DISCOVERY AND CHARACTERIZATION OF LINEAR MANNAN SPECIALIZED ENZYMES: A STRATEGY FOR AÇAÍ SEEDS VALORIZATION

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ABSTRACT

Annually, açaí production generates around 1.6 million tons of seeds, primarily composed of linear mannan, presenting an opportunity to produce mannan-derived value-added products through mannanases-mediated hydrolysis. However, little is known about mannanases that exhibit high specificity to linear mannan. Therefore, this study aimed to discover and characterize specialized fungal enzymes targeting this substrate, using a palm species mannanase (*Eg*Man5A), which naturally hydrolyzes mannans when germinating, as guide. After conducting bioinformatics analyses, genes encoding the plant and two fungal mannanases (*Ja*Man5A and *Sl*Man5A) were cloned and expressed, and the enzymes were purified for characterization. The enzymes demonstrated optimal pH and temperature ranges between 4.5-5.5 and 60-75 °C, respectively, with all three enzymes proven thermostable. Among them, *Sl*Man5A was the most thermotolerant. In kinetic parameters assessments, *Sl*Man5A was the most efficient enzyme when hydrolysing ivory nut. Specificity analysis revealed that JaMan5A and *Sl*Man5A could only hydrolyze linear mannans. *Sl*Man5A stood out when hydrolyzing linear mannans, making it the most specific enzyme for the hydrolysis of açaí seeds. This study will contribute to the molecular and biochemical understanding of linear mannans enzymatic hydrolysis, paving the way for future sustainable utilization of açaí seeds.

Keywords: Açaí seeds. Linear mannan. Endo-1,4 β-mannanase. Enzyme discovery. Enzyme characterization.

1 INTRODUCTION

The increasing consumption of açaí pulp has driven its production, reaching 1.9 million tons in 2022 in Brazil[1]. As the edible portion of the açaí fruit comprises only 15% of its total mass, and the remaining 85% represents the seed[2], around 1.6 million tons of seeds are discarded annually, contributing to stream eutrophication and increased flood risks in producing cities[3]. Chemical characterization has revealed that 50% of their composition comprises mannan[2], specifically linear mannan. Considering the mannan content, the high availability, and the fact that these seeds accumulate in a restricted geographic area, açaí seeds present a significant opportunity to establish the production of mannan-derived inputs, such as the mannano- oligosaccharides (MOS) and mannose[2,4,5]. The sustainable production of MOS and mannose could be facilitated by enzymes, notably endo-1,4 β-mannanases. These endo-1,4 β-mannanases are categorized into glycosyl hydrolase (GH) families GH5, GH26, GH45, and GH113, according to the Carbohydrate-Active EnZymes (CAZy) database. However, the structural features of linear mannan make it resistant to enzymatic degradation[6] and limited research has explored the enzymatic hydrolysis of raw açaí seeds, with reported mannose yield of less than 5%[2] and around 11% MOS yield after 72 hours of hydrolysis[7]. One possible source for finding efficient mannanases for linear mannan hydrolysis could lie in the genome of some palm plant, as many Arecaceae members accumulate linear mannan as energetic storage, which is hydrolyzed by the plant mannanases on the event of germination. Thus, this study aimed to: (1) identify linear mannan specialized enzymes from fungi species using CUPP[8,9] –a clustering tool that annotates the family, subfamily, and EC function of CAZymes - using the sequence from *Elaeis guineensis* mannanase (a closely related sequence since the açaí genome was not accessible during the study) as guide; (2) clone, express, and purify the palm and the fungal prospected enzymes; and (3) biochemically characterize these enzymes.

