ACTA CHEMICA SCANDINAVICA 24 (1970) 72–76

Glycosidases and Monosaccharides in Germinated Leguminous Seeds

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The water extracts of the germinated seeds of the five leguminous species: *Trifolium repens*, *Trifolium pratense*, *Medicago sativa*, *Anthyllis vulneraria*, and *Lotus corniculatus* contained the following monosaccharides: D-galactose, D-glucose, and D-fructose. D-Mannose could not be detected. The activity of β-mannosidase could neither be measured by the synthetic substrate, β-phenyl D-mannoside nor with the natural substrate, mannobiose. These results indicate that β-mannosidase does not participate in the utilization of the reserve polysaccharide, galactomannan.

Several leguminous species that have an endosperm contain galactomannans as reserve polysaccharides.[1] The structures of the galactomannans from some species have been examined by several authors. The amount of galactose varies to some extent, but all galactomannans appear to have the same structural features, with the exception of a few deviations. They consist of a backbone chain of D-mannopyranose units joined by β-1,4-linkages, to which side chains of single D-galactopyranose units are connected by α-1,6-linkages.

For the full utilization of the reserve galactomannan during germination of the seeds it has been postulated that at least three enzymes are involved in the hydrolysis of the polysaccharide.[2] These are: α-galactosidase (α-D-galactoside galactohydrolase; E.C. 3.2.1.22), β-mannanase (β-1,4-mannan mannanohydrolase; E.C. 3.2.1.), and β-mannosidase (β-D-mannoside mannohydrolase; E.C. 3.2.1.25).

α-Galactosidases and β-mannanases have been found to be present in microorganisms, animals, and plants including the *Leguminosae* and have been studied to some extent.[3–7] The distribution of β-mannosidases is restricted, and only a few studies have been carried out. Its presence in the *Leguminosae* has only been indicated vaguely, and to our knowledge its activity has never actually been measured.

The determination of the structure of leguminous galactomannans has mostly been carried out by non-enzymatic methods. In a few studies crude enzyme preparations from various sources have been used, but have not always provided much information.

It is obvious that the elucidation of the structure of a galactomannan, the understanding of its catabolism and its anabolism would be far more simple if one had the responsible enzymes available in a purified and well characterized form.

The present work has been carried out as a survey of the presence of glycosidases one would expect to find in the germinated seeds of some leguminous species with galactomannans as reserve polysaccharide.

The majority of the species examined in the present work (*Trifolium repens* L, *Anthyllis vulneraria* L, *Lotus corniculatus* L) contain galactomannans whose composition and structure have been described in papers published from this laboratory.[8-10]

MATERIALS AND METHODS

The seeds. The following five leguminous species, obtained from commercial sources, were used; *Trifolium pratense* L, *Trifolium repens* L, *Medicago sativa* L, *Antyhyllis vulneraria* L, and *Lotus corniculatus* L.

Preparation of crude extracts. The seeds (15 g) were soaked in water (24 h) and germinated (40 h) at room temperature in the dark on moist filter paper. The germinated seeds were homogenized in water (100 ml) with a Sorvall Omnimixer. The homogenate was centrifuged, and the clear supernatant, designated as crude extract, was used for further experiments.

Ammonium sulfate fractionation. The crude extract (100 ml) was brought to 80 % saturation by the addition of solid $(NH_4)_2SO_4$. After 24 h at 4°, the precipitate was collected by centrifugation, dissolved in citrate-phosphate buffer (0.01 M, pH 4.6) and dialysed (48 h) against the same buffer. The final volume was 20 ml.

Sephadex gel filtration. 10 ml of the ammonium sulfate fractionated extract was applied to a Sephadex G 200 column (2.5 × 40 cm) which was equilibrated and eluted with tris-acetate buffer (0.01 M, pH 7.2) containing NaCl (0.1 M) at 4°. Fractions of 2.5 ml were collected.

Enzyme substrates. *p*-Nitrophenyl α-D-galactoside, *o*-nitrophenyl β-D-galactoside, and *p*-nitrophenyl α-D-mannoside were commercial products. Phenyl β-D-mannoside was synthesized according to the method of Helferich and Winckler.[11] The mannobiose (4-*O*-β-D-mannopyranosyl-D-mannose) was obtained by partial acid hydrolysis of a galactomannan.[12] The ivory nut mannan was a gift from Dr. T. E. Reese.

