Biochemical properties of Recombinant Aspartyl Peptidase from *Rhizomucor miehei*

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ABSTRACT

Microbial enzymes, particularly fungal peptidases, have become crucial in various industrial applications such as food technology, pharmaceuticals, and bioenergy. Understanding these enzymes' biochemical properties is essential for optimizing their use and reducing costs. Filamentous fungi like *R. miehei* are especially promising due to their genetic variability and ability to produce enzymes under diverse conditions. The aim was to induce expression, purify the protein and conduct biochemical characterization assays of the recombinant aspartic peptidase (rAspPep-RmThe). Our findings revealed an aspartic peptidase with a molecular mass of approximately 39 kDa, exhibiting peak activity at pH 5.5-6.5 and 40°C, and maintaining stability above 70% across pH 3-7 and temperatures up to 55°C for 1 hour. The peptidase activity was reduced by certain metal ions (especially aluminum and copper (II) chloride) and by cationic surfactant (CTAB). On the other hand, it was minimally affected by SDS, Tween, Triton X-100, reducing agent (DTT), and chaotropic agents (guanidine hydrochloride and urea). Understanding the properties of rAspPep-Rm is essential, as these traits are critical for the biotechnological application of rAspPep-Rm, especially its notable role in cheese production through milk coagulation. This study concludes that rAspPep exhibits robust biochemical properties, making it suitable for applications.

Keywords: Aspartyl peptidase. Biotechnology. Cheese production. *Rhizomucor miehei*.

1 INTRODUCTION

Recently, the use of microbial enzymes in industrial applications has surged due to the discovery of novel enzymatic compounds with significant industrial potential. Among these, fungal peptidases have attracted considerable attention for their versatile applications in food technology, pharmaceuticals, and bioenergy production. Understanding the biochemical properties of these enzymes is essential for optimizing their operational parameters, enhancing their activity, and reducing costs. Furthermore, insights from enzyme stability studies and the identification of inhibitory agents are crucial for therapeutic development and industrial product design (Singh *et al.,* 2016).

The utilization of enzymes as biocatalysts in various industrial sectors is highly advantageous due to their rapid and efficient action, specificity, and low toxicity. Additionally, the environmental impact of enzymes is minimal because of the nature of the waste they generate. Microorganisms, especially filamentous fungi, offer significant potential for isolating enzymes for biotechnological applications due to their exposure to diverse environmental conditions. These fungi are excellent sources of industrial enzymes, attributed to their genetic variability and adaptability to adverse environments (Mesbah, 2022).

The recombinant methodology has emerged as a pivotal tool in enzyme characterization and application. By employing genetic engineering techniques, specific enzymes can be produced in host organisms, such as bacteria or yeast, under controlled conditions. This approach not only facilitates the production of large quantities of enzymes but also allows for the modification of enzyme properties to meet specific industrial requirements. Recombinant enzymes often exhibit improved stability, activity, and specificity, making them highly valuable for industrial applications. Moreover, the ability to produce enzymes recombinantly accelerates the discovery and optimization process, ultimately leading to more efficient and cost-effective industrial processes (Mattanovich *et al.,* 2012).

The filamentous fungus *R. miehei* is notable for its robust production of hydrolytic enzymes, particularly peptidases and lipases. In a recent study by Cozentino et al. (2020), the commercial lipase Lipozyme® RM IM derived from *R. miehei* was utilized in the acidolysis reaction of grape seed oil, showcasing its ability to synthesize triacylglycerol from oil fatty acids, potentially offering health benefits upon consumption. Additionally, emerging enzymes like aspartic peptidase, with meat tenderization potential, and α-amylase capable of producing high maltose levels from liquefied starch, have garnered attention for their prospective applications in the food industry (Nagarajan, 2023). Notably, genes encoding these enzymes were sourced from *R. miehei* CAU432, cloned, and expressed in *K. phaffii*. Understanding the characteristics of novel enzymes, including those from *R. miehei*, is crucial given their diverse potential applications. Moreover, the genome of *R. miehei* underscores its significance in producing industrially relevant enzymes. Using transcriptome data, this study selected a gene encoding aspartic peptidases for evaluation, emphasizing the importance of exploring the enzymatic capabilities of this fungus.

2 MATERIAL & METHODS

The pPICZαA vector was used to transform *K. phaffii* cells, containing the AOX1 promoter and Shble gene for Zeocin® resistance. The X-33 strain of *K. phaffii*, known for its efficient secretion system, was chosen for production. Colony PCR was employed for post-transformation screening of zeocin-resistant colonies, verifying the presence of the Aspartyl peptidase gene. Recombinant

clone 7 underwent production as per manufacturer instructions, starting with growth in BMGY medium at 30 °C for 48 hours, followed by transfer to BMMY medium with methanol induction every 24 hours. After 120 hours, supernatant was concentrated and diafiltered against a 50 mM MES pH 6.0 buffer using a FlexStand diafiltration system with a 10 kDa hollow fiber cartridge for compound removal and macromolecule concentration.

