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Additional Precipitation Reactions of Lectins with Human Serum Glycoproteins

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Dedicated in memoriam Dr. K. Heide

Summary: Highly purified human serum glycoproteins were treated with neuraminidase and examined for their cross reaction with several lectins with anti-galactosyl specificity: β -D-galactosyl structures are thought to be the main terminal sugar residues that become attached *de novo* after removal of neuraminic acid. The following lectins were tested: Tridacnin from the bivalve clams *Tridacna maxima* and *Tridacna gigas*, the agglutinin from the sponge *Axinella polypoides*, the lectin from the roach *Rutilus rutilus* and the plant lectins from *Ricinus communis*, *Ononis spinosa*, *Glycine soja* and *Abrus precatorius*. In agar gel diffusion, these purified and precipitating lectins gave more or less strong or negative results against the different neuraminidase-treated serum glycoproteins, thus indicating subtle differences with respect to their anti-galactosyl combining specificity. On the other hand, serum glycoproteins which reacted with the same lectin, did not always show complete identity lines. Finally, as revealed by these lectins, the carbohydrate moiety of serum glycoproteins may reflect a complex and broad spectrum of heterogeneity. This could lead to a more detailed understanding of the topographical and steric arrangement of the chemical structure and of the biological role of carbohydrate groups in these glycosubstances.

Zusätzliche Fällungsreaktionen von Lectinen mit Glykoproteinen aus Menschenserum

Zusammenfassung: Menschliche Serum-Glykoproteine wurden mit Neuraminidase behandelt und im Hinblick auf ihre Kreuzreaktionen mit verschiedenen Lectinen mit Anti-Galactosyl-Spezifität untersucht. Es wird angenommen, daß β -D-Galactosyl-Strukturen in der Regel diejenigen Zuckerreste sind, welche nach Entfernung der Neuraminsäure endständig werden. Folgende Lectine wurden getestet: Tridacnin aus den Muscheln *Tridacna maxima* und *Tridacna gigas*, das Agglutinin vom Schwamm *Axinella polypoides*, das Lectin aus der Plötze *Rutilus rutilus* und die Pflanzenlectine aus *Ricinus communis*, *Ononis spinosa*, *Glycine soja* und *Abrus precatorius*. Die gereinigten, präzipitierenden Lectine gaben mehr oder weniger starke Präzipitate, wenn sie im Agargel gegen verschiedene, Neuraminidase-behandelte Serum-Glykoproteine diffundierten. Auf diese Weise konnten feine Unterschiede bezüglich der Anti-Galactosyl-Spezifität aufgezeigt werden, während andererseits Serum-Glykoproteine, die mit demselben Lectin reagierten, nicht immer vollständige Identitätslinien ergaben. Die Ergebnisse deuten an, wie komplex und heterogen der Kohlenhydratanteil dieser Serum-Glykoproteine ist, wenn man ihn mit mehreren Lectinen untersucht. Aber nur so gewinnt man ein detailliertes Bild von der topographischen und sterischen Anordnung dieser Kohlenhydratketten in diesen Glykosubstanzen und kann etwas über ihre Chemie und biologische Bedeutung in Erfahrung bringen.

Introduction

In a previous communication (1) we have already described the reaction of an invertebrate lectin with various human serum glycoproteins, especially after they have been treated with neuraminidase. It was concluded that most, if not all, of the precipitin reactions were due to (the uncovering of) terminal, non-

reducing β -D-galactosyl residues. On the other hand, quite a number of different cross reactions, identity and non-complete-identity reactions could be observed. This led to the assumption, that many structural and steric differences do occur among these glycoproteins, also with respect to the serological availability of the carbohydrate groups. On the basis of these results, we investigated the possibility of further subdividing or classifying serum glycoproteins with the aid of lectin markers for their carbohydrate chains.

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²⁾ Paper XXXIII of the series Studies on Lectins.

Material and Methods

Material

Preparation of O-glycosylpolyacrylamide copolymers

Water soluble O-glycosylpolyacrylamide copolymers were prepared by copolymerization of allylglycosides with acrylamide, dialysis and lyophilization (2). These model substances had sedimentation coefficients in the range $s_{20,w}^0 = 3-4S$ and contained 10-25% sugar. Their physicochemical properties will be published elsewhere (V. Hořejší, J. Kocourek, in preparation).

