The Correlation between the Mono-, Oligo- and Polysaccharides and the Glycosidases present in Clover Seeds

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In the preceding article [1] it was concluded that it is unlikely that β-mannosidase participates in the utilization of the reserve polysaccharides,[2] the galactomannans. Five species were examined and it was impossible to measure the activity of the enzyme, and no mannose could be identified after the seeds had been germinated for two days. Prolongation of the germination period did not alter these results (unpublished data). In the extracts of germinated seeds the following monosaccharides were identified; D-galactose, which may arise from the galactomannan as the activity of α-galactosidase was high, furthermore D-fructose and D-glucose. Oligosaccharides were not detected.

From these findings it was considered to be of interest to examine the ungerminated seeds for the presence of other possible sources of reserve carbohydrates than the galactomannans, and also to find the enzymes responsible for the accumulation during germination of the monosaccharides mentioned.

The present paper reports the results from such an examination of one of the species, *Trifolium pratense* L. (red clover). *Trifolium repens* L. (white clover) was also examined, and it contained the same components and the same enzymes.

In the extracts of the ungerminated seeds it was possible to identify D-galactose, D-glucose, and D-fructose as in the germinated seeds. In addition D-ribose, sucrose, raffinose, stachyose, and verbascose were found, all of which were utilized during two days of germination as even highly concentrated extracts gave very weak spots on chromatograms.

The mentioned oligosaccharides are all found in a large variety of higher plants and are generally accepted as reserve carbohydrates.[3,4] To our knowledge the occurrence of D-ribose in leguminous seeds has not been reported earlier; small amounts have, however, been found to be present in the seeds of *Suaeda macrocarpa* Moq.[5] Furthermore two unidentified fractions (I and III, Fig. 1) were obtained by fractionation on Sephadex G 15; none of them contained mannose after total acid hydrolysis. Fraction III contained three different components, all giving ribose, glucose, and galactose as hydrolysis products, and all being metabolized during germination. Neither the ribose nor the ribose-containing oligocomponents are products formed as a result of the extraction technique employed, as an extract obtained by an enzyme inhibiting extraction method (boiling 80 % ethanol) contained the same components.

Table 1. Specific activities of the glycosidases in resting and germinated seeds.

Enzyme	Resting seeds	Germinated seeds
α-Galactosidase	9.45	50.30
β-Galactosidase	18.45	31.90
α-Glucosidase	0.13	0.21
β-Glucosidase	0.63	0.78
α-Mannosidase	8.10	20.00
β-Mannosidase	0.94	0.62
β-Fructofuranosidase	10.40	43.00
Protein content	8 mg/ml	7 mg/ml

Results from the analysis of the enzymes present in the extracts of resting and germinated seeds are given in Table 1. The increasing activities of β-fructofuranosidase and α-galactosidase during germination explain the rapid utilization of sucrose and the oligosaccharides of the stachyose-series as energy reserve, giving rise to free galactose, glucose, and fructose. Neither α- nor β-glucosidase showed significant activity and this is not surprising as the glucose present in the oligosaccharides is liberated by the combined action of the two formerly mentioned enzymes. As before the activity of β-mannosidase was not measurable. An explanation of the high α-mannosidase activity has not been found.

As mentioned before, it was obvious that ribose and the different oligosaccharides in the resting seeds were rapidly utilized during germination. The galactomannan content, however, was only insignificantly reduced after two days of germination. Heat-treated but not ethanol precipitated extracts of resting and germinated seeds were examined by gel filtration on

Sephadex G 100. These results support the former suggestion,[1] that the galactomannan may serve as a structural polysaccharide, for example in the regulation of the water balance of the seeds, rather as reserve polysaccharide. This conclusion is also supported by the lack of β-mannosidase.

Experimental. The methods used, when not otherwise stated, are the same as in the former publication.[1]

Enzyme extracts. The crude extracts of the dry, milled seeds or the germinated seeds (10 g to 100 ml water) were dialyzed (24 h) against water to obtain a glucose free extract for the determination of the activity of β-fructofuranosidase. The dialysis had no influence on the activity of the other enzymes.

Estimation of the activities of β- and α-glycosidases was carried out with the corresponding *o*- or *p*-nitrophenyl glycosides as chromogenic substrates. The β-mannosidase activity was tested by means of phenyl-β-D-mannoside, *p*-nitrophenyl-β-D-mannoside, and mannobiose as substrates.

The β-fructofuranosidase activity was estimated with sucrose as substrate (2.5 % in 0.01 M acetate buffer, pH 4.8). The appropriate diluted enzyme solution (0.2 ml) was incubated at 37° with 0.2 ml substrate for 60 min. The reaction was stopped by transfering the test tubes to a boiling water bath (2 min). The liberated glucose was estimated by the glucose oxidase method.

Definition of enzyme units. One unit of activity is the amount of enzyme required to hydrolyze 10^{-9} mol of substrate/min under the conditions described. *Specific activity* is number of enzyme units/mg protein.

Fractionation of the deproteinized (3 min, 100°) crude extract on Sephadex G 100 resulted in two well separated fractions. A polysaccharide fraction which is anticipated to be the galactomannan as it was eluted as an authentic sample, and gave only galactose and mannose by total acid hydrolysis. The second fraction, which was of the same size as the first one, contained all the mono- and oligosaccharides. The elution was followed by the determination of the total carbohydrate content by the phenol sulfuric acid method.

Concentrated mono- and oligosaccharide extracts were prepared by the following method: Crushed germinated or milled dry seeds (100 g) were extracted with water (1000 ml, 1 h), centrifuged, and deproteinized (100°, 5 min). The polysaccharide was removed by ethanol precipitation (2 volumes). The supernatant was concentrated to dryness and dissolved in 50 ml of water. This extract was gel filtrated on Sephadex G 15 and a partial separation was obtained (Fig. 1). The elution was followed by the phenol sulfuric acid method parallel with thin-layer chromatography on Kieselgel G in the solvent: propanol, nitromethane, water, 5:3:2.

