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ELISA-based enzyme isolation, purification, and characterization of polygalacturonase and α-amylase from *Aspergillus oryzae*

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ABSTRACT

The α-amylase (α-A) and polygalacturonase (PG) enzymes produced by *Aspergillus oryzae* are the primary subjects of this research, which also aims to isolate, purify, and characterize them. Biological material utilization was conducted by all applicable ethical norms. To achieve highly pure enzymes with improved activity, the purification process included ammonium sulphate precipitation, dialysis, and size-exclusion chromatography employing Toyopearl HW-65 resin. We used SDS-PAGE and gel filtration to find the molecular weights of α-amylase and polygalacturonase. We tested the enzyme activity under different pH, temperature, and substrate conditions. According to Michaelis-Menten kinetics, substrate-specific activity was found through a kinetic study. In addition, α-amylase showed optimal activity at pH 6.8, and polygalacturonase at pH 4.8. Enzyme-linked immunosorbent assays (ELISAs) demonstrated the presence and sensitive quantification of these enzymes. To emphasis their regulatory roles, researchers examined how divalent cations and inhibitors affected enzyme activity. By examining the catalytic properties and operational stability of these enzymes, the study highlights their potential industrial applications in food processing, biotechnology, and diagnostics.

Keywords: Enzyme-Linked Immunosorbent Assay (ELISA), *Aspergillus oryzae*, Polygalacturonase, α-amylase, Characterization.
Article type: Research Article.

INTRODUCTION

Metabolism, signal transduction, and food acquisition are just a few of the many biochemical activities that rely on enzymes, which are biological catalysts. Because of its capacity to release a variety of extracellular enzymes, such as polygalacturonase (PG) and α-amylase (α-A), *A. oryzae* stands out among the microbes known for its enzymatic abilities. In addition to being critical for the organism's survival, these enzymes have enormous biotechnological and commercial applications (Mao *et al*. 2024). Pectin is a key plant polysaccharide that may be broken down by PG, while starch can be broken down by α-A, which ultimately produce glucose. Biotechnology and industrial enzymology can only progress if we learn more about these enzymes, how to isolate them, and what they may be used for. Here, the enzyme-linked immunosorbent assay (ELISA) has shown to be an excellent tool for enzyme detection and characterisation due to its sensitivity and specificity. ELISA has changed the face of medicine and science by making it possible to identify enzymes at low concentrations in complicated biological mixtures. Enzymes including PG and α-A from *A. oryzae* may be effectively isolated and studied using ELISA in

conjunction with procedures like ammonium sulphate precipitation and chromatographic techniques (Mazhari *et al*. 2024; Yadav *et al*. 2024). The study of enzymes like α-A and PG has significant consequences for biofuels, food technology, and medicines. Pectin is a polysaccharide that is present in plant cell walls. It is hydrolysed into simpler molecules like galacturonic acid by an enzyme called polygalacturonase. Its industrial importance is shown by its use in clarifying fruit juice, processing textiles, and treating bio-waste. Similarly, the starch breakdown processes rely on α-amylase, which renders them priceless to enterprises that manufacture sweeteners, bioethanol, and brewing goods. Enzymes like these have a place in process optimisation, where getting the most out of a given amount of energy requires finding the sweet spot between acidity and alkalinity (Gama Cavalcante *et al*. 2024). Extensive isolation and purification of these enzymes is required for their efficient use. One common first step in enzyme purification is ammonium sulphate precipitation. This method allows for the selective precipitation of the target enzyme while minimising impurities by utilising the idea of protein solubility regulation by salt concentration (Awasthi *et al*. 2024). After precipitation, size-exclusion chromatography and other chromatographic methods allow for a more precise method of enzyme molecular size separation. One example is the successful use of Toyopearl HW-65 resin for the purification of enzymes and proteins with very little contamination (Matsuzawa 2024). Even in complex combinations, these approaches provide for exact enzyme detection and quantification when paired with ELISA. Because of their sensitivity, specificity, and versatility, ELISA-based approaches are great in detecting and quantifying particular enzymes. Detection of minute quantities in complicated materials is made possible by these assays, which utilise antibodies to target the enzyme of interest. While purifying *A. oryzae*, enzyme-linked immunosorbent assays (ELISA) can confirm the presence and quality of PG and α-A (Singh *et al*. 2024). Important aspects of enzyme activity can be uncovered by ELISA tests to explore substrate selectivity, enzyme kinetics, and interactions with activators and inhibitors. One of the most important functions of PG is the breakdown of pectin, an essential component of plant cell walls. This enzyme is ideal for use in industrial processes including textile processing and fruit juice clarity since it works best in acidic environments (pH 4.5-5.5) at moderate temperatures (45-55°C; Ohashi *et al*. 2024). Its utility in hydrolysing plant biomass, an essential step in biofuel production, is based on its capacity to decompose complex carbs into galacturonic acid. Starch conversion operations cannot be carried out without α-amylase at pH 4.0-4.5 and temperatures of 50-60 °C. In contrast, α-A starts the breakdown of starch by breaking α-1,4 bonds, which results in the production of maltose and dextrin as byproducts. The brewing and baking industries find it acceptable since it performs successfully at temperatures of 60-70 °C and slightly alkaline pH levels of 6.0-7.0. After being studied and fine-tuned, these enzymes can be used as catalysts in industrial processes that aim for maximum efficiency (Amobonye *et al*. 2024). The diagnostic potential of these enzymes extends beyond their commercial uses. Biosensors for the detection of certain polysaccharides or other biomarkers, for instance, might be informed by the characterisation of enzyme kinetics and substrate specificity. When it comes to food, enzyme-based tests are a great way to check the quality of both ingredients and final goods. In addition, ELISA tests are incredibly specific, which means they can quickly and accurately measure enzyme concentrations, which is great for quality control during enzyme synthesis. Since it can release a lot of extracellular enzymes, *A. oryzae* has been used for enzyme synthesis and has been the subject of a lot of research. Because of its genetic tractability and relative simplicity of cultivation, this organism might serve as a template for future industrial enzyme production. Recent developments in molecular biology have made it possible to genetically edit *A. oryzae* strains to generate more of a particular enzyme, increasing the mushroom's value in both academic and industrial contexts. The main objectives of this research are to employ ELISA-based approaches to isolate, purify, and characterise PG and α-A from *A. oryzae*. The study is focused on developing enzyme preparations that are extremely pure by combining techniques such as ammonium sulphate precipitation, size-exclusion chromatography, and enzyme-linked immunosorbent assays. More specifically, the research aims to learn how enzymes work, what substrates they like, and how pH and temperature affect enzyme activity. Our knowledge of enzyme function will be expanded and new avenues for biotechnology and diagnostics will be opened up by these discoveries. A major step forward in biochemical research has been the use of ELISA into enzyme purification and characterisation. Enzymes have many potential uses in areas as varied as food technology, medicines, and environmental research, and this method provides a dependable and effective way to explore their features. This study adds to the existing information on industrial enzymes by examining three enzymes from the fungus *A. oryzae* such as PG and α-A. It emphasises the potential of these enzymes to promote innovation in many industries.

