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Lectin purification from carp roe (*Cyprinus carpio* L.), investigation of its carbohydrate specificity and application in histochemistry

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Abstract

A method of lectin purification from carp roe (CCRA) was elaborated, which includes affinity chromatography on cross-linked ovomucoid and copolymers of polyvinyl alcohol and blood group-specific substances. That allowed obtaining lectin with electrophoretic purity and yielding of ≈ 42 mg/kg roe. Electrophoresis in 15% polyacrylamide gel in the presence of β-mercaptoethanol showed one band with molecular mass ≈ 15 kDa, whereas in the absence of β-mercaptoethanol, CCRA exposed band with molecular mass ≈ 60 kDa. The resulting lectin was thermostable, withstanding heating to 75^0C for 15 minutes, without noticeable loss of hemagglutinating activity. Gel column chromatography on Toyopearl HW-55 determined the lectin molecular weight of 120±3 kDa. For the lectin activity, divalent metal ions (Ca^{2+} and Mg^{2+}) were not necessary. CCRA showed the best agglutination titer with pigeon erythrocytes, weaker – with rabbit and dog erythrocytes, and significantly weaker – with human and rat erythrocytes. CCRA lectin was specific to N-acetyl-D-galactosamine and D-galactose group carbohydrates. The best lectin activity inhibition possessed alkaline phosphatase of calf intestine and fetuin. CCRA exposed highest affinity to complex oligosaccharide similar to the receptor of *Phaseolus vulgaris* erythroagglutinin (PHA-E). A comparative study on the histochemical specificity of CCRA and PHA-E using specimens of normal tissues, and that of colon neoplasia, showed similar, yet not identical binding properties. CCRA lectin rather differentially labeled adenoma and adenocarcinoma of colon, which suggests its prospective applicability in diagnostic histopathology.

Keywords: carp roe lectin (CCRA), purification, properties, application in histochemistry.

Introduction

Lectins are proteins/glycoproteins that selectively bind carbohydrates (including carbohydrate receptors of living tissues) without causing their chemical transformations. Due to their carbohydrate selectivity, lectins are widely used for detection of specific carbohydrate determinants on the cell surface or in the cellular compartments. For this purpose, lectins with well-defined carbohydrate specificity and preferably with high selectivity to carbohydrate determinants are required. Currently, only a limited number of lectins with such properties are renowned. Therefore, the search and investigation of new lectins with rare carbohydrate specificity remains an important issue [1–6].

Plants are considered the main sources for lectin purification, since they often contain lectins in significant amounts and can be cultivated under regular conditions, therefore methods of plant lectins purification are well documented [7, 8]. However, recently more attention is focused on fungal and animal lectins.

Earlier we reported purification protocol for a lectin from fruiting bodies of the fungus *Mycena pura* (Fr.) Kumm. (MPFA), which possess high affinity interaction with alkaline phosphatase of calf intestine [9]. The relative inhibitory intensity of MPFA-alkaline phosphatase interaction was highest among 20 tested lectins. However, this fungus is of a small size, its fruiting bodies can be found in sufficient quantities during very short seasonal period and only in particular places, therefore its availability is limited.

During our studies on lectins from different sources, we founded out that the lectin from carp roe (*Cyprinus carpio* L., CCRA) also interacts with high affinity with alkaline phosphatase. This lectin purification protocol was described in several publications [10, 11]; moreover, a lectin from roe of a very close species – gibel carp (*Carassius auratus gibelio*) also was purified [12]. In these papers, lectin purification was maintained by affinity chromatography on Sepharose 4B followed by elution of the adsorbed lectin by 0.4 M N-acetyl-D-glucosamine and its additional purification on Superdex G-75 [10], or ion-exchange chromatography on phosphocellulose, DEAE-52 and subsequent hydroxylapatite column chromatography [12]. As a result, from 500 g of common carp roe it was obtained 10 mg of galactose-type lectin with molecular mass of 26.68 kDa, which showed carbohydrate specificity to N-acetyl-D-glucosamine and, to a lesser extent, to L-rhamnose and L-fucose. For lectin activity, divalent metal ions (Ca^{2+} and Mg^{2+}) were not necessary [10].

According to data of other authors [11], rabbit erythrocyte agglutination by recombinant common carp

lectin was completely inhibited by D-mannose (25 mM), D-glucose (50 mM) and D-xylose (76 mM), but not by L-fucose, D-galactose, melibiose, lactose and maltose at a concentration of 100 mM. This lectin was Ca^{2+}-dependent.

From a gibel carp, it was obtained C-type Ca^{2+}-dependent lectin with molecular mass of 21.5 kDa; rabbit erythrocyte hemagglutination with this same lectin was inhibited by D-mannose, L-fucose and D-mannosamine, but not by N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-galactose or D-glucose [12].

As seen from above, literature data on physicochemical properties and carbohydrate specificity of *Cyprinus carpio* lectin are contradictory. At the same time, carbohydrate specificity is accounted among the most important functional characteristics of lectins, which determine the prospects of their further application in biological and histochemical research.

The aim of present investigation was to elaborate optimal protocol of lectin purification from carp roe (CCRA), to study its carbohydrate specificity and applicability in histochemical research.

Materials and Methods

Purification protocol

Purification of lectin was performed in our laboratory (Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv). Roe was obtained from one carp weighing 2.65 kg, which was caught in fish industry near Lviv, Ukraine. Freshly picked roe (560 g) was placed in a mixer and homogenized with 1.8 liters of 1% sodium chloride solution by stirring for 5 minutes at 3000 min^{-1}. The resultant homogenate was centrifuged at 6000 g for 10 minutes and pH of the supernatant adjusted to 4.5. After removal of sediment by centrifugation at 6000 g for 10 minutes, the pH of supernatant was again adjusted to 7. Then, solution was again centrifuged for 20 minutes, at 6000 g. The obtained supernatant was applied to the affinity sorbent column (cross-linked ovomucoid [13], h=25 cm, V=480 mL). The column was washed with 0.05 M phosphate buffer with 3% NaCl at a speed of 5 mL/min as long as the level of extinction (A_{280}) in the eluate did not decrease below 0.1. Lectin adsorbed on the column was removed by 2% solution of lactose dissolved in the previous buffer. Protein containing fractions were combined and the lectin was precipitated by ammonium sulphate in the ratio 560 g of salt for one liter of lectin solution (Figure 1).