Human serum glycoproteins

These were supplied in a highly purified state by the Laboratories of the Behringwerke Marburg. We are greatly indebted to the late Dr. K. Heide and to Mr. Heinz Haupt for their great efforts in purifying these materials. Carbohydratefree proteins were given under code numbers and showed no precipitin reaction with any lectin (for instance albumin or anaphylatoxin, the latter reacted with Tridacnin only because of its basic nature!). Only Fetuin was of bovine origin.

Neuraminidase-treatment of serum glycoproteins

Neuraminidase-treatment of purified samples of serum glycoproteins was performed as follows: 0.2 ml of a 4% solution of the serum glycoproteins was diluted 1:1 with neuraminidase (Behringwerke), so that we were incubating with 100 U of the enzyme for 4 h at 37°C. This solution was then used for the test, because control experiments with neuraminidase and the lectins were negative.

Techniques

Agar gel diffusion tests

These were performed in the usual way using Ouchterlony's technique (1, 3).

Haemagglutination and its inhibition with simple sugars

The haemagglutination activity was assayed by a serial dilution test tube method (4), starting with 1% lectin solution in the first tube. The inhibition activity of simple sugars was expressed as the lowest concentration of the sugar which completely inhibits the agglutination caused by diluted lectin solution (4 times more concentrated than the lowest active concentration (4)). Trypsinized erythrocytes were prepared by incubation of washed cells with 1% trypsin (Léciva, Praha) in saline at 37°C for 45 min.

Lectins

Tridacna haemolymph

This was prepared according to the first communication in this series (1). The precipitin is referred to as Tridacnin. *Tridacna maxima* and *Tridacna gigas* haemolymph was used, as described previously (5). The β -D-galactosyl specificity of the *Tridacna maxima* Tridacnin has already been described (6). The specificity of the *Tridacna gigas* Tridacnin is very similar, in that it is also inhibited by N-acetyl-D-galactosamine and D-galactose (unpublished results).

Lectin from *Rutilus rutilus*

Preparation of the lectin from *Rutilus rutilus* (roach): 150 g of the lyophilized *Rutilus rutilus* roe were pulverized and stirred 3 h with 750 ml of 0.1 mol/l acetate buffer (pH 5.5) containing 0.5 mol/l NaCl. The mixture was then centrifuged and the opalescent yellow extract was added to 300 ml of homogenized O- α -D-galactopyranosyl polyacrylamide gel (7, 8) and stirred 1 h. The gel was washed 3 times with 1 l of the buffer used for extraction, then poured onto the column (5 x 20 cm) and washed with the same buffer until the effluent did not con-

tain any UV absorbing material. The adsorbed lectin was eluted by 0.2 mol/l D-galactose solution in the buffer used during the whole isolation; fractions containing protein were dialysed against deionized water and lyophilized. Yield, 340 mg.

Specificity of the *Rutilus lectin:* The lectin was active against human O, A₁, and A₂ erythrocytes in a concentration of 60 mg/l, and against B group at a concentration of 30 mg/l. Trypsinization led to 4-8x enhancement of agglutinability of the cells.

The agglutination of trypsinized B group erythrocytes was inhibited by L-rhamnose (10 mg/l), D-galactose (625 mg/l), L-arabinose (625 mg/l) and lactose (2.5 g/l), but not D-glucose, D-mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and L-fucose (10 g/l). On addition of solid L-rhamnose or D-galactose to the lectin solution a slight turbidity developed, which was not observed with other sugars.

The lectin was soluble and stable in 0.1 mol/l acetate buffer pH 5.5) containing 0.5 mol/l NaCl. At lower ionic strength or higher pH the lectin rapidly aggregated and precipitated. The lectin precipitated strongly with acrylamide copolymers containing allyl- α -L-rhamnoside or α -D-galactoside, less with copolymers containing β -lactoside or L-arabinoside residues, and not all with copolymers containing D-glucose, D-arabinose, or D-xylose residues. It reacts with quite a number of glyco-substances and even lectins, including Tridacnin.

The physicochemical properties of this and other fish roe lectins will be published elsewhere (A. Krajhanzl, V. Hořejší, J. Kocourek, in preparation).

Lectins from *Ricinus communis*

Ricinus communis lectins were isolated by affinity chromatography of the haemagglutination active fraction from an extract of castor bean seeds (precipitated at 30-60% saturation by (NH₄)₂SO₄), on O- β -lactosylpolyacrylamide gel. The specifically adsorbed lectins were eluted by 0.2 mol/l D-galactose solution, dialysed and lyophilized (8). The agglutinin (M_r) 120,000 which was used as precipitin in this study was separated from toxin (M_r 60,000) by gel filtration on Sephadex G-100.

