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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF A RAW STARCH DIGESTING ALPHA-AMYLASE FROM THE DIGESTIVE JUICE OF THE SNAIL *LIMICOLARIA FLAMMEA* (MÜLLER 1776)

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ABSTRACT

An α-Amylase was purified to homogeneity from the digestive juice of *L. flammea* by Sephacryl S-200 HR gel filtration, DEAE-Sepharose CL-6B anion exchange and Phenyl-sepharose CL-6B hydrophobic interaction chromatography, with a 19.55-fold increase in specific activity and 7.8 % recovery. The molecular weight of the α-Amylase was estimated to be 62 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. The optimum temperature and pH were 45°C and 5.0 respectively. The purified enzyme belonged to the EDTA-non sensitive α-amylase, but its activity was slightly stimulated by the presence of Ca^{2+} ions. The end-products of starch hydrolysis were glucose, maltohexaose and mor e oligosaccharides.

INTRODUCTION

α-Amylases (endo-1,4 α-D-glucan glucohydrolase; E.C. 3.2.1.1) are among the most important enzymes in present-day biotechnology. This family of enzymes finds potential applications in a wide range of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries (Sajedi *et al*., 2004). These enzymes also play an important role in microbials, animals and plants carbohydrates metabolism (Hsieh, 2008). The amylolytic enzymes are composed of the following components: α-amylase (EC 3.2.1.1); β-Amylase (EC 3.2.1.2); glucoamylase (EC 3.2.1.3); iso amylase (EC 3.2.1.68); pullulanase (EC 3.2.1.41) and α-glucosidase (EC 3.2.1.20). Bulk enzymatic extracts are preferred for commercial applications but pharmaceutical and clinical formulations need highly purified enzymes (Sivaramakrishnan *et al*., 2006; Gangadharan *et al*., 2006; 2008). The main rule of the α-amylase is the saccharification of starch to glucose and this way is preferred to chemical hydrolysis because it offered many advantages such as low coast, reaction specificity and higher yields (Antranikian, 1992). Industrial enzymatic starch hydrolysis expect thermostable (90-105°C) amylases for simultaneous gelatinization and liquefaction processes (Uma *et al*., 2009; Chi *et al*., 2009). Therefore research for new thermostable and high yield enzymes including genetics to produce them is of great interest (Gupta *et al*., 2003). Amylases of many microorganisms have been studied to find enzymes corresponding to these applications (Van Der Maarel, 2002; Pardeep *et al*., 2010; Hmidet *et al*., 2009). Recently, the importance of enzymatic hydrolysis of raw starch without heating has become well recognized, mainly from the viewpoints of energy savings and effective utilization of the biomass, thereby reducing the cost of starch processing (Singh and Soni, 2001; Fu *et al*., 2005).

This has generated a worldwide interest in the discovery of several raw starch-digesting amylases that do not require gelatinization and can directly hydrolyze the raw starch in a single step below the gelatinization temperature (Kelly *et al*., 1995; Yetti *et al*., 2000; Zhang and Chi, 2007). Most of enzymes studied for raw starch digestion are reported for microorganisms and recombinants (Hostinova *et al*., 2003; Knox *et al*., 2004; Chi *et al*., 2009). Among animals, many reports indicated the presence of hydrolyzing enzymes in the digestive juice of snails (Colas, 1977). In the present study, we report on the purification and the characterization of a raw starch digesting α-amylase purified from the digestive juice of the snail *Limicolaria flammea (Müller 1776)*. The properties of the enzyme including the raw starch digestibility were reported.

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MATERIAL AND METHODS

Enzymatic source

The digestive juice of the snail *Limicollaria flammea (Müller 1774)* was the enzymatic source. The snails are grown in the University of Nanguy Abrogoua (Côte d'Ivoire). The digestive juice of the snail *Limicolaria flammea (Müller 1774)*, was extracted from three days unfed snails. The shell was broken and the digestive tube was isolated. The digestive juice was collected in the erlenmeyer by successive pressions on the digestive tube, centrifuged (10 000 g, 30 min, 4°C) and the supernatant was conserved at 4°C with sodium azide (0.02 % : w/v) as preservative.