The resultant solution was thereafter purified by affinity chromatography on a copolymer of polyvinyl alcohol and human blood group-specific substance A [14]. For this purpose, 12 mL of the obtained lectin solution was applied to a column (h=8 cm, V=50 mL), previously washed with 0.05 M phosphate buffer with 3% NaCl. Thereafter, the column was washed with 0.05 M phosphate buffer with 3% NaCl at a speed of 1.5 mL/min as long as the level of extinction (λ 280 nm) in the eluate did not decrease below 0.1. Lectin adsorbed on the column was removed by 2% solution of lactose dissolved in the previous buffer. Protein containing fractions were combined and lectin was precipitated by ammonium sulphate at the ratio 560 g of salt for one liter of lectin solution (Figure 2).

Lectin characteristics

The purity of the obtained lectin preparation was evaluated by disc electrophoresis in 10% polyacrylamide gel (PAGE – polyacrylamide gel electrophoresis) in alkaline buffer system (pH 8.6). Minimal molecular weight of lectin's polypeptide chains was determined by electrophoresis in 15% polyacrylamide gel with 0.1% sodium dodecyl sulphate (SDS-Na) in the presence and in the absence of β-mercaptoethanol [15]. As standard were used mixture of proteins with known molecular weight produced by Fermentas (Lithuania) (Figure 3).

Complete molecular weight of lectin was determined at a column of Toyopearl HW-55 (h=39 cm, d=1.5 cm) using egg lysozyme (14.3 kDa), *Pisum sativum* lectin (49 kDa), *Helix pomatia* lectin (79 kDa), *Phaseolus vulgaris* erythroagglutinin (126 kDa) as protein markers. Protein elution was performed in 0.1% phosphate buffer, pH 7.4, with elution rate 2 mL/min (Figure 4).

Carbohydrate specificity of the obtained lectin was determined according to inhibition of rabbit erythrocytes hemagglutination by carbohydrates and glycoproteins. Minimal concentration, which completely inhibited activity of lectin (1:4 titer), was estimated using step-by-step dilution of them [8]. For this purpose, it were used carbohydrates as follows: D-glucose, D-fructose, D-galactose, sucrose, maltose, lactose (Soyuzhimreaktiv, Russia), raffinose (Fluka, Switzerland), α- and β-methyl-D-galactose, L-rhamnose, cellobiose, N-acetyl-D-galactopyranoside and N-acetyl-D-glucopyranoside (Chemapol, Czech Republic), D-mannose, D-turanose, L-ribose (Bratislava Chemical Institute, Slovakia), melibiose, α-methyl-D-mannoside, L-fucose (Koch Light, UK).

Tissue specimens and lectin histochemistry

Biopsy and autopsy material was received from Department of Pathology, Dubno Central Regional Hospital (Rivne Region) and from Lviv Regional Pathology Bureau according to approval of the Ethics Committee of Danylo Halytsky Lviv National Medical University [PhD research program of R.V. Antonyuk entitled "Lectin histochemistry of colon under normal conditions and that affected by neoplasia" (Protocol # 4 of 22.04.2013) on the specialty of histology and embryology], based on the cooperation agreement between the Department of Histology and Embryology, Lviv Medical University and Dubno Central Regional Hospital (Rivne Region), and Lviv Regional Pathology Bureau. The investigation was carried out according to the ethical criteria adopted by the 1st National Congress on Bioethics (Kyiv, Ukraine, 2001).

In order to test the applicability of new lectin preparation for histochemical research, it was used a panel of human organ samples as follows: sublingual salivary gland, esophagus, stomach, small intestine (duodenum, jejunum, ileum), Meckel's diverticulum, colon (cecum, appendix, ascending, transverse, descending, sigmoid, rectum), neoplastic lesions of colon (adenomas, adenocarcinomas), liver, pancreas, gallbladder, lung, kidney, adrenal gland, spleen, heart, cerebral and cerebellar cortex.

Biopsy and autopsy specimens were collected from the Department of Pathology, Dubno Central Regional Hospital (Rivne Region) and Lviv Regional Pathology Bureau.

The histological material was fixed in 4% neutral formalin or Bouin's fluid, dehydrated in graded concentrations of ethanol (50^0, 60^0, 70^0, 80^0, 90^0, 96^0, 98^0), cleared in *o*-xylene and embedded in paraffin. Tissue sections 5–7 μm thick were subjected to Hematoxylin and Eosin (HE). Carbohydrate determinants of tissue glycoconjugates were labeled with *Cyprinus carpio* lectin, conjugated to horseradish peroxidase (HP) by method of Nakane & Kawaoi [16] in our modification [17]. Briefly, carbohydrate moiety of peroxidase was oxidized by sodium *meta*-periodate; thereafter, the appeared aldehydic groups of peroxidase interacted with CCRA amino groups in an alkaline medium (pH 8.4–9.8), with the formation of peptide bonds; for conjugation purposes, amounts of peroxidase and lectin were taken in equivalent quantity.

Lectin binding protocol included 45 minutes incubation of tissue section with lectin-peroxidase conjugate (10–25 μg/mL dilution in phosphate-buffered saline) with subsequent visualization of lectin receptor sites with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) in the presence of 0.015% H_2O_2. More detailed information concerning staining protocol, as well as control on the specificity of binding, is presented elsewhere [18]. CCRA labeling of tissue glycoconjugates was compared with those of phytohemagglutinin-E (PHA-E) – lectin which possess similar carbohydrate specificity. Microscopic investigation was performed using Carl Zeiss Ng microscope (Jena, Germany) equipped with Canon IXUS 700 digital camera (Canon, Tokyo, Japan).

Results

Carp roe contains large amounts of fat that made some difficulties for lectin purification. Fat forms an emulsion, which was partly precipitated by ammonium sulphate, and gradually washed with buffer during purification on the affine sorbent. Charts of CCRA purification on the cross-linked ovomucoid and copolymer of polyvinyl alcohol and blood group-specific substance A are represented respectively in Figure 1 and Figure 2. From 560 g of carp roe was received 23 mg lectin (≈ 42 mg/kg). The resulting lectin preparation was thermostable – it withstands heating for 15 minutes to 75^0C without noticeable loss of hemagglutinating activity. The lectin also withstand ethanol precipitation: its hemagglutination titer decreased only for ≈ 10% after precipitation in two volumes of cooled to 0^0C ethanol and with subsequent dissolution of the precipitate in 1% NaCl solution.

While subjected to disc electrophoresis in 7.5% polyacrylamide gel alkaline buffer system (pH 8.6), lectin moved as two closely located bands. Thereafter, gel was cut into pieces with subsequent lectin elution and measurement of its hemagglutination titer with pigeon erythrocytes. The proteins eluted from both strips expressed hemagglutinating activity (Figure 3), encompassing that the lectin consisted of two isoforms.

The effectivity of elaborated carp roe lectin purification protocol is represented in Table 1. Total activity is expressed in hemagglutinating units – result of multiplication volume of extract to agglutination titer of pigeon erythrocytes.