Enzymes assays. α-Galactosidase, β-galactosidase, and α-mannosidase activities were measured with the chromogenic substrates dissolved in citrate-phosphate buffer (0.05 M, pH 4.6). The appropriate diluted enzyme solution (0.2 ml) was incubated at 37° with 0.2 ml substrate (10 mM) for 15 min. The reaction was stopped by adding Na_2CO_3 (5.0 ml, 0.1 M), and the yellow colour of *o*- or *p*-nitrophenol, thus developed, was measured at 405 mμ.

β-Mannosidase activity was measured with phenyl β-D-mannoside at pH 5.2 and with varying times of incubation (15 min—24 h), and the phenol produced was measured at 285 mμ. Mannobiose was also used as substrate (10 mM), incubation at 37° for 24 h. The reaction mixture was examined by thin-layer chromatography.

β-Mannanase activity was measured in gel filtrated extracts with a fresh suspension (3 %) of ivory nut mannan in citrate-phosphate buffer (0.05 M, pH 4.6). The incubation was carried out at 37° for 30 min and the amount of reducing components measured.[13] The production of oligosaccharides was examined by thin-layer chromatography.

Definition of enzyme units. One unit of activity is defined as the amount of enzyme required to hydrolyse 10^{-6} moles of substrate/min under the conditions described.

Paper chromatography. The descending method on Whatman No. 1 paper was used in the following systems (v/v): a. Butanol, pyridine, water, 5:3:2; b. Ethyl acetate, acetic acid, fumaric acid, water, 18:3:1:4.

Thin-layer chromatography was carried out on kieselgur G impregnated with 0.1 M phosphate buffer pH 5 in the following system (v/v): c. Butanol, acetone, phosphate buffer pH 5, 40:50:10.

Aniline oxalate was used as spray reagents for the chromatograms.

High voltage electrophoresis (2000 V, 90 min) was carried out on Munktell filter paper No. 302 in borate buffer (0.1 M, pH 10), and germanate buffer (0.05 M, pH 10.7).[14]

The electropherograms were sprayed with *p*-anisidine hydrochloride.

RESULTS AND DISCUSSION

The assays of the enzymes were performed on the crude extracts and on the gel filtrated extracts. The activities of β-galactosidase and α-mannosidase which are also present in such extracts were for the purpose of comparison always measured parallel to the enzymes under test.

The relative activities of the different glycosidases present in the crude extracts are given in Table 1.

Table 1. Relative activity of the glycosidases in the crude extracts.

	α-Galacto-sidase	β-Galacto-sidase	α-Manno-sidase	β-Manno-sidase
Trifolium repens	1.0 :	0.6 :	1.0 :	0.0
Trifolium pratense	1.0 :	0.9 :	0.7 :	0.0
Medicago sativa	1.0 :	0.3 :	1.0 :	0.0
Anthyllis vulneraria	1.0 :	0.9 :	2.7 :	0.0
Lotus corniculatus	1.0 :	1.0 :	2.0 :	0.0

Examinations of the gel filtrated extracts were performed with approximately the same results for all five species. A typical picture of the enzymes present is given for *Trifolium repens* (Fig. 1).

The activity of β-mannosidase could neither be measured with the synthetic substrate, β-phenyl D-mannoside, nor with the natural substrate, mannobiose; the latter was only used in the examination of gel filtrated extracts. Both substrates were used as it is stated [2,15] that β-mannosidases from other sources than *Leguminosae*, have higher affinities for β-manno-oligosaccharides than for phenyl or methyl β-D-mannosides.

By the identification of the carbohydrates in the crude extracts one would expect to obtain some information as to which enzymes are present, in this case the enzymes which are responsible for the breakdown of the galactomannans. As a result of the action of the three postulated enzymes one would expect to find the following carbohydrates; D-galactose accumulated by the action of α-galactosidase; D-mannose accumulated by the action of β-mannosidase and, to some extent, by the action of β-mannanase.[7] Furthermore one would expect to find different manno-oligosaccharides accumulated by the action of the randomly splitting endopolysaccharase, β-mannanase.[3,2,7]

The water extracts were examined by paper chromatography (solvent a) and found to contain three monosaccharides. Oligosaccharides were not present in detectable amounts. The three monosaccharides were isolated by preparative paper chromatography and then examined separately by means of paper