Lectin from *Ononis spinosa*

Ononis spinosa lectin was prepared from the root of the plant. The dried and pulverized root was extracted with water and the extract was fractionated by (NH₄)₂SO₄ precipitation. The active fraction (0-50% saturation) was dialysed, lyophilized and then applied on the column of O- β -lactosylpolyacrylamide gel. The adsorbed lectin was eluted by 0.2 mol/l D-galactose or lactose, dialysed and lyophilized. The isolated lectin was relatively anti-H specific and the agglutinating activity was inhibited by N-acetyl-D-galactosamine, lactose or D-galactose.

Lectin from *Glycine soja*

Glycine soja lectin was isolated by affinity chromatography of the seed extract on N-acetyl- α -D-galactosaminylpolyacrylamide gel. The lectin was eluted with 0.2 mol/l D-galactose solution, dialysed and lyophilized.

Details of isolation and characterization of these and other lectins will be the subject of another publication (V. Hořejší, J. Kocourek, in preparation).

Lectin from *Abrus precatorius*

Abrus precatorius seeds (200 g), red variety collected from India, were suspended in 8.5 g/l NaCl (1 l) at 5°C for two days and then homogenized in a Waring Blender. The mixture was centrifuged at 8000 rpm in a Sorvall Ro-2 centrifuge. The clear supernatant (200 ml) was fractionated with (NH₄)₂SO₄ up to 80% saturation. The precipitate formed in each fraction was removed by centrifugation at 10,000 rpm for 30 min, dissolved in a minimum volume of water, then dialysed against distilled water until free from ammonium ions (negative to

Nessler's reagent). The precipitate formed during dialysis was removed at 18,000 rpm for 30 min and the supernatants were lyophilized. The fraction obtained between 36 and 55% saturation of $(\text{NH}_4)_2\text{SO}_4$ showed most haemagglutinating activity (titer 8×10^6) with human red cells. The lectin is inhibited by galactose and galactose-containing carbohydrates, like lactose. It has already been described in detail by other authors (9, 10).

Lectin from *Axinella polypoides*

Axinella polypoides lectin was gift from Dr. Hagen Bretting, Zoologisches Institut and Museum, University of Hamburg. It is a mixture of two agglutinins which are inhibited by glyco-substances with terminal *D*-galactosyl residues, preferably β -1 \rightarrow 6 linked (11, 12).

Mouse myeloma protein

The sample J 539, already described in a previous paper (13) was used.

Results

The results of our precipitin experiments are given in table 1. The different neuraminidase-treated serum glycoproteins are listed in the first column, and their

precipitin reactions with different lectins are shown in the neighbouring columns.

Whereas the results with *Tridacna maxima* lectin have already been discussed elsewhere (1), the experiments with the roach roe (*Rutilus rutilus*) lectin were quite interesting. Although it also reacts with *D*-galactose, only a few glycoproteins are precipitated, irrespective of whether they are treated with neuraminidase or not. This fact favours the assumption, that the corresponding receptor is not terminal, but innerchain linked *D*-galactose. This finding is also strongly supported by the result with β_2 -glycoprotein III, which only reacts with *Rutilus* lectin, showing no reaction with any of the other lectins, all of which, as far as we know, are directed against terminal, *D*-galactosyl residues. On the other hand, most of these lectins react with *Rutilus*-negative glycoproteins.

The reaction of all β -*D*-galactosyl specific lectins with serum glycoproteins, especially those treated with neuraminidase, is in agreement with our present knowledge of the structure of the glycoprotein carbohydrate chains (14, 15). Differences in the strength of inter-

Tab. 1. Precipitation of lectins with serum glycoproteins.