Table 1 – ***Control of lectin purification by stages***

No. of stage	Stage of lectin purification	Total protein* (V×C)	Total activity* (V×U)	Yield [%] by activity
1.	Crude extract	59280	12160	100
2.	After correlation of pH	18720	11520	94.7
3.	Affinity chromatography on cross-linked ovomucoid	530.4	3264	71.1
4.	Affinity chromatography on the copolymer	23.76	1408	57.9

*Total protein expressed in mg – result of multiplication of protein concentration (C) by Lowry in mg/mL on volume (V) of extract in mL. U: Hemagglutinating units.

Electrophoresis in 15% polyacrylamide gel, in the presence of *β*-mercaptoethanol, showed one band with molecular mass ≈ 15 kDa, whereas in the absence of *β*-mercaptoethanol CCRA showed one band with molecular mass ≈ 60 kDa (Figure 5). Gel column chromatography on Toyopearl HW-55 determined this lectin molecular weight of 120±3 kDa (Figure 4). Dialysis of lectin solution against 1% EDTA sodium salt during the night did not reduce its agglutination titer, indicating that for lectin activation divalent metal ions are not required.

The obtained CCRA lectin preparation exposed species specificity to red blood cells. Namely, it strongly agglutinated red blood cells of a pigeon, but not of carp, mice, cow, goat, frog (Table 2). It is noteworthy, that pigeon erythrocytes are quite rarely used to identify and quantify the lectin preparations; therefore, information about their agglutination titers with respect to different lectins is fragmental and incomplete.

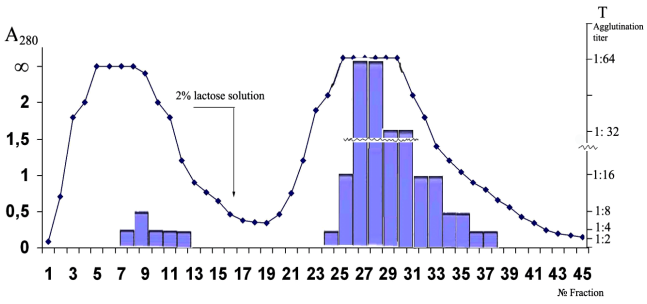

Figure 1 – ***Purification of the carp roe lectin (CCRA) by affinity chromatography on cross-linked ovomucoid. The arrow shows the place of application of 2% lactose solution.***

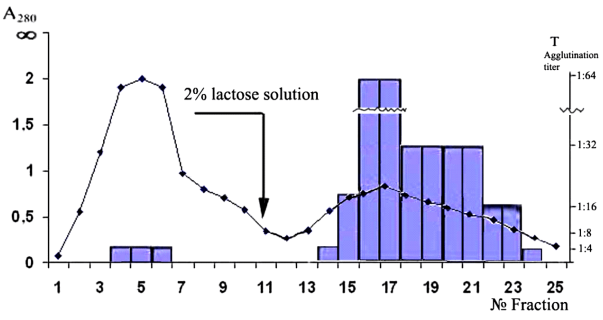

Figure 2 – ***Purification of the carp roe lectin by affinity chromatography on copolymer of polyvinyl alcohol and blood group-specific substances A. T: Rabbit erythrocyte agglutination titer.***

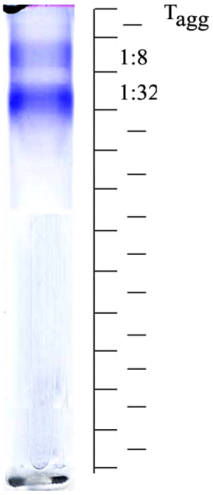

Figure 3 – ***Disc electrophoresis of CCRA in alkaline (pH 8.6) buffer system and hemagglutinating activity of individual fractions.***

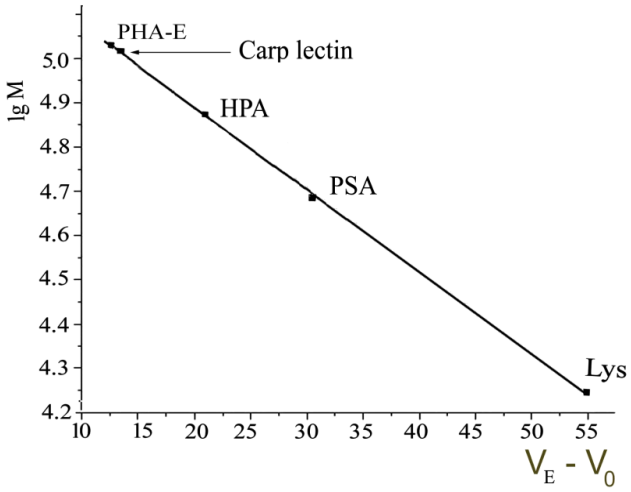

Figure 4 – ***Estimation of CCRA molecular weight by gel-chromatography on Toyopearl HW-55 column (39×1.5 cm), eluted by 0.1 M acetate buffer pH 6.4, supplemented with 0.5 M NaCl, elution rate 0.3 mL/min. PHA-E: Phaseolus vulgaris erythroagglutinin, Mr 126 kDa; HPA: Helix pomatia agglutinin, Mr 79 kDa; PSA: Pisum sativum agglutinin, Mr 49 kDa; Lys: Egg lysozyme, Mr 14.3 kDa.***

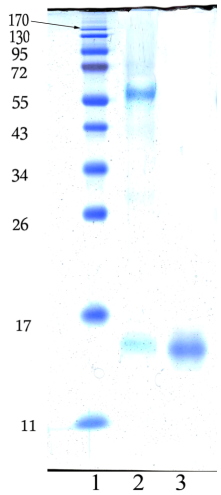

Figure 5 – ***CCRA electrophoresis in 15% polyacrylamide gel. Protein molecular weight markers (1) and of purified CCRA in the presence of 0.1% SDS-Na (2) and in the presence of 0.1% SDS-Na + β-mercaptoethanol (3).***

Table 2 – ***The minimum concentration of CCRA that agglutinated erythrocytes of different species***

No.	Erythrocyte type	The minimum concentration of lectin that agglutinated erythrocytes [μg/mL]
1.	Pigeon	1.25
2.	Rabbit	10.7
3.	Dog	10.7
4.	Hunan, A	43
5.	Rat	43
6.	Mice	– (>1000)
7.	Goat	– (>1000)
8.	Cow	– (>1000)
9.	Carp	– (>1000)
10.	Frog	– (>1000)

CCRA: Carp roe (*Cyprinus carpio*) lectin.

Our results on interaction of pigeon erythrocyte with various lectins are presented in Table 3. These data can provide additional information about the structure of carbohydrates, to which CCRA possess highest affinity, as well as demonstrate which of already known lectins stay close to CCRA due to their carbohydrate specificity.

