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Physicochemical properties and binding-site amino acid residues of galactoside-binding protein of human placenta

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Abstract. The galactose-binding lectin of human placenta has been purified to homogeneity by affinity chromatography on asialo-fetuin column. The protein, extractable from the tissue only with lactose is apparently membrane-bound. Molecular weight determination of native protein and subunit indicated a dimer of 13·4 kDa subunits. Inhibition of haemagglutination with various saccharides indicate that thiodigalactoside is the best inhibitor followed by lactose· However, *p*-nitrophenyl- and 1-O-methyl derivatives of galactose showed that α-anomers inhibited slightly better than *β*-anomer. Modification of amino acid residues indicated involvement of arginine, lysine and histidine residues at the saccharide-binding site. Cysteine residue modificatioin also abolished haemagglutinating activity. Amino acid composition of the lectin is also presented·

Keywords. Human placenta; galactose-binding lectin; binding-site amino acids; chemical modification·

Introduction

The role of carbohydrates in cellular recognition is well documented· It has become increasingly apparent that cell surface carbohydrates are intimately involved in lymphocyte 'homing', tumour invasiveness, trophoblast implantation and inter-cellular adhesion (Ashwell, 1977). The recently emerged field of carbohydrate-specific binding proteins, particularly, the galactose-binding one in hepatic tissue has been reviewed by Ashwell and Harford (1982)· Barondes (1984) has reviewed soluble galactose-binding protein from several animal tissues. Some lectins of certain animal species are also developmentally regulated. These *β*-galactose-binding lectins are isolated in mono-, di- or oligomeric forms (Carding *et al.,* 1985). The predominant lectins contain subunits in the range 13–26 kDa· A free SH-group is an essential feature of these lectins. The lectins are generally extracted from frozen or fresh tissue in presence of lactose and require no metal ions, with the exception of human hepatic lectin (Baenziger and Maynard, 1980). Recently, Hirabayashi and Kasai (1984) have reported a *β*-galactoside-binding lectin from human placenta·

In this communication we report the isolation and detailed physicochemical studies of the same lectin from normal human placenta. We have also examined the amino acids involved at the sugar-binding site by their chemical modification using specific reagents.

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Abbreviations used: pHMB, *p*-Hydroxymercuribenzoate; DTNB, dithionitrobenzene; SDS, sodium dodecyl sulphate; TNBS, trinitrobenzene sulphonic acid; PBS, phosphate buffered saline; WGA, wheat germ agglutinin; RCA1, caster bean agglutinin; WBA, winged bean agglutinin; PAGE, polyacrylamide gel electrophoresis; IgA, immunoglobulin A.

Materials and methods

Galactopyranosylamine, thiodigalactoside, galactosamine, melibiose, raffinose, stachyose, 1-O-methyl-α- and *β*-D-galactose, 1-O-methyl-α-D-glucose, chitin, 1-O-methyl-α-D-mannose, wheat germ, *p*-nitrophenyl derivatives of galactose, N-acetylimidazole, maleic anhydride, molecular weight markers (Aldolase, soybean trypsin inhibitor, ovalbumin, bovine serum albumin), citraconic anhydride, fetuin, *p*-hydroxymercuribenzoate (pHMB), dithionitrobenzoic acid (DTNB), sodium dodecyl sulphate (SDS) and trinitrobenzenesulphonic acid (TNBS) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. 2-Hydroxynitrobenzyl bromide, phenyl glyoxal hydrate, 2-methoxynitrobenzyl bromide, diethyl pyrocarbonate, 1,2-cyclohexanedione and N-ethyl morpholine were obtained from Fluka, Buchs, Switzerland. Sephadex and Sepharose were the products of Pharmacia Fine Chemicals, Sweden. Biogel P-60 and P-100 were purchased from BioRad Laboratories, USA. All other reagents were of analytical grades and obtained from local sources.

Normal full-term placenta tissue were collected from Trivandrum Medical College, in ice. The tissue was cleaned of blood and other adhereing tissue material and kept frozen at –20°C until use. The trophoblasts were separated from freshly collected placenta and kept overnight at 0°C in ice. All the operations were carried out at 0–4°C unless otherwise mentioned. The centrifugation was done wmyh SORVALL-RC-5B centrifuge. Frozen or fresh tissue was used for the isolation of galactose-binding protein. The homogeneous protein from both the types of tissue were found to be electrophoretically and immunologically identical. The buffer used for all the experiments was 20 mM phosphate-150 mM sodium chloride-4 mM mercapto-ethanol, pH 7·2 (phosphate buffered saline, PBS) unless otherwise mentioned·

