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1,2-α-D-MANNOSIDASE FROM A WOOD-ROTTING BASIDIOMYCETE, *PYCNOPORUS SANGUINEUS*

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Key Word Index—*Pycnoporus sanguineus*; *Trametes sanguinea*; Basidiomycete; wood-rotting fungi; α-D-mannosidase; mannosidase.

Abstract—An acidic α-D-mannosidase has been isolated from the culture filtrate of a wood-rotting Basidiomycete, *Pycnoporus sanguineus* and the molecular and enzymatic properties of the enzyme determined. The extracellular mannosidase was homogeneous on PAGE at pH 9.4. The M_r as determined by SDS-polyacrylamide disc gel electrophoresis was 64 000, and the pI was pH 4.7 using electrofocusing. The purified enzyme had a pH optimum of 4.5 with Baker's yeast mannan and had no activity towards *p*-nitrophenyl-α-mannoside. The K_m and k_{cat} values for Manα1-2Man at pH 4.5 and 30° were 0.9 mM and 1.9 sec. the enzyme had no activity towards Manα1-3Manα1-2Man, and it cleaved specifically the 1,2-α-linked side chain of yeast α-mannan, producing free α-D-mannose.

INTRODUCTION

α-Mannosidases (EC 3.2.1.24) are found in many higher plants [1]. The level of α-mannosidase increases during seed germination of a plant [1]; however, the physiological role of the enzyme is not clear. It may be important in removing a major part of the mannose residues from storage glycoproteins and exposing the core for further glycosidase action, thus facilitating the mobilization of the proteins.

The growth of wood-rotting Basidiomycetes depends on having cell bound and extracellular glycolytic enzymes, because the fungi feed entirely by adsorption.

In previous papers [2, 3], the purification and mode of action of an acidic α-D-mannosidase from *Aspergillus saitoi*, a food microorganism, were investigated with Baker's yeast mannan [2] and various oligosaccharides liberated from glycoproteins [3].

The present report deals with the isolation of an acidic α-D-mannosidase from *Pycnoporus sanguineus* and its mode of action on mannooligosaccharides.

RESULTS

Purification of α-D-mannosidase

A crude enzyme preparation obtained from the submerged culture filtrate of *P. sanguineus* ATCC 14622 (= *Trametes sanguinea* IFO 7045 [4] or *Pycnoporus coccineus* [5, 6] by a previously published method [4] using 0.01 M sodium acetate buffer, pH 5.0, was precipitated with cold methanol. The 40–60% methanol precipitate was collected at 4°, and then dissolved in 0.01 M sodium acetate buffer, pH 5.0. Ion-exchange filtration of this preparation on a DEAE-Sephadex A-50 column (4 × 60 cm) equilibrated with the enzyme extraction buffer resulted in a 23.5-fold purification. Further column chromatography (2 × 65 cm) using SP-Sephadex C-50 yielded an additional 10-fold purification. The enzyme was eluted with 0.01 M sodium acetate buffer, pH 4, containing 0.2 M sodium chloride. Rechromatography on DEAE-Sephadex (1 × 50 cm) gave an active fraction; the enrichment of the enzyme was 1.6-fold. Finally, SP-Sephadex chromatography (1 × 30 cm) brought about an additional 4.7-fold purification. The enzyme was eluted with 0.01 M sodium acetate buffer, pH 4.0, which contained 0.2 M sodium chloride. The final preparation of the α-D-mannosidase was devoid of other glucosidase activities.

The results of the purification are summarized in Table 1. The active fraction from the final purification step was dialysed against distilled water and freeze-dried. The protein (2 μg) obtained from SP-Sephadex C-50 migrated as a single band on PAGE at pH 9.4 and on SDS-disc gel electrophoresis.

General properties

The substrate used for determining the general properties of the enzyme was Baker's yeast α-D-mannan. The purified enzyme was active between pH 3.5 and 5.5, showing a well-defined optimum pH of 4.5.

At 30°, the enzyme was stable for 1 hr at pHs between 3.0 and 6.0. In 0.01 M sodium acetate buffer, pH 4.5, the activity was stable at 60° for 10 min. It decreased by *ca* 75% at 70° after 10 min and 90% or more was lost at 80° for 10 min.

