MYO-INOSITOL, A COFACTOR IN THE BIOSYNTHESIS OF VERBASCOSE

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Sugars of the raffinose family are fairly common as reserve and transport material in higher plants (1,2,3). The biosynthesis of stachyose (0-α-D-galactopyranosyl-(1→ 6)-0-α-D-galactopyranosyl -(1→ 6)-0-α-D-glucopyranosyl-(1→ 2)-ß-D-fructofuranosid) in extracts from Phaseolus vulgaris seeds has recently been reported (4) to proceed via galactinol (0-α-D-galactopyranosyl-(1→ 1)-myo-inositol) according to the following two equations:

(I) UDP-gal + myo-inositol ⇌ galactinol + UDP

(II) Galactinol + raffinose ⇌ stachyose + myo-inositol

Galactinol was already isolated and its structure determined in 1953 (5,6). Its biosynthesis according to equation I was described later by Frydman and Neufeld (7).

Extracts from Ph.vulgaris seeds are able to catalyze both reactions (I and II). However, the galactinol:raffinose-6-galactosyl-transferase (equ.II) from this plant does not recognize stachyose as galactosyl acceptor and is unable, therefore, to catalyze the biosynthesis of verbascose, the next higher homologue of the sugars of the raffinose family.

It has been possible now to obtain an enzyme from seeds of Vicia faba which is able to catalyze the galactosyl transfer from galactinol not only to raffinose but also to stachyose, in the latter case giving rise to verbascose.

Materials and methods:

Two tests have been used to measure the enzyme activity: test (A) with galactinol-C^{14} was carried out as described previously (4). Test (B) is based on the determination of the myo-inositol set free due to the galactosyl transfer. Myo-inositol was determined enzymatically according to Weissbach (8). Protein was determined by the biuret method. Radioactive spots were located by a strip scanner or by radioautography, and the radioactivity measured directly on the paper with a methane flow counter (Frieseke and Hoepfner 407 A).

Authentic verbascose was isolated from V.faba seeds by carbon column chromatography (9). Galactinol-C^{14} (12,6 uC/umole; 95.5 % of the radioactivity in the galactosyl moiety) was prepared as described previously (4). All other substances were commercial products.

Results

Purification of the enzyme and kinetics of verbascose formation:

Table 1 shows the purification procedure which yielded a 55-fold increase in specific activity. 1oo g of seeds were homogenized in 24o ml of 0,1 M sodium phosphate buffer pH 7.0 with an Ultra Turrax blendor at high speed for 3 minutes. The extract was pressed through cheese cloth, centrifuged at 17000 x g for 10 minutes and then treated as described in table 1.

The ratio $\frac{\text{galactosyl transfer to raffinose}}{\text{galactosyl transfer to stachyose}}$ was 2.68 with fraction 1 and 2.54 with fraction 4, indicating that one and the same enzyme catalyzes both reactions. This is also supported by the strong mutual inhibition which was observed when the enzyme was incubated with both acceptors simultaneously (table 2).

Table 1: Enzyme purification

Fraction	Total Protein mg	Total Activity umoles/hr	Spec.Activ. umoles/hr/ mg Prot.
1.Supernatant (17000xg)	12 444	57	o.oo46
2.$(NH_4)_2SO_4$ (33-50 %)	1 188	55	o.o46o
3.Ca-phosphate gel	313	32	o.1o2o
4.$(NH_4)_2SO_4$ (4o-48 %)	39	9,8	o.251o

The $(NH_4)_2SO_4$ precipitate obtained between 33 and 5o % saturation from the supernatant was taken up in 0.1 M phosphate buffer, pH 7.0 and dialyzed overnight against 3 l of the same buffer 0.05 M. Then calcium phosphate gel was added (8o mg/1oo mg protein). After centrifugation the activity was found in the supernatant. Finally $(NH_4)_2SO_4$-treatment (4o-48 % saturation) yielded the fraction which has been used for all experiments reported without further dialysis. During the purification the activity was measured using test (B) with raffinose as acceptor, since it gives the highest rates (see table 3).

Table 2: Mutual inhibition with both raffinose and stachyose being present as acceptors.

Acceptor added	Sugars produced (cpm)	
	Stachyose	Verbascose
Raffinose	5 244	17o
Stachyose	195	2 125
Raffinose + Stachyose	3 312	99o

In a total volume of 4o ul 0.1 M phosphate buffer pH 7.0 galactinol -C^{14} (17ooo cpm) was incubated for 3.5 hrs at 32°C with o.o8 mg protein and o.5 µmole of the acceptors indicated. The products were separated on Whatman Nr.1 paper in the solvent system ethyl acetate : butanol : acetic acid : water = 3:4:2.5:3.

