Purification and Properties of an α-Amylase Produced by a Cassava-Fermenting Strain of Micrococcus luteus

Article *in* Folia Microbiologica · February 1997

Impact Factor: 1 · DOI: 10.1007/BF02826551 · Source: PubMed

3 authors:

Matthew Olusoji Ilori
University of Lagos
73 PUBLICATIONS **956** CITATIONS
SEE PROFILE

Olukayode Amund
University of Lagos
77 PUBLICATIONS **688** CITATIONS
SEE PROFILE

Olusesan Omidiji
University of Lagos
24 PUBLICATIONS **1,010** CITATIONS
SEE PROFILE

Folia Microbiol. 42 (5), 445–449 (1997)

Purification and Properties of an α-Amylase Produced by a Cassava-Fermenting Strain of *Micrococcus luteus*

M.O. ILORI, O.O. AMUND and O. OMIDIJI

Department of Biological Sciences, University of Lagos, Nigeria

Received February 7, 1997

ABSTRACT. An extracellular α-amylase produced by a cassava-fermenting strain of *Micrococcus luteus* was purified 26-fold by gel filtration and ion-exchange chromatography. The molar mass was estimated to be approximately 56 kDa. The optimum temperature of the enzyme was 30 °C, optimum pH 6.0 and optimum substrate concentration was 0.6 % (*W/V*). Treatment of the enzyme at 70 °C for 10 min resulted in 70 % loss of activity. The activation energy was determined to be 34.8 kJ/mol. The activity of the enzyme was enhanced by Mg^{2+}, Ca^{2+}, K^+, Na^+ and inhibited by EDTA, KCN and citric acid. The enzyme may find some application in local food processing.

Utilization of starch by microorganisms during fermentation is due to the ability of the organisms to produce a wide range of extracellular enzymes. Several such enzymes from various microorganisms have been reported (Hopkins 1946; Welker and Campbell 1963; Ingle and Erickson 1978; Champ *et al.* 1983; Sen and Chakrabarty 1984; Tsvetkov and Emanuilova 1989; Ali and Hossain 1991). Depending on the source, amylases are known to exhibit similarities and differences in properties which include pH and temperature dependence, molar mass, Michaelis constant (K_m), thermostability, activation energy and other physico-chemical parameters. As a result, different amylases have found specific application in many industries (Chandra *et al.* 1980).

Cassava (*Manihot esculenta*), an important staple food crop in West Africa, is processed into 'gari' and 'foufou' in Nigeria (Amund and Ogunsina 1987). Microbiological changes taking place during the traditional processing of cassava have been reported (Okafor 1977). Although biochemical changes occurring during cassava fermentation are mediated by microbial enzymes (Amund and Ogunsina 1987), few studies have been carried out on the properties of these enzymes.

In this paper we report the purification and physico-chemical properties of an α-amylase produced by a cassava-fermenting strain of *Micrococcus luteus* with a view to further development of the organism as a starter culture for cassava fermentation.

MATERIALS AND METHODS

Microbial strain. Micrococcus luteus used here was isolated from cassava steep liquor. It was a Gram-positive, nonsporing coccus, occurring singly or in clusters. The cells were oxidase-negative, catalase-positive, nonmotile, did not produce acid from sugars and gave neutral reaction in Hugh and Leifson medium. The organism grew in the minimal salts medium and produced extracellular proteins with amylolytic properties.

Crude enzyme preparation from Micrococcus luteus. *M. luteus* was isolated from cassava steep liquor by a plate-culture technique described by Amund and Ogunsina (1987) and identified with the schemes of Collins and Lyne (1976) and Buchanan and Gibbons (1974). Stock cultures were maintained on nutrient agar slants. Young cultures were used in all experiments by subculturing the organisms for 1-d before inoculation. The organism was grown at 30 °C for 2 d in a mineral salt medium which contained (g/L): $NaNO_3$ 3, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, KH_2PO_4 1, $FeSO_4 \cdot 7H_2O$ 0.02, $CaCl_2 \cdot 2H_2O$ 0.1, peptone 10. Starch was autoclaved separately and added to the medium to a final concentration of 1 % (*W/V*). Cells were harvested by centrifugation at 10 000 *g* for 30 min at 4 °C. The supernatants were used as sources of crude extracellular enzyme.

