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PLANT PROTOPLAST AGGLUTINATION BY ARTIFICIAL CARBOHYDRATE ANTIGENS

P. J. LARKIN
Department of Agronomy, The Waite Agricultural Research Institute, Glen Osmond, South Australia, 5064

SUMMARY

The existence of β-lectins on protoplast surfaces is confirmed by the agglutination of protoplasts by those Yariv antigens that have sugar specificities which also interact with isolated β-lectins. Agglutination by β-maltosyl but not by β-D-mannosyl Yariv antigens is used to identify some of the structural features required of the ligand for β-lectin binding. Inhibition of agglutination by phenolic glycosides and the effect of protoplast fixation are also investigated.

INTRODUCTION

In a preceding report (Larkin, 1977) both normal mammalian sera and β-glucosyl Yariv antigen (a triple-liganded synthetic β-D-glucosyl antigen) were found to interact with plant protoplasts causing agglutination. A new class of plant lectin called all-β lectin (first described by Jermyn & Yeow, 1975) was inferred to be present on protoplast surfaces and to be responsible for both types of agglutination (Larkin, 1977).

In this paper the above observations are extended to include a larger range of Yariv antigens. The specificities required to induce agglutination are shown to conform to the specificities required for all-β lectin binding (Jermyn & Yeow, 1975) and for arabinogalactan-protein binding (Anderson *et al.* 1977). Yariv antigen/protoplast interactions and agglutination inhibition studies are used to draw inferences concerning the structural characteristics required of a receptor molecule before β-lectin can bind. The effect of glutaraldehyde fixation on protoplast agglutination is also examined.

MATERIALS AND METHODS

Protoplast isolation

Plants were grown and protoplasts isolated as described previously (Larkin, 1976, 1977). The osmoticum used throughout these experiments was either OSI or V47ml, which have mannitol or NaCl, respectively, as the major osmotic stabilizer (Larkin, 1977). Viability was determined using the fluorescein diacetate technique (Larkin, 1976).

Yariv antigens

Yariv antigens are red-coloured phenylazo-glycosides with 3 glycoside moieties on each molecule (Fig. 1). These were a generous gift of Dr M. A. Jermyn (C.S.I.R.O. Division of Protein Chemistry); Yariv, Rapport & Graf (1962) and Jermyn & Yeow (1975) give details of

their synthesis. The following abbreviations will be used to refer to the various Yariv antigens: $(\beta\text{-D-GLU})_3$ for the Yariv antigen with 3 β-D-glucosyl units; similarly $(\beta\text{-D-GAL})_3$ has β-D-galactosyl units, $(\beta\text{-MAL})_3$ has β-maltoside units, $(\beta\text{-D-XYL})_3$ has β-D-xyloside units, $(\beta\text{-LAC})_3$ has β-lactosyl units, $(\beta\text{-CELL})_3$ has β-cellobiosyl units, $(\alpha\text{-L-RHA})_3$ has α-L-rhamnosyl units, $(\beta\text{-D-MAN})_3$ has β-D-mannosyl units, $(\alpha\text{-D-NAcGAL})_3$ has α-D-*N*-acetylgalactosaminyl units; $(\alpha\text{-NAcGLU})_2(\beta\text{-D-GLU})_1$ for the Yariv antigen with a β-D-glucosyl unit on one arm and α-*N*-acetyl-glucosaminyl units on the other two arms; $(\beta\text{-D-GLU})_2(NO_2)_1$ has two β-D-glucosyl units and the third arm has only a $-NO_2$ unit in place of a glycoside; (β-D-GLU) is *p*-β-D-glucosyloxyphenylazo-4-hydroxylbenzene. Other designations follow this pattern.