Galactose-binding protein was isolated according to Hirabayashi and Kasai (1984) with modification. One-hundred gram of tissue was homogenised with 500 ml of PBS, pH 7·2 in SORVALL Omnimixer at 25% of its maximum speed for 2 min. The homogenate was centrifuged at 6000 *g* for 20 min. The pellet was extracted twice with 100 ml of PBS, pH 7·2 containing 100 mM lactose for 30 min. After each extraction the supernatant was collected by centrifugation as above. From the combined lactose extracts, proteins were precipitated with solid ammonium sulphate (56 *g*/100 ml) and dissolved in minimum volume of PBS, pH 7.2. The clear solution was dialyzed against the same buffer with 2 changes for 18 h.

The dialyzed solution was applied on a (2 × 18 cm) asialofetuin-Sepharose column. The column was prepared according to deWaard *et al.* (1976). The column was equilibrated and washed with PBS, pH 7·2 until the affluent had A_{280} below 0·02. The galactose-binding protein was eluted with the same buffer containing 20 mM lactose and 3 ml fractions were collected. The haemagglutinating activities of the dialyzed fractions were examined with trypsinised rabbit or human B + ve erythrocytes. The active fractions were pooled, dialyzed against PBS, pH 7·2 for 18 h and concentrated by ultrafiltration through Amicon PM-10 membrane·

Protein concentration was estimated by the method of Lowry *et al.* (1951) or by Bradford's protein assay (1976). Electrophoresis was carried out on polyacrylamide gel according to Davis (1964) and SDS polyacrylamide slab gel according to Laemmli (1970). The chemical modification of various amino acids were undertaken to obtain an idea of the sugar-binding region of the lectin. Amino groups were modified with TNBS (Fields, 1972), maleic anhydride (Butler *et al.,* 1969) and

citraconic anhydride (Atassi and Habeeb, 1972). Sulphydryl group was estimated and modified with DTNB according to Habeeb (1972) and also with N-ethylmaleimide and pHMB (Riordian and Vallee, 1972). Tyrosine was titrated and modified with N-acetylimidazole (Riordian and Vallee, 1972). Tryptophan was modified with dimethyl sulphoxide/acetic acid/HCl (Savige and Fontana, 1977). Tryptophan was also modified with 2-hydroxy- and 2-methoxynitrobenzyl bromide (Riordian and Vallee, 1972). Arginine was modified with phenyl glyoxal hydrate (Takahashi, 1968), 1,2-cyclohexanedione (Smith, 1977) and histidine by the method of Miles (1977). Carbohydrate residues were modified by periodic acid (Spiro, 1966) and with periodate and sodium cyanoborohydride (Thorpe *et al.,* 1985). Total carbohydrate was estimated by phenol-sulphuric acid method (Dubois *et al.,* 1965). The molecular weight of the protein under undenaturing condition was carried out on Biogel P-100 in PBS, pH 7·2 containing 2·5 mM 2-mercapatoethanol according to Andrews (1965).

Concanavalin A was purified and immobilised to Sepharose 4B by CNBr-methods of Porath as modified by Surolia *et al.* (1973). Wheat germ agglutinin (WGA) was purified on chitin column according to Bloch and Burger (1974). It was immobilised to Sepharose 4B by CNBr-method (Axen *et al.,* 1967). Castor bean agglutinin (RCA1) was purified and immobilised according to the method of Appukuttan *et al.* (1977). Winged bean agglutinin (WBA) and Jack fruit seed lectin (Jacalin) were purified according to methods published earlier (Appukuttan and Basu, 1981; Sureshkumar *et al.,* 1982).

Results and discussion

The affinity-eluted galactose-binding protein was homogeneous as confirmed by polyacrylamide gel electrophoresis (PAGE). The molecular weight of the undenatured lectin by gel filtration was found to be 26·9 kDa (figure 1). The subunit molecular weight of 13·4 kDa (figure 2) as revealed by SDS-PAGE was in agreement with the observation of Hirabayashi and Kasai (1984). These results suggest that the lectin is a dimmer. We have not observed any molecular weight higher than 27 kDa even by gel filtration on Biogel P-60 or P-100. The lectin did not contain any glycohydrolase activity·

The protein could agglutinate several native and trypsinised animal and normal human B + ve cells. It agglutinated native calf, pig, rat, mice and rabbit erythrocytes but not those of sheep, goat and dog. But after trypsinization of the cells it agglutinated rabbit, calf, pig, sheep, dog, rat, mice and normal human B + ve erythrocytes but not that of goat. Trypsinised human cells of A, B, AB and O groups were also agglutinated by placenta galactose-binding lectin. This lectin is non-mitogenic towards human lymphocytes.