Serum glycoprotein after neuraminidase-treatment	Precipitating lectins from							
	<i>Tridacna maxima</i>	<i>Tridacna gigas</i>	<i>Rutilus* rutilus</i>	<i>Ricinus communis</i>	<i>Glycine soja</i>	<i>Ononis spinosa</i>	<i>Axinella polypoides</i>	<i>Abrus precatorius</i>
Antithrombin III	+	-	-	+	-	-	-	+
Haemopexin	++	+	-	+	++	++	-	+
Transferrin	-	-	-	+	-	-	-	-
α_1 S-Glycoprotein	+	-	-	++	+	++	-	+
Fetuin	+	-	-	++	+	++	-	+
β_2 -Glycoprotein I	++	+	-	++	++	++	++	++
Cholinesterase	++	+	++	++	++	++	+	++
α_1 B-Glycoprotein	-	-	+	+	-	-	-	+
3,1S- α_2 -Glycoprotein	++	-	-	++	+	++	-	++
α_1 -Antitrypsin	-	+	-	++	-	-	-	+
α_2 HS-Glycoprotein	-	-	-	+	-	-	-	+
α_2 -Macroglobulin	++	+	+	++	+	++	++	++
α_1 -Antichymotrypsin	++	+	++	++	++	++	-	++
8S- α_3 -Glycoprotein	++	++	++	+	++	++	-	++
β_2 -Glycoprotein III	=	-	++	-	-	-	-	-
Gc-Globulin	-	-	-	+	-	=	-	-
Secretory component	++	+	+	++	++	++	-	++
Lactoferrin	++	+	-	+	+	++	-	++
Prothrombin	=	-	++	+	-	+	-	-
Inter- α -trypsininhibitor	+	-	++	++	+	++	-	+
C3-Activator	++	+	-	++	+	-	+	++
3,8S- α_2 -Glycoprotein	-	-	=	++	-	-	-	+
Coeruloplasmin	-	+	++	++	+	+	-	+
C1-Inactivator	+	+	++	++	+	++	-	++
9,5S- α_1 -Glycoprotein	-	-	++	++	-	-	+	++
Haptoglobin	++	+	+	++	++	++	-	++
Thyroxin binding globulin	++	+	+	++	+	++	-	++
IgA	+	-	-	++	-	++	-	++
IgM	+	+	+	+	-	++	-	++
IgD	-	=	+	++	-	-	-	+
IgG	+	-	-	-	-	-	-	-
IgE	++	++	+	++	++	++	++	++

- = no precipitin reaction

+ = weak, but good visible precipitin reaction

++ = strong, sharp precipitation line

* = Precipitation identical with both native and neuraminidase-treated glycoproteins

action of various *D*-galactosyl specific lectins clearly reflects different requirements of these lectins for steric arrangements of the interacting carbohydrate groups.

In this respect, some of these glycoproteins give very strong precipitation reactions, and carry polyvalent lectin receptors within or on their carbohydrate groups: Cholinesterase, α_1 -antichymotrypsin, 8S- α_3 -glycoprotein, secretory component (IgA), inter- α -trypsin-inhibitor, haptoglobin and IgE.

The precipitin reactions of IgE are very interesting; this was obtained from a myeloma patient and was more than 98% pure. A characteristic precipitin pattern with a typical set of anti-galactose lectins is presented in figure 1.

In addition, we have observed a weak reaction of IgE with *Pneumococcus* type XIV antiserum (unpublished results).

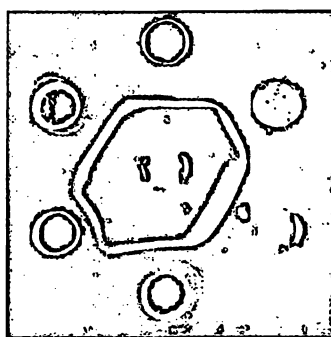


Fig. 1. Typical precipitin pattern between a glycoprotein (neuraminidase-treated IgE, in the middle) and different anti-galactosido-specific lectins (from 12 o'clock on clockwise):

- 1 = *Tridacna maxima* Tridacnin
- 2 = *Tridacna gigas* Tridacnin
- 3 = *Glycine soja* lectin
- 4 = *Ricinus communis* lectin
- 5 = *Axinella polypoides* lectin
- 6 = *Ononis spinosa* lectin

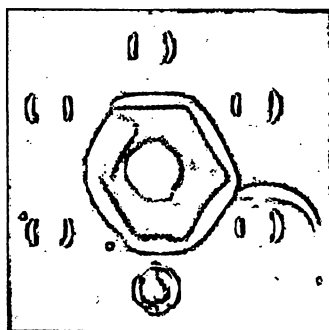


Fig. 2. Precipitin reactions of the *Rutilus rutilus* lectin (middle) with certain (serum) glycoproteins (from 12 o'clock on clockwise):

- 1 = Prothrombin
- 2 = Coeruloplasmin
- 3 = 9,5S- α_1 -glycoprotein
- 4 = β_2 -glycoprotein III
- 5 = Uromuroid
- 6 = C₁-inactivator