Table 3 – ***Agglutination titers of pigeon erythrocyte with different lectins (at a concentration of 10 mg/mL)***

No.	Lectin	Carbohydrate specificity of lectin	Titer with pigeon erythrocytes
1.	*Galanthus nivalis* lectin	DManp	–
2.	*Hippeastrum hortorum* lectin	DManp	–
3.	*Narcissus poeticus* lectin	DManp	1:8
4.	*Pisum sativum* lectin	DManp > DGlcp	1:16
5.	*Vicia sativa* lectin	DManp > DGlcp	–
6.	*Lens culinaris* lectin	DManp > DGlcp	–
7.	Concanavalin A	DManp > DGlcp	1:16
8.	*Polygonatum multiflorum* lectin	DManp > DGlcp	1:32
9.	*Solanum tuberosum* lectin	Oligosaccharide structure containing DGlcNAcp	1:128
10.	Wheat germ agglutinin	DGlcNAc	1:512
11.	*Helix pomatia* lectin	α-DGalNAcp	–
12.	*Arachis hypogaea* lectin	Galβ1-3GalNAc	–
13.	*Amaranthus caudatus* lectin	Galβ1-3GalNAc	1:2
14.	*Sophora japonica* lectin	GalNAcβ1-6Gal	1:32
15.	*Artocarpus integrifolia* lectin (Jacalin)	Galβ1-3GalNAc	1:128
16.	*Soja hispida* lectin	GalNAcα1-3Galβ1-6Glc	1:1024
17.	*Caragana arborescens* bark lectin	Galβ1-4GlcNAc	1:1024
18.	*Robinia pseudoacacia* bark lectin	DGalNAcp > DGlcNAcp	1:1024
19.	*Clitocybe nebularis* lectin	DGalNAcp > DGalp	1:1024
20.	*Ricinus communis* lectin	Neu5Acα2-6Galβ1-4Glc	1:64
21.	*Sambucus nigra* bark lectin	Neu5Acα2-6Galβ1-4Glc	1:1024
22.	*Viscum album* lectin	GlcNAcβ1-6Gal	1:512
23.	*Amanita virosa* lectin	Galβ1-4Glc	1:16
24.	*Phaseolus vulgaris* erythroagglutinin (PHA-E)	GlcNAcβ1-2Man	**1:16384**
25.	*Phaseolus vulgaris* leucoagglutinin (PHA-L_4)	GlcNAcβ1-2Man	**1:8192**
26.	*Cyprinus carpio* lectin	DGalNAcp	**1:8192**
27.	*Mycena pura* lectin	Man(α1-3)Man	1:512
28.	*Perca fluviatilis* lectin	Fucα1-2Ga1β1-4Glc	1:8
29.	*Laburnum anagyroides* bark lectin	Fucα1-2Gaβ1-4Glc	1:1024

The best inhibitors of CCRA activity were carbohydrates of D-galactose group, in particular, N-acetyl-D-galactosamine. However, CCRA weakly interacted with mannose and some disaccharides, that contain glucose (Table 4), indicating that this lectin possess a highest affinity to complex structure oligosaccharides. 4-Nitrophenyl carbohydrate derivatives (namely, 4-nitrophenyl-β-D-galactopyranoside) were the best inhibitors of CCRA

(Table 5), while the same 4-nitrophenyl radicals did not interact with PHA-E lectin, which was in the closest neighborhood to CCRA with respect to pigeon hemagglutination test.

Table 4 – ***The interaction of MPFA, CCRA and PHA-E with some mono- and disaccharides***

No.	Carbohydrate	Minimum carbohydrates concentrations that inhibits the activity of 4 units of lectin [mM]		
		CCRA	*MPFA*	*PHA-E*
1.	D-glucose	–	100	–
2.	*α*-Methyl-D-glucopyranoside	–	–	–
3.	D-mannose	100	50	–
4.	*α*-Methyl-D-mannopyranoside	25	12.5	–
5.	Melibiose (Gal*α*1,6Glc)	6.25	–	–
6.	Lactose (Gal*β*1,4Glc)	6.25	25	–
7.	Gentiobiose (Glc*β*1,6Glc)	–	100	–
8.	Turanose (Glc*β*1,3Fru)	–	100	–
9.	N-acetyl-D-galactosamine	0.2	–	–
10.	*α*-Methyl-D-galactopyranoside	0.78	–	–
11.	*β*-Methyl-D-galactopyranoside	12.5	–	–
12.	L-arabinose	12.5	–	–
13.	L-rhamnose	6.25	–	–

CCRA: Carp roe (*Cyprinus carpio*) lectin; MPFA: *Mycena pura* fungus lectin; PHA-E: *Phaseolus vulgaris* erythroagglutinin. These lectins did not interact with N-acetyl-D-glucosamine, D-xylose, D-fructose, L-fucose, sucrose. Dash –: Absence of interaction at the concentration of 100 mM.

Table 5 – ***Interaction of lectins with nitrophenyl derivatives***

No.	Carbohydrates	Minimum carbohydrates concentrations that inhibits the activity of 4 units of lectin [mM]		
		CCRA	*MPFA*	*PHA-E*
1.	4-Nitrophenol-*β*-D-glucopyranoside	– (40)	0.6	– (40)
2.	4-Nitrophenol-*β*-D-galactopyranoside	0.01	5	– (40)
3.	4-Nitrophenol-*α*-D-galactopyranoside	0.08	– (40)	– (40)
4.	4-Nitrophenol-*α*-D-mannopyranoside	– (40)	10	– (40)
5.	4-Nitrophenol-*α*-L-fucopyranoside	10	– (40)	– (40)
6.	4-Nitrophenol-*β*-D-glucopyranoside	– (20)	10	– (40)
7.	Phenyl-*α*-N-acetyl-D-glucopyranoside	36	18	– (72)

CCRA: Carp roe (*Cyprinus carpio*) lectin; MPFA: *Mycena pura* fungus lectin; PHA-E: *Phaseolus vulgaris* erythroagglutinin.

At the mono- and disaccharides level, CCRA showed very little similarity to lectin from fruiting bodies of *Mycena pura* fungus (MPFA), similarly to which it demonstrated highest affinity towards alkaline phosphatase of calf intestine and calf fetuin. However, the studies on interaction of CCRA, MPFA and PHA-E with polysaccharides and glycoproteins evidenced more significantly about similarities of these lectins carbohydrate receptors (Table 6).

