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PURIFICATION AND PROPERTIES OF A β-MANNANASE FROM ALFALFA SEEDS

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Summary

A β-mannanase (EC 3.2.1.25) has been purified from germinating Alfalfa seeds by successive chromatography steps; on hydroxyapatite, DEAE-cellulose and ECTEOLA-cellulose. The enzyme preparations were homogeneous as judged by gel electrophoresis. A 5000-fold increase in specific activity (from the crude extract) was obtained.

The purified enzyme has a molecular weight of 40 000. Several of its properties were determined: pH optimum 5.2 and optimal temperature of activity 50°C.

The hydrolysis of galacto- and gluco-mannans (with various ratios of mannose to galactose and glucose) as well as that of mannooligosaccharides was studied in detail. A prefered point of attack at the third position from the non-reducing end was shown. Comparative results from the hydrolysis of intact galactomannans, or galactomannans previously hydrolysed by galactosidase, suggest that galactose hinders the accessibility of the mannan backbone to the enzyme.

Introduction

Several investigations on β-mannanases (EC.3.2.1.25, β-D-mannoside mannohydrolase) from various sources have been so far reported. Mannanases from plants have been described by Hylin and Sawai [1], Beaugiraud and Percheron [2], McCleary and Matheson [3] and by Shimara et al. [4]. These enzymes have also been found in animal tissues, by Kooiman [5], Courtois et al. [6,7].

In fungi, formation of mannanase through induction was demonstrated by Lyr [8], Ritter [9], Reese and Shibata [10] and Eriksson and Winell [11].

Reese and Shibata [10] have purified the β-mannanase of various strains of *Penicillium* and *Aspergillus* and studied their substrate specificity. Courtois et al. [12] have shown hydrolysis of Alfalfa galactomannan by a β-mannanase from *Aspergillus orizae* which also contained α-galactosidase.

A mannanase from *Aspergillus* was purified and its properties studied by Eriksson and Winell [11].

Innami [13] has isolated a β-mannanase from human gastro-intestinal bacteria; Williams and Doetsch [14] have characterized a mannanase from rumen *Streptococci.* Recently, Emi et al. [15] reported the purification, cristallization and properties of a β-mannanase from *Bacillus subtilis.*

The importance of studies on the specificity of β-mannanase for the determination of the structure of mannans prompted us to investigate the enzyme from Alfalfa seeds.

The present paper describes a procedure for the purification of this enzyme and gives data about its properties and the specific hydrolysis pattern of various substrates such as galactomannans, glucomannans, mannans and oligomannosides.

Materials and Methods

Chemicals

Hydroxyapatite was prepared according to the modification by Levin [16] of the method of Tiselius et al. [17].

DEAE-cellulose (DE 52) was purchased from Pharmacia, ECTEOLA-Cellex E-cellulose was from Biorad. The reagents used for the acrylamide gel electrophoresis were purchased from Eastman Chemical Co. (Rochester, N.Y.): *N,N,N',N'*-tetramethylethylenediamine, *N,N'*-methylenebisacrylamide and acrylamide. Coomassie brilliant blue was from Mann.

Substrates

Galactomannans having different ratios of mannose to galactose were prepared from various vegetable species (Table I). A glucomannan (glucose/mannose, 1 : 3) was isolated from Salep (*Orchis militaris* or *Orchis intacta*). The oligosaccharides mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaose were separated after acid hydrolysis of a mannan extracted from *Phoenix canariensis.* This mannan was also used as a substrate. The linkage between the mannosyl residues of the galactomannans, glucomannans and oligomannosides was of the β 1—4 type.

Enzyme assay

The standard assay procedure was carried out in 0.03 M citrate/phosphate buffer, pH 5.2, with 0.4% final concentration of substrate, in presence of 3—10 μg of enzyme protein. After 10 min incubation at 37° C, the reducing sugar (mannose) was determined by the Somogyi-Nelson method [18,19]. Specific activity was expressed as the amount of mannose (μM) liberated per mg of enzyme per min under the above conditions.

Protein was determined according to the Zamenoff micromethod [20] with biuret as reagent.