The raw starch

The raw starches of corn, cassava and young growing palmyrah Palm were extracted according to the method of Banks and Greenwood (1975) modified by Amani (1993)

Chemicals

From Sigma Chemical Company

Soluble starch, ammonium persulfate, acrylamide, N,N'-methylene-bis acrylamide, Tris, chlorhydric acid, N,N,N'N' tetramethylethylenediamine (T.E.M.E.D), β-mercaptoethanol, Sodium Dodecyl Sulfate (S.D.S.), glycerol, bromophenol blue, glycine, Coomassie blue R-250, methanol, acetic acid, sodium acetate.

From Prolabo

Sodium thiosulfate, Folin-ciocalteus, D (+) anhydre glucose, ammonium sulfate, copper sulfate, sodium carbonate, sodium hydroxyde.

From Pharmacia Biotech

Sephacryl S-200 HR, DEAE-Sepharose CL-6B, and Phenyl-sepharose CL-6B gels.

Amylase activity and protein concentration essays

α-Amylase activity was measured by the dinitrosalicylate (DNS) method of Bernfeld (1955). The reaction mixture (0.3 mL) in 0.05 M acetate buffer (pH 5.0) contained 0.1 mL of 1% starch and 0.05 mL of enzyme solution. The mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 0.3 mL of dinitrosalicylic acid solution. The reaction mixture was then heated in boiling water for 5 min, and the absorbance at 540 nm was measured in a spectrophotometer (Spectra physics) after cooling in ice and diluting with 3 mL of distilled water. The reducing sugars released by the enzyme were estimated from a glucose standard graph. One unit of α-amylase activity was defined as the amount (µmol) of reducing sugar released by minute under standard assay conditions. Protein concentration was measured with Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Purification procedure of the alpha amylase

Sephacryl S-200 HR gel chromatography

One ml of the digestive juice was applied onto a Sephacryl S-200 HR column (1.6 x 63 cm) previously equilibrated with acetate buffer 20 mM (pH 5.0), and proteins were eluted with the same buffer at a flow rate of 0.25 ml/min. one (1) ml fractions were collected.

DEAE-Sepharose CL 6B Fast Flow

The active enzyme fractions were pooled and applied onto a DEAE-Sepharose CL 6B Fast Flow column (3.14 x 7 cm) previously equilibrated with sodium acetate buffer 20 mM (pH 5.0). After washing through all unbound proteins, the enzyme was eluted by step gradient of NaCl (0 to 1 M) in the same buffer at a flow rate of 2 ml/min. Fractions of 2 ml were collected. The active fractions were dialyzed and concentrated.

Phenyl-Sepharose CL 6B chromatography

Sodium thiosulfate was mixed to the concentrated active fraction of enzyme to reach 1.7 M and loaded on a phenyl-Sepharose CL-6B column (1,5 x 4,5 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1.7 M sodium thiosulfate. The active fractions were eluted with a multiple steps gradient of sodium thiosulfate from 1.7 M to 0 M in the same buffer at a flow rate of 0.5 ml/min. The active fractions were dialyzed against 20 mM sodium acetate buffer (pH 5.0) and used for the characterization as the pure enzyme.

Determination of molecular weight and homogeneity

The relative molecular weight of the native purified enzyme was estimated by gel Filtration on a Sephacryl S-200 HR column (1.6 x 63 cm) using β-amylase (204100Da), bovine serum albumine (67000), amyloglucosidase (62900 Da), ovalbumine (45000 Da), lysozyme (20800), cytochrome C (12400 Da) as MW markers. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the α-amylase as described by Laemmli (1970). Proteins were silver stained (Blum *et al*., 1987). The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Amersham Biosciences) consisting of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa)

Effect of pH and temperature on the activity and the stability of the enzyme

The effect of pH on α-amylase activity was performed in the pH range of 3.5-8.0 at 37°C. For the measurement of pH stability, the enzyme was incubated at 37°C for 2 hours in different buffers and the residual activity was determined under the enzyme assay conditions. The following buffer systems were used: sodium acetate buffer 100 mM, pH 3.6-5.50; phosphate buffer 100 mM, pH 5.6-8.0; citrate buffer 100 mM, pH 3.5-8.0. The effect of temperature on α-amylase activity was determined in 100 mM sodium acetate buffer (pH 5.0) over the range of 35-80°C under the standard assay conditions. Thermal stability of the purified enzyme was examined by pre-incubating the enzyme aliquots (100 µl) at temperature range of 40-70 °C for 80 min. Then aliquots were cooled in ice and the remaining activity was measured at 37°C in 100 mM sodium acetate buffer pH 5 under enzyme assay

conditions. The non-heated enzyme activity was taken as 100%.

