"Subsite Mapping and Biotechnological Applications of Recombinant Aspartyl Peptidase"

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ABSTRACT

The increasing utilization of enzymes as biocatalysts in various industrial sectors is driven by their operational benefits and essential role in fostering a sustainable economy. Enzymes offer advantages like enhanced selectivity, specificity, reduced operational costs, and minimal toxicity, thereby lowering environmental impact. The catalytic site of an enzyme, particularly in peptidases, is divided into subsites, each interacting with specific substrate residues, influencing enzyme specificity and efficiency. Subsite mapping is crucial for understanding these interactions and is valuable for drug design, enzyme engineering, and the development of substrate-specific inhibitors. Filamentous fungi, notably *Rhizomucor miehei*, are promising sources of industrial enzymes due to their ability to grow in low-cost media and produce extracellular enzymes. Recombinant production of these enzymes ensures high purity and yield, facilitating detailed studies and industrial applications. This study cloned and expressed an aspartic peptidase from *R. miehei*, followed by purification and kinetic analysis. Results indicated that amino acid properties significantly impact the enzyme's catalytic efficiency and affinity, providing insights for future enzyme modifications and inhibitor design. The comprehensive subsite mapping underscores the intricate nature of enzyme-substrate interactions, crucial for advancing biocatalytic applications.

Keywords: Catalytic specificity. Aspartyl peptidase. *Rhizomucor miehei*. FRET.

1 INTRODUCTION

The use of enzymes as biocatalysts in various industrial sectors is growing annually due to their operational advantages and their crucial role in promoting a green and sustainable economy. Enzymes offer benefits such as selectivity and specificity, reduced operational costs, and lower toxicity, resulting in a reduced environmental impact. However, there is still a market demand for enzymes that can function under the adverse conditions typical of industrial processes, such as variations in pH and temperature (Aruna *et al.,* 2023; Mesbah, 2022; Gurumallesh *et al*., 2019).

The catalytic site of an enzyme has dual roles: binding the substrate and catalyzing reactions. This efficiency dictates the enzyme's activity towards specific substrates and its overall specificity. Peptidases have catalytic sites divided into subsites, each accommodating an amino acid residue of the substrate. These subsites are numbered based on the substrate's amino acid residue, and the substrate itself is called labeled according to cleavage sites. Furthermore, the subunits of the catalytic site vary in their ability to bind amino acid residues, with some showing specific preferences. This allows peptidases to distinguish between cleavage sites, enhancing specificity. Peptidase specificity relies on substrate recognition at the catalytic site, making subsite mapping essential for characterizing enzyme preferences. This information is invaluable for various applications, including drug design, enzyme engineering, understanding physiological processes, and the development of substrate-specific inhibitors (Hamin Neto, 2018).

Microorganisms offer substantial potential for sourcing enzymes for biotechnological uses since they grow in diverse environmental conditions. Among microorganisms, filamentous fungi have industrial potential, as they grow in media containing low-cost agricultural by-products, have easy enzymatic extraction, secrete enzymes extracellularly, and their genetic variability allows adaptation to adverse conditions. The secretion of active enzymes by these fungi results in enzyme extracts with minimal impurities, which fuels research into scaling up fermentation processes, improving enzyme purification, and enhancing strains, thereby boosting their use in industrial applications. This process is even more efficient when the enzyme is recombinant. A recombinant enzyme is crucial for ensuring high purity, consistency, and yield, which are essential for detailed studies of its catalytic specificity. This approach allows for precise genetic modifications to enhance or alter enzyme properties. Additionally, it facilitates large-scale production, making it viable for industrial and research applications (Song *et al*., 2023; Mesbah, 2022; Meyer *et al*., 2020).

The filamentous fungus *Rhizomucor miehei* is well-known for its abundant production of hydrolytic enzymes, notably peptidases and lipases. Recent commercial use of Lipozyme® RM IM, a lipase derived from *R. miehei*, demonstrates its effectiveness in the acidolysis reaction of grape seed oil (Cozentina, 2020). Within the enzymes produced by this fungus, the aspartic peptidase is important for various sectors of the industry, including cheese production, casein hydrolysates, peptide synthesis, and the reduction of protein turbidity in fruit juice. In cheese production, microbial aspartic peptidases can replace chymosin derived from newborn ruminants. Ethical concerns and production issues associated with extracting chymosin from ruminants make microbial enzymes a cost-effective and faster alternative. Moreover, the genome of *R. miehei* underscores its significance in producing industrially relevant enzymes (Wang *et al*., 2021; Sun *et al*., 2018). Thus, genes encoding aspartic peptidase were sourced from *R. miehei* CAU432, cloned, and expressed for further evaluation, highlighting the importance of exploring the enzymatic capabilities of this fungus.