The saccharide-inhibition studies of haemagglutination were carried out with trypsinised rabbit and human B + ve erythrocytes. The inhibition studies are shown in table 1. Thiodigalactoside was found to be the best inhibitor followed by lactose, as also shown by Hirabayashi and Kasai (1984). Agglutination of trypsinised human cells was inhibited by 8-times lower concentration of thiodigalactoside as compared to rabbit cells. Similarly lactose concentration was 4-fold lower under indentical conditions for human cells compared to rabbit cells. It has also been observed that galactose aminated at 1-position (*i.e.* galactopyranosylamine) was a better inhibitor than galactose and galactosamine (2-position aminated). Alpha-anomer of galactose

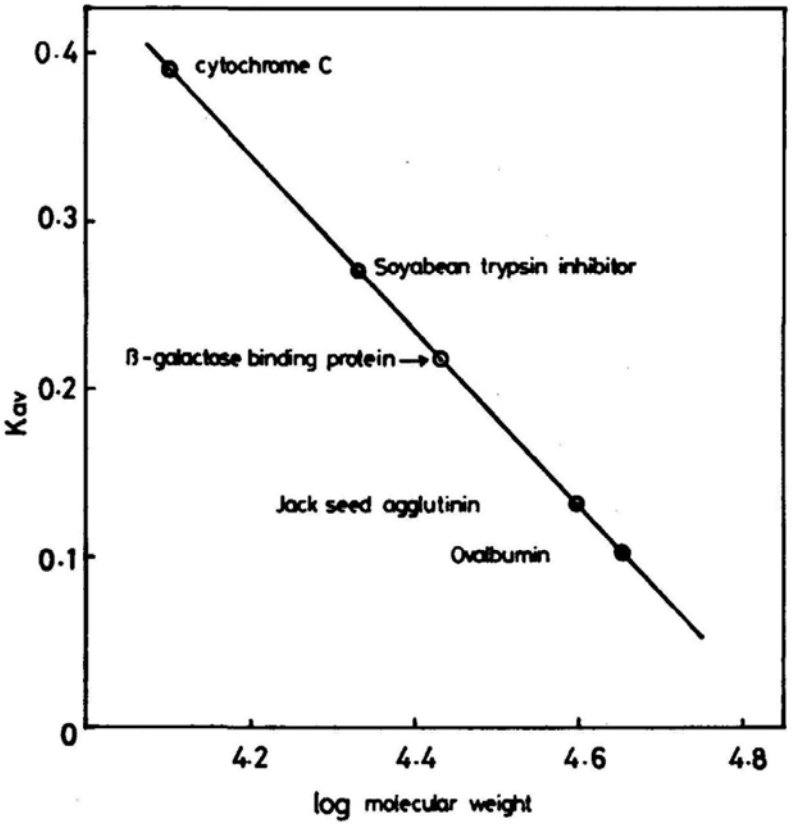

Figure 1· Gel filtration was carried out on Biogel P-100 (2 × 78 cm) in PBS, pH 7·2 containing 2 mM mercaptoethanol at 25°C. 2·5 ml fractions were collected at a flow rate of 12 ml/h· The standard proteins employed are cytochrome C, soybean trypsin inhibitor, Jack seed agglutinin (Sureshkumar *et al.,* 1982) and ovalbumin·

was found to be slightly better inhibitor than its *ß*-anomer. But glucose or mannose did not inhibit agglutination with both the cell types. In the present study we have observed that the saccharide concentrations required for the inhibition of agglutination were much lower than reported by Hirabayashi and Kasai (1984). Our observations were repeated with several batches of tissue extracts. It has been observed with fresh or frozen tissue, as well as trophoblasts cells of normal tissue. We did not require any protease inhibitor addition during the isolation of the lectin. The lectin retained its agglutinating activity when kept in the presence of 1 mM mercaptoethanol in PBS for several weeks at a concentration of 1 mg/ml at 0°C. Protein concentration higher than 1 mg/ml resulted in aggregation after 3–4 days at 0°C· The lectin lost about 80% of its agglutinating activity within a week when kept frozen at – 20°C. But the cleaned, whole placenta tissue retained activity under similar conditions for 2 weeks. The agglutination of trypsinised human erythrocytes required 6-fold higher amount of lectin as compared to rabbit cells. The concentrations of inhibitory saccharides required for inhibiting agglutination were also lower in case of human cells. It is assumed to be due to weaker binding of lectin to human cells relative to rabbit cells·