Substrate specificity

The purified enzyme was examined for its ability to hydrolyze various carbohydrates such as corn starch, amylose, glycogen, cellulose, hemicelluloses at a final concentration of 1% (w/v) in 100 mM sodium acetate buffer (pH 5). Enzyme activity was measured under the standard assay conditions. The hydrolysis of the soluble starch was taken as 100%.

Effect of metal ions and chemicals on the enzyme activities

The effects of various metal ions and reagents at 1 mM on α-amylase activity were determined by preincubating the enzyme with the individual reagents in 100 mM sodium acetate buffer pH 5 at 37°C for 30 minutes. Activities were then measured at 37°C in the presence of the metal ions or reagents. The enzyme activity without metal ions or reagents was recorded as 100%.

Kinetic parameters

Kinetic parameters (Km and Vmax) were determined from the Line weaver and Burk (1934) plots. The activity was determined in 100 mM sodium acetate buffer pH 5 with the substrate concentration varying from 0.25 to 0.75 % (w/v) under the standard assay conditions.

Enzyme adsorption

The adsorption rate on different starches was determined. A protein concentration of 10U was mixed with starch 1% (w/v) in the final volume of 5 ml buffered at pH 5.0 with 50 mM sodium acetate buffer. The resulting samples were shaken at ambient temperature (25°C) for 20 min and then centrifuged 15 min at 5000 g to sediment the insoluble substrate. The residual activity in the supernatants (A) was measured. The adsorption rate (AR) was defined by the following equation (Mitsuiki *et al*., 2005):

AR (%) = [(B-A)/B] x 100 where B represents the activity in the original enzyme solution.

Raw starch concentration effect on enzyme activity

Hydrolysis process was carried out by varying the starch concentration from 1% to 20% (w/v) in the reaction mixture (total volume of 5.0 mL) containing 10 U enzyme preparation at 40°C and 110 rpm. After 6 hours incubation, aliquots were withdrawn, boiled in hot water to stop reaction, and centrifuged at 5 000 g for 10 min. The reducing sugars in the supernatants were quantified (DNS method) to determine the extent of starch hydrolysis.

Raw starch hydrolysis

The raw starch grains of corn, cassava and palmyrah palm were desiccated. Each starch grain was suspended 1% (w/v) in a buffered (100 mM acetate buffer, pH 5.0) total volume of 8.0 mL containing 1 ml of enzyme solution (15 U). The mixtures were incubated in rotary water bath shaker at 40°C and 110 rpm. After 3h, 6h and 12 h, aliquots were taken, heated in hot water to stop reaction and centrifuged at 5 000 g for 10 min. The reducing sugars in the supernatant were quantified (DNS method). The extent of hydrolysis of raw starch (Rh) was defined by the following formula (Mitsuiki *et al.,* 2005): Rh (%) = (A_t/A_o) x 100, where A_t was the amount of sugar in the supernatant after the hydrolysis reaction and A_0 was the amount of raw starch before the reaction.

Identification of the reaction products

The products of the starch enzymatic hydrolysis were subjected to Thin-Layer Chromatography (TLC) with a precoated silica gel plate 60 (10 × 5 cm, Merck, Germany) according to the method of Hsieh and al. (2008). Developed in a solvent system composed of butanol/acetic acid/water (9:3,75:2,25 v/v/v), the spots were visualized by spraying TLC plates with sulfuric acid/ethanol (1:1 v/v) followed by heating at 105°C for 5 min.