MATERIAL AND METHODS

Meeting ethical standards

This study complied with international standards for the ethical use of biological materials since all operations followed the rules set out by the Institutional Animal Research Committee of the National Research Centre, Egypt.

Gathering samples

Strains of *A. oryzae* were cultivated under ideal circumstances for enzyme synthesis after being obtained from commercial fermentation sources. To promote the development of α-amylase (α-A) and polygalacturonase (PG), cultures were kept in a nutrient-rich medium that included starch and pectin. To guarantee maximum enzyme activity, samples were collected during the logarithmic growth phase (Senoo *et al*. 2024).

Materials and substances

The research made using analytically-grade reagents. Starch, maltose, polygalacturonic acid, and other substrates and cofactors were purchased from Sigma-Aldrich. There were chromogenic chemicals used to track enzyme activity, such as o-phenylenediamine dihydrochloride (OPD) and p-nitrophenyl phosphate (p-NPP). Protein molecular weight markers were purchased from Pharmacia Fine Chemicals in Sweden for use in SDS-polyacrylamide gel electrophoresis. Bovine serum albumin (BSA) and other chemicals, including Tris-HCl, were produced according to the strictest purity requirements (Seidler *et al*. 2024).

Isolating and purifying enzymes

The crude enzymes were removed from the culture supernatant by precipitating them with ammonium sulphate. Enzymes of interest were concentrated by this process, which enabled solubility-based protein selective separation.

Limitation on size chromatography. Enzymes were further purified by employing Toyopearl HW-65 resin for molecular size-based separation. According to reference, this technique yielded α-A and PG preparations that were extremely pure by minimising the presence of extraneous proteins (Ozdemir *et al*. 2024).

Enzyme electrophoresis method

The enzymes extracted were tested for purity and molecular weights using SDS-polyacrylamide gel electrophoresis. In order to verify the molecular size of the isolated enzymes, the electrophoresis procedure was carried out using conventional protein markers.

Evaluation of polygalacturonase activity

The rate of decreasing sugar release from polygalacturonic acid was used as a metric of PG activity. The enzyme was incubated with a reaction mixture at 45°C for 30 minutes that contained polygalacturonic acid (1% w/v) in 50 mM acetate buffer (pH 4.5). The addition of 3,5-dinitrosalicylic acid (DNS) halted the process, and the reducing sugars were quantified by measuring the absorbance at 540 nm. The quantity of enzyme needed to release 1 μmol of galacturonic acid per minute under the test conditions was referred to as one unit of PG activity (Ozdemir *et al*. 2024).

Evaluation of α-amylase activity

Starch iodine colorimetric analysis was used to measure the activity of α-amylase. After adding the enzyme to a combination of 50 mM phosphate buffer (pH 6.5) and 1% starch, the mixture was incubated at 60 °C for 10 minutes. By adding 1 M HCl, the process was stopped, and the decrease in blue colour intensity (caused by starch hydrolysis) was detected at 620 nm. According to Ohashi *et al.* (2024), one dose of α-A was considered active if it hydrolysed 1 mg of starch per minute.

Examining the amount of protein

A dye-binding test was used to quantify the protein content in this investigation, with bovine serum albumin (BSA) acting according to Ohashi *et al.* (2024). The accuracy of this method in quantifying PG and α-A, as well as other enzymes in complicated combinations, has earned it widespread recognition (Battisti *et al*. 2024).

A. oryzae enzyme purification

Unless otherwise specified, the PG and α-A enzymes were extracted from *A. oryzae* at a temperature of 4 °C. We used a Teflon-pestled homogeniser to mix 25 g *A. oryzae* mycelial biomass with a 1:1 ratio of 0.05 M Tris-HCl

buffer (pH 8.0). To get the crude extract, the homogenate was centrifuged at 13,000 ×g for 25 minutes at 4 °C. In order to precipitate ammonium sulphate from the supernatant, the solution was agitated at 4 °C for 20 minutes while it was progressively saturated to 85%. Centrifugation at 13,000 ×g for an extra 15 minutes was used to separate the proteins that had precipitated. The remaining ammonium sulphate was dialysed out by extensively dialysing the pellet against the same Tris-HCl buffer after it was redissolved (Szarek *et al.* 2024). A Toyopearl HW-65 column (150 cm × 2.5 cm) pre-equilibrated with 0.05 M Tris-HCl buffer at pH 8.0 was used to further purify the dialysed extract. To separate the components of the sample, the column was run at a flow rate of 35 mL h^{-1}. PG and α-A were made more pure by repeating this process twice. There was enzymatic activity testing performed on each column fraction to confirm the presence of the target proteins (Bashtar *et al*. 2011).