TABLE I

β-MANNANASE SUBSTRATES (GALACTOMANNANS)

Origin	Ratio of constituent sugars (Galactose : mannose)
Alfalfa (*Medicago sativa*) seeds	1.0
Guar	0.5
Gleditschia ferox	0.25
Carob (*Ceratonia siliqua*)	0.25

Amino acid composition

The β-mannanase was completely hydrolysed with 6 M HCl at 110°C for 16 h in a sealed tube under vacuum. The amino acid separation and titration was carried out in a Technicon apparatus.

Gel electrophoresis

Electrophoresis was performed on 7.5% acrylamide separating gel according to the technique described by Maizel [21]. Experiments were carried out at 4°C with a current of 6 mA per gel. The gels were stained with Coomassie brilliant blue (0.25% in methanol/acetic acid/water, 50 : 10 : 40, v/v) for 2 h and bleached at 40°C in a 25% methanol and 7% acetic acid solution.

Sodium dodecyl sulfate gel electrophoresis

The electrophoresis was carried out on 7.5% acrylamide gel following the procedure described by Weber and Osborn [22]. A current of 3 mA per gel (5 mm in diameter and 110 mm length) was used. Electrophoresis was performed overnight at room temperature. The gels were stained with Coomassie brilliant blue for 5 h.

Paper chromatography

After hydrolysis of the substrates, the products were identified by ascending chromatography on Whatman No. 3 paper or on Schleicher and Schüll No. 2043 A paper. Three kinds of solvent were used:

(1) butanol/pyridine/water (9 : 5 : 4) for the separation of mono- , di- , tri- and tetra-mannosides.

(2) Isopropanol/methylethylketone/ethyl acetate/*n*-butanol/water (6 : 5 : 3 : 2 : 6) for the separation of pentamannosides.

(3) Isoamyl-alcohol/isopropanol/water (3 : 9 : 8) for oligomannosides containing more than five mannose residues in their molecule.

To locate these mannosides, paper chromatograms were sprayed with β-naphtylamine or orthophenylenediamine [23].

Results

Purification of β-mannanase

Alfalfa seeds were germinated for 2 days at 25°C. All further operations were carried out at 4°C. The seeds were ground in 9‰ NaCl solution and the

TABLE II

PURIFICATION OF β-MANNANASE FROM ALFALFA SEEDS

Enzyme activity was tested under standard conditions as described in Materials and Methods. Protein concentration was estimated by the microbiuret technique [20]

Step	Volume (ml)	Total units	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Enrichment (fold)
Crude extract (Supernatant I)	1300	137.8	18 200	0.0076	100	1
Acid Supernatant II	1225	118.8	857.5	0.139	86.2	18.3
$(NH_4)_2SO_4$ precipitation (Supernatant III)	90	101.16	360	0.281	72.8	36.9
Hydroxyapatite (Fraction IV)	35	17.4	25.5	0.682	22.5	89.7
DEAE-Cellulose (fraction V)	35	6.26	2.13	2.94	4.5	386.8
ECTEOLA-Cellex E (Fraction VI)	35	3.88	0.09	42.73	2.8	5622

