Expression of α-Amylase in a Tropical Strain Penicillium rubrum with Bread as Growth Substrate

Adekunle Odunayo Adejuwon1, 2, Anthonia Olufunke Oluduro2, Femi Kayode Agboola3, Patrick Ojo Olutiola2, 4, Melissa Jane Robbiani5, Sheldon Jerome Segal6

1Department of Microbiology, Faculty of Sciences, Lead City University, Ibadan, Nigeria
2Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria
3Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria
4Department Biological Sciences, Bowen University, Iwo, Nigeria
5Director, Biomedical HIV Research, Centre for Biomedical Research, The Population Council, Manhattan, New York, United States of America
6Director, Division of Population Sciences, The Rockefeller Foundation, Manhattan, New York, United States of America/Adjunct Professor of Clinical Pharmacology, Weill Medical College, Cornell University, Manhattan, New York, United States of America

Corresponding author: Adekunle Odunayo Adejuwon, Department of Microbiology, Faculty of Sciences, Lead City University, Ibadan, Nigeria

Abstract: Background: Penicillium rubrum is an Ascomycete known to produce the toxic metabolites rubratoxins A and B associated with jaundice and convulsions in infected patients. The fungus is known to contaminate grains and grain products in the tropics. Materials and methods: In this current investigation, sterile fresh bread was inoculated with spore suspensions of approximately 6 x 10⁵ spores of isolate per ml of a tropical strain Penicillium rubrum. Incubation was at 25°C. Extracellular proteins produced were subjected to partial purification by ammonium sulphate precipitation and dialysis. Further purification using gel permeation and ion-exchange chromatography was employed. Results: The proteins produced by Penicillium rubrum in the inoculated medium exhibited α-amylase activity. The molecular weights of the α-amylase fractions obtained and estimated by gel filtration using Sephadex G-100 were approximately 47, 316 Daltons and 15, 849 Daltons. The apparent Michalis-Menten constant (K_m)
values for the hydrolysis of starch by the purified α-amylase fractions were approximately 10 mg/ml, 5 mg/ml, 25 mg/ml and 16 mg/ml. Optimum activities were at 35°C for all the fractions and were at pH 6.0. The activities of the α-amylase fractions produced by the fungus were stimulated at varying degrees by NaCl, KCl, CaCl₂ and MgCl₂ but inhibited by ethylene diamine tetraacetic acid (EDTA), mercuric chloride (HgCl₂) and 2,4-dinitrophenol (DNP). The α-amylase fractions were sensitive to heat, losing all their activities within twenty minutes of heating at 80°C. Conclusion: Apart from the toxic metabolites rubratoxins produced by Penillium rubrum, the fungus produces α-amylases in contaminated bread at 25°C in the tropics. These α-amylases are stable at 35°C and at pH 6.0.

Key words: Penicillium rubrum, bread, purification, characterization.
1. Introduction

α-Amylases are hydrolytic enzymes which catalyze the degradation of starch molecules into dextrins (Aiyer, 2005). Various microorganisms including fungi are capable of production of α-amylases (Pandey, 2000; Adejuwon, 2013). It has recently been demonstrated that *Penicillium rubrum* produces α-amylase in bread as carbon source (Adejuwon, 2015a).

In this present investigation, a tropical strain *Penicillium rubrum* was grown in a bread medium as sole growth source. The extracellular α-amylase produced was purified and characterized. The properties of the enzyme are presented.

2. Materials and Methods

2.1 Source and Identification of Isolate

The tropical strain *P. rubrum* for this research was isolated from mouldy bread and identified at the Seed Health Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria using techniques contained in the illustrated Handbook of Fungi (Cannon and Kirk, 2007). The identification was done by observing cultural and morphological characteristics of the fungus. Identity was also confirmed by genetic DNA sequencing using Polymerase Chain Reaction (PCR) at the Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The isolate was cultured on Potato Dextrose agar plates and slants.

2.2 Bread as a Source of Carbon

Freshly baked loaves of bread were obtained at the bakery of the Department of Food Science and Technology, Obafemi Awolowo University, Ile-Ife, Nigeria. Bread loaf was soaked in distilled water (1% w/v), mercerated with a homogenizer and autoclaved at 15 psi (121°C) for 15 minutes. One hundred millilitre of the bread medium in conical flasks (250 ml) was inoculated with 1 ml of aqueous spore suspension containing
approximately $6 \times 10^5$ spores per ml of isolate. Incubation was at 25°C.