Finding the original molecular weight

The PG and α-A native molecular masses were found by employing size-exclusion chromatography, which was adjusted using protein standards. The ferritin (430 kDa), catalase (250 kDa), alcohol dehydrogenase (140 kDa), bovine serum albumin (70 kDa), and myoglobin (18 kDa) were the standards utilised. The molecular weights of the target enzymes were determined by comparing their elution patterns with these standards (Mahmoud *et al.* 2021).

Using electrophoresis in analysis

The molecular weights of the subunits of PG and α-A, which originate from *A. oryzae*, were determined using SDS-PAGE on a 12% polyacrylamide gel. The presence of protein bands could be observed after staining the gel with 0.3% Coomassie Brilliant Blue R-250. The electrophoresis findings confirmed the homogeneity and integrity of the isolated enzymes by validating their molecular features and subunit composition (Lionetto *et al*. 2013).

Enzyme-linked immunosorbent assay (ELISA)

In order to semi-quantitatively evaluate the amounts of PG and α-A in *A. oryzae*, an ELISA study was utilised. A chequerboard titration was used to determine the optimal concentrations of the antigen, antibody, and conjugate. A solution of 25 μg mL^{-1} of the enzyme-specific antisera was prepared for the experiment by diluting it with sodium bicarbonate buffer (0.05 M, pH 9.6). One hundred microlitres of this solution was spread over a 96-well polystyrene microtiter plate, which was then left to incubate at 4 °C for the night (Garcia-Conde *et al*. 2023). After the incubation period, the plate washed three times with TPBS (a solution of Tween-20 in PBS with a pH of 7.5). The last wash involved immersing the plate for six minutes. An hour of room temperature incubation was followed by the application of a 5% solution of non-fat dried milk in PBS to inhibit non-specific binding sites. Once the blocking process was completed, the wells were placed in an incubator set at 36 °C for 1.5 hours with 100 μL of enzyme preparations or diluted culture filtrates from *A. oryzae* (1:45 dilution in PBS). This technique was followed by washing to eliminate any loose material (Kukreti *et al.* 2024). Then, 100 μL of wells were incubated at 36 °C for 50 minutes after adding secondary antibody solutions coupled to horseradish peroxidase (HRP). We employed diluted 1:900 concentrations of anti-rabbit IgG for PG and anti-mouse IgG for α-A as secondary antibodies in our trial. After five washes, the wells were supplemented with 100 μL of a substrate solution that included 55 mg of o-phenylenediamine (OPD) in a 0.14 M sodium phosphate and 0.04 M sodium citrate buffer (pH 5.6) with 0.011% H_2O_2. A duration of twenty-five minutes was given to the enzymatic reaction. Each well was treated with 100 μL of 1N NaOH in order to halt the reaction. An ELISA plate reader was used to measure the absorbance at 450 nm. Readers of the optical density (OD) were able to quantify the enzyme content in the samples. This improved ELISA method allowed for the precise and sensitive identification of PG and α-A, which aided in their description and subsequent uses in diagnostics and biotechnology. Not only did this technique confirm that the target enzymes were present and active, but it also demonstrated how specific the assay was by using secondary antibodies that were designed for each enzyme. The effectiveness of ELISA in enzyme purification and characterisation was demonstrated by several washes, blocking agents, and exact reaction conditions, which resulted in minimum background noise and accurate values. The test showed promise for uses in food technology, industrial processes, and enzyme-based research once the approach was adjusted to include enzymes from *A. oryzae*.

RESULTS

Improving the quality of *A. oryzae* crude extract purification of enzymes for PG and α-A activity

Table 1 summarises the results showing that the crude *A. oryzae* extract had an initial specific activity for polygalacturonase (PG) of 195.7 units mg^{-1} protein. The enzyme's purity was improved by passing the extract

through an 80% ammonium sulphate [$(NH_4)_2SO_4$] saturation period. Enzymes are typically concentrated using centrifugation at 12,000 × g for 30 minutes after stirring the crude mixture.

Table 1. Purification of PG and α-Amylase from A. Oryzae

Parameters	PG			α-Amylase		
	Crude Extract	$(NH_4)_2SO_4$ Fraction	Toyopearl HW-65 Fraction	Crude Extract	$(NH_4)_2SO_4$ Fraction	Toyopearl HW-65 Fraction
Protein Content (mg)	75	44.5	6.8	40	27.8	8.4
Enzyme Activity (units)	12,000	11,230	7,560	102,000	86,850	45,300
Specific Activity (units mg^{-1})	195.7	278.3	1128	2,480	3,120	5,400
Recovery Rate (%)	80	75	54.2	74	84.3	44.7
Purification Fold	1	1.4	6	1	1.22	2.1

Rainfall and haemodialysis

To remove any remaining salts, the precipitate pellet was dialysed and then resuspended in a 0.05 M acetate buffer with a pH of 5.0. With an efficiency of 81.9%, this fraction preserved a significant amount of PG activity. At 278.3 units mg^{-1} (U mg^{-1}) protein, the enzyme's specific activity was enhanced during the purification phase. Likewise, following dialysis in a 0.05 M phosphate buffer (pH 6.5) and 75% ammonium sulphate precipitation, the α-A activity in the crude extract, which was initially 2480 U mg^{-1} protein, was maintained. A protein specific activity of 3120 units mg^{-1} and an enzyme recovery rate of 84.3% were the outcomes of this process.