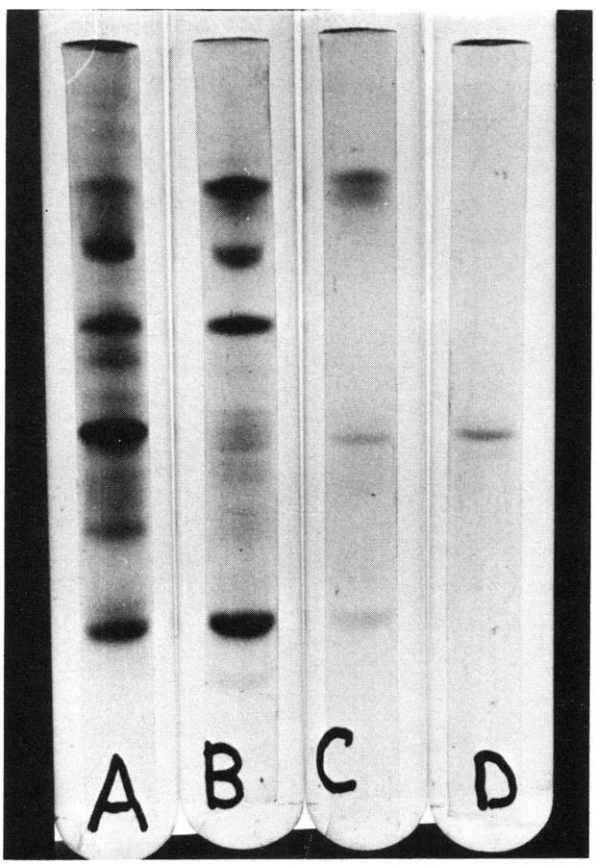

Fig. 1. Polyacrylamide gel electrophoreses of a β-mannanase preparation at various stages of purification. (A), $(NH_4)_2$ SO_4 fraction; (B), hydroxyapatite eluate; (C), DEAE-cellulose eluate; (D), ECTEOLA-cellulose eluate.

mixture stirred during 3 h with a magnetic stirrer. After centrifugation for 30 min at 10 000 × *g*, the precipitate was discarded and the supernatant (Supernatant I) was collected and dialysed for 24 h against a phosphate/citric acid buffer, pH 3.5. The precipitate that appeared during dialysis was removed by centrifugation at 10 000 × *g*. The supernatant, called Supernatant II, was concentrated by ammonium sulfate addition to 80% saturation. The precipitate obtained was suspended in distilled water and dialysed against distilled water for 24 h. A precipitate which appeared in the dialysis bag was removed by centrifugation at 15 000 × *g* for 20 min and the supernatant (Supernatant III) was collected, adjusted to 0.001 M phosphate concentration by addition of 1 M potassium phosphate buffer, pH 6.0, and adsorbed on to a hydroxyapatite column previously equilibrated with 0.001 M potassium phosphate buffer, pH 6.0. Stepwise elution was carried out with 0.001, 0.006 and 0.035 M potassium phosphate buffer, pH 6.0. The elution profile at 0.035 M phosphate buffer shows a double peak; 80% of the mannanase activity was found in its first part whereas the second fraction had a galactosidase activity that we call α-galactosidase I (this enzyme has also been purified; details about it will be reported elsewhere). It should be noted also that when elution is carried out with 0.05 M potassium phosphate buffer, pH 6.0, containing 3 M KCl, another galactosidase activity is eluted: this α-galactosidase II is not yet purified and will form the subject of a forthcoming publication.

The fraction which possessed β-mannanase activity (Fraction IV), after con-

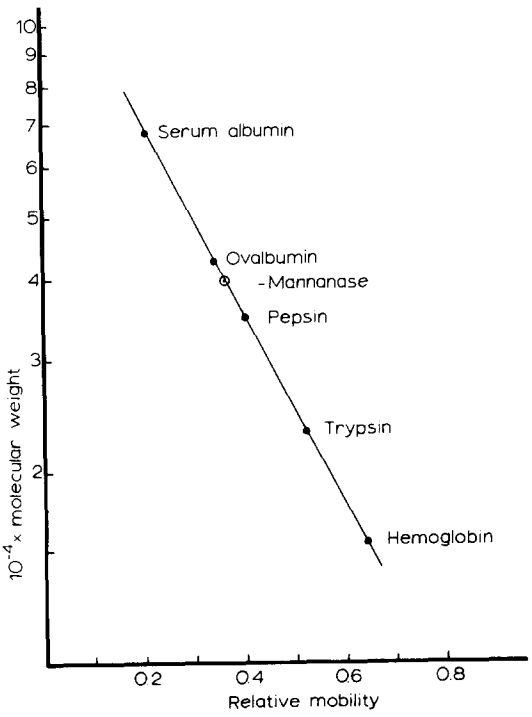

Fig. 2. Estimation of molecular weight of β-mannanase by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

centration in an Amicon apparatus and dialysis against 0.01 M Tris · HCl buffer, pH 6.4, was chromatographed on a DEAE-cellulose column previously equilibrated with the same buffer. The column was eluted with 0.01, 0.05 and 0.1 M NaCl. The enzyme activity was found in the fraction eluted by 0.1 M NaCl (Fraction V). After concentration and dialysis against 0.01 M potassium phosphate buffer, pH 6.8, Fraction V, which is contaminated with some α-galactosidase I and other proteins, was applied on an ECTEOLA Cellex-E column previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The column was eluted stepwise with 0.01 M potassium phosphate buffer, pH 6.8, containing 0.01, 0.1 and 0.2 M NaCl. The β-mannanase activity was found in the 0.2 M NaCl fraction (Fraction VI). After this last column chromatography step the enzyme is purified 5600-fold (Table II). Polyacrylamide gel electrophoresis showed only one protein band possessing β-mannanase activity (Fig. 1).