Table 6 – ***Interaction of lectins with polysaccharides and glycoproteins***

No.	Polysaccharide or glycoprotein	Minimum concentration of glycoprotein that inhibits the activity of lectin [%]		
		CCRA	*MPFA*	*PHA-E*
1.	*Transferrin*	0.06	0.125	0.125
2.	*Bovine thyroglobulin*	0.25	0.125	0.06
3.	*Alkaline phosphatase from calf intestine*	0.008	0.002	0.015
4.	*Orosomucoid (α-AGP)*	0.015	0.125	0.25
5.	*Ovomucoid*	0.125	0.06	0.06
6.	*Asialoovomucoid*	–	0.015	0.004
7.	*Sheep submandibular mucin*	0.125	0.125	0.125
8.	*Bovine submandibular mucin*	0.125	1	–
9.	*Desyalizated bovine submandibular mucin*	–	0.5	–
10.	*Human immunoglobulin G*	–	–	–
11.	*α_2-Macroglobulin*	–	1	0.25
12.	*Group-specific substance H*	0.125	0.125	0.25
13.	*Group-specific substance A*	0.125	0.125	0.25
14.	*Group-specific substance B*	0.125	0.25	0.25
15.	*Ovalbumin*	1	0.125	0.5
16.	*Glycogen of pig liver 1%*	–	0.5	–
17.	*Yeast mannan 1%*	0.5	–	–
18.	*Potato starch 1%*	0.25	1	–
19.	*Calf fetuin*	0.015	0.008	0.008

CCRA: Carp roe (*Cyprinus carpio*) lectin; MPFA: *Mycena pura* fungus lectin; PHA-E: *Phaseolus vulgaris* erythroagglutinin; *α*-AGP: *α*-1-Acid glycoprotein. Dash –: Absence of interaction at the concentration of 1%.

It should be noted, that highest similarity between three above-mentioned lectins was demonstrated by their interaction with intestinal alkaline phosphatase and calf fetuin, although most of other tested glycoproteins interacted with similar, yet weaker affinities with these three lectins.

Lectin histochemistry

Results on comparative labeling of human organ tissues with CCRA and PHA-E are presented in Table 7.

Table 7 – ***Comparative results on lectin labeling of human organ tissues***

Organ	Lectin	
	CCRA	*PHA-E*
Esophagus	–	–
Stomach	–	+/-
Small intestine	+/-	++/-
Large intestine	+/-	++/-
Meckel's diverticulum	++/-	++/-
Sublingual salivary gland	++/+	++/+
Pancreas	–	–
Liver	–	–
Gallbladder	+/-	+/-
Heart	–	–
Cerebellar cortex	–	–
Cerebral cortex	–	–
Kidney	+/-	++/-
Adrenals	+/-	+/-
Spleen	–	–
Lung	–	–

CCRA: Carp roe (*Cyprinus carpio*) lectin; PHA-E: *Phaseolus vulgaris* erythroagglutinin. Intensity of binding: - negative; + weak; ++ moderate; +++ strong; +/- variable reactivity.

In the stomach, it was revealed weak reactivity of chief cells luminal plasma membrane with PHA-E, these same structures being completely negative with CCRA. In the small intestine, CCRA receptor sites were found on the luminal plasma membrane of surface enterocytes, while PHA-E additionally labeled these cells Golgi complex. In the colon, both lectins faintly labeled luminal surface of colonocytes, membranous elements of goblet cell secretory granules (this label being more pronounced with PHA-E); moreover, PHA-E interacted also with Golgi zone of some surface colonocytes) (Figure 6).

Sample of Meckel diverticulum (evagination of small intestinal wall, present in newborn children and as a vestigial remnant of the omphalomesenteric duct in approx. 2% of the adult population [19]), obtained during appendectomy was also studied. In within the Meckel diverticulum, it was revealed intestinal type mucosa with characteristic villi and goblet cells predominance over columnar enterocytes. Weak to moderate reactivity was detected of both lectins with luminal plasma membrane, apical cytoplasm of columnar enterocytes and supra-nuclear areas of goblet cells. It is noteworthy, that mucin-containing secretory granules in small and large intestine were negative with both lectins used.

In sublingual salivary gland, it was detected binding of both lectins with secretory granules and perinuclear cytoplasm of striated duct, but not with acinar cells (Figure 7). In the kidney, CCRA receptor sites were detected exclusively in cortico-medullary stromal elements, while PHA-E also interacted with the brush border of proximal and distal tubules (Figure 8), that is consistent with data of others [20].

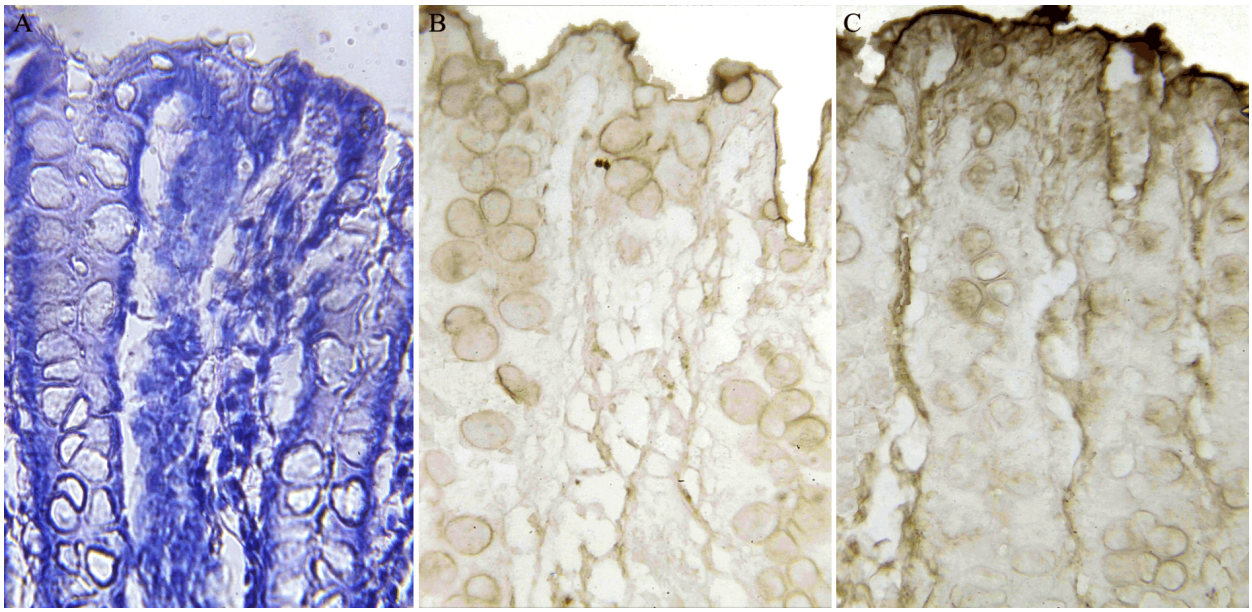

Figure 6 – ***Colon: (A) HE staining, ×400; (B) CCRA labeling: rather weak reactivity of luminal plasma membrane of surface colonocytes and goblet cell mucin membranous investment, ×400; (C) PHA-E reactivity similar to CCRA, though more intense; some surface colonocytes expose additional label of Golgi zone, ×400.***