2.3 Extraction of Enzyme

Daily, the contents of each flask were carefully filtered through glass fibre filter paper (Whatman GF/A). The protein content of the filtrates was determined using the Lowry et al. (1951) method. The filtrates were assayed for $\alpha$-amylase activity using the method of Pfueller and Elliott (1969).

2.4 Enzyme Assay

2.4.1 $\alpha$-amylase

$\alpha$-Amylase activity was determined using the method of Pfueller and Elliott (1969). Reaction mixtures consisted 2 ml of 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 ml of enzyme. Controls consisted only 2 ml of the prepared substrate. The contents of both experimental and control tubes were incubated at 35°C for 30 min. The reaction in each tube was terminated with 3 ml of 1 N HCl. 0.5 ml enzyme was then added to the control tubes. 2ml mixture from each set of experimentals and controls was transferred into new sets of test tubes. 3ml of 0.1 N HCl was added into the contents of each test tube after which 0.1 ml of iodine solution was added. Optical density readings were taken at 670nm. One unit of $\alpha$-amylase activity was defined as the amount of $\alpha$-amylase which produced 0.1 percent reduction in the intensity of the blue colour of starch-iodine complex under conditions of the assay. Specific activity was calculated as enzyme units per milligram protein.

2.5 Ammonium Sulphate Fractionation

The crude enzyme, on day of optimum activity during daily sampling, was treated with ammonium sulphate (analytical grade, BDH) at 40-90% saturation. Precipitation was allowed to continue at 4°C for 24 h. The mixture was centrifuged at 6,000g for 30
minutes at 4°C using a high speed cold centrifuge (Optima LE-80K Ultracentrifuge, Beckman, USA) at the Central Laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. The supernatant was discarded. The precipitate was reconstituted in 0.2 M citrate phosphate buffer, pH 6.0. The protein content of the fractionated enzyme was determined (Lowry et al., 1951). α-Amylase activity was also determined (Pfueller and Elliott, 1969).

2.6 Dialysis

Using acetylated dialysis tubings (Visking dialysis tubings, Sigma) (Whitaker et al. 1963) and a multiple dialyser, the ammonium sulphate fractionated enzyme preparation was dialysed under several changes of 0.2 M citrate phosphate buffer, pH 6.0 at 4°C for 24 h. The protein content of the dialysed enzyme was determined using the Lowry et al. (1951) method while α-amylase activity was determined using the method of Pfueller and Elliott (1969).

2.7 Fractionation of Enzyme Using Sephadex G-100 Column

Dialysed α-amylase from *P. rubrum* was subjected to further purification using Sephadex G-100.

2.7.1 Preparation and packing of Sephadex G-100 column

The Sephadex G-100 resin was suspended in buffer (0.2 M citrate phosphate, pH 6.0 containing 5 mM sodium azide) and allowed to swell for seventy two hours. Fine particles on the suspension were decanted. The suspension was deaerated under vacuum pressure until a slurry of air-free gel particles was obtained. The buffer was also deaerated. The column was half filled with the buffer and the gel slurry added until the column was almost filled. The gel was left to settle in the column. The top of the column was connected to a reservoir and more gel added until the column was
filled. The buffer was allowed to flow continuously through the gel throughout the process. When the column had been well equilibrated with the same buffer and resin and a constant bed height obtained, the enzyme was applied. A sample applicator (2.3 cm x 5.0 cm) was placed on top of the gel to prevent distortion when applying sample. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

2.7.2 Calibration of Sephadex G-100 column

The column was calibrated with proteins of known molecular weight (Andrews, 1964; Olutiola and Cole, 1976). Five milligram per ml each of the standard proteins: catalase (mol. wt., 240,000), glucose oxidase (mol. wt., 150,000), human haemoglobin (mol. wt., 68,000), egg albumin (mol. wt., 45,000), horse myoglobin (mol. wt., 17,000) and cytochrome C (mol. wt., 12,400) was dissolved in 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM sodium azide. The total sample volume of each standard protein was 5 ml. The solution was applied to the column. Fractions (5 ml/tube) were collected in tubes using an automated LKB fraction collector (700A Ultra Rac). Protein content of eluted fractions was monitored at 280nm. The elution volume of each of the standard protein was plotted against the logarithm of its molecular weight to obtain a standard calibration curve (Andrews, 1964).