Molecular properties

The pI of the enzyme was 4.7, using isoelectric focusing. The M_r was determined by SDS-disc gel electrophoresis and the apparent value was 64 000. The marker proteins used were cytochrome *C* and cross-linked cytochrome oligomers from horse heart (monomer, 12 400; dimer, 24 800; trimer, 37 200; tetramer, 49 600 and hexamer, 74 400).

Table 1. Purification of *Pycnoporus* α-D-mannosidase

Step	Enzyme activity (nkat)	Total protein (mg)	Specific activity (mkat/kg)	Purification (fold)	Yield (%)
1. Crude enzyme extract	284	18 700	0.015	1	100
2. MeOH precipitate	187	7 300	0.026	1.7	66
3. DEAE-Sephadex A-50	75	213	0.35	24	26
4. SP-Sephadex C-50	23.2	6.3	3.7	245	8
5. DEAE-Sephadex A-50	4.60	0.77	6.0	400	2
6. SP-Sephadex C-50	1.45	0.145	10.0	667	0.5

Specificity and mode of action

The enzyme did not act on artificial substrates such as *p*-nitrophenyl-α-D-mannoside (pH 5.0). It hydrolysed Baker's yeast α-D-mannan (pH 4.5) producing free D-mannose. The anomeric configurations of the reaction products of the enzyme from Baker's yeast α-D-mannan as a substrate were examined. The optical rotation increased gradually after the enzyme was added, in a positive direction in this case. However, an immediate decrease in optical rotation was observed upon the addition of ammonium hydroxide, indicating that the reaction product was an α-anomer.

A survey of the action of the enzyme on various oligosaccharides liberated from yeast α-D-mannan showed that the enzyme hydrolysed the Manα1-2Man linkage in Manα1-2Man (pH 4.5) and Manα1-2Manα1-2Man but did not hydrolyze Manα1-3Manα1-2Man (pH 4.5) linkages at all.

Kinetic studies

The determined kinetic parameters of the release of α-D-mannose from Manα1-2Man by the purified enzyme at pH 4.5 and 30° were K_m 0.9 mM and k_{cat} 1.9/sec.

DISCUSSION

An enzyme with mannosidic activity in the acidic pH range found in the culture filtrate of the wood-rotting Basidiomycete, *P. sanguineus* (= *Trametes sanguinea*) does not act on *p*-nitrophenyl α-D-mannoside. It can readily convert Baker's yeast α-D-mannan to free α-D-mannose. The enzyme seems to be highly specific for the Manα1-2Man linkage and can be used as a reliable reagent both to detect the Manα1-2Man linkage in the sugar chain and to remove D-mannose. Although α-mannosidases are generally very heat labile, the *Pycnoporus* enzyme retained most of its activity after heating at 60° and pH 4.5 for 10 min. This stability is an asset because prolonged incubation is often necessary in the study of certain intact glycoproteins.

A 1,2-α-D-mannosidase from *Aspergillus saitoi* [2, 3] with an M_r of 51 000 was purified from koji culture [2]. The optimum pH of *A. saitoi* α-mannosidase was 5.0; it had a K_m of 0.45 mM with Baker's yeast mannan and no activity towards *p*-nitrophenyl-α-D-mannoside. The specificity of the newly found enzyme resembles that of 1,2-α-mannosidase from *A. saitoi*, which can cleave specifically the 1,2-α-linked side chain of yeast α-mannan producing free mannose [2], and can readily convert Manα1-2Manα1-3Manβ1-4GlcNAc to Manα1-3Manβ1-4GlcNAc [3].

Tabas and Kornfeld [7] reported that the rat liver Golgi α-D-mannosidase shows specificity for α-1,2 linked mannose residues and is effective in converting the $Man_9GlcNAc$ oligosaccharide to the $Man_5GlcNAc$ oligosaccharide. *Pycnoporus* α-D-mannosidase shows a more restricted specificity than *Aspergillus niger* α-D-mannosidase [8] and jack bean α-D-mannosidase [9, 10].

Swaminathan *et al.* [8] reported that an α-D-mannosidase from *A. niger* shows relatively high specificity for the Manα1-2Man linkage, although it can cleave Manα1-3Man and Manα1-6Man linkages at 1% of the rate of the Manα1-2Man linkage. Although the α-mannosidase from *A. niger* has been characterized, it does not hydrolyse Baker's yeast mannan or *p*-nitrophenyl-α-mannoside [8].