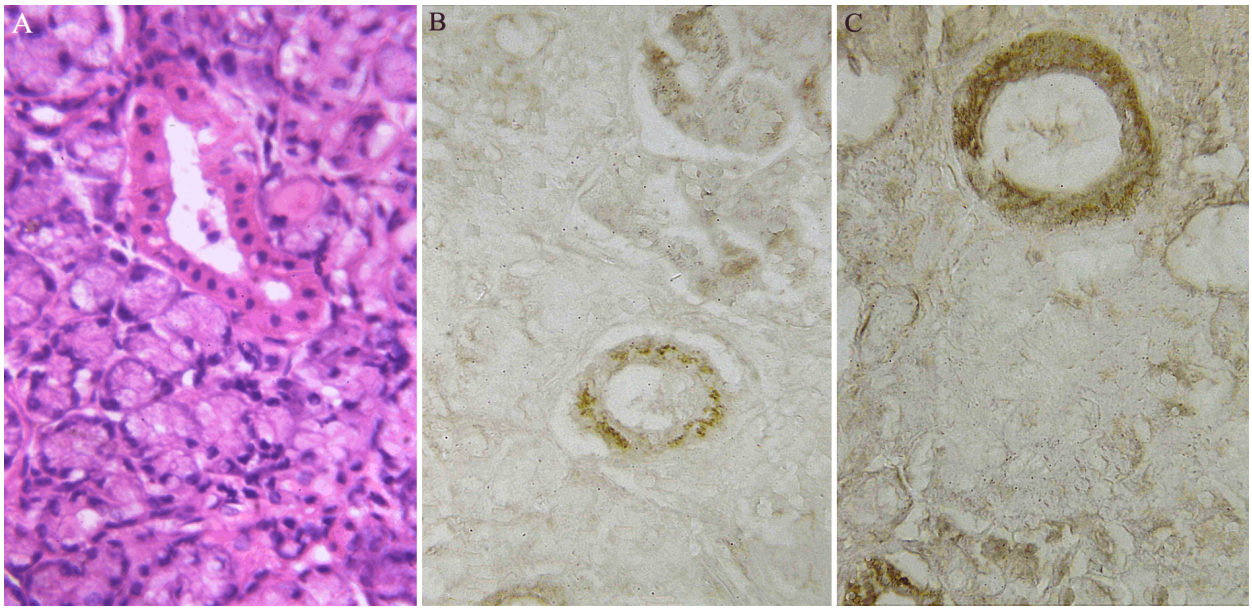

Figure 7 – ***Sublingual salivary gland: (A) HE staining, ×400; (B) CCRA binding: lectin receptor sites located in perinuclear (Golgi) zone of striated duct cells, acini and stromal components completely negative, ×400; (C) PHA-E: Selective reactivity of striated duct cell cytoplasmic glycoconjugates, ×400.***

Additionally to normal human tissues, we examined also preparations of colonic tumors (adenocarcinomas G1–G3, adenomas). Out of these, CCRA receptor sites were detected in 11 of 21 adenocarcinoma samples, PHA-E label – in 15 of 21 samples. The intensity of binding was low enough, and in most cases both lectins label was restricted to apical plasma membranes of colonocytes. Interestingly, adenomas and well-differentiated adenocarcinomas exposed more lectin reactive sites (Figure 9) (seven of nine samples for CCRA, and eight of nine for PHA-E) in comparison with moderate and low differentiated adenocarcinomas (Figure 10). A higher intensity staining was also noted of CCRA and PHA-E receptor sites in adenomatous mucosa in comparison to decreased reactivity of adjusting adenocarcinomatous lesions.

As seen from Table 7, CCRA reactivity with several organs was completely negative (esophagus, stomach, pancreas, liver, heart, cerebellar and cerebral cortex, spleen, lung) or faint enough (small intestine, gallbladder, adrenals) – therefore, micrographs of these organs are not presented in this article.

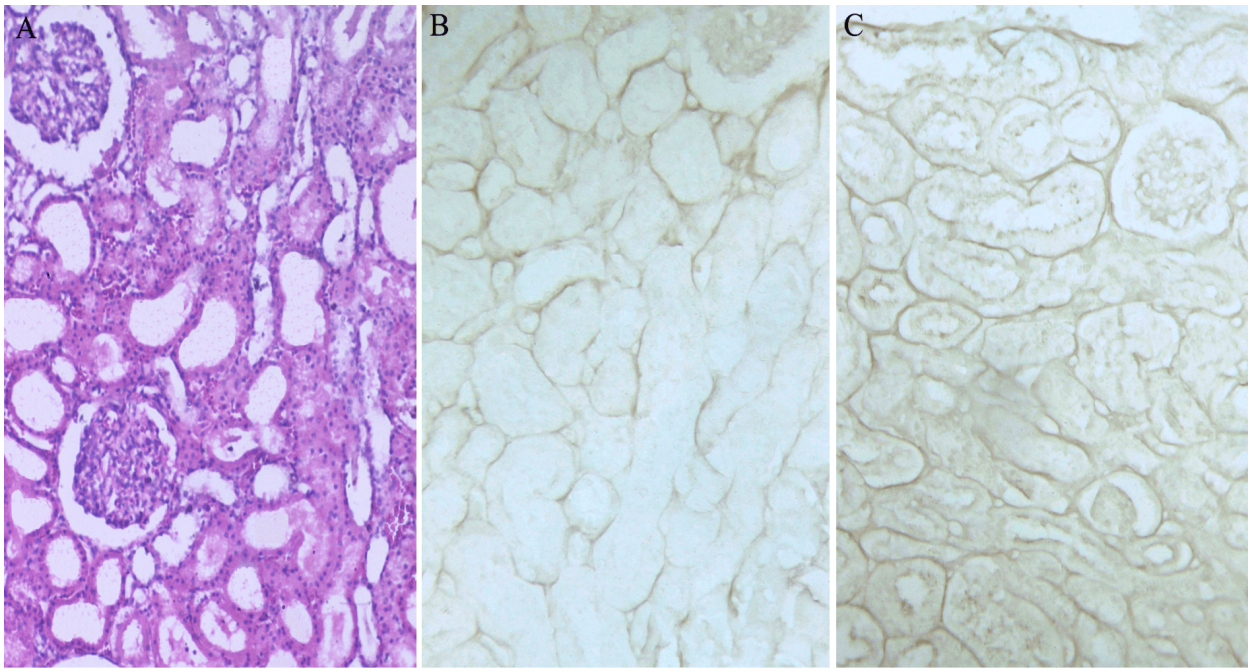

Figure 8 – ***Kidney: (A) HE staining, ×100; (B) CCRA label located in stromal elements and in the basement membranes enveloping renal tubules, ×100; (C) PHA-E binding, besides basement membranes and stromal elements, adjusted to brush border of proximal tubules and to luminal surface of distal tubules, ×100.***