Protein and amylase assays. Protein assay was carried out by Lowry method with bovine serum albumin as standard or by absorbance measurement at 280 nm. Amylase activity was determined using the dextrinogenic assay method of Pfuller and Elliot (1969). One unit of amylase activity was defined as the amount of enzyme in 1.0 mL reaction mixture which produces 0.01 % reduction per min of the intensity of blue-black color of starch–iodine complex.

Qualitative estimation of amylolytic products. Products of amylolysis were identified by descending paper chromatography using the solvent system of 1-butanol–acetic acid–water (4:1:5,

V/V/V). Starch (1.0 %, *W/V*) was incubated with the purified enzyme in a water bath at 30 °C for 10–120 min. The digest was applied to a Whatman no. 1 chromatography paper (250 × 450 mm) mounted in an equilibrated tank and allowed to run for 12 h. The paper was air-dried and the sugars were located by spraying with aniline hydrogen-phthalate solution (Harborne 1984). Standard sugars (maltose and glucose) and the products of amylolysis were then observed in normal light and also under UV light.

Enzyme purification. Sephadex G-100 (*Pharmacia*) suspended in sodium phosphate buffer (50 mmol/L, pH 6.0) was packed into a glass column (12 × 400 mm) at room temperature. The gel in the column was further washed with the same buffer at a constant flow rate of 18.0 mL/h. Ammonium sulfate (analytical grade) was added to the culture supernatant (100 mL) to 80 % saturation and was allowed to precipitate for 2 h at 4 °C. It was then centrifuged at 10 000 ***g*** for 30 min at 4 °C.

The precipitate was redissolved in the phosphate buffer and dialyzed against four changes of the same buffer for 18 h at 4 °C. The dialyzed protein solution was then introduced to the top of the Sephadex column and eluted with the phosphate buffer. Fractions (3 mL) were collected and assayed for protein content and enzyme activity. Fractions that showed amylase activity were pooled and rechromatographed on a DEAE-cellulose column using sodium phosphate buffer (50 mmol/L, pH 6.0) containing a NaCl concentration gradient (0.1–0.5 mmol/L). The fractions collected were similarly assayed for protein content and amylase activity.

Molecular mass determination. The molar mass of the enzyme was estimated by gel filtration on Sephadex G-100 columns as described by Andrews (1964). Proteins were eluted with the phosphate buffer at the same flow rate of 18.0 mL/h. The column was calibrated with proteins of known molar mass: bovine serum albumin (25 kDa), ovalbumin (45), chymotrypsinogen (25) and ribonuclease (13). The void volume was determined with blue dextran (2.0×10^6, *Pharmacia*).

Effect of pH and temperature on amylase activity. The effect of pH was determined with acetate buffer (50 mmol/L, pH 3.5–5.5) and sodium phosphate buffer (50 mmol/L, pH 6.0–8.5). The effect of temperature on enzyme activity was assayed between 15 and 70 °C. The effect of heat on enzyme stability was investigated by maintaining the enzyme at 70 °C for 2 h with assays of enzyme activity at 10-min intervals. The effect of temperature on reaction velocities (activation energy) was also determined at different concentrations of the substrates and at various temperatures (Morris 1971).

Effect of substrate concentration on enzyme activity. Different concentration of starch (0.1–1.5 %, *W/V*) were used as substrate for amylase activity assays.

Effect of cations and inhibitors on enzyme activity. The effect of cations on amylase activity was determined using concentrations ranging from 0.5 to 60 mmol/L of chloride salts of Ca^{2+}, Mg^{2+}, Na^+ and K^+. The salts were added to the substrate employed for enzyme assay. The effects of inhibitors including EDTA, KCN, citric acid and L-cysteine at different concentrations (5–50 mmol/L) on amylase activity were also examined. The inhibitors were added to the substrate used for the enzyme assay.

RESULTS

Maltose and glucose were detected as the products of amylolysis by paper chromatography, indicating the enzyme to be an α-amylase. The results of the enzyme purification steps are shown in Table I. A 26-fold purification was achieved. Amylase activity was eluted as a single peak from Sephadex G-100 and DEAE-cellulose columns (Figs 1, 2). The molar mass of the enzyme was estimated to be approximately 56 kDa. Peak activity of the purified enzyme was found at pH 6.0 and at 30 °C (Table II); little enzyme activity was detected above 45 °C.

