PURIFICATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF AN ENDO-β-1,4-MANNANASE PRODUCED BY *Aspergillus niger* 1234

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ABSTRACT

Endo-*β*-1,4-mannanases (EC 3.2.1.78) are hydrolases that catalyze the hydrolysis of the internal *β*(1,4) bonds of the mannan backbone to release *β*-1,4-manno-oligosaccharides. *β*-mannanases are of increasing industrial importance, as they can be used in the processing of mannan-rich plant biomass to obtain bioproducts, such as mannan-oligosaccharides (MOS). This work aims to purify and characterize endo-β-1,4-mannanases produced by *Aspergillus niger* 1234. The purification strategy consisted of concentrating the crude enzyme by ultrafiltration, followed by size exclusion and ionic exchange chromatography, using Sephadex® G-75 and DEAE-Sephacel resins. The concentrate eluted in G-75 showed two peaks of enzymatic activities (MAN1234A and MAN1234B). After elution in DEAE, the purification of MAN1234B was verified by electrophoresis and mass spectrometry. The molecular mass of MAN1234B was estimated at 41.2 kDa, with optimum temperature and pH of 62ºC and 3.9, respectively, and a K_m of 3.03 mg/mL. Furthermore, it maintains activity for 72 hours when incubated at up to 62ºC. The ability to produce MOS with a degree of polymerization of up to 6 mannoses was demonstrated. The results provided more information about these enzymes, which are scarce compared to other hydrolases, and will facilitate their efficient application.

Keywords: Endo-β-1,4-mannanase. Purification. *Aspergillus*. Mannan. Mannan-oligosaccharides.

1 INTRODUCTION

Enzymatic processing is particularly important for plant biomass polysaccharides utilization in a Bioeconomy context. After initial deconstruction through various pretreatments, polysaccharides become more accessible and can be hydrolyzed by enzymes to produce oligosaccharides and monosaccharides, which can serve as a platform for a range of products. However, the distinct compositions and structures of different raw materials require specific enzymes that act synergistically during the enzymatic processing of each biomass fraction. Lignocellulosic biomasses, such as sugarcane bagasse and straw, require a pool of enzymes, rich in cellulases and xylanases, while non-cellulosic biomasses rich in mannans, such as açaí, juçara and macaúba seeds, as well as coffee beans, require mannanases. While some agricultural co-products, such as bagasse and sugarcane straw, already play a significant economic role, many mannan-rich raw materials remain underutilized or are simply improperly discarded, leading to serious environmental issues[1].

Mannan is a polysaccharide with typically two primary functions in plant cell walls: a structural component of the plant cell wall or energy storage in certain seed. Mannan is categorized into four subfamilies based on the sugar residues in the polysaccharide structure: linear mannan, galactomannan, glucomannan, and galactatoglucomannan. Linear mannan consists solely of mannose residues in the backbone, linked by β-1,4-type linkages, with minimal or no branching. Galactomannan features a main chain of mannose with galactose branches linked by α-1,6-type linkages. Glucomannans are formed by mannose and glucose residues in the main chain, in a 2:1 ratio, connected by β-1,4 glycoside linkages. Galactoglucomannan is similar to glucomannan but has additional galactose branches connected by α-1.6 linkages[2].

Although there are several enzymes involved in the complete hydrolysis of mannan, depending on the type of mannan, endo-β-1,4-mannanases (EC 3.2.1.78) are the main endo-acting enzymes that act in the catalysis of mannan hydrolysis to release *β*-1,4-manno-oligosaccharides[2]. Surprisingly, compared to other hydrolases, there is a scarcity of studies addressing the purification, characterization and application of these enzymes in detail. This is a technological bottleneck that can impede its efficient use. In fact, one of the limiting factors for industrial-scale plant biomass processing is the precise characterization of the enzymes required and the formulation of efficient enzyme mixtures that can enable high conversion rates of polysaccharide to monosaccharides and oligosaccharides at low cost. Therefore, the motivation for developing this work was the possibility of carrying out a detailed study of the purification and characterization of endo-β-1,4-mannanases produced by *Aspergillus niger* 1234. This information will contribute for understanding its efficiency and specificity, facilitating the development of customized and optimized processes to its target products. This work begins with the strategy to isolate the target enzyme, followed by the characterization and application of the crude enzyme and the endo-β-1,4-mannanase, aiming to produce mannose and mannan-oligosaccharides (MOS).