Microscopic analysis of the raw starch

All starch grains were washed two times with pure ethanol before and after the enzymatic hydrolysis. After air dried they were examined by scanning optical microscopy (mark Ceti) at an adequate (x 400) magnification. The microscope is controlled by a computer via the kappa software that allows direct pictures taking.

RESULTS AND DISCUSSION

Purification of the alpha amylase

Chromatography of the crude enzyme on Sephacryl S-200 HR column gave two active peaks of amylase activity (Fig.1). The fractions of the highest peak of α-amylase activity were pooled and applied on a DEAE sepharose CL-6B column (Fig. 2). Unbound proteins exhibited no α-amylase activity. Only the peak of proteins eluted by 0.25 M NaCl exhibited α-amylase activity. When loaded on Phenyl-Sepharose CL 6B, the dialyzed and concentrated α-amylase fraction reached to three proteins peaks. The only one with amylase activity was eluted at 0.5 M ammonium thiosulfate gradient step (Fig. 3). This final step provided about 19.55 fold purification of the enzyme over the crude extract (Table 1). The specific activity (1109.2 U/mg) was inferior to that of *Bacillus sp thermophile* (1483 U/mg) (Mamo and Gessesse, 1999) or *Eleusine coracana* (1773 U/mg) (Nirmala and Muralikrishna, 2003). But this activity is higher than the α-amylases of *Nocardiopsis sp* 7326 (548 U/mg) (Zhang and Zeng, 2008) and *Haliotis sieboldii* (853 U/mg) (Hsieh *et al*., 2008).

Effect of pH and temperature on the activity and the stability of the enzyme

The purified enzyme was highly active between pH 4.50 and 5.50 with an optimum at pH 5. However, the activity increased from pH 3 to 4.5 and decreased behind pH 5. The purified α-amylase has the same optimal pH than the glucoamylase of *Bacillus sp.* (Gill and Kaur, 2004) and its optimum pH is lower than pH 7 exhibited by the α-amylase of *thermobifida fusca* (Chao-Hsun *et al*., 2009). The optimal pH of the purified α-amylase is in the range (pH 5-7) reported for most of

Table 1. Summary of purification steps of *L. flammea* α-amylase

Purification steps	Total protein (mg)	Total activity (UI)	Specific activity (UI/mg)	Purification fold	Yield (%)
Digestive juice	218	12365	56,72	1	100
Sephacryl S-200 HR	40	10725	268,12	4,73	86,74
DEAE Seph. CL 6B	9	2736	304	5,36	22,13
Phényl-seph CL 6B	0,87	965	1109,2	19,55	7,8

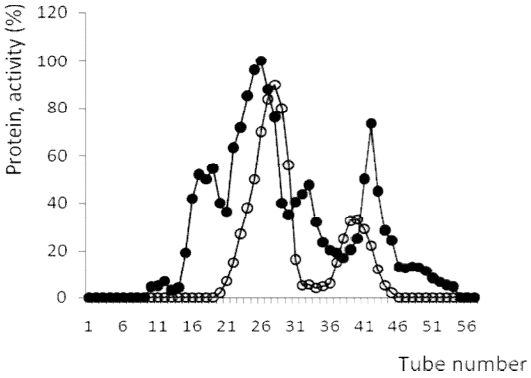


Figure 1. Gel filtration chromatography of the α-amylase on a sephacryl S-200 HR column previously equilibrate with 20 mM acetate buffer pH 5. Proteins were eluted with the same buffer (0.25 ml/min) and 1 ml fractions collected.
(Absorbance: -●-; Activity: -o-)

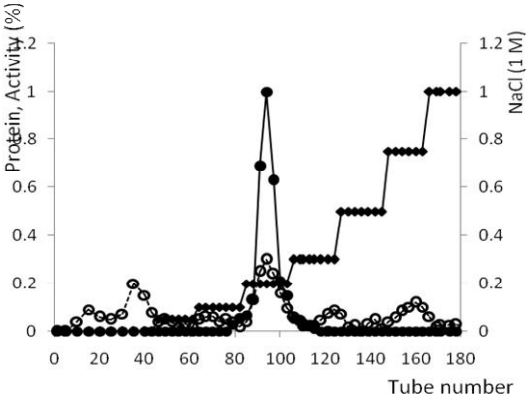


Figure 2. Anion exchange chromatography of the α-amylase A1 on DEAE-Sepharose CL 6B column previously equilibrated with 20 mM acetate buffer pH 5. Elution (2ml/min) with the same buffer containing step gradient (0 to 1 M) of NaCl. Fractions of 2 ml were collected. (Absorbance: -o-; Activity: -●- ; NaCl: -♦-)