Agglutination tests

The test for agglutination involved incubation in drops of test solution plus protoplast suspension in plastic Petri dishes for 20–40 min with occasional rocking at 22–27 °C. The protoplast suspensions and test solutions were always at pH 7·2 and the osmolality about 0·5 osmol. OSI was the most frequent osmoticum. Particular care was taken in the inhibition experiment to maintain a constant osmolality even as the concentration of test glycoside varied. This was achieved by altering the concentration of mannitol. The test drops were examined microscopically and rated for agglutination on a subjective scale,

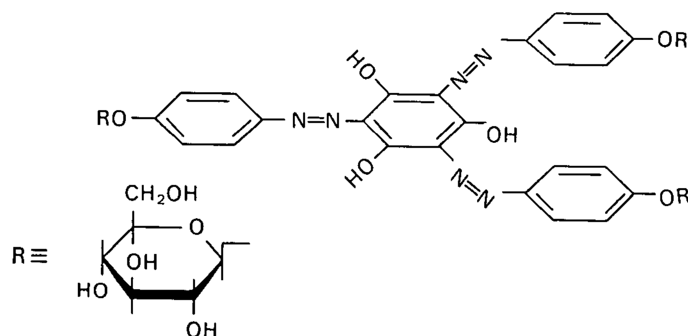


Fig. 1. 1,3,5-Tri-(*p*-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene or $(\beta\text{-D-GLU})_3$.

RESULTS AND DISCUSSION

Agglutination by Yariv antigens

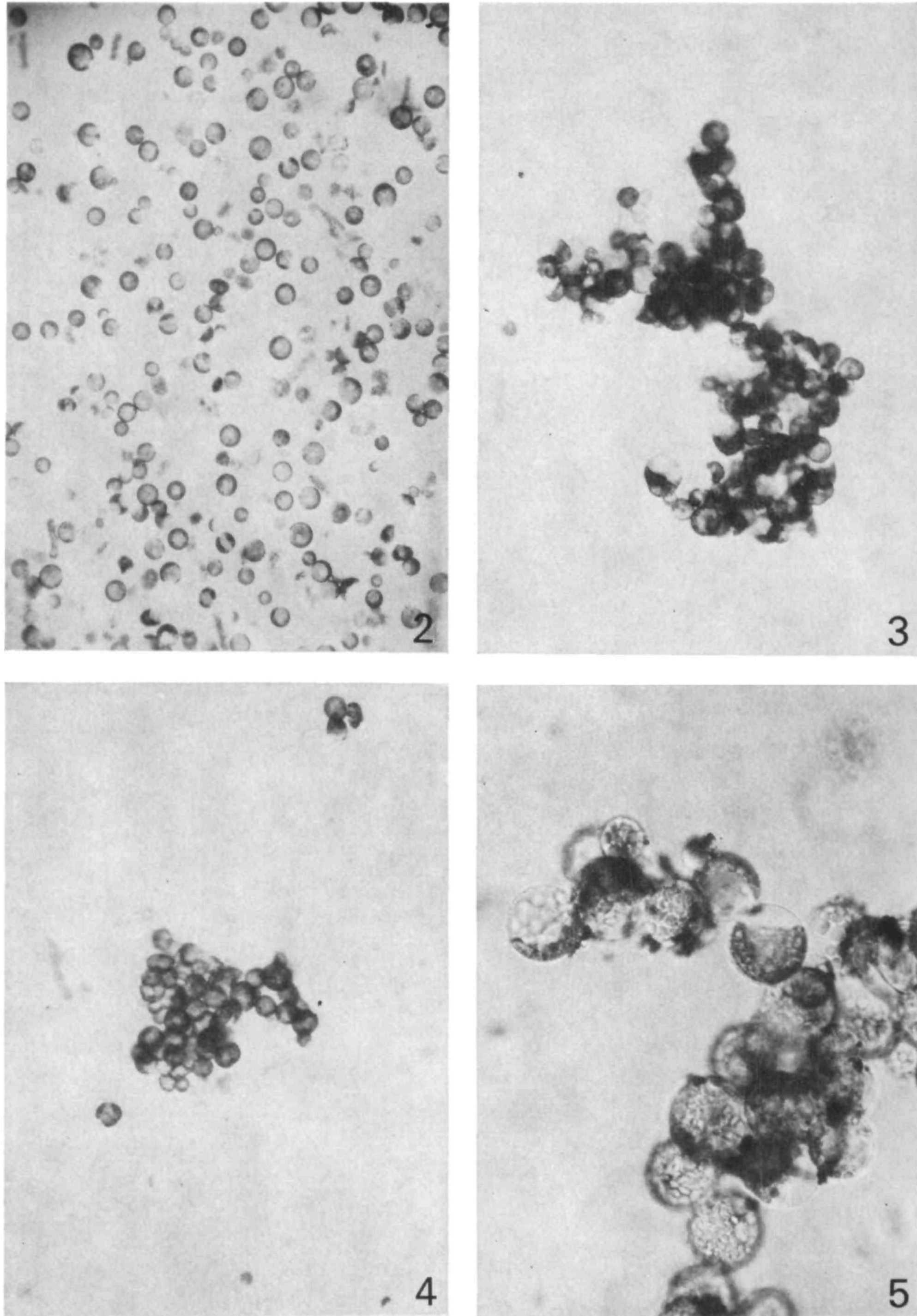
Table 1 summarizes the results of agglutination in the presence of different Yariv antigens. Figs. 2–5 exemplify the nature of the cell interactions observed. In some preparations the leaf protoplasts of *Nicotiana tomentosa*, *N. alata* and *N. miersii* had unusually large diameters (> 100 μm). When this was the case, $(\beta\text{-D-XYL})_3$ was unable to mediate agglutination. In other preparations (even from the same plants 2 or 3 weeks later) the diameters were similar to those of other species (< 50 μm) and agglutination with $(\beta\text{-D-XYL})_3$ was normal.