Sodium dodecyl sulfate gel electrophoresis

After electrophoresis of β-mannanase on 1% sodium dodecyl sulfate gel, only one protein stained band appeared. This result suggests that our enzyme contains only one type of polypeptide chain. As shown in Fig. 2, where logarithms of the molecular weight of reference proteins are plotted versus electrophoretic mobility on 7.5% gel with 1% sodium dodecyl sulfate, the molecular weight of our protein band corresponds to 40 000.

Disc gel electrophoresis

The value of 40 000 for the molecular weight was confirmed by disc gel electrophoresis [24]. Since in this technique native enzyme is used, it seems evident that Alfalfa seed β-mannanase is made up of only one polypeptidic chain.

TABLE III

AMINO ACIDS COMPOSITION

Amino acid	%
Lysine	5.76
Histidine	1.318
Arginine	1.109
Aspartic acid	11.5
Threonine	4.14
Serine	11.572
Glutamic acid	11.885
Proline	3.158
Glycine	10.10
Alanine	16.70
Valine	4.838
Methionine	0.955
Isoleucine	2.828
Leucine	5.492
Tyrosine	3.635
Phenylalanine	4.90

Amino acid composition

Table III lists the amino acid residues detected after acid hydrolysis of the purified β-mannanase preparation. The monoamino acids are in large amounts (60%), which is characteristic of plant seed proteins; methionine, histidine and arginine, on the contrary, do not represent more than 1% each of the total recovery.

Properties

Effect of temperature on enzyme activity

The inactivation of the enzyme was measured at 37, 45, 50 and 55°C as a function of incubation time (5, 10, 20 and 30 min) as shown in Fig. 3.

The effect of temperature on the initial velocity was also studied. The experiments were carried out during 10 min at 10, 15, 20, 25, 30 and 37°C. The calculated energy of activation for β-mannanase was 8.10 kcal/mol. The optimal temperature was determined by incubation under standard conditions for the enzyme assay; 10 min at different temperatures with and without Ca^{2+} in the

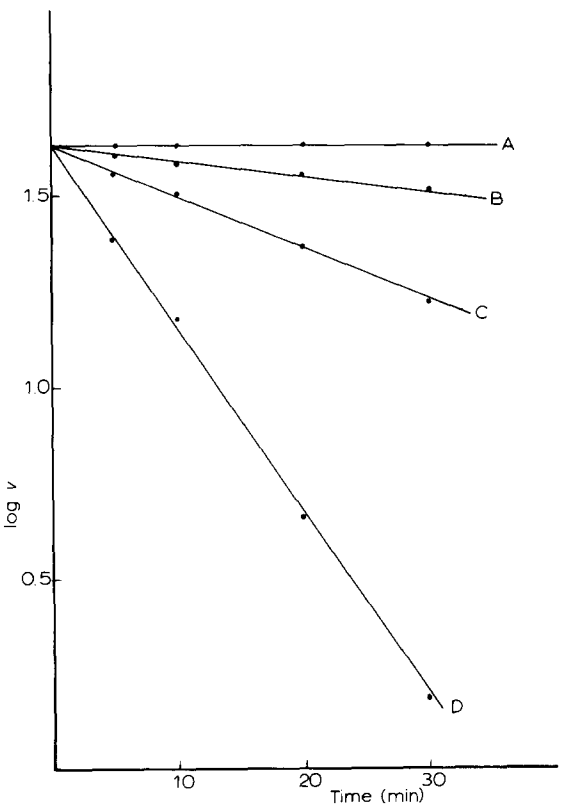

Fig. 3. Heat inactivation of β-mannanase. Aliquots of the purified enzyme preparation were heated at 37, 45, 50 and 55°C for the indicated time (A, B, C and D, respectively). Activity was measured under standard enzyme assay conditions (Materials and Methods). The ordinate represents the logarithm of the residual activity.