Table I. Purification of amylase produced by *M. luteus*

Purification step	Volume mL	Protein μg/mL	Enzyme activity U/mL	Specific activity U/μg protein	Purification factor
Crude enzyme	100	321	330	1.03	1.00
$(NH_4)_2SO_4$	6	175	455	2.60	2.52
Dialysis	5	160	470	2.94	2.85
Sephadex G-100	9	90	660	7.33	7.12
DEAE-cellulose	9	38	995	26.18	25.42

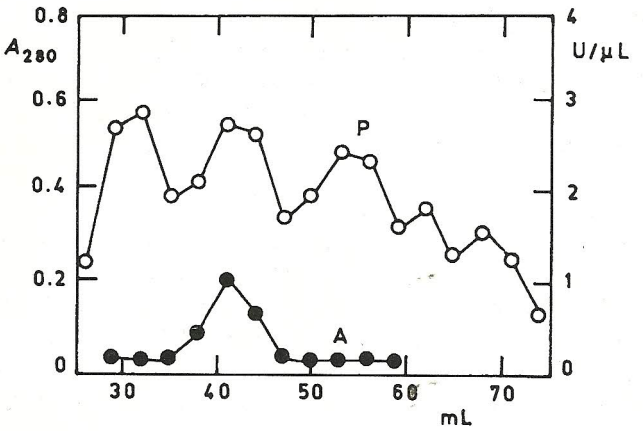

Fig. 1. Elution of proteins (elution volume, mL) in concentrated culture filtrates of *M. luteus* on Sephadex G-100; P — protein concentration (absorbance A_{280}), A — amylase activity (U/μL).

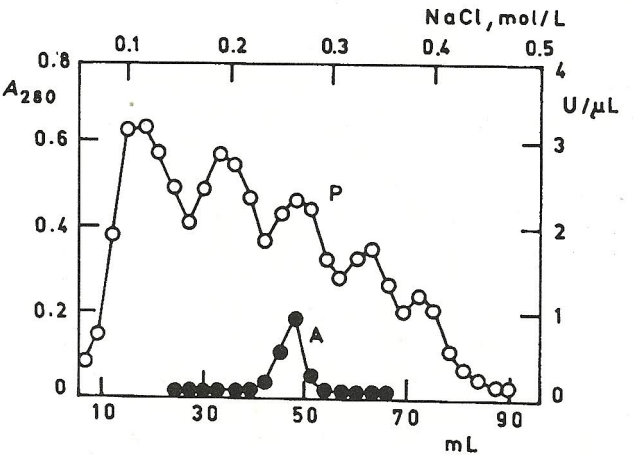

Fig. 2. Ion-exchange chromatography of enzyme fractions (elution volume, mL) obtained from the culture of *M. luteus* on DEAE-cellulose; P — protein concentration (absorbance A_{280}), A — amylase activity (U/μL).

Table II. Effect of pH and temperature on the activity of *M. luteus* amylase

pH	Amylase activity U/mL	Temperature °C	Amylase activity U/mL
3.0	50	15	90
3.5	90	20	210
4.0	340	25	320
4.5	380	30	580
5.0	510	35	240
5.5	570	40	220
6.0	650	45	170
6.5	500	50	60
7.0	450	55	40
7.5	410	60	25
8.0	360	65	20
8.5	200	70	10

Heat treatment of the enzyme at 70 °C for 10 min resulted in 70 % loss in activity (Table III). Virtually complete denaturation was observed after 40 min.

The optimum substrate (starch) concentration was found to be 0.6 % (*W/V*) (Table IV). The K_m and *V* values were derived from a typical Lineweaver–Burk plot as 0.29 mg/mL and 555.6 U/mL, respectively.

The activation energy of the enzyme was calculated from a typical Arrhenius plot to be approximately 34.8 kJ/mol.

The activity of the enzyme was increased by Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions up to their optimal concentrations of 15, 10, 5 and 10 mmol/L, respectively (Fig. 3). Furthermore, EDTA, KCN, citric acid and L-cysteine at a concentration of 25 mmol/L caused a 92, 70, 54 and 26 % inhibition of *M. luteus* amylase, respectively (Fig. 4).