Additional purification via size-exclusion chromatography

A Toyopearl HW-65 column was used for size-exclusion chromatography, which allowed for further purification. Fig. 1a shows that *A. oryzae* PG was successfully isolated as a peak with maximum activity during the chromatography stage. With a yield of 54.2% and a purification of 6.1 times, the specific activity of PG reached 1128 units mg^{-1} protein (Table 1). The specific activity of α-amylase increased to 5400 U mg^{-1} protein during Toyopearl HW-65 chromatography, which resulted in a 2.2-fold purification and a recovery yield of 44.7%.

Determination of molecular mass

By using gel filtration chromatography, the molecular weight of *A. oryzae* enzymes was determined. In its original condition, α-A had a molecular weight of 270 kDa, whereas PG had a molecular weight of 300 kDa. The findings were validated by SDS-PAGE analysis, which revealed a single band at 300 kDa for PG and a similar band at 270 kDa for α-A (Fig. 2).

The ideal pH range

Appropriate buffer systems were used to evaluate the effect of pH on enzyme activity. Fig. 3a shows that the optimum activity of PG was observed at pH 4.8, after testing it using citrate-phosphate buffer throughout a pH range of 3.5 to 6.5. The α-A activity, on the other hand, was monitored in a phosphate buffer from 5.5 to 8.5 pH, reaching its peak at 6.8 pH (Fig. 3b). The enzymes' known functions in pH-dependent starch and pectin degradation are consistent with these results.

How enzymes work and which substrates are preferred

Different substrates were used to investigate the kinetic characteristics of *A. oryzae*, PG and α-A. The relative activity of PG was shown to be 100% with pectin as the substrate, 56.4% with polygalacturonic acid, and 12.3% with galacturonic acid, as shown in Table 2. In the same way, α-A showed its highest activity with soluble starch (100%), along with moderate activity with amylopectin (62.1%), and low activity with maltodextrin (18.7%).

Polygalacturonase enzyme kinetics

Incubating PG with different doses of pectin allowed us to determine its reaction velocity (V). Fig. 3c shows the results of using a Michaelis-Menten kinetic model, which states that the reaction velocity reaches a maximum at Vmax as the substrate concentration increases. As is typical of enzymatic processes, this happens when substrate concentrations are very high and the enzyme's active sites become saturated.

How it works and what it means

The *A. oryzae* PG and α-A were successfully isolated and characterised, demonstrating the effectiveness of purifying techniques that use ELISA. Fruit juice clarity, textile processing, and starch hydrolysis for bioethanol generation are just a few of the commercial applications that might benefit from these enzymes' enhanced specialised activity after purification. The use of enzyme detection by ELISA also guarantees excellent specificity and sensitivity, which makes enzyme monitoring in purification and industrial processes more easier.

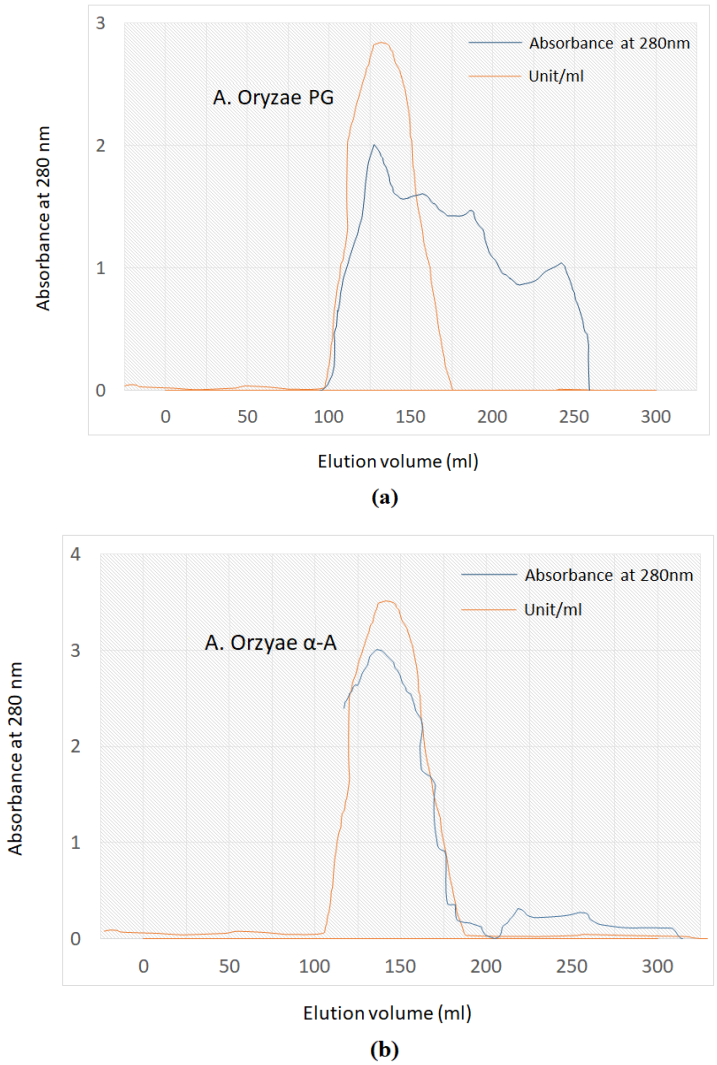

Fig. 1. The Toyopearl HW-65 column (150 cm × 2.5 cm), pre-equilibrated with 0.05 M Tris-HCl buffer at pH 8.0, was used to chromatographically separate the ammonium sulphate fraction of *A. oryzae*. The primary enzymes that were examined in this study were PG and α-A, which are both produced by the fungus *A. oryzae*.

The *A. oryzae* α-amylase's kinetic behaviour

The kinetic parameters of α-amylase were studied by incubating the enzyme with increasingly higher amounts of starch. The correlation between substrate concentration ([S]) and response velocity ([V]) is shown in Fig. 3d, the activation profile that consequently follows. The results demonstrated that, in accordance with Michaelis-Menten kinetics, the reaction velocity increased linearly by elevating substrate concentrations, reaching a plateau at the enzyme's maximum velocity (Vmax). For starch, the Km value—the substrate concentration at which the reaction rate approached half of Vmax—was found to be around 1.5 mM. Under the experimental circumstances, our results emphasize the substrate selectivity and kinetic behaviour of *A. oryzae* α-amylase.