2 MATERIAL & METHODS

The fungus *Aspergillus niger* 1234 (CFAM/Fiocruz - Amazon Fungi Collection) was selected for the present study based on literature data and previous studies conducted at the Bioethanol Laboratory (IQ – UFRJ), demonstrating its high capacity for endo-β-1,4-mannanase production[3]. To produce β-mannan-degrading enzymes, *A. niger* 1234 was cultured at 30 ºC for 7 days

in a liquid-state medium containing the commercial galactomannan locust bean gum (Sigma) as carbon source. Flasks of 1 L containing the carbon source (30 g/L) and supplemented medium (1,2 g/L $NaNO_3$, 3,0 g/L KH_2PO_4, 6,0 g/L K_2HPO_4, 0,05 g/L $CaCl_2.2H_2O$, 0,2 g/L $MgSO_4 \cdot 7H_2O$, 0,002 g/L $FeSO_4 \cdot 7H_2O$, 0,016 g/L $CoCl_2.6H_2O$, 0,005 g/L $MnSO_4 \cdot 4H_2O$, 0,0014 g/L $ZnSO_4 \cdot 7H_2O$ and 12 g/L yeast extract at pH 4.8) were inoculated with spore suspension (1%) from a routine subculture. After the growth procedure, the crude extract was filtered using a vacuum system and stored at 5°C for subsequent use as a source of endo-β-1,4-mannanase activity. Endo-β-1,4-mannanase activity was determined by mixing 250 µl of enzyme solution with 250 µl of locust bean gum (0,5%) in 50 mM citrate buffer at pH 4.8 for 10 min at 50ºC. The release of reducing sugar was measured using the dinitrosalicylic reagent method[4]. Endo-β-1,4-mannanase activity was expressed as µmol reducing sugar formed per min^{-1} ml^{-1} enzyme solution, i.e. as IU ml^{-1}. Mannose was used as the standard. Protein concentration was measured by the method of Lowry[5], using bovine serum albumin as standard.

The crude extract was concentrated by ultrafiltration using an Amicon system (Millipore®) with a 10 kDa cut-off point membrane. Concentrate samples were fractionated by gel filtration on Sephadex G-75 (60 x 2.6 cm) column pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. Fractions of 4.0 mL were collected at a flow rate of 20 mL/h. Fractions with endo-β-1,4-mannanase activity were pooled, and loaded onto a DEAE-Sephacel column, equilibrated with 50 mM sodium acetate buffer, pH 5.0. Fractions of 4.0 mL were collected at a flow rate of 40 mL/h by eluting the column with buffer followed by a linear gradient of NaCl (0–1 M), pH 5.0. Fractions corresponding to endo-β-1,4-mannanase activity were pooled and stored for later use at 4 ºC. SDS-PAGE electrophoresis was used to evaluate protein fractionation, potential isoforms, and the degree of homogeneity of the crude enzyme and the fractions obtained from the purification steps. The SDS-PAGE electrophoresis was carried out using a 12% gel and stained with colloidal Coomassie[6]. Replicate SDS-PAGE containing 0.1% locust bean gum was performed following by incubation in the reaction buffer to promote the enzymatic hydrolysis of locust bean gum and stained endo-β-1,4-mannanase activity using Congo Red (zymogram)[6]. A qualitative mass spectrometry assay was also performed to analyze the homogeneity of the purified enzyme and define its molecular mass. The purified fraction was subjected to trypsin digestion, and the mass spectrum (MS) was acquired using the MALDI-TOF/TOF Autoflex II spectrophotometer (Bruker Daltonics, Germany) operating in reflector mode with positive ion detection.