It is interesting that the large-diameter protoplasts could still be agglutinated by the other β-D-glycosyl Yariv antigens. It may be that the highly distended state causes a conformational restraint on the membrane-located β-lectin which interferes with $(\beta\text{-D-XYL})_3$ binding. The osmolality and pH were unchanged between the experiments in which the average diameters varied. The interactions shown in Table 1 are consistent with the hypothesis in Larkin (1977) that β-lectins exist in protoplast surfaces. In particular, agglutination occurred only in the presence of the Yariv

Table 1. *The interaction of protoplasts with Yariv antigens*

		Protoplast species										
Yariv antigen	Final conc., mg/ml	*Nicotiana tobacum* leaf	*Nicotiana tomentosa* leaf	*Nicotiana miersii* leaf	*Nicotiana alata* leaf	*Petunia hybrida* leaf	*Vicia faba* leaf	*Triticum aestivum* leaf	*Avena sativa* leaf	*Bromus inermis* leaf	*Daucus carota* culture	Other species† leaf
$(\beta\text{-D-GLU})_3$	0·05	+	+	+	+	+	+	+	+	+	+	+
$(\beta\text{-L-GLU})_3$	0·05	−	.	.	.	−	.	.	−	.	.	.
$(\beta\text{-D-GAL})_3$	0·05	+	+	+	+	+	+	+	+	+	.	.
$(\alpha\text{-D-GAL})_3$	0·05	−	−	.	.	−	.	.	−	−	.	.
$(\beta\text{-MAL})_3$	0·05	+	+	.	.	+	+	+	.	.	.	.
$(\beta\text{-D-XYL})_3$	0·05	+	*	*	*	+	+	.	.	.	.	.
$(\beta\text{-L-XYL})_3$	0·05	−	.	.	.	−	.	.	.	.	.	.
$(\beta\text{-LAC})_3$	0·05	+	+	.	.	+	+	.	.	.	.	.
$(\beta\text{-D-MAN})_3$	0·05	−	−	−	.	−	−	.	−	−	.	.
$(\alpha\text{-D-NAcGAL})_3$	0·05	−	−	.	.	−	−	.	.	.	.	.
$(\alpha\text{-L-RHA})_3$	0·05	−	.	.	.	−	.	.	.	.	.	.
$(\beta\text{-CELL})_3$	0·05	+	+	.	.	+	+	+	.	.	.	.
$(\beta\text{-D-GLU})_1$ $(\alpha\text{-NAcGLU})_2$	0·05	−	.	.	.	−	.	.	−	−	.	.
$(\beta\text{-D-GLU})_1$ $(\alpha\text{-D-GAL})_2$	0·05	−	.	.	.	−	.	−	−	−	.	.
$(\beta\text{-D-GLU})_2$ $(\alpha\text{-D-GAL})_1$	0·05	+	+	.	.	.	+	.	.	.	.	.
$(\beta\text{-D-GLU})_2$ $(NO_2)_1$	0·05	+	.	.	.	.	+	.	.	.	.	.
$(\beta\text{-D-GLU})_1$ $(NO_2)_2$	0·05	−	.	.	.	.	−	.	.	.	.	.
$(\beta\text{-D-GLU})_1$	0·25	−	−	.	.	−	.	.	.	.	.	.