The protein was found to contain one SH-group when titrated with DTNB by

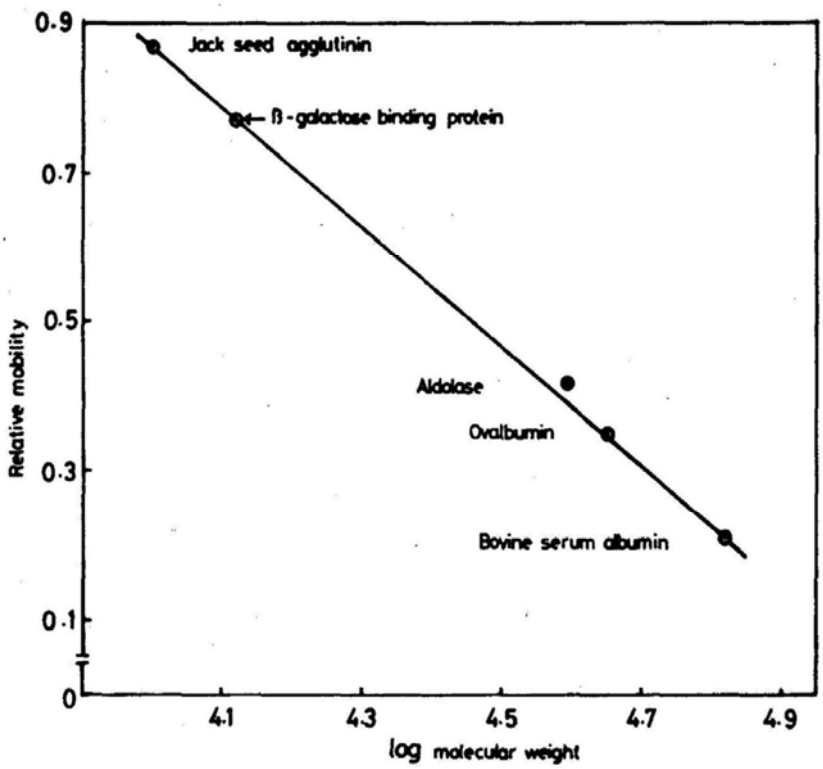

Figure 2. SDS-Polyacrylamide gel (10% gel) was carried out according to Laemmli (1970). The standard proteins are Jack seed agglutinin, aldolase, ovalbumin and bovine serum albumin.

Ellman's method (Ellman, 1959) or even under denaturating condition as described by Habeeb (1972). Tryptophan residue could not be detected by the methods of Savige and Fontana (1977) or by spectral shift (Bencze and Schmid, 1957). Tyrosine residues were determined by O-acetylation with N-acetyl imidazole (Riordian and Vallee, 1972). The lectin contained 3 tyrosine residues. This was confirmed by aminoacid analysis (table 3). Total carbohydrate content estimated by phenol-sulphuric acid method (Dubois *et al.,* 1965) with galactose as Standard was found to be 16·7%.

There is no information in the literature about the amino acids involved in the saccharide-binding site of *ß*-galactose-binding protein. In order to get some insight into the binding site we have chemically modified a few amino acid residues. The glycoprotein did not contain any tryptophan residue. Similar observation has been made by Hirabayashi and Kasai (1984) from amino acid analysis. Tyrosine residues were found not to be involved in the saccharide binding. On the other hand arginine residue modification resulted in complete inhibition of agglutination. Histidine modification by diethyl pyrocarbonate showed inhibitory effect on agglutination. The amino group modification by various method had inhibitory effect. Similar results were obtained by SH-group modifications·

The oxidation of saccharide side chains by periodic acid had inhibitory effect. This treatment was carried out at 0°C for 16 h. Treatment of the glycoprotein with a

Table 1. Inhibition capacity of common saccharides on agglutination of erythrocytes by galactose-binding lectin of human placenta·

Saccharides	Minimum concentration (mM) required to inhibit twice the haemagglutinating amount of lectin	
	Trypsinised human B + ve cells	Trypsinised rabbit cells
Thiodigalactoside	0·019	0·16
Lactose	0·20	0·78
Lactitol	4	4
D-Galactose	25	50
Melibiose	12·5	50
Raffinose	25	100
Stachyose	12·5	25
D-Galactosamine	25	50
D-Galactopyranosylamine	0·39	6·25
N-Acetyl-D-galactosamine	25	100
1-O-Methyl-α-D-galactose	3·13	25
1-O-Methyl-β-D-galactose	12·5	50
p-Nitrophenyl-α-D-galactose	1·5	2·5
p-Nitrophenyl-β-D-galactose	3·13	5·0
L-Fucose	NI	NI
1-O-Methyl-α-D-glucose	> 200	NI
1-O-Methyl-α-D-mannose	> 200	NI
Protein concentration	**350 ng**	**60 ng**