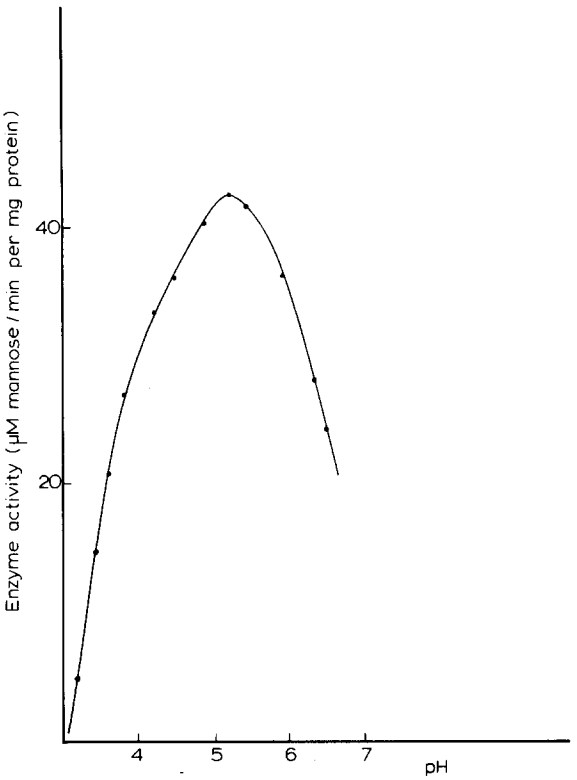

Fig. 4. Dependence of enzyme activity on pH. Phosphate/citrate buffer was used from pH 3 to pH 7 at a concentration of 0.03 M. The other conditions were those of the standard assay.

mixture. Activity is highest at 50° C and decreases at 55° C. In presence of Ca^{2+}, a similar curve was obtained with an increased rate of hydrolysis. This indicates that Ca^{2+} acts as an activator of the enzymic reaction.

pH optimum

As shown in Fig. 4, the enzyme displays the highest activity at pH 5.2 in 0.03 M phosphate/citrate buffer. At pH 6.5, 4.0 and 3.5 the respective activities are 58, 70 and 33% of that observed at pH 5.2. The optimum pH is sharp between 5.0 and 5.4, as opposed to galactosidases which show a broad optimum pH.

Effect of substrate concentration

The molecular weight of the substrates being very high and not exactly known, the K_m is expressed as the percentage of hydrolysis measured (as described under Enzyme assay in Material and Methods). The K_m was determined with different substrates as shown in Fig. 5. The enzyme has a higher affinity for the glucomannan from Salep (K_m, 0.11%) than for the galactomannan from Carob (0.33%) or *Gleditschia* (0.33%) and much higher than for Guar galactomannan (3,96%). It seems that increasing the galactose residues renders the substrate molecule increasingly resistant to hydrolysis.

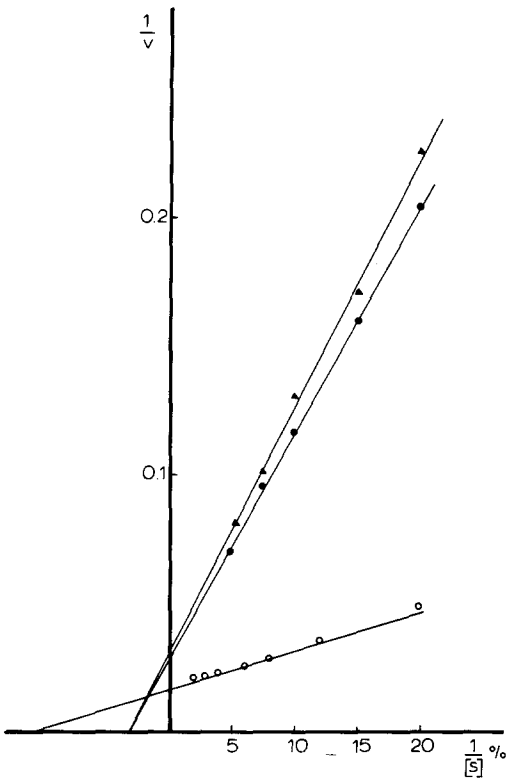

Fig. 5. K_m values of β-mannanase. The enzyme preparation was incubated at 37°C for 10 min in 0.03 M Phosphate/citrate buffer (pH 5.2) with various substrates: galactomannan from Carob, ▲——▲; galactomannan from *Gleditschia ferox*, ●——●; glucomannan from Salep, ○——○.