† *Hordeum vulgare*, *Sorghum vulgare*, *Triticale*, *Zea mays*, *Linum usitatissimum*, *Tulipa* sp., *Brachycome* sp., *Brassica napus*.
* When protoplasts < 50 μm diameter, + = 25–100 % agglutination; when protoplasts > 100 μm diameter, − = no agglutination.



Figs. 2–5. *Triticum aestivum* mesophyll protoplasts.

Fig. 2. Control in OSI (pH 7·2). × 150.

Fig. 3. Agglutination in 0·05 mg/ml $(\beta\text{-CELL})_3$. × 150.

Fig. 4. Agglutination in 0·05 mg/ml $(\beta\text{-MAL})_3$. × 150.

Fig. 5. Agglutination in 0·05 mg/ml $(\beta\text{-D-GAL})_3$. × 600.

antigens which Jermyn & Yeow (1975) found interacted with β-lectins and which Anderson *et al.* (1977) found interacted with arabinogalactan-proteins. These are namely (β-D-GLU)$_3$, (β-D-GAL)$_3$, (β-LAC)$_3$, (β-CELL)$_3$, (β-MAL)$_3$ and (β-D-XYL)$_3$.

A general conclusion is that activity is dependent on a β-D-glycosyl moiety. Yariv antigens with β-L, α-D- or α-L-glycosyl moieties do not interact with the β-lectins and hence do not mediate protoplast agglutination. An exception to this generalization is (β-D-MAN)$_3$ which did not cause agglutination. Dr Jermyn (personal communication) has also remarked on the inactivity of (β-D-MAN)$_3$ with isolated β-lectins.

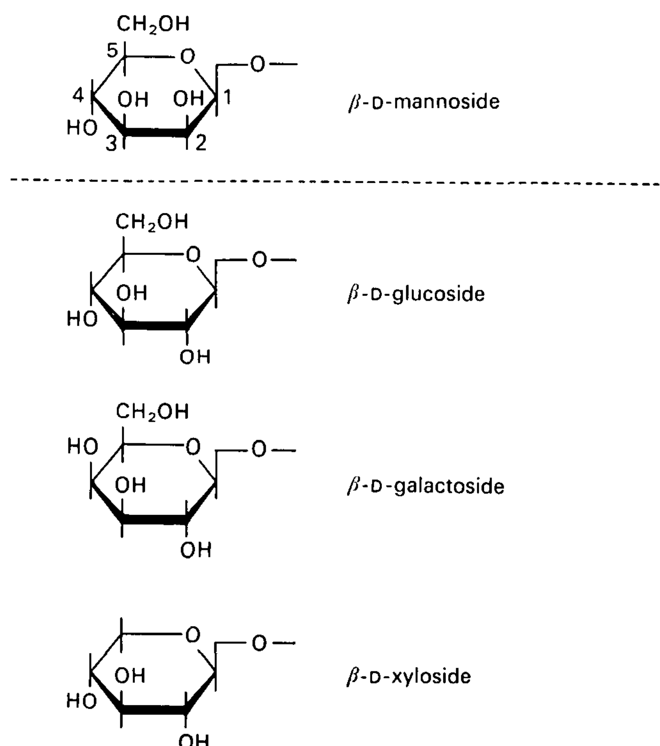


Fig. 6. Comparison of the structures of β-D-mannoside, β-D-glucoside, β-D-galactoside and β-D-xyloside.

Fig. 6 shows a comparison of the structures of β-D-mannoside, β-D-glucoside, β-D-galactoside and β-D-xyloside. The configuration at C_4 varies amongst the active structures and therefore may be inferred to be unimportant. The existence of a 6th carbon is obviously not necessary since (β-D-XYL)$_3$ is bound. The configuration at C_3 is the same in all 4 structures and therefore there is no clue as to the importance of this carbon to β-lectin binding. The configuration of C_2 is the same in all the acceptable structures and opposite in β-D-mannoside. This implies that C_2 configuration and C_1 configuration (conferring α- and β- linkage) are important in β-lectin binding.

