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Note

Purification and characterization of a β-mannanase from the digestive tract of the edible snail *Helix lucorum* L.

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D-Mannose in mannose-containing polysaccharides in plants is usually linked β-(1 → 4) [1]. β-Mannosidase (EC 3.2.1.25) and β-mannanase (EC 3.2.1.78) enzymes cleave the β-mannosidic linkage in (1 → 4)-β-mannans to yield D-mannose and manno-oligosaccharides, respectively. β-Mannanase enzymes have been reported to occur in a wide range of plant tissues [2,3] as well as in culture filtrates or mycelia of several micro-organisms [3,4]. They are endohydrolases that cleave randomly within the (1 → 4)-β-mannan chain of galactomannans, glucomannans, galactoglucomannans, and linear mannan [3].

It is known that galactomannans are found in the endosperm of many legume species [5]. The proportion of D-galactose varies from 10 to 50% according to the species [2] and the extent of hydrolysis of galactomannans by β-mannanase, free of α-galactosidase and β-mannosidase, is governed by the galactose content of the polysaccharide [5,6]. Apart from the usefulness of β-mannanases in the analysis of the fine structures of galactomannans [7,8] it is known, from an industrial standpoint, that such enzymes as β-mannanases (which are able to hydrolyse crystalline and/or amorphous mannan to oligosaccharides of low degree of polymerization) could find applications in processes such as coffee extraction and in the enzymatic treatment of wood pulp [6].

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Studies concerning terrestrial gastropods (snails and slugs) have revealed that their digestive juices contains an extremely complex mixture of glycosidases [9–13]. However, only limited studies have been performed on β-mannanases [2,3] and β-mannosidases [14,15] present in the digestive tract of herbivorous terrestrial snails.

This study concerns the purification, characterization and properties of β-mannanase from the gut of the edible snail *Helix lucorum* L. (Gastropoda: Pulmonata). Additionally, it is a part of a study concerning the physiology of nutrition of this snail, which is commercially important.

1. Experimental

Origin of the animals.—Adult snails (diameter > 35 mm) originate from the region of Logos, Edessa in Northern Greece where the ecology and biology of *Helix lucorum* have been studied [16]. Before transportation in cardboard boxes the animals were kept under aestivation for 3 months in controlled chambers (14 × 10 cm, 20 ± 1°C, 0% relative humidity).

Substrates.—Carob galactomannan, lucerne galactomannan, and insoluble mannan were kindly provided by the Laboratory of Biochemistry, University of Paris V. Xylan, lichenan, laminaran, carboxymethylcellulose, native cellulose, *p*-nitrophenyl (PNP) β-D-mannopyranoside and PNP β-D-galactopyranoside were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of the crude extract.—All operations were performed at 4°C. After killing of the snails according to the method of Chung [17], their digestive tracts (oesophagus, salivary glands, and crop) were removed. Guts from 25 animals (~ 7 g fresh weight) were homogenized in ~ 8 vol. (60 mL) of ice-cold distilled NaCl 0.9% (w/v) solution. The homogenate was then centrifuged at 20 000 *g* for 20 min. After centrifugation, the supernatant was precipitated by addition of solid ammonium sulfate to 80% saturation. The suspension was stirred for 12 h and the precipitate collected by centrifugation at 20 000 *g* for 20 min. The precipitate was dissolved in a minimum volume of ice-cold distilled water and dialysed overnight against 2 mM ice-cold potassium phosphate buffer, pH 5.3. The dialysed solution constituted the crude extract.

Enzyme assays.—During the purification procedure, mannanolytic activities were assayed by incubation (100 μL enzyme solution, 100 μL carob galactomannan (8 mg/mL) and 50 μL of McIlvaine buffer [18], pH 5.45). After 20 min at 37°C, the reducing sugars were determined by the Somogyi–Nelson micromethod [19]. Proteins were determined by the Coomassie Brillant Blue G250 technique [20] using bovine serum albumin (Sigma) as a standard. Specific activity was expressed as μmol mannose liberated per min (U) and per mg protein. Other enzyme assays were performed according to the methods described in a previous study [21].

Thin-layer chromatography (TLC).—Products of the enzymatic hydrolysis of mannan were detected by TLC (silica gel-coated F 1500 Schleicher and Schüll, Dassel, Germany). The solvent system was 1-propanol–EtOH–EtOAc–AcOH–pyridine–water (7:3:3:2:2:3). Chromatograms were sprayed with an alcoholic solution of β-naphthylamine followed by a treatment with 2% H_2SO_4 in EtOH [22].