***A. oryzae* polygalacturonase's kinetic behaviour**

Similarly, the activity of *A. oryzae* PG was investigated with different concentrations of pectin. At low substrate concentrations, the results showed a linear rise in reaction velocity, which plateaued when the enzyme reached

Vmax, exhibiting a conventional Michaelis-Menten kinetic profile. Polygalacturonase has a strong affinity for pectin, as seen by the Km value at 1.2 mM substrate concentration. The substrate choice and reaction kinetics of PG, as well as its other enzyme characteristics, are significantly illuminated by these findings.

Analysis via comparison

Their substrate specificity and catalytic effectiveness were confirmed by the distinctive Michaelis-Menten behaviour seen by both PG and α-A. Finding the sweet spot of substrate concentrations for optimum enzyme activity in industrial settings is a key finding of the study. The enzymes' kinetic characteristics were pinpointed using size-exclusion chromatography and ELISA-based approaches, demonstrating the reliability of these methods for enzyme characterisation.

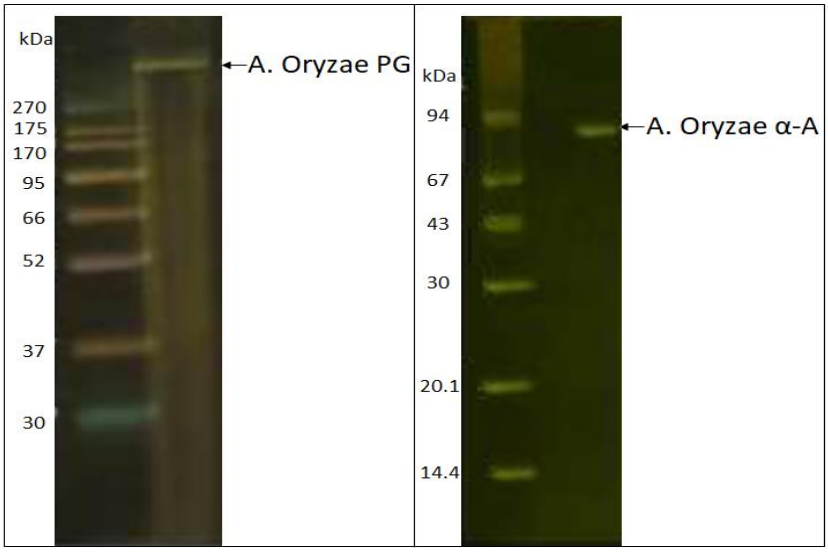

Fig. 2. The molecular weights of the *A. oryzae* enzymes α-amylase and purified polygalacturonase. Using a 12% SDS-PAGE examination, the molecular mass of each enzyme component was determined. Subfigure (a) shows the evaluation of polygalacturonase molecular weight, whereas subfigure (b) centres on α-amylase.

How divalent cations and inhibitors affect enzyme activity

After being isolated from *A. oryzae*, PG and α-A were pre-incubated at 37 °C with different doses of divalent cations (1.5 mM and 3.5 mM, respectively) before the enzyme activity was measured. It was shown that α-amylase relies on certain metal cofactors for its best catalytic function, since the addition of 3.5 mM$CoCl_2$, $MnCl_2$, $NiCl_2$, and $MgCl_2$ greatly increased its activity. On the other hand, $ZnCl_2$, $CuCl_2$, and $CaCl_2$ significantly reduced α-amylase activity. Similarly, whereas $MgCl_2$ and $CaCl_2$ reduced polygalacturonase activity, $CoCl_2$, $ZnCl_2$, and $NiCl_2$ increased it. The results show that divalent cations are crucial for regulating the activity of *A. oryzae* enzymes.

The impact of enzyme inhibitors

Additionally, the impact of several inhibitors on the activities of PG and α-A was investigated. Enzymes were tested for inhibition sensitivity by pre-incubating them with particular inhibitors at 37 °C for 5 minutes. The α-amylase activity was decreased by 71.2% when EDTA, a famous chelating agent, was added, and by 91.3% when DL-dithiothreitol (DTT) was added. Notably, the inhibitory effects of glutathione and bipyridyl on α-A activity were not substantial. The activity of PG was reduced by 89.5% when exposed to EDTA, 48.3% when exposed to bipyridyl, and 68.4% when exposed to rivastigmine. Glutathione and DTT showed moderate inhibitory effects. The results show that various enzymes have variable inhibitor sensitivity and underlying biochemical properties, which should be taken into account when using enzymes in diagnostic and commercial applications.

PG and α-A protein quantification in *A. oryzae* samples

ELISA was used to quantify PG and α-A proteins from *A. oryzae* in samples from wheat, maize, and rice substrates. The optical density (OD) values differed among substrate types and treatment groups, indicating that environmental and processing variables affect enzyme synthesis.