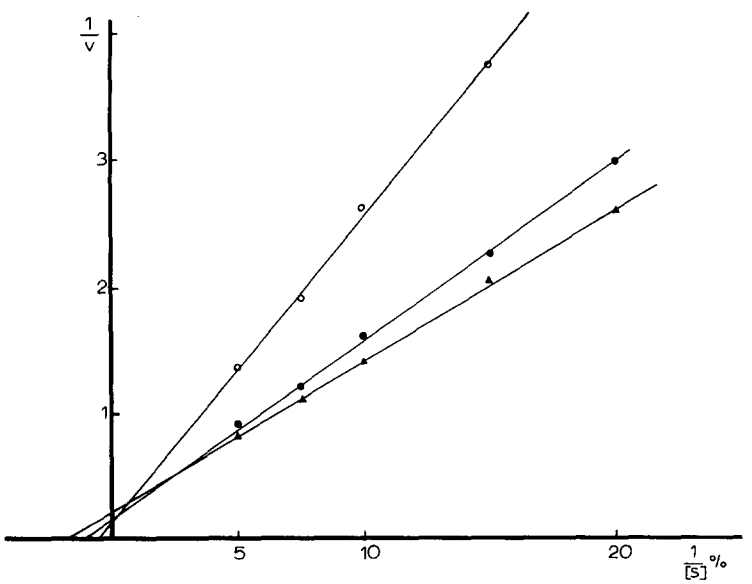

Fig. 6. K_m values of β-mannanase with Alfalfa galactomannan previously treated (10, 20 and 30 min) with galactosidase II. Assays and symbols as for Fig. 5.

The galactomannan from Alfalfa seeds, the molecule of which is composed of successive mannose residues for the backbone and a galactose residue attached to each mannose, is not hydrolysed. To allow for this observation, the kinetics of hydrolysis by galactosidase II (mentioned above in the description of the purification) were studied and the K_m for those substrates was determined. It was observed that the affinity of the enzyme increases as the galactose residues are removed (Fig. 6).

Mechanisms of action of β-mannanase

Phoenix canariensis. 1,4-β-Mannan was subjected to hydrolysis by this enzyme for varying time intervals with constant stirring (since this mannan is insoluble) at 37° C in 0.03 M phosphate/citrate buffer, pH 5.2.

The hydrolysis products were identified by paper chromatography as described under Materials and Methods. After 5 min, mannotetraose, mannotriose and mannobiose appeared, whereas no liberation of mannose was observed. Mannotriose appeared first and was found, as expected, in larger amounts than mannotetra- and mannobiose at the very beginning of the hydrolysis; later on, they are found in the same proportions. Even after 48 h of incubation, no significant amounts of free mannose were obtained.

Salep glucomannan. After 10 min hydrolysis by β-mannanase, mannobiose and mannotriose were liberated from Salep glucomannan. Even after 24 h no glucose-containing oligosaccharides were detected.

Manno-oligosaccharides. The hydrolysis of the manno-oligosaccharides was carried out for 2 and for 24 h at 37° C (Fig. 7). Mannobiose and mannotriose were not attacked. Hydrolysis of mannotetraose was slow; after 2 h, about

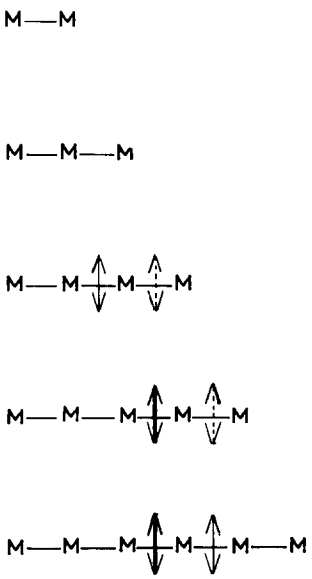

Fig. 7. Action of β-mannanase on mannooligosaccharides: preferential point of attack ↕; secondary point of hydrolysis, ↕; very light hydrolytic attack, •.