Another inference arising from Table 1 is that in the case of Yariv antigens with disaccharide moieties only the residue closest to the phenolic structure need be in acceptable form. (β-LAC)$_3$ has 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside

moieties, (β-CELL)$_3$ has 4-*O*-β-D-glucopyranosyl-β-D-glucopyranoside and both of these are agglutinating. This is to be expected since both residues of these disaccharides are in acceptable configuration. However (β-MAL)$_3$ with 4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside moieties is also agglutinating. Only the residue closest to the phenolic group is in β-linkage and yet it is apparently just as effective a ligand for β-lectins as (β-CELL)$_3$ and (β-LAC)$_3$.

It was further demonstrated by the hybrid Yariv antigens (where the 3 arms are not identical) that agglutination was only possible where at least 2 of the arms carried an acceptable glycoside. This was to be expected since 2 protoplasts will adhere when the surface lectins are binding to the one molecule. The failure of (β-D-GLU)$_1$ (α-NAcGLU)$_2$ and (β-D-GLU)$_1$ (α-D-GAL)$_2$ and (β-D-GLU)$_1$ (NO_2)$_2$ to cause protoplast agglutination may indicate that these molecules are not aggregating spontaneously into complexes which would be multi-liganded with suitable glycosides.

Glutaraldehyde fixation and protoplast agglutination

It was reported earlier (Larkin, 1977) that fixation prevents serum- or (β-D-GLU)$_3$-mediated agglutination. This was reinvestigated and extended. *Bromus inermis* leaf protoplasts were used to test the effect of glutaraldehyde fixation. A sample of protoplasts was suspended in 3% glutaraldehyde in V47ml (pH 7·2) for 2 h before

Table 2. *Glutaraldehyde fixation and protoplast agglutination*

	Bromus inermis leaf protoplasts	
Test treatment	Glutaraldehyde-fixed, 0% FDA viable	Untreated, 90% FDA viable
V47ml(pH 7·2)	+	−
0·05 mg/ml (β-D-GLU)$_3$	+	+ + + +
0·05 mg/ml (β-D-GAL)$_3$	+ +	+ + + +
0·05 mg/ml (α-D-GAL)$_3$	+	−
0·05 mg/ml (β-D-MAN)$_3$	+	−
1/10 normal rabbit serum	+ +	+ + + +
1/10 normal kangaroo serum	+ +	+ + + +
1/6 normal sheep serum	+ +	+ + + +

+ + + + = 75–100% agglutination; + + = 25–50%; + = < 25%; − = negligible.

thoroughly washing in osmoticum. This sample had no FDA fluorescing protoplasts as compared to 90% fluorescing protoplasts in an unfixed control suspension. There was a low level of spontaneous aggregation of the fixed protoplasts which were not responsive to significant further agglutination by either Yariv antigens or animal sera (Table 2). Glimelius, Wallin & Eriksson (1974) also observed some spontaneous aggregation of glutaraldehyde-fixed carrot protoplasts and a subsequent reduction of Con A-induced agglutinability.

Fixation of animal cells has often been reported to reduce agglutination by plant lectins (Nicolson, 1974; Rutishauser & Sachs, 1975) though not always (Marquardt &

Gordon, 1975). This effect may be the result of a loss of lectin receptor mobility in the fixed animal cells resulting in a lack of clustering of sites, which in turn may be necessary for strong adhesion of cells at contact points. This principle may apply to protoplasts in that membrane-located β-lectins may also need to cluster at contact points to facilitate agglutination. Alternatively fixation may simply reduce the deformability of protoplasts and thus reduce cell-to-cell surface contact which is essential for aggregation. This has also been suggested as an explanation for fixed animal cell behaviour (Nicolson, 1974; Gibson, Marquardt & Gordon, 1975; Van Blitterswijk *et al.* 1976).