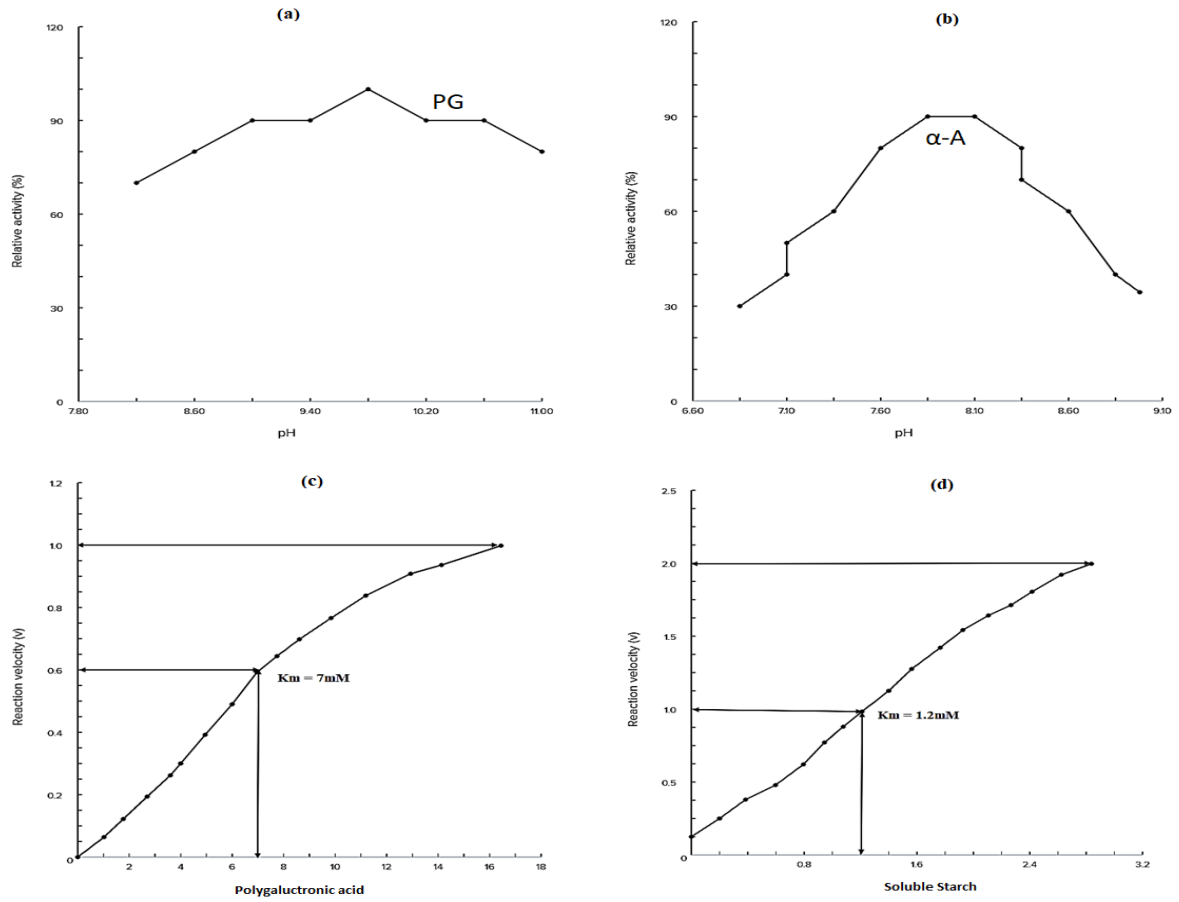

Fig. 3. How different circumstances affect enzyme activity in *A. oryzae* PG and α-A. These investigations examined pH, substrate concentration, and reaction velocities to determine optimum enzymatic conditions; (a) An acetate buffer system was used to investigate PG activity from pH 4.0 to 6.5; (b) The pH dependency of α-A activity was examined using a phosphate buffer system from 6.0 to 8.5; (c) PG was evaluated at 0.1-5 mg mL^{-1} polygalacturonic acid concentrations to determine substrate specificity and reaction velocity; (d) The reaction velocity of α-A was measured using soluble starch as a substrate at concentrations from 0.5 to 10 mg mL^{-1}.

Table 2. Lists the substrate specificity of *A. oryzae* PG and α-A.

Enzyme	Substrate	Density (mM)	Hydrolysis rate	Activity (%)
PG	p-NPP	5	0.512	100
	G-6-P	5	0.092	18
	AMP	5	0.076	14.8
	ADP	5	0.046	8.9
	ATP	5	0.134	26.2
	NaphP	5	0.101	19.7
	CP	5	0.044	8.6
	PEP	5	0.031	6.1
α-A	AcSChI	2	0.476	100
	PrSChI	2	0.306	64.3
	BuSChI	2	0.121	25.4
	BzSChI	2	0.082	17.2

A. oryzae Extract PG

The ELISA OD values for PG in *A. oryzae* extracts from wheat samples ranged from 0.220 to 0.450. OD measurements in maize samples varied from 0.480 to 1.300, showing greater enzyme expression in maize-based substrates.

Different rice processing methods produced different OD readings:

- $CaCl_2$-treated samples had OD values ranging from 0.470 to 0.880.
- Thermally processed enzyme extracts have OD values of 0.630-0.850.
- Chemical solvent-treated samples read 0.520–0.710.

α-A levels in *A. oryzae* extracts

Quantification of α-A in *A. oryzae* extracts showed substrate- and treatment-dependent variation. OD values varied from 0.210 to 0.430 in wheat samples and 0.460 to 1.200 in maize samples.

Rice samples treated differently had the following OD readings:

- In saline solutions, OD values ranged from 0.480 to 0.860.
- OD values for zinc-treated enzyme extracts varied from 0.610 to 0.820.
- Pesticide-exposed samples had OD values of 0.500–0.690.