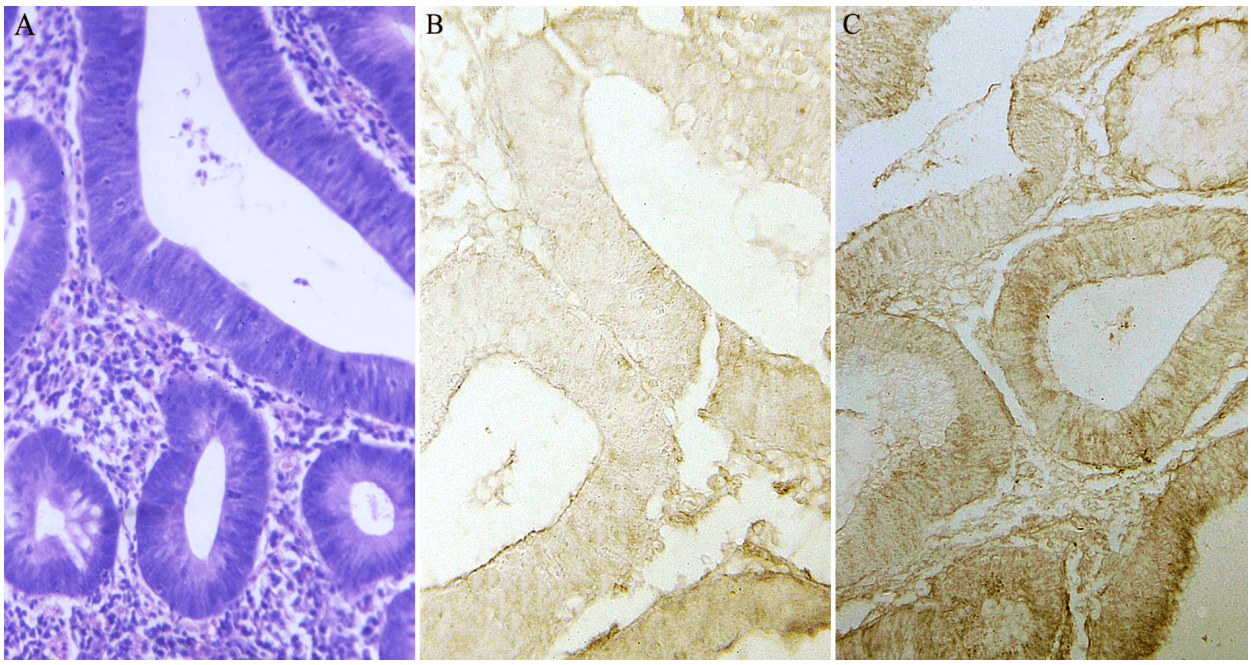

Figure 9 – ***Well differentiated adenocarcinoma of rectum: (A) HE staining, ×200; (B) CCRA label restricted to the lumenal plasma membrane, cytoplasmic glycoconjugates faint positive, ×400; (C) PHA-E binding to the luminal plasma membrane and supranuclear (Golgi) zone of tumor cell, ×400.***

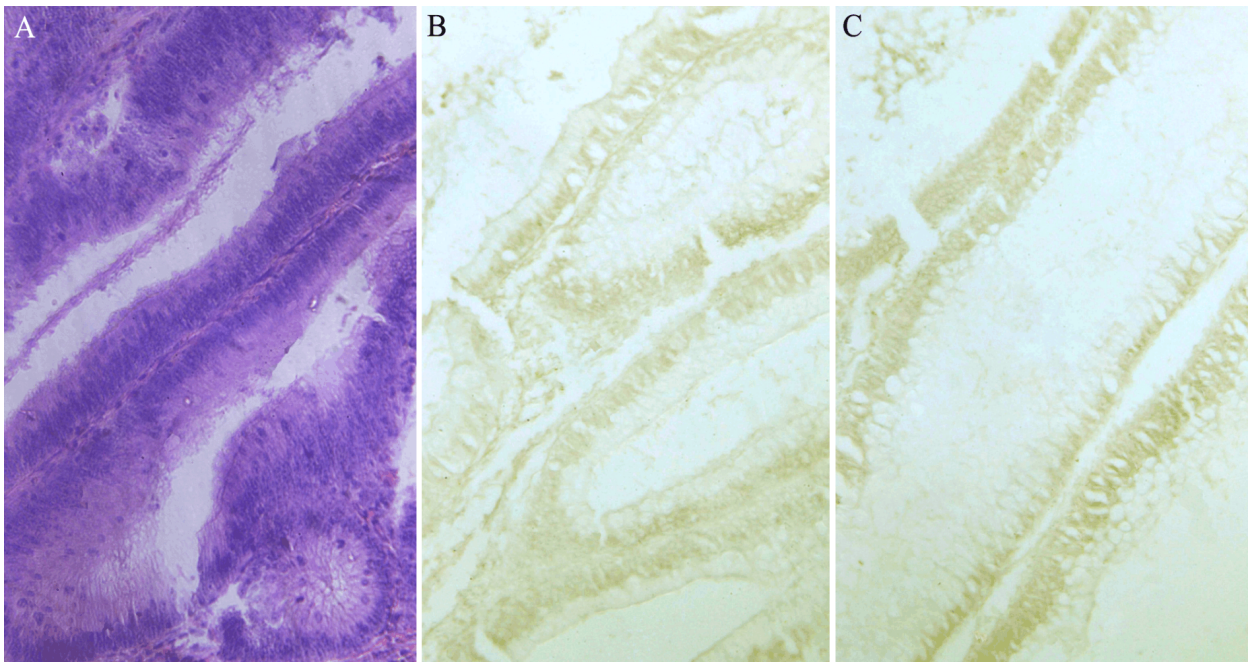

Figure 10 – ***Rectal adenoma: (A) HE staining, ×400; (B) Intense CCRA reactivity of glycoconjugates located in the basal part of transformed colonocytes (granular endoplasmic reticulum zone), luminal surface faint positive, ×400; (C) PHA-E receptor sites possess similar distribution, ×400.***

Discussion

Attempts to purify the lectin on partially hydrolyzed Sepharose 6B as described Galliano *et al.* [10] gave a very low output, therefore we rejected this sorbent. Cross-linked ovomucoid [13] was much more effective sorbent for CCRA preparation. However, while using it the resulting lectin preparation needed further purification. Better results were obtained by ion-exchange chromatography on DEAE-Toyopearl, yet the end product of this method consisted of two fractions with varying degree of purity, therefore to our current thinking optimal solution for CCRA purification was to use affinity chromatography on a copolymer of polyvinyl alcohol and blood group-specific substances A [14].