2 MATERIAL & METHODS

a. Sequence analysis, cloning, expression, and purification of plant and fungal mannanases

The mannanase sequence from *E. guineensis* (oil palm) (*Eg*Man5A) was used to identify similar fungal mannanases. We conducted a blastp (protein-protein BLAST) search, focusing on fungi. We analyzed the top 250 resulting sequences with CUPP[8, 9]. Sequences into the same CUPP group as plant mannanases were further examined for sequence quality and organism pathogenicity. Two fungal GH5 endo-1,4 β-mannanase sequences, one from *Jaapia argillacea* (*Ja*Man5A) and the other from *Serpula lacrymans* (*Sl*Man5A), were chosen for further experiments.

Genes encoding *Eg*Man5A, *Ja*Man5A, and *Sl*Man5A were synthesized chemically, excluding their predicted signal peptides. These genes were cloned into the pPICZαA vector (Invitrogen, Cergy-Pontoise, France). Plasmids were used for the transformation of *P. pastoris* X-33 via electroporation. After expression, cultures supernatants underwent concentration and the concentrated supernatant was applied to a Ni2+ Sepharose resin (GE Healthcare, Chicago, IL, USA). Resulting proteins were concentrated and diafiltrated with a 10 kDa MWCO PES membrane (Sartorius, Goettingen, Germany). Protein purity was

assessed via SDS-PAGE gels with a 4-12% gradient and Western blotting, using the Trans-Blot Turbo Transfer System (Bio-Rad, Copenhagen, DK). Protein aliquots were prepared by adding 10% glycerol and stored at -80 °C.

b. Enzyme biochemical characterization

Mannanase activity was assessed using the p-hydroxybenzoic acid hydrazide (PAHBAH) method for reducing sugars and ivory nut mannan as substrate. All the results of enzyme characterization were assessed at initial rate. One unit of mannanase activity was defined as the amount of enzyme required to produce 1 µmol of reducing end carbohydrate per minute. The investigation of enzyme's optimal pH and temperature and was conducted at pH values of 3.5 to 6.5, alongside temperatures set at 55 to 85 °C. To evaluate thermostability, the purified enzymes underwent one-hour incubation at their respective optimal temperature, and 10 °C above and below the optimum. To explore the specificity of the purified enzymes and draw comparisons with GH26 endo-1,4-β-mannanase (*C. japonicus*) (Megazyme) and the commercial enzyme BGM "Amano" 10 (Amano Enzymes, Nagoya, Japan), different substrates were assessed, including: (1%) linear mannan (Ivory nut), low DP linear mannan (1,4-β-D-Mannan) (Megazyme), glucomannan (Konjac gum) (Megazyme), and galactomannan (locust bean gum) (Sigma-Aldrich). Hydrolysis assays were conducted with 1% substrate, 0.2 µM of purified enzyme in a 20 µL reaction mixture in eppendorf tubes under agitation. To ascertain the Michaelis-Menten kinetics of the purified enzymes, 2 to 0.1% concentrations of ivory nut mannan were employed. Additionally, açaí seeds were subjected to water-ethanol extraction to remove seed's polyphenols and 1 min ball milling to homogenise the substrate. Açaí seeds were hydrolysed using 1% of substrate and 1 µM of purified enzyme. BGM "Amano" 10 was used as a comparison.

3 RESULTS & DISCUSSION

The selected mannanases, *Eg*Man5A, *Ja*Man5A, and *Sl*Man5A were successfully cloned, expressed, and purified. The predicted molecular masses for *Eg*Man5A, *Ja*Man5A, and *Sl*Man5A were 41.5, 49.2, and 49.7 kDa, respectively. SDS-PAGE, EndoH treatment, and western blot analysis indicated that all three enzymes exhibited the expected mass. After purification, the impact of pH and temperature on enzyme activity was assayed. The substrate chosen for characterizing the mannanases was ivory nut due to its structural similarity to the açaí seed mannan. All enzymes proved to be thermostable during assayed time and showed optimal activity between 60-75 °C and at pH 4.5-5.5. Following the enzyme characterization, the enzyme rates at different concentrations of ivory nut mannan were assayed, plotted, and modelled using the Michaelis-Menten equation. The three catalytic sites of the enzymes became saturated between 10-20 mg/mL of substrate. Despite *Ja*Man5A and *Sl*Man5A displaying similar K_m for ivory nut linear mannan, SlMan5A demonstrated a nearly doubled catalytic constant (K_{cat}) when compared to *Ja*Man5A. This is reflected in the efficiency constant (K_{cat}/K_m), with *Sl*Man5A presenting twice the efficiency compared to *Ja*Man5A. *Sl*Man5A was the most efficient mannanase to catalyze ivory nut linear mannan.