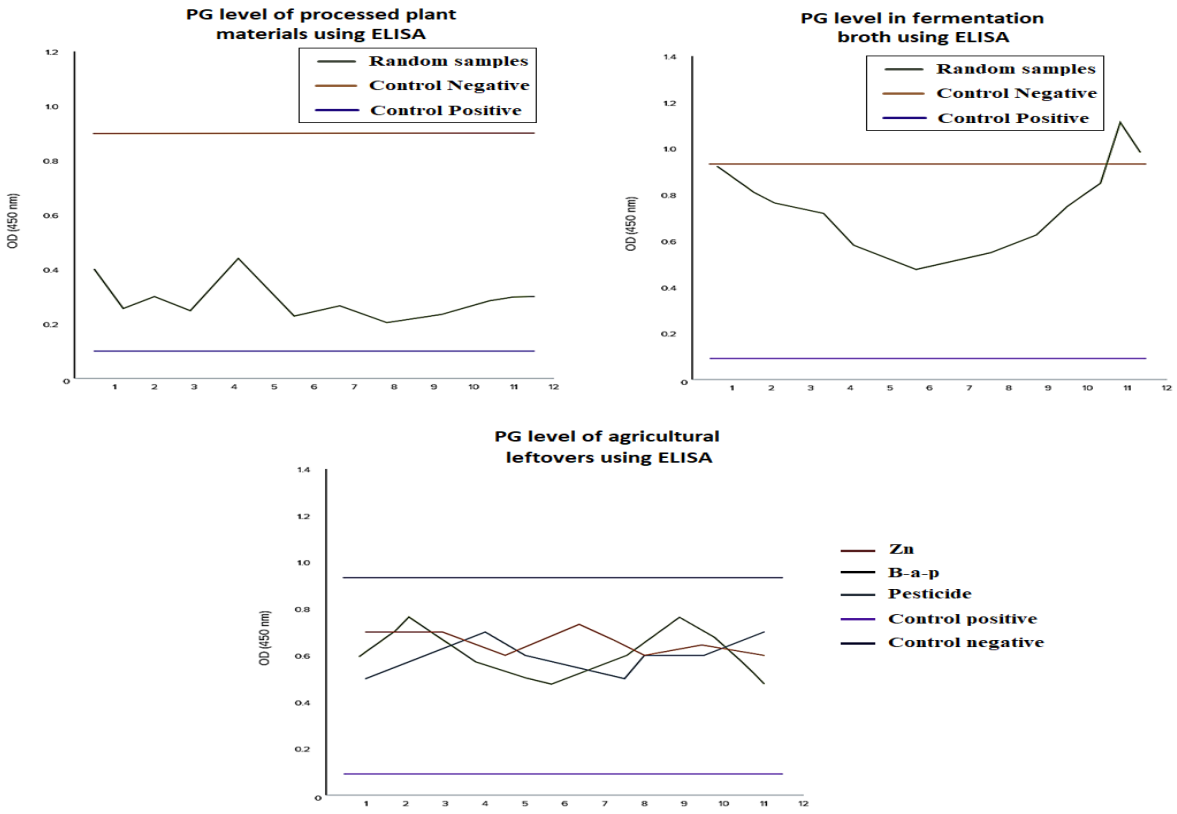

Fig. 4. ELISA quantification of PG enzyme.

Different substrate PG levels

ELISA levels of *A. oryzae* PG differed greatly between substrates. In randomly selected rice samples, enzyme concentrations varied from 0.180 to 0.570 OD units. Wheat has somewhat greater PG activity, with OD values of 0.580–1.100.

Levels of α-A in different substrate samples

Variable α-A enzyme concentrations were also observed. ELISA findings showed considerable α-A activity in maize samples, with OD values ranging from 0.140 to 0.490. In contrast, wheat samples exhibited high α-A activity (OD values: 0.510-1.230), indicating the *A. oryzae* potential in producing enzymes in wheat-based substrates.

Significant variation in α-A optical density (OD) values was observed in fermentation broth samples across treatments.

- Maltose-enriched substrates increased enzyme activity and substrate hydrolysis, with OD values of 0.720 to 0.940.
- Samples with inhibitory chemicals, such as pesticide residues, had OD values of 0.580 to 0.800, indicating partial enzymatic inhibition.

• Samples treated with metal ions, particularly zinc ions, had the lowest OD values, between 0.440 and 0.610, indicating significant enzyme inhibition.