Polyacrylamide gel electrophoresis (PAGE).—Electrophoresis was usually performed on 7.5% polyacrylamide disc gels (100 × 5 mm) [23] with a current intensity of 6 mA. The gels were stained with Coomassie Brillant Blue [21]. The molecular weight of the purified enzyme was determined by electrophoresis on polyacrylamide gels using the Hedrick and Smith method [24] and by SDS-PAGE following the procedure of Laemmli [25]. Reference proteins used were phosphorylase a monomer (94 kDa), bovine serum albumin monomer (68 kDa), pepsin (35 kDa), and trypsin (23.3 kDa). The purified mannanase was tested for carbohydrate content by periodic acid–Schiff staining [26].

2. Results

Purification of mannanase.—The crude extract was applied to a column (2.5 × 10 cm) of hydroxylapatite [27,28] equilibrated with potassium phosphate buffer (2 mM, pH 5.3). Mannanase was eluted by the same phosphate buffer (50 mM). After dialysis, the active fraction was applied on a DEAE-Sepharose column (1.8 × 12 cm) equilibrated with 2 mM phosphate–citrate buffer, pH 7.6. Mannanase was eluted with the equilibration buffer. Purification was achieved by ion exchange chromatography on a QA-Trisacryl column (1.8 × 10 cm). The β-mannanase was eluted with the equilibration buffer (2 mM potassium phosphate buffer, pH 7.0). At this stage, a single protein band appeared on gel electrophoresis (Fig. 1), with which the mannanase activity was associated.

The final mannanase was purified 26-fold (13% yield). Its specific activity was 29 U/mg protein (Table 1).

Kinetic properties of the purified mannanase.—Rate curves of the hydrolysis of carob galactomannan showed linearity up to 30 min when mannanase diluted 20-fold was used. Therefore, this dilution and an incubation time of 30 min was used for all other experiments.

Specificity of mannanase.—The purified mannanase attacked carob galactomannan (29 U/mg) and insoluble mannan (3.7 U/mg). The enzyme also hydrolysed lucerne galactomannan, but to a lesser extent (0.924 U/mg). The purified mannanase also showed a slight activity towards cellulose (CMC: 0.440 U/mg; native cellulose: 0.240 U/mg) and PNP β-D-mannopyranoside (0.210 U/mg) but had no activity on xylan, laminarin, lichenan or PNP β-D-galactopyrannoside. TLC revealed that the purified

Table 1
Purification of β-D-mannanase from the digestive tract of *Helix lucorum*

Steps	Volume (mL)	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Crude extract	10	192	208	1.1	100	1
Hydroxyapatite	100	27	76	2.8	37	2.6
DEAE-Sepharose	111	2.44	30	12.3	14	11.2
QA-Trisacryl	50	0.93	27	29	13	26.4

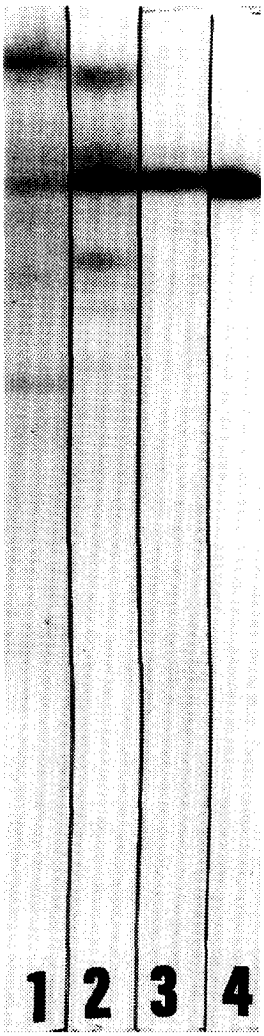

Fig. 1. Polyacrylamide (7.5%) gel electrophoresis of the purified mannanase under non-denaturating conditions. Successive stages of purification. Lane 1: crude extract; lane 2: hydroxyapatite column; lane 3: DEAE sepharose column; lane 4: QA trisacryl column.

mannanase hydrolysed the insoluble mannan to give di- and tri-saccharides as the major products as well as tetrasaccharides. Higher homologues (e.g. penta-, hexa-saccharides) were apparent but to a lesser extent, whereas mannose was never present. It can be concluded, then, that the purified mannanase is an *endo*-β-D-mannanase (EC 3.2.1.78).

Effect of pH on the rate of hydrolysis of carob galactomannan.—The effect of pH on the activity of the purified mannanase was studied using McIlvaine buffers of pH 2.8–8.8 at 37°C. Maximum mannanase activity was recorded at pH 6.6.

Thermal denaturation.—The purified mannanase remained stable at 4°C for nearly 1 month. Moreover, it was proved to be stable enough to freeze (3 months at −20°C).

Temperature stability was measured by incubating purified mannanase in McIlvaine buffer at 30–60°C for 5 min. The mannanase remained stable from 30–45°C. The preincubation of the enzyme for 5 min at 50°C, 55°C and 60°C caused approximately 40, 60 and 90% loss of activity, respectively.