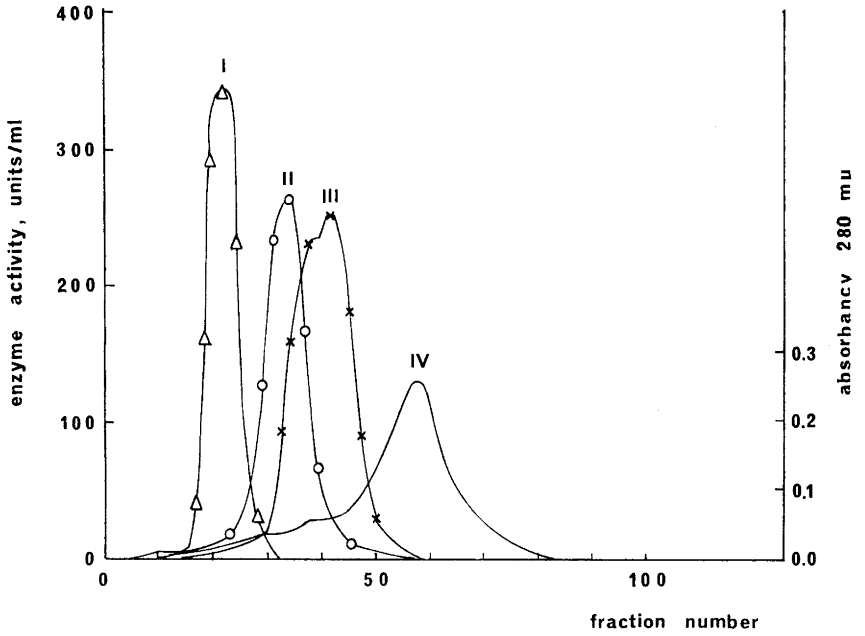

Fig. 1. Elution pattern of enzyme components of *Trifolium repens* (red clover) on Sephadex G 200. Curve I, α-mannosidase activity; Curve II, β-galactosidase activity; Curve III, α-galactosidase activity; Curve IV, protein, absorbancy at 280 mμ.

chromatography, and high voltage electrophoresis. The monosaccharides detected by the combination of these methods were undistinguishable from the reference substances; D-galactose, D-glucose, and D-fructose. D-Mannose was not present in detectable quantity. Parallel electrophoretic experiments with reference sugars demonstrated that D-mannose was so well removed from the other sugars present that even traces of D-mannose would not be overlapped by the more important amounts of the other monosaccharides.

To obtain higher concentrations of the products from the enzymatic reactions, the crude extract (100 ml), which contained enzymes and their substrates under what one would expect to be fairly optimal conditions, was put in a dialysis bag and dialyzed against water at room temperature. Care was taken to prevent bacterial growth. The water (900 ml) was changed twice, each time after 24 h, and concentrated under reduced pressure. The concentrates (10 ml) were examined by the same methods as used with the crude extracts, and it was confirmed that no D-mannose was detectable. The concentrates contained galactose, glucose, fructose, and also oligosaccharides which were present in detectable amounts. They were not further investigated.

These results indicate that β-D-mannoside mannohydrolase does not participate in the hydrolysis of the galactomannans of the five leguminous species examined during the germination of the seeds.

A β-mannanase has been reported in the germinated seeds of two leguminous plants, guar [3] and fenugrec.[7] These enzymes are endopolysaccharases, splitting mannans of the β-(1,4)-type at random to lower oligosaccharides.

Using ivory nut mannan as substrate it was not possible to record any β-mannanase activity in the gel filtrated extracts of germinated red clover seeds.

A typical endo-β-(1,4)-mannanase, if present in some leguminous seeds, would not account for a complete hydrolysis of the mannan chains to monosaccharide units, which could be further metabolized according to general pathways. The absence of β-D-mannoside mannohydrolase during germination may bring into consideration the possibility that only the "outer coat" of galactose of the galactomannan has the function of a carbohydrate reserve, whereas the backbone of the β-1,4-mannan chain is a structural polysaccharide. Another possible explanation of the absence of the appropriate set of hydrolytic enzymes in the germinating seeds is that the mannan part of the polysaccharide is utilized at a later stage of the young plant's development. The author is, however, inclined to prefer the hypothesis that the mobilization of the mannose of the galactomannan during the germination might not necessarily depend upon the presence of a typical β-mannosidase. The possible interaction of phosphorolysis and isomerization should also be considered in further research on the utilization of the galactomannans during germination.

Acknowledgements. The author is indebted to Dr. Elwyn T. Reese, U. S. Army Natick Laboratories, Natick, Massachusetts, for the gift of a sample of ivory nut mannan, and to Mrs. Gry Sletten, Vollebekk, for skilful technical assistance.

Technical equipment which made it possible to carry out the present work, was supplied by *Norges almenvitenskapelige forskningsråd.*

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Received May 30, 1969.