amongst several other glycoproteins (fetuin, ovomucoid, hen-egg albumin, α_1-acid glycoprotein, immunoglobulin, transferrin, submaxillary mucins) because it reacts with a large number of lectins and was easily available. Native porcine thyroglobulin gave a precipitation band with Con A, SBA and PHA-Els. After acidic treatment (0.1 N, 1 h, 80°C) the desialylated thyroglobulin reacted also with PNA, abrin and RcA. Agglutination inhibition studies gave the same results. Glycosylated cytochemical markers were tested by the same methods, and the results are summarized in table 2. Native horseradish peroxidase (IBF or type VI, Sigma) which is a mannose-containing glycoprotein reacts with Con A, but failed to react with the other lectins tested. However with horseradish peroxidase (Type II, Sigma) binding occurred also with WGA [21].

Native ferritin which does not contain any sugar does not react with any lectin. CB-Ferritin and CB-horseradish peroxidase (di-*N*-acetyl chitobiose derivatives) reacted with WGA, StA and with anti-di-*N*-acetyl-chitobiose antibodies [22] as expected on the basis of the specificity of these lectins. L-Ferritin and L-horseradish peroxidase reacted with SBA, PNA and RcA, but not with PHA-ELs or with Con A. PTG-Ferritin and PTG-horseradish peroxidase reacted with Con A, PHA-Els and SBA but not with RcA, PNA and Limulin; the desialylated PTG-markers did react with PNA and RcA. These results are in agreement with the known specificities of the lectins; PNA and RcA bind galactose residues if they are not substituted, Limulin binds NANA-GalNAc but NANA-Gal very poorly.

3.3. *Specificity of the glycosylated markers*

Glycosylated horseradish peroxidase had 95% of the activity of the native protein, even after six months storage. The precipitation band of lectins/glycosylated cytochemical markers was visualized

Table 2
Precipitation of lectins-glycosylated cytochemical marker complexes[a]

Lectins	Simplest inhibitors [1]	HRP	CB-HRP	L-HRP	PTG-HRP	Fer	CB-Fer	L-Fer	PTG-Fer
Con A	Man, Glc	+	+	+	+	–	–	–	+
PHA-Els	Gal→GlcNAc→Man [23]	–	–	–	+	–	–	–	+
SBA	Gal, GalNAc	–	–	+	+	–	–	+	+
PNA	Gal, Gal→GalNAc [24,25]	–	–	+	–(+) b)	–	–	+	–(+) b)
RcA	Gal	–	–	+	–(+) b)	–	–	+	–(+) b)
WGA	GlcNAc, CB	–	+	–	–	–	+	–	–
StA	CB, CT [10,2]	–	+	–	–	–	+	–	–
Limulin	NANA→GalNAc [11]	–	–	–	–	–	–	–	–

[a] *Abbreviations* (as in table 1): CT tri-*N*-acetyl chitotriose, Fer ferritin
[b] With desialylated glycopeptide of procine thyroglobulin ferritin derivative

both by the Coomassie Blue method and the specific reaction for peroxidase activity. Therefore, glycosylated-horseradish peroxidase keeps its activity even as a three-dimensional complex with lectins. Finally, the precipitation reaction could be inhibited by a ligand specific of each lectin (table 2).

4. Conclusion

Glycosylated-horseradish peroxidase and glycosylated-ferritin are easy to prepare. Substitution of these cytochemical markers by diazophenyl derivatives did not destroy the enzymatic activity of horseradish peroxidase nor the oligomeric structure of ferritin. The glycosylated markers bind lectin specifically according to the nature of carbohydrate substituents. The binding of a glycosylated marker and lectin is strong enough to induce a precipitation reaction. Finally, these glycosylated markers have been shown to be usable both in light and electron microscopy [7,8]. Preparation of glycosylated cytochemical markers able to react with *N*-acetyl neuraminic acid and fucose-binding lectins are in progress.

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