Table III. Effect of heat (70 °C) on activity of *M. luteus* amylase

Time min	Enzyme activity %[a]	Time min	Enzyme activity %[a]
0	100	70	0
10	30	80	0
20	25	90	0
30	10	100	0
40	5	110	0
50	0	120	0
60	0		

[a]Initial enzyme activity (100 %) = 580 U/mL.

Table IV. Effect of starch concentration on the activity of *M. luteus* amylase

Starch % (*W/V*)	Amylase activity U/mL	Starch % (*W/V*)	Amylase activity U/mL
0.1	60	0.7	600
0.2	120	0.8	570
0.3	260	0.9	540
0.4	440	1.0	500
0.5	480	1.5	320
0.6	670		

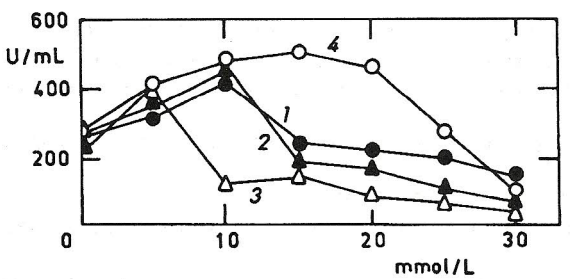

Fig. 3. Effect of cations (mmol/L) on the activity of partially purified amylase (U/mL) of *M. luteus*; *1* — Mg^{2+}, *2* — K^+, *3* — Na^+, *4* — Ca^{2+}.

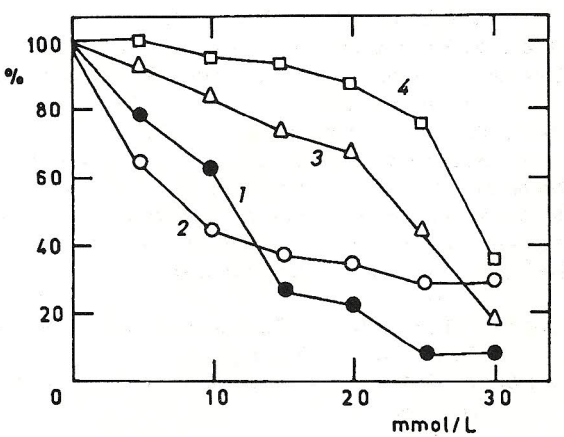

Fig. 4. Effect of inhibitors (mmol/L) on the activity of partially purified amylase (%) of *M. luteus*; *1* — EDTA, *2* — KCN, *3* — citric acid, *4* — L-cysteine.

DISCUSSION

M. luteus produced an α-amylase during cassava fermentation which hydrolyzed starch to maltose and glucose. These products are utilized for growth and further conversion to lactic acid by *Lactobacillus* species which have been reported to be associated with cassava fermentation (Okafor 1977). The presence of amylase in *M. luteus* is of physiological importance since it enables it to

hydrolyze starch in the medium to assimilable sugars. Production of a similar enzyme had been reported in *M. varians* (Adeleye 1990*a*). The molar mass of the enzyme estimated to be approximately 56 kDa is almost similar to what was reported for the amylase of *B. brevis* (Tsvetkov and Emanuilova 1980) but higher than what was reported for the amylase of *B. subtilis* (Fisher *et al.* 1960). The enzyme was fairly active over a wide range of pH and temperature. Observations similar to this had been reported by Ghosh *et al.* (1991) and Champ *et al.* (1983). The rapid denaturation of the amylase at elevated temperature may be the result of the mesophilic nature of the organism. A similar observation was reported in *M. varians* (Adeleye 1990*b*).