30% of this substrate disappeared with concomitant appearance of mannotriose, mannobiose and free mannose. After 24 h, the percentages of these compounds in the mixture were respectively 10, 80 and 10%. Mannopentaose on the contrary is 94% hydrolysed after 2 h, giving equal amounts of bi- and tri-mannosides (49%) and mannose (2%). Mannohexaose disappeared after 2 h with liberation of mannotriose (50%), mannotetraose (25%) and mannobiose (25%). No free mannose was obtained from this mannoside after 2 h; 24 h later, mannose was produced by hydrolysis of the mannotetraose to give mannobiose (41%), mannotriose (55%) and mannose (4%).

These results suggest a mechanism of action of the β-mannanase which is presented in Fig. 7.

Discussion

In the present work, the isolation of a β-mannanase from Alfalfa seed, as a purified protein made up of one polypeptidic chain only, with a molecular weight of 40 000, is reported.

The study of the hydrolysis pattern of various substrates by Alfalfa β-mannanase gave the following results.

This enzyme does not hydrolyse 1,4-β-mannobiose or mannotriose and only acts on mannotetraose slowly. On the other hand it hydrolyses mannopentaose and mannohexaose quickly. The traces of mannose that appear during β-mannan hydrolysis probably result from degradation of mannopentaose and mannotetraose.

The Alfalfa seed galactomannan with a ratio of mannose/galactose of 1 : 1 (Table I) is not attacked by this enzyme. However, the same substrate when previously treated with α-galactosidase becomes available to the β-mannanase. Kinetic studies were carried out on the galactosidase prehyrolysed-substrate, as well as on the *Gleditschia ferox* and *Ceratonia siliqua* galactomannans. Results suggest that galactose hinders the accessibility of the mannan backbone to the enzyme.

Reese and Shibata [10] who studied the hydrolysis pattern of a partially purified β-mannanase from fungi, reported that their enzyme is unable to hydrolyse a "partially degraded" Guar galactomannan with mannose/galactose ratio of 2 : 1. They suggested that a single unsubstituted mannose unit between galactose branches did not give sufficient room for the enzyme to approach the hydrolytic site. This does not seem to be the case for our enzyme which hydrolyses Guar mannan, even if its affinity for this substrate is 10-fold lower than that for Gleditschia or Carob galactomannan. Yamamoto et al. [15] purified a β-mannanase from *B. subtilis*. Its mode of action is also that of an endolytic attack on mannan. Nevertheless with 1,4-β-mannohexaoses or heptaoses as substrates, it preferentially attacks the fourth position from the non-reducing end, whilst Alfalfa seed β-mannanase always hydrolyses those oligomers at the third position from the non-reducing end. This observation again shows that significant differences exist as regards the mechanism of action of mannanases from various sources. Another difference between the β-mannanases of Alfalfa seeds and *B. subtilis* concerns the effect of Ca ions. Ca^{2+} seems to protect the *B. subtilis* enzyme from heat inactivation, whilst it increases the rate of hydrolysis of

Alfalfa seed β-mannanase and thus acts as an activator of the enzymic reaction.

After hydrolysis of Konjac glucomannan (which has a mannose/glucose ratio of 61 : 38) by *B. subtilis* mannanase, Emi et al. [15] obtained mannobiose, -triose, -tetraose, -pentaose and an oligomer containing glucose and mannose. Salep glucomannan (which contains 3 mannose residues to 1 glucose) when hydrolysed by our β-mannanase, released only mannobiose and mannotriose, which demonstrates that the glucose is irregularly distributed in the molecule, and consequently the existence of numerous long sequences of mannosyl residues in the unbranched chain.

Recently, McCleary and Matheson [3] reported the separation of four β-mannanases from Alfalfa seeds after several steps of column chromatography. This is not in agreement with our results. Not one of these mannanases has the molecular weight of our β-mannanase (40 000). At each step of our purification procedure, every protein peak was tested for β-mannanase activity and we have found such activity only in one of them. Contrary to these authors [3], after Sephadex G-100 chromatography β-mannanase activity was detected in only one peak. They determined the molecular weight of their enzyme preparations by gel filtration; they could not use a more accurate method such as polyacrylamide gel electrophoresis because, as they reported, their β-mannanase preparations showed more than one protein band in gel electrophoresis.

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