For the kinetic experiments, locust bean gum was used as substrate in a concentration range of 0.25 – 5.0 mg/mL. K_m and V_{max} values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program[7]. The determination of optimum temperature and pH of purified endo-β-1,4-mannanase was evaluated using a Central Composite Rotatable Design (CCRD 2^2). The optimal temperature and pH for enzymatic activity were evaluated in the range of 30°C to 90°C and pH 3.0 to 7.0, as observed in studies previously described in the literature and considering the enzyme's thermal stability range. The stability of purified endo-β-1,4-mannanase was carried out by preincubating the enzyme solution at 50ºC, 60ºC and 70ºC and samples were taken at defined intervals to measure its activity. The effect of some reagents ($AgNO_3$, $CaCl_2$, $CoCl_2$, $CuSO_4$, EDTA, $FeCl_3$, $FeSO_4$, KCl, $MgSO_4$, $MnCl_2$, NaCl, SDS and $ZnSO_4$) at 1 mM and 10 mM final concentrations in the purified endo-β-1,4-mannanase activity was also evaluated. Additionally, a study was conducted on the application of the pure fraction and the crude enzyme in the hydrolysis of locust bean gum to analyze the release of mannose and mannan-oligosaccharides (MOS) using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)[8].

3 RESULTS & DISCUSSION

The fungus *Aspergillus niger* 1234 was cultured in a liquid medium using galactomannan (locust bean gum) as a carbon source. The kinetic production of endo-β-1,4-mannanase was monitored, with the highest production observed on the sixth day of cultivation, reaching 15.30 IU/mL. The crude enzyme was concentrated to 57.13 IU/mL and subsequently used in chromatographic steps to purify the endo-β-1,4-mannanase. The concentrate enzyme was subjected to gel filtration chromatography on Sephadex G-75 (Figure 1A). The profile exhibited two peaks of endo-β-1,4-mannanase activity (MAN1234A and MAN1234B). The fractions from the second peak of activity (MAN1234B) were pooled and applied to an ion exchange DEAE-Sephacel column (Figure 1B), resulting in a purified endo-β-1,4-mannanase as determined by SDS-PAGE (Figure 1C) with a molecular mass estimated as 45.6 kDa. The fraction MAN1234A was also applied to ion exchange chromatography, but the fractions with mannanases activity have high protein heterogeneity. In support to SDS-PAGE result, zymogram of MAN1234B also revealed one protein band coincident with that staining for endo-β-1,4-mannanase activity (Figure 1C). Zymography and SDS-PAGE analysis suggested that fractions MAN1234A and MAN1234B present endo-β-1,4-mannanase isoforms with very similar molecular masses. Mass spectrometry analysis of MAN1234A (after ion exchange) confirmed the homogeneity of the purified enzyme, identified by homology an endo-β-1,4-mannanase from *Aspergillus niger* (accession number A0A0N7AZ90), precisely defined the molecular mass as 41.2 kDa and estimate an isoelectric point of 4.6.

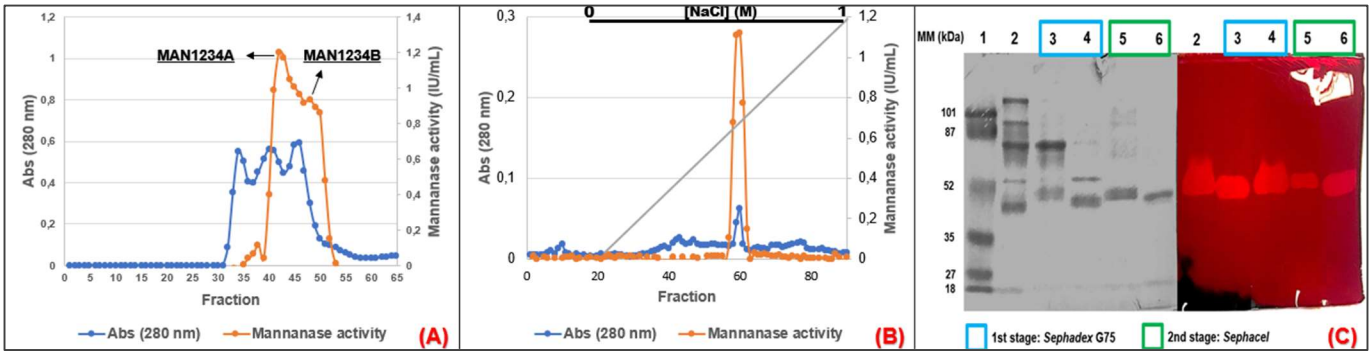

Figure 1 Elution profile on Sephadex G-75 (A) and DEAE-Sephacel (B) resins. Blue curve: absorbance of fractions at 280nm. Orange curve: enzymatic activity of the collected fractions. (C) SDS-PAGE electrophoresis (gel in gray) and zymogram (gel in red) of the purification steps: 1 -

protein standard, 2 - crude extract concentrate, 3 - MAN1234A (G-75), 4 - MAN1234B (G-75), 5 - MAN1234A (DEAE) and 6 - MAN1234B (DEAE). Blue square represents the first purification step and the green square the second purification step.