2.7.3 Application of enzyme to Sephadex G-100 column

Ten milliliter of the dialysed enzyme was applied to the column and eluted with 0.2 M citrate phosphate buffer, pH 6.0 containing 5 mM sodium azide (NaN₃). Fractions (5 ml/tube) were collected. Protein was monitored at 280nm. α-Amylase activity was determined as described. Fractions with α-amylase activity with peak ranges were pooled. Also the molecular weight of the unknown enzyme was extrapolated from the standard curve.
2.8 Further Fractionation Using Ion-Exchange Chromatography

CM-Sephadex C-25 and CM-Sephadex C-50 columns were prepared as described in Pharmacia manual. The resin were swollen in distilled water and equilibrated in the elution buffer. A column (2.5 x 40 cm) was used. Ten milliliter of pooled fractions from the Sephadex G-100 column which exhibited α-amylase activity was applied to the prepared columns of CM-Sephadex C-50 and CM-Sephadex C-25. The columns were first washed with 0.2 M citrate phosphate buffer pH 6.0 containing 5 mM sodium azide to remove unbound proteins followed by elution with 0.2 M citrate phosphate buffer, pH 6.0, with linear gradients of 0.1 – 0.5 M NaCl. Fractions (5 ml/tube) collected were monitored spectrophotometrically at 280nm. α-Amylase activity was determined using the method of Pfueller and Elliott (1969).

2.9 Properties of the Partially Purified Enzymes

The effects of temperature, pH, salts and some chemicals on the activities of the purified α-amylases from *P. rubrum* were investigated after the fractionation.

2.9.1 Effect of temperature

The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme. Incubation was at a range of 4-50°C for 1 hr. α-Amylase activity was there after determined.

2.9.2 Stability test at 80°C

The effect of heat (80°C) on the stability of the partially purified enzymes at different periods, 0, 2, 10, 15 and 20 minutes was examined. The activities of the heated enzymes were determined by incubating 0.5 ml of each enzyme with 2 ml of the citrate phosphate buffered 0.2% starch substrate (pH 6.0) at 35°C for 1 hr. α-Amylase activity was there after determined.
2.9.3 Effect of pH

The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer at different pH values ranging from pH 3.0 – 8.0. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme. Incubation was at 35°C for 1 hr. α-Amylase activity was then determined.

2.9.4 Effect of substrate concentrations

Different concentrations, 0.08 – 0.4% (w/v) of starch (Sigma) in 0.2 M citrate phosphate buffer, pH 6.0 were used as substrates. The reaction mixture was 2 ml of substrate and 0.5 ml of enzyme, incubated at 35°C for 1 hr. α-Amylase activity was afterwards determined.

2.9.5 Effects of salts and some chemicals

The effects of NaCl, KCl, CaCl$_2$ and MgCl$_2$ at varying concentrations (0, 2, 10, 15, and 20 mM) on the activities of the purified α-amylases were examined. Each salt was prepared in 0.2% starch in citrate phosphate buffer pH 6.0.

The reaction mixture, 2 ml of substrate and 0.5 ml of enzyme, was incubated at 35°C for 1 hr. α-Amylase activity was then determined.

Different concentrations (0, 2, 4, 6 and 8 mM) each of mercuric chloride, 2,4-dinitrophenol and EDTA were prepared in 0.2% starch in citrate phosphate buffer, pH 6.0. These were used as substrates. α-Amylase activity was determined.

3. Results

The isolate *P. rubrum* grew and exhibited α-amylase activity in the bread medium used in this research at 25°C.
Gel filtration of the dialysed enzyme on Sephadex G-100 gave five peaks of protein designated F, G, H, I and J. Only the components of peaks H and I expressed α-amylase activity. The molecular weights of components of peaks H and I estimated from their elution volumes were approximately 47,316 Daltons and 15,849 Daltons respectively.