The specific activity (U/mg of enzyme) of the expressed enzymes against various mannan substrates was tested and compared to two commercially available mannanases. The ivory nut linear mannan and low DP linear mannan were hydrolyzed by all enzymes. Interestingly, *Ja*Man5A and *Sl*Man5A did not exhibit hydrolytic activity towards galactomannan and glucomannan within the tested period, whereas the other three enzymes demonstrated catalytic activity on these substrates. The GH26 commercial mannanase showed the highest specific activity when hydrolyzing konjac gum and locust bean gum, but *Sl*Man5A surpassed GH26 mannanase when hydrolyzing the linear mannan substrates. Among the expressed enzymes, *Sl*Man5A proved to be the most efficient enzyme when hydrolyzing linear mannan, whereas *Eg*Man5A was the least efficient. The low efficiency of EgMan5 is probably due to it being a plant enzyme, which are normally "good enough" to maintain plant metabolism[10]. *Ja*Man5A and *Sl*Man5A proved to be specific enzymes for the hydrolysis of unsubstituted mannans, and this specificity could also represent a higher efficiency of those enzymes when hydrolysing the açaí seeds. Then, we decided to test the specific activity of these enzymes using *in nature* milled açaí seeds as substrate. The efficiency of enzymes to hydrolyze the açaí seeds followed the pattern of ivory nut mannan hydrolysis, and *Eg*Man5A was the less effective enzyme. *Ja*Man5A and the commercial enzyme provided similar specific activity, while *Sl*Man5A proved to be the most effective enzyme when hydrolyzing açaí seeds, achieving almost the double effect of the commercial enzyme. As far as we know, this is the first study that engaged to assess more specific enzymes for the hydrolysis of mannan from açaí seeds, which could contribute to the development of optimized enzymatic processing of those seeds.

4 CONCLUSION

A pipeline for fungal enzyme discovery using the germination process of a seed as inspiration was tested and validated through the cloning, expression, purification, and characterization of three uncharacterized mannanases (*Eg*Man5A, *Ja*Man5A, *Sl*Man5A). The enzymes showed robustness when tested different pHs and temperatures, with *Ja*Man5A and *Sl*Man5A, the fungal enzymes, proving to be stable, highly specialized, and efficient enzymes for the hydrolysis of linear mannan. Interestingly, *Ja*Man5A and *Sl*Man5A were unable to catalyze galactomannans and glucomannans, which will be further investigated. The specificity of expressed enzymes towards linear mannan was reflected in the hydrolysis of açaí seeds mannan, with *Sl*Man5A exhibiting twice the specific activity than the commercial mannanase used for comparison. This study will contribute to the molecular and biochemical understanding of enzymatic hydrolysis of linear mannans, providing basis for the upgraded use of açaí seeds, paving the way for unlocking the full potential of this biomass.

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ACKNOWLEDGEMENTS

The authors would like to acknowledge all the faculty, staff, and students from the Biocatalysis Laboratory (INT) and from the Section for Protein Chemistry and Enzyme Technology (DTU Bioengineering). The program Institutional Program for Internationalization - CAPES/PRINT - Call nº 41/2017, to the UFRJ, CAPES and the Serrapilheira Institute are thanked for providing for the sandwich doctorate and doctorate scholarships, research funding and financial aid (grant number Serra-1708-15009).