Table 3. *Inhibition of protoplast agglutination by simple glycosides*

Glycoside	Minimum conc. causing 50 % reduction in agglutination (mM)	Agglutinating agent employed	Protoplasts employed
Glucose	> 500	0·05 mg/ml (β-D-GLU)$_3$, 1/8 serum	Carrot culture, tobacco leaf
Methyl-β-D-glucoside	> 500	0·05 mg/ml (β-D-GLU)$_3$, 1/8 serum	Carrot culture, tobacco leaf
Methyl-β-D-galactoside	> 500	0·05 mg/ml (β-D-GLU)$_3$, 1/8 serum	Carrot culture, tobacco leaf
Methyl-α-D-glucoside	> 500	0·05 mg/ml (β-D-GLU)$_3$, 1/8 serum	Carrot culture, tobacco leaf
O-hydroxymethylphenyl-β-D-glucoside (salicin)	140	0·05 mg/ml (β-D-GLU)$_3$, 1/40 serum	*N. tabacum* leaf, *N. tomentosa* leaf
Thiodigalactoside	> 25	0·05 and 0·005 mg/ml (β-D-GLU)$_3$, 0·005 mg/ml (β-D-GAL)$_3$, 1/20 serum	Tobacco leaf
p-nitrophenyl-α-D-glucoside	> 12·5	0·05 and 0·005 mg/ml (β-D-GLU)$_3$, 0·005 mg/ml (β-D-GAL)$_3$, 1/20 serum	Tobacco leaf
p-nitrophenyl-β-D-glucoside	10	0·05 and 0·005 mg/ml (β-D-GLU)$_3$, 0·005 mg/ml (β-D-GAL)$_3$, 1/20 serum	Tobacco leaf
Indoxyl-β-D-glucoside	25	0·05 and 0·005 mg/ml (β-D-GLU)$_3$, 0·005 mg/ml (β-D-GAL)$_3$, 1/20 serum	Tobacco leaf

Inhibition of agglutination by glycosides

A number of simple sugars and glycosides were tested for the ability to inhibit agglutination mediated by serum or β-D-glycosyl Yariv antigens (Table 3). The substances were tested at varying concentrations and in each case the sum of the concentrations of mannitol and test-substance was 500 mM and the pH was maintained

at 7·2. Even at 500 mM the non-phenolic glycosides showed no detectable inhibition. In addition to those of Table 3, lactose and cellobiose were not inhibitory up to 150 mM.

Some of the glycosides were much less soluble, and this restraint prevented thiodigalactoside and *p*-nitrophenyl-*β*-D-glucoside being tested beyond 25 and 12·5 mM respectively. They were not inhibitory up to these concentrations. However 50% reduction in agglutination was caused by 3 phenolic-*β*-D-glycosides, namely salicin (140 mM), *p*-nitrophenyl-*β*-D-glucoside (10 mM) and indoxyl-*β*-D-glucoside (25 mM). The structures of these glycosides are shown in Fig. 7. These glycosides did not reduce the viability of the protoplasts.