Elution of components of peak H of *P. rubrum* on CM Sephadex C-50 produced three peaks of protein designated Ha, Hb and Hc. Only the components of peak Ha and Hb exhibited α-amylase activity. When the components of peak I were separated on CM-Sephadex C-25, three peaks of protein designated Ia, Ib and Ic were obtained. Only the components of peaks Ia and Ib possessed α-amylase activity.

Purification folds of approximately 37, 27, 27 and 25 were obtained for peaks Ha, Hb, Ia and Ib respectively (Table 1).

Investigations were carried out to determine the influence of some factors on the activities of the partially purified enzymes (CM-Sephadex C-25 and CM-Sephadex C-50 fractions). The fractions employed were components of Ha, Hb, Ia and Ib.

The α-amylase activities of the components of peaks Ha, Hb, Ia and Ib obtained from *P. rubrum* increased gradually as concentrations of starch increased (Fig. 1). The Lineweaver-Burk plots indicated that the apparent K_m for the enzymic hydrolysis of starch were approximately 10 mg/ml, 5 mg/ml, 25 mg/ml and 16 mg/ml for fractions Ha, Hb, Ia and Ib respectively.

The pH of the reaction mixtures had affect on the activities of the enzymes produced by *P. rubrum*. The activities of the enzymes increased with increase in pH and generally decreased after the optimum. Optimum α-amylase activities of fractions Ha, Hb, Ia and Ib were at pH 6.0 (Fig. 2).

The temperature of the reaction had effect on the activities of α-amylases produced by the fungus. The activities of the enzymes increased with increase in temperature of incubation until an optimum was attained. Thereafter there was gradual decline. Optimum α-amylase activities of fractions Ha, Hb, Ia and Ib (Fig. 3) for *P. rubrum* were
at 35°C.

Subjection of the α-amylases to heat at 80°C for a period of twenty minutes had significant effects on the activities of the enzymes. The activity of each enzyme decreased with increase in time of heating. The α-amylase fractions Ha, Hb, Ia and Ib of *P. rubrum* lost activities of approximately 73%, 83%, 81% and 77% within 2 minutes. Complete inactivations were observed within 10 minutes (Fig. 4).

The salts employed in this research investigation stimulated α-amylase activities produced by *P. rubrum*. There was increase in activity of enzyme with increase in concentration of each cation.

For α-amylase fractions Ha, Hb, Ia and Ib (Figs. 5-8), the highest stimulations were with KCl (for fraction Ha), NaCl (for fraction Hb), MgCl₂ (for fraction Ia) and CaCl₂ (for fraction Ib) at 20 mM concentrations. The least stimulations were with NaCl (for fractions Ha and Ia) and KCl (for fraction Hb, Ia and Ib) at 20 mM concentrations.

Mercuric chloride (HgCl₂), ethylene diamine tetraacetic acid (EDTA) and 2,4-dinitrophenol (DNP) inhibited α-amylase activities of *P. rubrum* used in this investigation. As the concentrations of the chemicals increased, α-amylase activities gradually decreased. Inhibition of approximately 100%, 93% and 87% (for fraction Ha); 93%, 85% and 90% (for fraction Hb); 100%, 92% and 89% (for fraction Ia); 100%, 84% and 92% (for fraction Ib) were observed at 8mM concentrations of HgCl₂, DNP and EDTA respectively (Figs. 9-12).

4. Discussion

Enzymes are influenced by several factors (Dixon and Webb, 1971; Nelson *et al.*, 1993). In this investigation, pH affected α-amylase activity produced by *P. rubrum*. Activities were optimum at pH 6.0. Amadioha (1998) reported *Rhizopus oryzae* as producing amylase with maximum activity at 6.0. In another investigation α-amylase from *Lasiodiplodia theobromae* was observed stable at pH 6.0 (Adejuwon, 2011b).
Fersht (1977) reported enzymes as being unstable outside their pH of optimum activity.

α-Amylase produced by P. rubrum expressed optimum activities at 35°C in this study. This is in line with a recent investigation with starch and potassium nitrate supporting growth and expression of α-amylase in Penicillium rubrum at 35°C (Adejuwon, 2015a).

There were approximately 73%, 83%, 81% and 77% loss of activity within 2 minutes when α-amylases from Penicillium rubrum were subjected to heat at 80°C. Complete inactivations were observed within 10 minutes. Enzymes are proteins which lose their structural integrity when subjected to heat (Nelson et al., 1993). A heat labile β-amylase from Aspergillus flavus was reported to have lost 90% activity when subjected to heat at 80°C within 25 minutes (Adejuwon, 2011a).