2 MATERIAL & METHODS

The pPICZαA vector was used to transform K*omagataella 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.

To determine the kinetic parameters (K_M, K_{cat}, and K_{cat}/K_M) of the enzyme rAspPep-Rm, fluorescent substrates employing fluorescence resonance energy transfer (FRET) were utilized. These substrates were modified at positions P1 (Abz-KL**X**SSKQ-EDDnp), P2 (Abz-K**X**RSSKQ-EDDnp), P3 (Abz-**X**LRSSKQ-EDDnp), P’1 (Abz-KLR**X**SKQ-EDDnp), P’2 (Abz-KLRS**X**KQ-EDDnp), and P’3 (Abz-KLRSS**X**Q-EDDnp), with X representing variations including acidic residues (Asp and Glu), basic residues (His, Lys, and Arg), neutral residues (Asn, Gln, and Tyr), or hydrophobic residues (Ala, Ile, Leu, Val, Gly, Pro, Phe, and Trp). The enzyme (rAspPep-Rm, concentration 43.4 nM) was incubated with various substrate concentrations under optimal pH and temperature conditions. Fluorescence measurements were performed using a Fluoroskan spectrophotometer (Thermo Scientific), with excitation wavelength (λ_{ex}) at 355 nm and emission wavelength (λ_{em}) at 460 nm. Kinetic parameters were determined from the collected data using GraphPad Prism 7 software (V_{max} and K_M) or were calculated (K_{cat} and K_{cat}/K_M).

3 RESULTS & DISCUSSION

The results demonstrate that the specificity of the catalytic site in the rAspPep-Rm varies significantly with the nature of the amino acids present in the substrate. The highest catalytic efficiency was observed with glycine at the P1 position, followed by lysine and proline. Glycine, being small and flexible, likely enhances catalytic efficiency by easily accommodating itself within the catalytic site. Proline also showed better catalytic efficiency and greater affinity, suggesting that its cyclic structure leads to favorable conformational interactions in the active site. In contrast, valine and leucine displayed low catalytic efficiency and affinity, likely due to their larger isopropyl side chains, which can cause steric hindrances and reduce compatibility with the catalytic site's microenvironment. This preference for proline at the S1 position was similarly observed in the serine protease from *Aspergillus fumigatus* (Da Silva et al., 2014).

For the modifications at P2 position, the highest catalytic efficiency was observed with the amino acid glutamine, followed by glycine and serine. Glutamine not only demonstrated superior catalytic efficiency but also the highest affinity, indicating that the presence of a lateral amide group favors both catalysis and binding. Glycine once again proved preferable for enzyme hydrolysis, while serine is similar to glutamine, polar and uncharged. Valine, on the other hand, was again unfavorable, possibly due to its size and hydrophobic characteristics that do not align well with the active site properties. This lower preference for valine was also noted in the aspartyl protease from *Phanerochaete chrysosporium* (Da Silva et al., 2017).

Considering substrates with modifications at P3 position, threonine and phenylalanine exhibited the highest catalytic efficiency and affinity. Threonine's hydroxyl group and polar nature, along with phenylalanine's phenyl group, likely favored interactions with the catalytic site. Conversely, methionine, glutamate, and tyrosine showed lower affinity, which may be related to their size, polarity, and potential for less favorable interactions within the catalytic site.

Modifications at the P’1 position revealed that glutamine again had the highest catalytic efficiency, followed by tyrosine and glycine. However, bulky amino acids like phenylalanine and tryptophan exhibited low affinity, likely due to steric hindrance. In the P’2 position, the best hydrolysis was achieved with leucine and lysine. Leucine's hydrophobic side chain and lysine's basic chain with an amine group likely promote specific interactions within the active site, enhancing catalytic efficiency and affinity. Tyrosine’s low affinity suggests that the S’2 site may not favor interactions with aromatic groups.

Finally, the highest catalytic efficiency for substrates with modifications at the P’3 position was observed with lysine, followed by alanine and aspartate. Interestingly, leucine showed the highest affinity but lower catalytic efficiency, indicating that its intense interaction with the active site might hinder hydrolysis. This comprehensive mutational analysis and biochemical characterization provide valuable insights into the structural preferences and functional dynamics of the fungal aspartyl protease catalytic site.

4 CONCLUSION

This study highlights the importance of individual amino acid characteristics in the catalytic efficiency and affinity of an aspartyl protease, enhancing our understanding of enzyme-substrate interactions. The findings can guide future enzyme modifications and the design of specific inhibitors. Mapping the catalytic site reveals the complexity of the interaction between the protease and its substrates. Amino acids with different sizes, polarities, and abilities to form specific interactions significantly influence catalytic efficiency and affinity. These properties must be considered in the design of inhibitors or modified substrates for protein engineering studies and drug development.

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