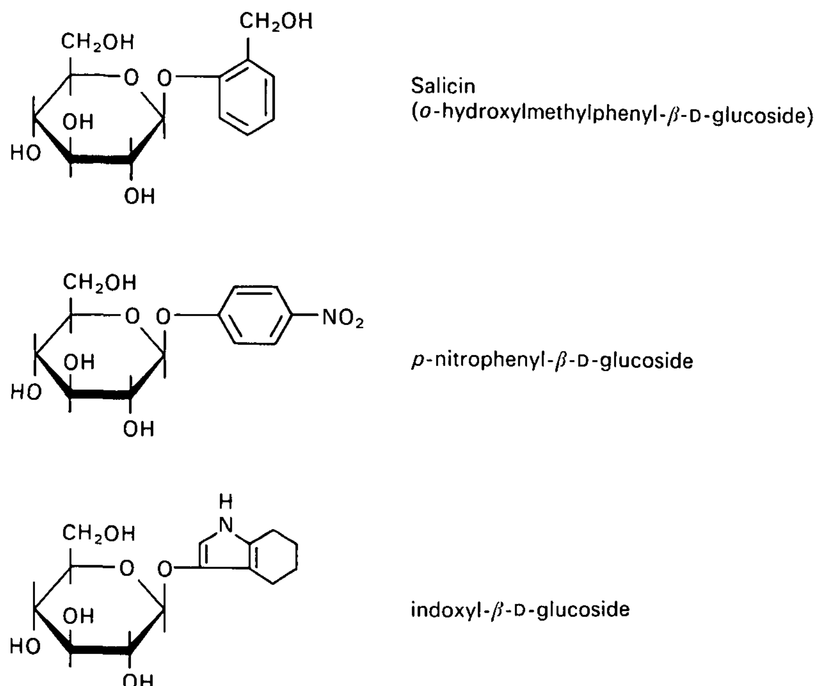


Fig. 7. The structures of salicin (*o*-hydroxylmethylphenyl-*β*-D-glucoside), *p*-nitrophenyl-*β*-D-glucoside and indoxyl-*β*-D-glucoside.

The Yariv antigens themselves are of course phenolic glycosides. It is interesting that the structural requirements for binding by *β*-lectins not only involve the sugar moiety but also the nature of the phenolic moiety to which it is linked. Too little data are as yet available to be able to define the necessary form of the phenolic structure.

CONCLUSIONS

Protoplast agglutination in the presence of a range of Yariv antigens has helped to define more fully the peculiar specificities of *β*-lectins located on protoplast surfaces. The preferred ligand for *β*-lectin binding is a phenolic glycoside with the sugar residue

closest to the phenolic being in D-conformation and in β-linkage. Carbon 1 configuration defines β-linkage and it is important that in addition the hydroxyl of C2 be in *trans*-orientation relative to the linkage of C1.

There have been a number of recent reports of lectins isolated from hypocotyl cell walls and the membranes of plasmalemma, Golgi apparatus, endoplasmic reticulum and mitochondria (Kauss & Glaser, 1974; Bowles & Kauss, 1975, 1976; Kauss & Bowles, 1976; Bowles, Schnarrenberger & Kauss, 1976). After isolation these lectins were assayed by their ability to agglutinate trypsinized rabbit erythrocytes and their specificities investigated by inhibition studies. That these can be grouped with classical lectins is suggested by their ability to agglutinate erythrocytes strongly, and by the fact that they are inhibited by non-phenolic glycosides. Indeed the lectin from the inner mitochondrial membrane of *Ricinus communis* is likely to be identical to the classical castor bean lectin (Bowles *et al.* 1976).

It is not yet clear whether the lectins described by these authors as being extractable from isolated plasmalemma exist together with β-lectins on protoplast surfaces. A number of classical lectins have been shown to be able to interact with Yariv antigens of appropriate sugar specificities (Larkin, in preparation). Since the membrane-extracted classical lectins were almost invariably inhibited by α-D-galactosides it was conceivable if they also exist on protoplasts that (α-D-GAL)$_3$ may cause agglutination. Such agglutination was not observed with (α-D-GAL)$_3$ nor any other α-glycosyl Yariv antigen (Table 1). It may be that the lectins described by these authors are bound to the membranes by their own binding activity. This possibility was suggested by the fact that the addition of lactose to the extractant greatly improved the yield of activity from *R. communis* mitochondrial membranes, since lactose is also a strong inhibitor of the lectin (Bowles *et al.* 1976). If this is the case and these lectins are bound to the protaplast plasmalemma by their own binding sites then obviously, unlike the β-lectins, they would not be able to react with exogenous substrates such as Yariv antigens. Alternatively the lectins described by Bowles & Kauss (1975, 1976) and Kauss & Bowles (1976) may be lost from the protoplasts during the enzyme incubation.

It remains to be seen whether lectin activities are artifacts of assay or whether they are meaningful representations of the true roles of these molecules. The β-lectins are apparently universally distributed in plants and located at cell surfaces. It is enticing to speculate that they may be part of the recognition systems for pollen compatibility, pathogenicity and symbiosis.

The physiological role may be assumed to involve interactions with phenolic glycosides many of which exist in plant tissues (Harborne, Mabry & Mabry, 1975; Harborne, 1967; Ribéreau-Gayon, 1972). D-conformation sugars are almost invariably in β-linkage in plant phenolics. Initial investigations with crude phenolic extractions from various plant tissues have shown that some are indeed able to inhibit β-lectin-directed protoplast agglutination.

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