Nl, No inhibition

mixture of sodium metaperiodate and sodium cyanoborohydride at pH 3·5 resulted in the oxidative cleavage of the carbohydrates and the reduction of the aldehyde groups to primary alcohols. By conducting the procedure at acid pH, the possibility of Schiff's base formation between the aldehyde groups and amino groups in the protein and the non-specific oxidation of amino acids was minimised· The modified lectin had lost its haemagglutinating activity. The results of chemical modifications of amino acids and saccharide side chains are summarised in table 2.

Native unmodified lectin of our preparation did not bind to any of the immobilised lectins, concanavalin A, wheat germ agglutinin, and β-galactose-specific RCA1 nor to cross-linked guar gum. But the protein could be bound to α-galactose-specific jack-fruit seed agglutinin (Jacalin) which binds specifically the immunoglobulin A (IgA) containing D-galactose residues (Roque-Barriera and Campos-Neto, 1985). It is difficult to explain the non-binding to RCA1, since the lectin binds to Jacalin as both these plant agglutinins recognise terminal D-galactose residues. This result does not necessarily suggest a terminal α-linked galactose in the placenta lectin. IgA which contains only β-linked terminal galactose (Baenziger and Kornfeld, 1974) binds to Jacalin (Roque-Barriera and Campos-Neto, 1985)·

Human placenta galactose-binding protein is different from human hepatic lectin. Hepatic lectin consists of a single subunit of 41 kDa glycoprotein and required detergent (Triton-X-100) for extraction from the tissue. It also required detergent and Ca^{2+} for binding to the ligand (Baenziger and Maynard, 1980). Placenta protein resembled more the galactose-binding lectin of bovine heart and brain (Carding *et al·,* 1985). One differences noted between bovine heart lectin and the present one is that in the latter we were unable to locate any high molecular weight fraction by gel

Table 2. Chemical modification of amino acid groups and oligosaccharide chains.

Modifying reagent	Amino acid group or oligosaccharide modified	Minimum concentration of protein (ng) required for agglutination			
		Trypsinised rabbit cells		Trypsinised human cells (B + ve)	
		Control*	Test	Control*	Test
None	—	60	60	350	350
Trinitrobenzene sulphonate	$-NH_2$	830	NA	3300	NA
Citraconic anhydride	$-NH_2$	560	NA	2240	NA
Maleic anhydride	$-NH_2$	540	NA	2150	NA
Dithionitrobenzene	$-SH$	260	NA	1050	NA
p-hydroxymercuribenzoate	$-SH$	142	NA	1050	NA
N-Acetyl imidazole	Tyr	580	580	2300	2300
Diethyl pyrocarbonate	His	500	NA	2000	NA
Phenyl glyoxal hydrate	Arg	1700	NA	4300	NA
2-Hydroxy nitrobenzyl bromide	Trp	580	580	2300	2300
2-Hydroxy nitrobenzyl bromide	Trp	580	580	4600	4600
2-Methoxy nitrobenzyl bromide	Trp	580	580	4600	4600
Sodium metaperiodate, pH 4·5	Oligosaccharides	500	NA	2200	NA
Sodium metaperiodate (40 mM) and sodium cyanobrohydride (80 mM) pH 3·5	Oligosaccharides	600	NA	ND	ND

*Identical conditions as required for test without the modifying reagent
NA, No agglutination·
ND, Not done·

Table 3. Amino acid composition of human placenta galactose-binding protein·

Amino acid	Mol/mol of protein[a]
K	11·36
H	3·08
R	6·60
B	19·72
T	9·49
S	12·24
Z	20·60
P	9·07
G	18·31
A	20·86
C[b]	2·0
V	9·51
M	1·41
I	6·25
L	13·12
Y	2·51
F	12·85
W[c]	0·0

Amino acid residues are denoted by single letter.
[a]Minimum molecular weight 26,900·
[b]Cysteine was determined by DTNB method.
[c]Tryptophan was estimated by dimethyl sulphoxide/HCl method·

electrophoresis or by gel filtration on Biogel and Sephadex G-200. The protein could not be extracted with bluffer solutions alone but required lactose. It may be presumed that the lectin remains attached to the oligosaccharide chains of the glycoconjugates on the membrane and lactose replaces the oligosaccharide chains during extraction process·

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