The optimum substrate concentration of 0.6 % (*W/V*) was observed for the amylase. Reduction of activity at higher substrate concentrations appeared to be due to inhibition of the enzyme by the substrate. The K_m value of the enzyme put its affinity for starch in the same class with those reported from other microorganisms. However, the K_m value was lower than reported for the amylase of *Bacillus brevis* (Tsvetkov and Emanuilova 1989) and *Aspergillus terreus* (Ghosh *et al.* 1991). The increase in amylase activity in the presence of metal ions might be due to their role in stabilizing the enzyme from denaturation and proteolytic degradation (Hasegawa *et al.* 1976) since many other enzymes are secreted into the culture medium. Inhibition by EDTA, a metal-chelating agent, further confirms the role of metal ions in the stabilization of the enzyme activity. This is in agreement with the observation of Ali and Hossain (1991) and Adeleye (1990*b*). Further work is in progress on the utilization of the microorganism as starter culture for 'gari' and 'foufou' preparation.

The authors wish to thank Mr. Cyril Esedo of the *Computer Science Department* for typing the manuscript.

REFERENCES

ADELEYE A.I.: Production and control of extracellular α-amylase in *Micrococcus varians*. *J.Basic Microbiol.* **30**, 723–727 (1990*a*).

ADELEYE A.I.: Purification and properties of α-amylase from *Micrococcus varians*. *J.Basic Microbiol.* **30**, 717–722 (1990*b*).

ALI S., HOSSAIN Z.: Characteristics of glucoamylase from *Aspergillus terreus*. *J.Appl.Bacteriol.* **71**, 144–146 (1991).

AMUND O.O., OGUNSINA O.A.: Extracellular amylase production by cassava fermenting bacteria. *J.Industr.Microbiol.* **2**, 123–127 (1987).

ANDREWS P.: Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem.J.* **91**, 222–233 (1964).

BUCHANAN R.E., GIBBONS N.E.: *Bergey's Manual of Determinative Bacteriology*. Williams & Wilkins, Baltimore 1974.

CHAMP M., SZYLIT P., RAIBAUD P., AIT-ABDELKADER N.: Amylase production by three *Lactobacillus* strains isolated from chicken crop. *J.Appl.Bacteriol.* **55**, 487–493 (1983).

CHANDRA A.K., MEDDA S., BHADRA A.I.C.: Production of extracellular thermostable α-amylase by *Bacillus licheniformis*. *J.Ferment.Technol.* **58**, 1–10 (1980).

COLLINS C.H., LYNE P.M.: *Microbiological Methods*. Butterworth and Co., New York 1976.

FISHER E.H., SUMMERWELL N.W., JUNGE J.M., STEIN E.A.: *Bacillus subtilis* α-amylase properties. *Proc.Internat.Congr.Biochem.* **8**, 124 (1960).

GHOSH H., CHATTERJEE B., DAS A.: Purification and characterization of glucoamylase of *Aspergillus terreus* NA-170 mutant. *J.Appl.Bacteriol.* **71**, 162–169 (1991).

HARBORNE J.B.: *Phytochemical methods*. Chapman and Hall Publ., London 1984.

HASEGAWA A., MIWA A., OSHINA T., IMAHORI K.: Studies on α-amylase from a thermophilic bacteria. I. Purification and characterization. *J.Biochem.* **79**, 469–477 (1976).

HOPKINS R.H.: Action of the amylases. *Adv.Enzymol.* **6**, 389–414 (1946).

INGLE M.B., ERICKSON R.J.: Bacterial α-amylase. *Adv.Appl.Microbiol.* **24**, 257–278 (1978).

MORRIS J.G.: *A Biologist's Physical Chemistry*. Edward Arnold, London 1971.

OKAFOR N.: Microorganisms asociated with cassava fermentation for gari production. *J.Appl.Bacteriol.* **42**, 279–284 (1977).

PFUELLER S.L., ELLIOT W.H.: Extracellular α-amylase activity in avocado fruit pulp. *J.Amer.Soc.Hortic.Sci.* **103**, 673–675 (1969).

SEN S., CHAKRABARTY S.L.: Amylases from *Lactobacillus cellobiosus* isolated from vegetable wastes. *J.Ferment.Technol.* **62**, 407–413 (1984).

TSVETKOV V.T., EMANUILOVA E.I.: Purification and properties of heat stable α-amylase from *Bacillus brevis*. *Appl.Microbiol. Biotech.* **31**, 246–248 (1989).

WELKER N.E., CAMPBELL L.L.: Effect of carbon sources on formation of α-amylase by *Bacillus stearothermophilus*. *J.Bacteriol.* **86**, 681–686 (1978).