Effect of substrate concentration.—The effect on the reaction rate of varying substrate concentration was studied on carob galactomannan. The apparent Michaelis constant (K_m was calculated by Lineweaver–Burk [29] plots. The mean K_m calculated was 0.13 ± 0.015 mg/mL.

Molecular weight.—The molecular weight, determined by the method of Hedrick and Smith [24], was ~ 35 000 kDa and was confirmed by SDS-PAGE, suggesting that the purified mannanase was monomeric.

Glycoprotein nature.—The purified mannanase was tested for the presence of carbohydrate by periodic acid/Schiff staining on polyacrylamide gel electrophoresis. It did not give a positive response, suggesting a non-glycoprotein nature for this enzyme.

3. Discussion

In this study, the purification and characterization of mannanase from the gut of the pulmonate snail *H. lucorum* are described.

The purified mannanase attacked insoluble mannan by releasing various oligosaccharides, mainly di- and tri-saccharides. The action of the enzyme on insoluble mannan — an unbranched homoglycan — and the fact that it shows very little activity on PNP β-D-mannopyranoside, allows us to classify it as an *endo*-β-D-mannanase (EC 3.2.1.78) [14].

Concerning the purification procedure followed in this study, preliminary experiments had revealed that the use of hydroxyapatite as a first column led to a considerable loss of mannanase activity (Table 1) but provided good separation of the different glycosidases (e.g. β-mannosidase, β-glucosidase, cellulases) present in the digestive juice of *H. lucorum* [11]. Moreover, hydroxyapatite has been used with success for the purification of cellulase and β-mannosidase in snails (*H. pomatia* [30], *Achatina fulica* [15]) and of cellulases in different insect species (*Ergates faber* [31], *Macrotermes mülleri* [32]).

The purification procedure resulted in 26-fold purification, which is comparable with the 15-fold purification of mannanase from another pulmonate snail, *H. pomatia* [3]. The low purification rate obtained could be due to the multiplicity of glycanases present in the digestive tract of *H. lucorum* [11,13]. The same has been proposed for an exo-(1 → 3)-β-glucanase purified from the gut of *H. pomatia* [33], which had the ability to cleave (1 → 4)-β-and (1 → 6)-β-glucosidic linkages in addition to (1 → 3)-β-linkages. Thus, removal of other glycanases is most probably a factor contributing to the apparently low yield of mannanase in our study. Additionally, it can not be excluded that there is a synergism between β-mannosidase and the purified β-mannanase of *H. lucorum*. Such synergism is apparent in another snail, *H. aspersa* [34], and has been reported to occur between β-glucosidase and cellulases of termites [35].

The apparent molecular weight of the purified mannanase (35 kDa) is in accordance with the results obtained from two other pulmonate snails: *H. pomatia* [3] and *H.*

aspersa [34]. The molecular weight determined for *H. lucorum* mannanase is also comparable with mannanase purified and characterized from eukaryotic (*Aspergillus niger* [3], *Streptomyces* sp. [4]) and prokaryotic (*Bacillus subtilis* [3]) microorganisms.

The mannanase purified from *H. lucorum* showed maximum activity at pH 6.6, a value is higher than that reported for *H. pomatia* [3] and those for the mannanases purified from various sources other than snails (*Medicago sativa, Cyamopsis tetragonolobus, Aspergillus niger, Bacillus* sp., *Irpex lacteus*) [3]. A similar optimum pH (6.8) has been found for *Streptomyces* sp. [4].

Our studies have shown that *H. lucorum* mannanase remained stable only up to 45°C, and thus it could be concluded that the enzyme was not thermostable, at least in comparison with other glycosidases [14,15,31,32,36]. The relative non-thermostability of the enzyme could be explained by its non-glycoproteinic nature, as is suggested by its negative reaction with periodic acid–Schiff reagent. Generally, *endo*-β-D-mannanases have not been proven to be extremely thermostable (*H. pomatia*, *M. sativa*, *C. tetragonolobus*, *Bacillus* sp. [3]; *Streptomyces* sp. [4]).

The mean K_m value determined for *H. lucorum* mannanase with carob galactomannan as substrate (0.137 ± 0.015) is lower than that reported with the same substrate for *H. pomatia* [3].

Experiments concerning the enzyme specificity revealed that the purified mannanase from *H. lucorum* does not attack the two galactomannans tested in the same way. Lucerne galactomannan, with a 1:1 galactose–mannose ratio [6], was hydrolysed to a much lesser extent than carob galactomannan (1:4 galactose–mannose ratio [6]). The higher hydrolysis rate of carob galactomannan is in accordance with that found for two other snails, *H. pomatia* [9] and *H. aspersa* [12], in crude extract preparations from the digestive tracts of the animals. According to McCleary [6], the extent and products of hydrolysis of galactomannans are a consequence of both the source of the particular *endo*-β-mannanase employed, as well as the sugar composition and physical state of the substrate (e.g. the galactose content of galactomannan).

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