The physicochemical characterization tests allowed us to observe that *A. niger* 1234 produces an endo-β-1,4-mannanase (MAN1234B) with an optimum temperature and pH of 62°C and 3.9, respectively. The K_m value of the purified enzyme against locust bean gum as the substrate were 3.03 mg/mL. Interestingly, the purified enzyme exhibited a lower apparent K_m value than endo-β-1,4-mannanase from *Aspergillus foetidus*[9] and *Aspergillus terreus*[12]. Furthermore, it maintains enzymatic activity for 72 hours when incubated at 52°C and 62°C, which is a relevant property for its application. However, the activity rapidly dropped at 72 °C. It was not possible to evaluate influences on the activity of MAN1234B by metal ions and phenolic compounds, as they were found to interfere with the methodology for quantifying reducing sugars by DNS. Comparison of some physicochemical properties of MAN1234B produced by *Aspergillus niger* 1234 and endo-β-1,4-mannanases from other microorganisms of wild fungal strains of the genus *Aspergillus* are listed in Table 1. Such enzymes had optimal activity varying from 60–70ºC and pH values between 3.9 and 7.0. They also differed in their molecular weight and K_m values.

Table 1. Comparison of the main defined physicochemical characteristics of the endo-β-1,4-mannanase produced by the *Aspergillus niger* 1234 of the present study with the end-β-1,4-mannanases produced by fungi of the genus *Aspergillus* of some studies.

Microorganism	Optimal pH	Optimal temperature (ºC)	MW (kDa)	Km (mg/mL)	Reference
Aspergillus foetidus	4.0	60	30 - 45	3,29	9
Aspergillus niger	5.5	50	66	0.11	10
Aspergillus oryzae	5.0	60	34	2.70	11
Aspergillus terreus	7.0	70	49	5.90	12
Aspergillus niger 1234	3.9	62	41.2	3.03	This work

The ability to produce MOS with a degree of polymerization of up to 6 mannoses was demonstrated, using the pure enzyme and the crude enzyme after hydrolysis of locust bean gum and analysis of the oligosaccharides by thin-layer chromatography. The enzymatic hydrolysis of galactomannan, a polysaccharide present in locust bean gum (LBG), releases mannan-oligosaccharides (MOS) through the action of endo-β-1,4-mannanase. Furthermore, the synergistic action with accessory enzymes present in the fungal extract can release a large amount of mannose, the most abundant monosaccharide in the structure of LBG, which can be used in fermentative processes. The prebiotic effect of MOS has been reported, as it beneficially affects human health by selectively stimulating the growth of intestinal microflora such as *Bifidobacterium spp*. and *Lactobacillus spp*., while limiting the growth of pathogenic bacteria in the colon[13]. The results prove the potential of this enzyme in the animal feed, food, and pharmaceutical industries.

4 CONCLUSION

An endo-β-1,4-mannanase (MAN1234B) was isolated from the mannan-degrading system of *A. niger* 1234 using a combination of chromatographic procedures and confirmed by electrophoresis, zymography, and mass spectrometry. The purified endo-β-1,4-mannanase has a molecular mass of 41.2 kDa and an isoelectric point (PI) of 4.6. Maximum enzymatic activity was found at 62°C and pH 3.9. The enzymatic activity remained stable after 72 hours at 52°C and 62°C, but the enzyme was denatured at 72°C. The enzyme has a K_m of 3.03 mg/mL. Enzymatic hydrolysis tests revealed that both the crude enzyme and MAN1234B have the capacity to produce mannose and mannooligosaccharides (MOS) with a degree of polymerization (DP) up to 6. These characteristics enable applications in processes that require monosaccharides as raw materials, as well as in processes for MOS production, which are relevant in the animal, food, and pharmaceutical industries.

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