The kinetics of the formation of verbascose as well as of D-galactose which arises in the same incubation mixture by hydrolysis of galactinol is shown in Fig.1.

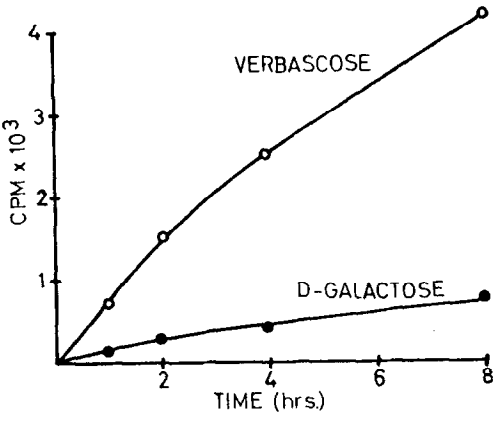

Fig.1. Kinetics of verbascose formation and galactinol hydrolysis. The reaction mixture contained in a total volume of 3o ul: galactinol-C^{14} (1oooo cpm), stachyose (1 umole), phosphate buffer. Other conditions as in table 2.

The reaction products stachyose-C^{14} and verbascose-C^{14} obtained from incubations of galactinol-C^{14} with raffinose and stachyose, respectively, were identified by paper chromatography and by enzymatic degradations as described previously (4).

Acceptor specificity of the enzyme:

Besides raffinose and stachyose, melibiose was the best galactosyl acceptor (table 3). As compared to raffinose less than 5 % transfer to lactose, D-galactose and D-glucose was observed. There was no transfer to glycerol, D-fructose, D-glucose-1-phosphate, D-glucose-6-phosphate, sucrose, maltose, cellobiose, trehalose, gentiobiose, and melizitose. Verbascose also did not serve as a galactosyl acceptor, which means that the enzyme cannot catalyze the biosynthesis of ajugose, the following member of the raffinose family.

Michaelis constants and maximal velocities:

The K_M for galactinol has been determined with raffinose as acceptor ($2,5 \times 10^{-2}$ M) by test (B) and the K_M for raffinose,

stachyose and melibiose by the galactinol-C^{14} test (A). The constants are given in table 3 together with the relative maximal velocities (raffinose as acceptor taken as 100). For comparison the corresponding values for the enzyme of Ph.vulgaris measured under identical conditions are included. The K_M-values for galactinol are rather high. However, the concentration in seeds is in an order of magnitude of 5×10^{-3}M (10).

Table 3: A comparison of the K_M- and V_{Max}-values of the enzymes from V.faba and from Ph.vulgaris.

Substrate	Enzyme from Vicia		Enzyme from Phaseolus	
	K_M	V_{Max}	K_M	V_{Max}
Raffinose	$8,5 \times 10^{-4}$	100	$8,4 \times 10^{-4}$	100
Melibiose	$3,9 \times 10^{-3}$	80	$1,2 \times 10^{-2}$	46
Stachyose	$3,3 \times 10^{-3}$	35	-	< 0,5 [1]
Galactinol	$1,1 \times 10^{-2}$	-	$7,3 \times 10^{-3}$	-

1) Incubation of the enzyme from Ph.vulgaris with stachyose as acceptor for 8 hours yielded a very weak radioactive spot on the chromatogram in the verbascose region.

Discussion:

In vivo results obtained with a wide variety of plant leaves first led to the suggestion that galactinol might be the galactosyl donor in the biosynthesis of the sugars of the raffinose family (11,12).

Thus, together with the in vitro results, there seems to exist sufficient evidence that the biosynthesis at least of stachyose and verbascose proceeds via galactinol. For raffinose biosynthesis, however, it has been shown (13,14) that at least in vitro UDP-gal can serve as galactosyl donor.

The acceptor specificity of the enzyme from Ph.vulgaris (4) and that from V.faba is extremely similar except for the recognition of stachyose. This difference, however, agrees very well with the actual sugar content of the two seeds. There are only traces of verbascose present in seeds of Ph.vulgaris, whereas this sugar is the major oligosaccharide in the seeds of V.faba (unpublished results).

Finally the unique role of myo-inositol in these reactions should be emphasized. In the overall reaction (equ.I + II) myo-inositol cancels out and thus behaves like a true enzymatic cofactor. Future work will have to show, whether myo-inositol acts more generally as a cofactor of glycosyl transfer reactions. The occurrence of a water-soluble O-α-D-mannopyranosyl-myo-inositol in yeast (15), however, indicates such a possibility.

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