The cations of KCl, NaCl, CaCl₂ and MgCl₂ used in this investigation stimulated α-amylase activities produced by P. rubrum. Stimulations were at varying degrees. α-Amylases are metalloenzymes stabilized by calcium ions (Aiyer, 2005). Also Ladokun and Adejuwon (2011) reported amylase from a strain of Aspergillus fumigatus stimulated by Al³⁺.

EDTA, 2,4-dinitrophenol and HgCl₂ in this investigation inhibited α-amylase activities expressed in P. rubrum. Previous investigations corroborate with reports on α-amylases from Candida albicans and Fusarium sp. inhibited by EDTA and HgCl₂ (Adejuwon et al., 2013).

α-Amylase activities from P. rubrum steadily increased as concentration of starch as substrate increased. α-Amylase in fungi is induced by starch (Adejuwon, 2015b). Starch is also a good substrate for α-amylase assays (Sarikaya and Cirakoglu, 1989).

Conclusively, Penicillium rubrum produces α-amylase in bread at 25°C. This can be explored in the industrial production of the enzyme.
Table 1: Partial purification of α-amylase obtained from bread deteriorated by *Penicillium rubrum*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity (Units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (Units/mg protein)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1625</td>
<td>34.3</td>
<td>47.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>90% (NH₄)₂SO₄ Precipitation</td>
<td>1388</td>
<td>23.1</td>
<td>60.1</td>
<td>85.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Sephadex G-100 Gel filtration Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak H</td>
<td>1141</td>
<td>3.2</td>
<td>356.6</td>
<td>70.2</td>
<td>7.5</td>
</tr>
<tr>
<td>I</td>
<td>936</td>
<td>7.3</td>
<td>128.2</td>
<td>57.6</td>
<td>2.7</td>
</tr>
<tr>
<td>CM-Sephadex C-50 Ion-Exchange Chromatography</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Peak Ha</td>
<td>517</td>
<td>0.3</td>
<td>1723.3</td>
<td>31.8</td>
<td>36.4</td>
</tr>
<tr>
<td>Hb</td>
<td>628</td>
<td>0.5</td>
<td>1256</td>
<td>38.6</td>
<td>26.5</td>
</tr>
<tr>
<td>CM-Sephadex C-25 Ion-Exchange Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Ia</td>
<td>256</td>
<td>0.2</td>
<td>1280</td>
<td>15.8</td>
<td>27</td>
</tr>
<tr>
<td>Ib</td>
<td>465</td>
<td>0.4</td>
<td>1162.5</td>
<td>28.6</td>
<td>24.5</td>
</tr>
</tbody>
</table>
Fig. 1: Effect of concentration of starch on the activity of partially purified α-amylases from *Penicillium rubrum*. 
Fig. 2: Effect of pH on the activity of partially purified α-amylases from *Penicillium rubrum*
Fig.3: Effect of temperature on the activity of partially purified α-amylases from *Penicillium rubrum*
Fig. 4: Heat stability test at 80°C of partially purified α-amylases from *Penicillium rubrum*
Fig. 5: Effect of salts on the activity of partially purified α-amylase (fraction Ha) obtained from *Penicillium rubrum*
Fig. 6: Effect of salts on the activity of partially purified α-amylase (fraction Hb) obtained from *Penicillium rubrum*
Fig. 7: Effect of salts on the activity of partially purified α-amylase (fraction Ia) obtained from *Penicillium rubrum*
Fig. 8: Effect of salts on the activity of partially purified α-amylase (fraction Ib) obtained from *Penicillium rubrum*
Fig. 9: Effect of some chemicals on the activity of partially purified α-amylase (fraction Ha) obtained from *Penicillium rubrum*
Fig. 10: Effect of some chemicals on the activity of partially purified α-amylase (fraction Hb) obtained from *Penicillium rubrum*
Fig. 11: Effect of some chemicals on the activity of partially purified α-amylase (fraction Ia) obtained from *Penicillium rubrum*
Fig. 12: Effects of some chemicals on the activity of partially purified α-amylase (fraction Ib) obtained from *Penicillium rubrum*

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