Tai *et al.* [9] reported that Manα1-2Man and Manα1-6Man linkages are cleaved at least 15 times faster than Manα1-3Man linkages by jack bean α-mannosidase. Berman and Allerhand [10], using natural abundance ^{13}C NMR spectroscopy, reported that the Manα1-2Man(α) linkage of Manα1-6(Manα1-3Man)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn is the first one to be digested, and the Manα1-3Man(β) linkage is hydrolysed next, faster than the Manα1-6Man(α) linkage. The Manα1-3(α) linkage is hydrolysed very slowly. They concluded that the reported 'rule' by Tai *et al.* [9] was based on studies of a limited number of model oligosaccharides.

It can be concluded that *Pycnoporus* α-D-mannosidase is highly specific for the 1,2-α-mannosidic linkage and that it therefore readily hydrolyses Baker's yeast mannan and Manα1-2Man. The trivial name *Pycnoporus* 1,2-α-D-mannosidase is suggested for this new enzyme.

EXPERIMENTAL

Materials. The crude enzyme preparation was the gift of K. Tomoda, Laboratory of Fermentation Products, Takeda Chemical Industries Ltd., Osaka. Baker's yeast mannan was purchased from Sigma (Lot 77C-0238). *p*-Nitrophenyl-α-D-mannoside (CH-8157) was from Wako Pure Chemicals, Osaka. Manα1-2Man was prepared by controlled acetolysis of Baker's yeast mannan, followed by gel filtration of the deacylated products on a Sephadex G-25 column according to the method of Kocourek and Ballou [11]. Mannotrioses containing Manα1-3Manα1-2Man and Manα1-2Manα1-2Man were also prepared by this method. Manα1-3Manα1-2Man was obtained by the complete digestion of Manα1-2Manα1-2Man in the mannotriose

fraction with *A. saitoi* 1,2-α-D-mannosidase [2]. Marker proteins of cytochrome *c* from horse heart and cross-linked cytochrome oligomers for SDS-disc gel electrophoresis were purchased from the Oriental Yeast Co., Ltd., Osaka.

Enzyme assay. *p*-Nitrophenyl-α-D-mannosidase activity was determined according to the published method at pH 5.0 [8]. For α-D-mannosidase activity, baker's yeast mannan dissolved in 0.1 M NaOAc buffer, pH 5.0, was used according to ref. [2]. The amount of mannose liberated was determined by the method of Somogyi-Nelson [12]. One katal of 1,2-α-D-mannosidase activity was defined as the amount of enzyme required to liberate 1 mol mannose from Baker's yeast mannan per sec at 30° and pH 5.0.

The anomeric configuration of D-mannose residue in the reaction products at pH 4.5 and 24° was analysed using a Perkin–Elmer model 141 polarimeter.

Determination of kinetic parameters. To determine the initial rates of enzyme activity for Manα1-2Man in 100 μl 0.1 M NaOAc buffer, pH 4.5, a procedure enabling the respective estimation of 0.32 μg of the enzyme in 20 μl 0.1 M NaOAc buffer pH 4.5 was developed. After the reaction was stopped by heating to 100° for 2 min, mannose liberated from Manα1-2Man was determined by the method of Somogyi-Nelson [12]. By this means, five points could be determined during the first 30 min of incubation at 30°, and the initial rate could be quite precisely determined. In all cases, satisfactory Michaelis–Menten kinetics were observed and a plot of $1/V$ vs. $1/[S]$ permitted the fitting of unambiguous straight lines.

Analytical determinations. Protein concns were usually estimated from the *A* at 280 nm. Protein was also determined by the method of Lowry *et al.* [13], using bovine serum albumin (fraction V, Daiichi Pure Chemicals Co.) as a standard.

PAGE was performed at 4° with the standard pore formulation of Davis at pH 9.4 and 3 mA constant for gel [14]. Gels were stained with 0.4% Coomassie brilliant blue dissolved in 50% MeOH–9.5% HOAc and destained in 5% MeOH–9.5% HOAc and 7% HOAc [15]. SDS-disc gel electrophoresis was performed at 4° with 10% acrylamide gel as described in ref. [15].

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