The purification process involved ion exchange chromatography using a Tricorn 5/50 column packed with SOURCE 15S cation exchange resin, using AKTA Purifier system. Equilibration and washing used the same dialysis buffer, while elution utilized a linear gradient of 50 mM MES buffer with 500 mM NaCl, pH 6. Eluted fractions were subjected to enzymatic activity assay with bovine serum albumin substrate and SDS-PAGE. Purified fractions were consolidated, concentrated, and dialyzed using a Vivaspin 20 system (5 kDa) with 10 mM MES buffer pH 6.

The biochemical properties of the enzyme rAspPep-Rm were investigated. The pH dependence was studied across a range of buffer solutions spanning from pH 3.0 to 9.0. Enzyme reactions were conducted at 35 °C with defined enzyme and substrate concentrations. The pH stability was assessed following a 1-hour incubation period at 25 °C. Optimal temperature was determined by performing assays at temperatures from 25 to 60 °C with 5 °C increments. Thermal stability was evaluated over temperatures ranging from 40 to 65 °C for exposure times of 10, 30, and 60 minutes. Residual activity was measured relative to the initial enzyme activity. Additionally, the impact of ions, surfactants, reducing agents, and chaotropic agents was examined by pre-incubating the enzyme with these compounds, followed by activity assays under optimal conditions.

3 RESULTS & DISCUSSION

Our results highlight the successful production, efficient purification, and biochemical characterization of rAspPep. Colony PCR confirmed the presence of the gene insert encoding aspartic peptidase (rAspPep) in *K. phaffii*, and gel electrophoresis of these clones revealed bands corresponding to the expected sizes. Purification using ion exchange chromatography showed rAspPep-Rm elution at 178 mM NaCl concentration, consistent with its protein profile. The expected 39 kDa molecular mass of rAspPep-Rm, determined *in silico* analysis, was also confirmed by SDS/PAGE.

The optimal pH range for rAspPep-Rm activity was found to be between 5.5 and 6.5, with MES buffer at pH 6 selected for subsequent assays. Stability studies revealed that residual activity exceeded 50% under all conditions, with a notable decrease in stability observed under alkaline conditions (pH 7.5 and above). These findings are consistent with previous studies on the optimal pH for aspartic proteases, which are typically active in acidic to neutral pH ranges (da Silva *et al.*, 2020). The apparent optimal temperature was estimated at 40°C, lower than that of aspartic peptidases from *Rhizopus microsporus* (da Silva *et al.*, 2020). This temperature was then used for subsequent experiments. Regarding thermal stability, the enzyme retained over 75% activity for up to 60 minutes at temperatures of 40, 45, 50, and 55°C. At temperatures of 60 and 65°C, residual activity remained above 50% for up to 30 minutes.

The impact of ions on rAspPep-Rm enzyme activity was examined by incubating the enzyme with 1 mM of various salts. Results show that salts including $MgSO_4$, $NiSO_4$, KCl, NaCl, $ZnSO_4$, $CoCl_2$, $CuCl_2$, and $AlCl_3$ led to decreased enzymatic activity, with reductions of up to 99.6% observed in the presence of $CuCl_2$ and $AlCl_3$. In contrast, $CaCl_2$, LiCl, $BaCl_2$, and $MnCl_2$ did not affect enzymatic activity significantly. These findings align with previous studies reporting varied effects of ions on aspartic peptidase activity (da Silva *et al.,* 2020) and on serine peptidase (da Silva *et al*. 2019). Despite no significant effect observed in this study, the positive influence of $CaCl_2$ on milk coagulation highlights its potential application in enzyme performance enhancement (da Silva *et al*., 2017).

Surfactant impact on rAspPep-Rm enzyme activity was assessed with concentrations ranging from 0.01% to 0.1% for each selected surfactant. Results revealed that 0.1% SDS reduced enzyme activity by approximately 20%. Conversely, CTAB exhibited a gradual effect, with residual activity of around 26% at 0.1% concentration. SDS, an anionic surfactant, and CTAB, a cationic one, may disrupt the enzyme's three-dimensional structure by interacting with charged amino acid residues. The enzyme's linear sequence contains 72 charged amino acids, comprising 19.1% of the total, possibly contributing to observed effects. Additionally, aspartic peptidases typically feature two negatively charged aspartic acid residues in their catalytic site. Non-ionic surfactants Triton X-100 and Tween 20 were also evaluated. While 0.01% Triton X-100 increased enzyme activity by approximately 20%, Tween 20 showed no significant effect. These findings suggest that surfactants, regardless of charge, may interact with different protein regions, affecting enzyme conformation and activity (Aguirre-Ramírez *et al.*, 2021).