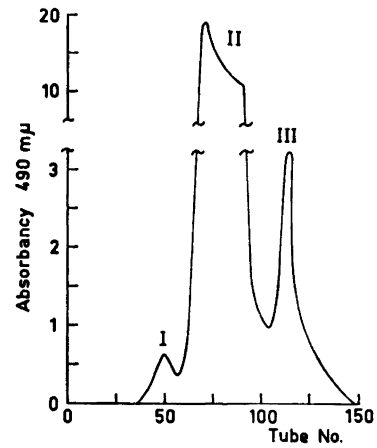

Fig. 1. Fractionation of the concentrated extract on Sephadex G 15.

Fraction I contained an unknown component. By strong acid hydrolysis galactose, glucose, arabinose, and xylose were liberated, it was difficult to obtain total hydrolysis. The component is non-dialysable and it has no mobility in paper or thin-layer chromatographic systems, which indicates a fairly high degree of polymerisation.

Fraction II contained mono- and oligosaccharides which were separated by preparative paper chromatography on Whatman No. 3 MM in the system: butanol, pyridine, water, 5:3:2. The following substances were indistinguishable from reference substances by paper-, thin-layer chromatography, and high voltage electrophoresis in borate buffer (0.1 M, pH 10); *a*, verbascose, *b*, stachyose, *c*, raffinose, *d*, sucrose, *e*, D-galactose, *f*, D-glucose, *g*, D-fructose, and *h*, D-ribose.

Treatment of *a*, *b*, *c*, and *d* with β-fructofuranosidase and treatment of *a*, *b*, and *c* with α-galactosidase gave the same split products as authentic samples treated with the same enzymes. Component *h*, D-ribose, was further analyzed, after reduction and acetylation, by gas liquid chromatography and mass spectrometry and was unambiguously identical with an

authentic sample examined in a parallel analysis.

Fraction III contained small amounts of three unidentified components with chromatographic mobilities in the regions of raffinose and stachyose. By development of the chromatograms with *p*-anisidine HCl the components had a pink colour. The total acid hydrolysis resulted in the liberation of galactose, glucose, and ribose.

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Kristallographische Computer-Programme für die CDC-3300

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Kürzlich wurde in dieser Zeitschrift eine Serie von kristallographischen Computer-Programmen in Mass Storage FORTRAN für eine CDC-3300-Anlage beschrieben.[1] Wir stellten einen entsprechenden Programmcyclus zusammen [2] und möchten diesen hier kurz vorstellen.

Alle Programme wurden für den MS-FORTRAN-Compiler und das Betriebssystem MASTER einer CDC-3300-Rechenanlage geschrieben. Die übersetzten Programme befinden sich auf Plattenfiles, von denen sie zur Benutzung einzeln oder im Job-Sequence-Verfahren in den Kernspeicher abgerufen werden können; variable Unterprogramme können in FORTRAN beigefügt werden. Die Daten-Ein- und Ausgabe kann wahlweise BCD über Lochkarten oder binär über Magnetband oder Plattenfile erfolgen. Im einzelnen bestehen die folgenden Programme.

POWDER. Dieses Programm dient zur Indizierung von Pulveraufnahmen aus vorgegebenen Gitterkonstanten und zu deren Verfeinerung und stammt in seinen Grundzügen von Lindqvist und Wengelin.[3] Wir führten die Möglichkeit zur Standardisierung mittels der Werte eines inneren Standards und die Berechnung von Indizierungshilfen nach dem Verfahren von Hesse [4] und Lipson [5] ein.

CORIN. Dieses Programm dient zur Korrektur der Daten eines Äquiinklinationstechnik benutzenden automatischen Diffraktometers und stammt von H. Paulus, Darmstadt. Durchgeführt werden können Untergrundkorrektur, Kontrolle berechnete-gemessene Diffraktometereinstellung, Lorentz-Polarisationskorrektur, zweidimensionale Absorptionskorrektur nach dem Verfahren von Busing und Levy [6] und Gewichtssetzung.

ORABS. Dieses Programm dient zur dreidimensionalen Absorptionskorrektur nach dem Verfahren von Busing und Levy [6] und wurde unter Benutzung einer von Schultze-Rhonhof, Bonn, bearbeiteten Fassung des Absorptionsprogramms von Wehe, Busing und Levy [7] und in Anlehnung an die Programmführung in CORIN neu geschrieben.

LSQ1MZ. Dieses Programm dient einerseits zur statistischen Erfassung des reziproken Gitters [8] und andererseits zur Datenvorbereitung für Patterson-Synthesen, Strukturfaktorenberechnungen und "least squares"-Verfeinerungen.

FSY. Dieses Programm zur Berechnung von Patterson-Synthesen, Fourier-Synthesen und Differenz-Fourier-Synthesen stammt von Schultze-Rhonhof.[9] Gegenüber der Originalfassung, die für jede Raumgruppe austauschbare Teile der Summierungsfolgen im Hauptprogramm vorsah, wurden diese Teile fest eingebaut. Die Fourier-Summation erfolgt nach einer Faktorisierung der allgemeinen Elektronendichte-Summationsformel in einem austauschbaren Unterprogramm, von dem 67 Fassungen für alle Raumgruppen bestehen.

MSFOUR. Dieses Programm zum Aufsuchen der Maxima und Minima einer dreidimensionalen Fourier-Synthese stammt in seiner Grundform ebenfalls von Schultze-