As an example of a precipitating lectin we have chosen the *Rutilus rutilus* lectin. In figure 2 we can see the precipitin reactions with different glycosubstances, which give more or less identical reactions. But this is not so with the other lectins listed in Table 1: they do not all give identical reactions with other cross reacting glycoproteins, and in many cases only partial identity is observed. This fact may subdivide these glycoproteins further and demonstrate some new relationships among them, as has been already shown in comparative studies with Tridacnin, certain serum glycoproteins and cross reacting polysaccharides or other glycosubstances (1). Also, in Figure 2, a precipitin reaction is shown between coeruloplasmin and 9,5 S- α_1 -glycoprotein.

In this connection it is worth mentioning, that with the exception of *Rutilus rutilus* lectin, these lectins give no or only very faint precipitates with serum proteins that have not been treated with neuraminidase. This may be due to the fact that *Rutilus rutilus* lectin can also detect certain innerchain *D*-galactose units.

The precipitin reactions of the *Axinella* precipitin fraction are remarkable: obviously some of the reactive glycoproteins seem to have terminal β -(1-6)-*D*-galactosyl structures. However, when the same glycoproteins were tested with the mouse myeloma protein, which has a very similar combining specificity (13), no precipitin reaction was observed.

The strong reaction of the *Abrus precatorius* lectin with most serum glycoproteins was to be expected, because of its known anti-Pneumococcal Type XIV polysaccharide cross-reactivity, i. e. it has a great affinity for terminal β -1-4-linked *D*-galactose (for instance to N-acetyl-*D*-glucosamine) (10), a substructure which has been suggested for the neuraminic acid free cryptic core of many serum glycoproteins (15, 16).

Discussion

The reaction of serum glycoproteins with certain lectins offers a new possibility of characterizing serum glycoproteins with respect to their slightly differing carbohydrate moieties. This may be of importance not only for elucidating the chemical structure of the oligosaccharide chains (16) but also for studying the metabolism and fate of glycoproteins, for instance their elimination by the liver (17, 18).

The reactivity of lectins with glycoproteins offers a suitable means for purification of these glycoproteins using affinity chromatography on immobilized lectins. So far, mostly Con-A Sepharose has been used for these purposes (19), but lectins with specificities different from Concanavalin A may be useful complementary tools. Most lectins used in the present study seem not to react with native, terminal sialic acid-containing glyco-

proteins, which presents a certain limitation. The obstacle was overcome by pretreatment of the sample with neuraminidase. The most promising lectin seems to be that of the roach (*Rutilus rutilus*), which also reacts with untreated glycoproteins.

Finally, our observations are of clinical interest, as neuraminidase action does occur in vivo in certain cases of polyagglutinability (20). Here, in addition to the lowered or altered electrophoretic mobility, the de novo reaction with specific lectins can be of diagnostic value.

Very few, but promising studies have been performed so far with serum glycoproteins and lectins; in this connection the precipitation activities of *Phaseolus vulgaris* lectins with different sera should be mentioned (21). Extensive studies with *Phaseolus vulgaris* lectin and different immunoglobulin classes and serum proteins have also been reported and reviewed elsewhere recently (22–26).

The fact, that the various comparative cross-reactions of serum glycoproteins, before and after removal of neuraminic acid, may lead to a new relationship between them, and may even reveal new heterogeneities among them.

At the present time, lectins may render a valuable service in comparative studies (e. g. in detection of slight structural differences between different glycoproteins, polysaccharides etc.), but one must always keep in mind that it is not possible to deduce the absolute structure of carbohydrate groupings on the basis of their reactions with a lectin. The results of inhibition experiments with different carbohydrates need not reflect the true situation during the interaction of lectins with more complex glycoproteins, cell receptors etc. Similarly, no straight-forward deduction can be made about the reactivity of terminal *D*-galactose residues with our lectins, simply based on the fact that removal of terminal sialic acid and uncovering penultimate β -*D*-galactoside residues is necessary for the "activation" of the glycoprotein. Other explanations are possible, and such a statement would be wrong and over simplified.

In a broader sense, lectins can be regarded as anti-carbohydrate reagents of non antibody-like nature. They occur in plants, invertebrates and vertebrates, but we do not know very much about their function and importance in various living organisms. Their behaviour as carbohydrate recognition molecules can be exploited as a tool, and as a basis for the study of their biological function.

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