The impact of reducing agents on rAspPep-Rm was assessed using varying concentrations (0.05-1 mM) of dithiothreitol (DTT). Results showed increased enzyme activity with DTT, suggesting a potential role in enhancing enzymatic hydrolysis. Notably, while the enzyme contains cysteine residues, the maintenance of its structural conformation may not rely heavily on disulfide bonds. This finding contrasts with Osuna-Ruiz *et al.* (2019), where DTT did not affect the activity of another aspartic enzyme, indicating variability in structural dependencies. The influence of chaotropic agents on rAspPep-Rm was examined by incubating the enzyme with urea or guanidine hydrochloride at concentrations ranging from 0.05 to 5 mM. Both agents demonstrated an activating effect, increasing enzyme activity by approximately 20%. This enhancement could be attributed to alterations in the solvation of water molecules surrounding the protein structure, facilitating substrate access to the enzymatic active site. However, it is important to note that higher concentrations of these agents may lead to protein denaturation, highlighting the delicate balance required for enzymatic activity modulation.

4 CONCLUSION

This study focused on purifying and characterizing a recombinant aspartic peptidase (rAspPep-Rm). Aspartic peptidase is crucial for protein processing and has diverse applications, including cheese production through milk coagulation. The enzyme demonstrated strong pH and thermal stability, with its catalytic activity influenced by various metal ions and the cationic surfactant CTAB. Additionally, rAspPep showed low sensitivity to chaotropic agents, surfactants, and DTT under the study's conditions. These biochemical traits support the use of rAspPep in biotechnological processes, facilitating product development and technological advancements.

REFERENCES

1 SINGH, R., KUMAR, M., MITTAL, A., MEHTA, P. K. 2016. Microbial enzymes: industrial progress in 21st century. 3 Biotec., v. 6, p. 174.

2 MESBAH, N. M. 2022. Industrial Biotechnology Based on Enzymes from Extreme Environments. Front. Bioeng. Biotechnol., v. 10, p. 2.

3 COZENTINO, I. S. C, RODRIGUES, M. F., MAZZIERO, V.T., CERRI, M. O., CAVALLINI, D. C. U., DE PAULA AV. 2022. Enzymatic synthesis of structured lipids from grape seed (*Vitis vinifera* L.) oil in associated packed bed reactors. Biotechnol Appl Biochem., v. 69, p.101-109.

4 NAGARAJAN E. R. 2023. Proteases: An overview on its recent industrial developments and current scenario in the revolution of biocatalysis. Materials Today: Proceedings, v. 2, p. 565-573.

5 DA SILVA, R. R., SOUTO, T. B., GONSALES DA ROSA, N., DE OLIVEIRA, L. C. G., JULIANO, M. A., JULIANO, L., ROSA, J. C., CABRAL, H. 2020. Evaluation of the milk clotting properties of an aspartic peptidase secreted by *Rhizopus microsporus*. Prep Biochem Biotechnol., v. 50, p.226-233.

6 DA SILVA, R. R., DA ROSA, N. G., DE OLIVEIRA, L. C. G., JULIANO, M. A., JULIANO, L., ROSA, J.C., CABRAL, H. 2019. Biochemical Properties and Catalytic Specificity of a Novel Neutral Serine Peptidase Secreted by Fungus *Pyrenochaetopsis* sp. Appl Biochem Biotechnol., v. 187, p.1158-1172.

7 DA SILVA, R. R., DE OLIVEIRA, L. C., JULIANO, M. A., JULIANO, L., DE OLIVEIRA, A. H., ROSA, J. C., CABRAL, H. Biochemical and milk-clotting properties and mapping of catalytic subsites of an extracellular aspartic peptidase from basidiomycete fungus *Phanerochaete chrysosporium*. Food Chem, v.15, n. 225, p.45-54.

8 AGUIRRE-RAMÍREZ, M., SILVA-JIMÉNEZ, H., BANAT, I. M., DÍAZ DE RIENZO, M. A. 2021. Surfactants: physicochemical interactions with biological macromolecules. Biotechnol Lett., v. 43, p. 523-535.

9 OSUNA-RUIZ, I., ESPINOZA-MARROQUIN, M. F., SALAZAR-LEYVA, J. A., PEÑA, E., ÁLVAREZ-GONZÁLEZ, C.A., BAÑUELOS-VARGAS, I., MARTÍNEZ-MONTAÑO, E. 2019. Biochemical characterization of a semi-purified aspartic protease from sea catfish *Bagre panamensis* with milk-clotting activity. Food Sci Biotechnol., v. 28, p. 1785-1793.

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