As seen from Table 6, alkaline phosphatase of calf intestine proved to be the best inhibitor of CCRA activity. It should be noted that carbohydrate chains of alkaline phosphatase of calf intestine and fetuin have been studied, their structures is presented in Figures 11 and 12. Calf fetuin is rich for carbohydrates, their total content reaches 22%. As shown in Figure 11, oligosaccharide chains are attached to polypeptide core in six locations. However, it should be noted that they are characterized by micro-heterogeneity, in particular, instead of Galβ1-4GlcNAc oligosaccharides may consist Galβ1-3GlcNAc links.

The common structure of alkaline phosphatase and fetuin are branched type oligosaccharide residues. If in fetuin they are tri-antennary, in alkaline phosphatase they are two- and four-antennary type. Terminal sites of the antenna contain disaccharide links of Galβ1-4GlcNAc connected to mannose core. The same disaccharide links are present in two-antennary terminal residues of transferrin and tri-antennary terminals of thyroglobulin and in chains of human blood group-specific substances. Perhaps, less affinity interaction with CCRA is due to lower carbohydrate content of these glycoproteins (5.5% in transferrin and 8% in thyroglobulin) in comparison to 17% in alkaline phosphatase.

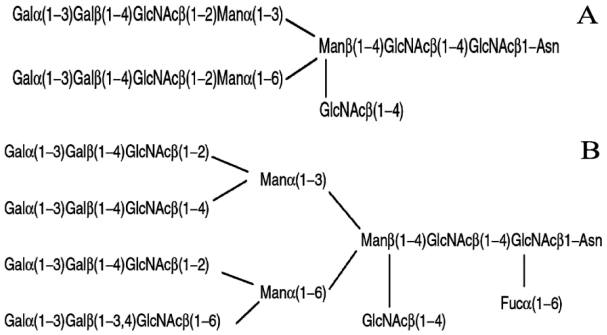

Figure 11 – ***(A) Structure of the carbohydrate chain attached to Asp249 alkaline phosphatase of calf small intestine; (B) Structure of the carbohydrate chain attached to Asp410 alkaline phosphatase of calf small intestine (after desyalization) [21].***

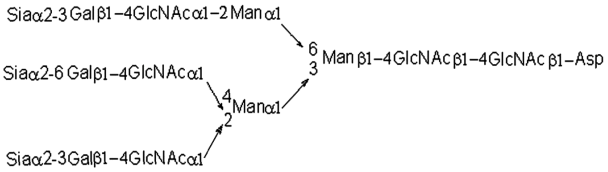

Figure 12 – ***Structure of the carbohydrate chain of calf fetuin [22].***

Ovalbumin contains 3.2% carbohydrates, which form tri- and tetra-antennary structures lacking Gal, but rich in GlcNAcβ1-2Man residues, which are also consistent in fetuin and alkaline phosphatase. It is likely, that presence of oligosaccharide chains with 2–3 carbohydrate residues comprising GlcNAc, Man, Gal/GalNAc in glycoproteins is critical for strong interaction of all three lectins under discussion (CCRA, MPFA and PHA-E).

As seen from Table 3, pigeon erythrocytes were preferably agglutinated by *Phaseolus vulgaris* erythroagglutinin (PHA-E), this observation being indicative of presence on the surface of pigeon erythrocytes of carbohydrate receptors, to which this lectin expose highest affinity. Yamashita *et al.* [23] deciphered structure of oligosaccharide, required for high affinity PHA-E binding (Figure 13). It is branched tri-antennary type octasaccharide, where R_1 and R_2 are hydrocarbon links and R_3 – GlcNAc-Asn or Fucα1-6GlcNAc. For effective binding, presence of Galβ1-4GlcNAcβ1-2Man chains linked with α(1-6)Man is quite necessary. The second antenna, representing GlcNAcβ1-2Man, does not need for lectin interaction presence of β-galactose groups. Therefore, it could be suggested, that CCRA would also well interact with similar oligosaccharide structure.

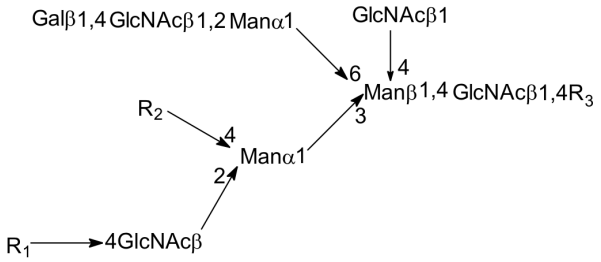

Figure 13 – ***Structure of oligosaccharide, to which PHA-E possess the highest affinity [23].***

Lectin histochemistry analysis demonstrated considerable similarities, yet not complete identity of CCRA and PHA-E specificity of binding with organ tissues under investigation (see Table 7). In our previous publication [24], we reported CCRA labeling of secretory granules in rat enterocytes, but not these in humans or other vertebrates, while PHA-E labeled mucin in frog intestines, indicating the species-specific histotopography of lectin receptor sites distribution.

Conclusions

A method of carp roe lectin (CCRA) purification was elaborated and its main biochemical and histochemical properties were studied. This lectin highest affinity was directed towards complex structure carbohydrate receptor, similar to PHA-E specific ligand that is a branched tri-antennary type octasaccharide with mannose core and DGal or DGalNAc epitopes on its ends. Glycoproteins which contain complex tri-antennary carbohydrate chains (calf fetuin or alkaline phosphatase of calf intestine) are strong inhibitors of carbohydrate binding activity of both – CCRA and PHA-E. Unlike the PHA-E, CCRA well interacted with DGal residues. CCRA showed highest agglutinability with pigeon erythrocytes, little weaker – with rabbit and dog erythrocytes, and much more weaker – with human and rat erythrocytes. Histochemical studies demonstrated considerable similarities, yet not complete identity of the receptor sites structure, to which interacted both lectins. In general, CCRA and PHA-E showed similar reactivity to cell and tissue glycoconjugates of a wide panel of organ samples, but PHA-E dominated in the intensity of binding and its receptor sites exposed wider distribution. CCRA rather differentially labeled adenomatous and adenocarcinomatous lesions of human colon. However, further investigations on this lectin prospective use in diagnostic histopathology are required.

Conflict of interests

The authors declare that they have no conflict of interests.

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