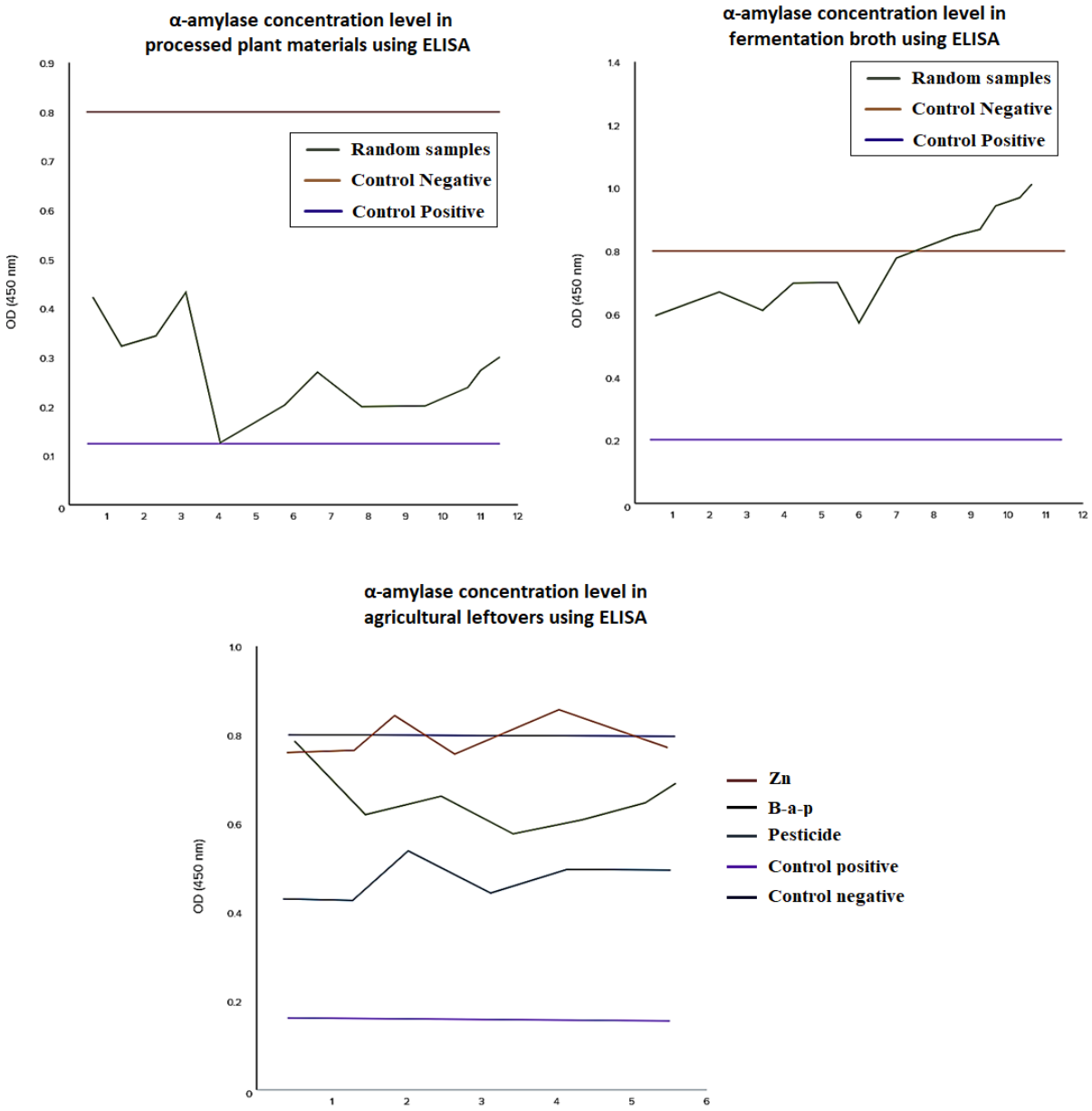

Fig. 5. ELISA results for α-amylase concentrations in samples from various sources, such as processed plant materials, fermentation broths, and agricultural leftovers.

DISCUSSION

The extracellular matrix of *A. oryzae* cultures is a significant source of polygalacturonase (PG) and α-amylase (α-A) enzymes. These enzymes catalytic efficiency and adaptability make them popular in industrial and molecular biology applications. PG, derived from microorganisms and plant tissues, degrades pectin, making it essential in fruit juice clarity, wine production, and waste management (Rani *et al.* 2024). α-A is also used in starch hydrolysis to produce maltose and glucose for brewing, baking, and bioethanol (Chaib *et al.* 2024). The α-A dose-dependent enzymatic activity and environmental sensitivity make it a useful reagent for assessing substrate quality in food and industrial operations. Toyopearl HW-65 chromatography purified PG and α-A from *A. oryzae*, showing separate activity peaks for each enzyme. PG had a specific activity of 4300 U mg^{-1}, 3.1-fold purification, and 55% yield, whereas α-A had 1050 U mg^{-1}, 4.7-fold purification, and 48% yield. These results demonstrate the purification protocol ability to isolate enzymes with minimum contamination. According to SDS-PAGE and gel

filtration, PG is a 120-kDa dimeric enzyme, similar to its structure in other fungal species (Farazi *et al*. 2024). In contrast, α-A was found as a 54 kDa monomer, supporting previous research on fungal α-A. The biological characteristics of metal ions were revealed by their effect on enzyme activity. Ca^{2+} and Mn^{2+} considerably increased PG activity, while Zn^{2+} and Cu^{2+} inhibited it. The α-A catalytic efficacy improved with Co^{2+} and Ni^{2+}, while Mg^{2+} and EDTA reduced its activity, suggesting its metal ion requirement for structural and functional stability. These findings are consistent with other fungal enzyme studies and emphasize the significance of optimising reaction conditions for commercial applications (Vashisth & Raghav 2024). Industrial usage, ELISAs, and other biochemical tests require PG and α-A. Its involvement in pectin hydrolysis makes PG an attractive choice for substrate-specific tests to assess pectinolytic activity in diverse samples (Huang *et al*. 2024). In addition, α-A may be used to assess food starch quality and biotechnology enzymatic activity. The purification and characterisation of PG and α-A from *A. oryzae* improve our understanding of their enzymatic characteristics and enable their use in many sectors. These enzymes may be quantified in complicated combinations quickly and sensitively using ELISA. Researchers can increase enzyme use in diagnostics and industrial biocatalysis by merging ELISA with modern purification methods as Toyopearl HW-65 chromatography to achieve excellent specificity and repeatability (Thabet *et al*. 2024). These findings show that PG and α-A have distinct biochemical properties affected by their parent organism, emphasising *A. oryzae* as a model system for enzyme synthesis. The genetic and structural basis of these traits may be studied to build enzymes with improved stability, activity, and substrate selectivity for specialised applications.

CONCLUSION

An optimized methodology for isolating, purifying, and characterizing polygalacturonase (PG) and α-amylase (α-A) from *Aspergillus oryzae* was effectively created in the study. Enzymes with high activity and purity were obtained through using size-exclusion chromatography, ammonium sulphate precipitation, and SDS-PAGE, demonstrating remarkable precision in enzyme purification. The promise of ELISA-based tests for diagnostic and industrial purposes was demonstrated by their ability to further validate the presence and specificity of the target enzymes. By demonstrating the best substrate concentrations and enzymatic conditions (pH, temperature, etc.), kinetic studies validated Michaelis-Menten behaviour. Important biochemical features needed for tailoring enzyme applications in bioethanol generation, textile processing, and food technology were also uncovered by studying the effect of divalent cations and inhibitors on enzyme activity. The results demonstrate the enzymes adaptability and practical use in industry, opening the door to better biotechnological procedures. Scientific progress and practical utility are guaranteed by this research's thorough framework for the effective utilization of fungal enzymes in varied applications, which leverages robust purification processes and sensitive detection methods.

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