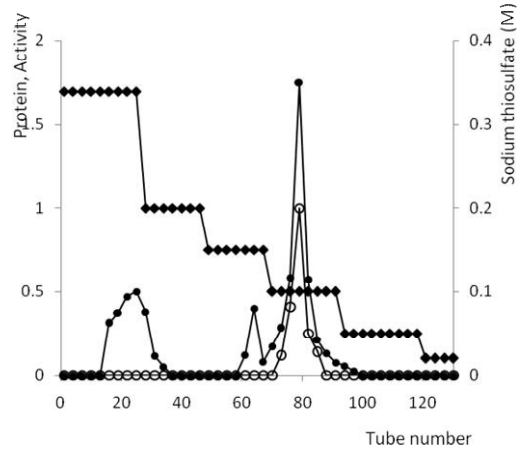


Figure 3. Hydrophobic interaction chromatography of the α-amylase A1 on Phenyl-Sepharose CL 6B column previously equilibrate with 20 mM acetate buffer pH 5 containing 1.7 M Sodium thiosulfate. Elution (0.5 ml/min) by a step gradient (1.7 to 0 M) of sodium thiosulfate.
(Absorbance: -●-; Activity: -o-; Sodium thiosulfate: -♦-)

α-amylases (Leveque *et al*., 2000). The α-amylase was very stable at pH 4.0 to 6. In that range of pH 100 % of activity was retained after 2 h incubation at 37°C (Fig 5). This range of pH stability was included in that of the α–amylase of *Nestereukonia sp* (pH 4-10) (Shafiei *et al*., 2010). However, the activity stability decreased sharply above pH 6 and was 55 % and 0 % of the initial activity at pH 6.50 and 7.5 respectively. The purified α-amylase had optimum activity at 45°C. The activity of α-amylase fell sharply at temperatures above 45°C. This temperature was similar to that of *Streptomyces gulbargensis* (Dastager *et al*., 2009), lower than optimal temperature of the α-amylase from *Pyrodyctium abyssi* (100°C) (Siquiera *et al*., 1997) and higher than that of the antarctic *Nocardiopsis sp.7326* (35°C) (zhang and Zeng, 2008). The relative activities at 40 and 50°C were about 80% and 40%, respectively.

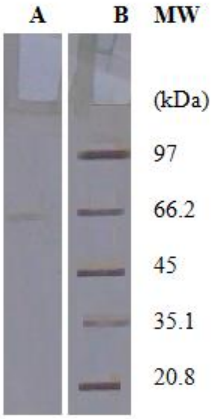


Figure 4. SDS-PAGE for homogeneity and molecular weight determination of *L. flammea* α-amylase. Lane A: α-amylase A1; Lane B: MW markers

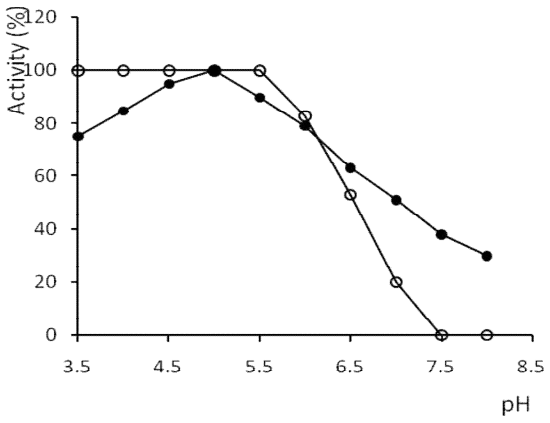


Figure 5. pH influence on activity and stability of α-amylase A1 The pH profile was determined in different buffers by varying pH values (3,5 to 8,0) at 37°C. The maximum activity obtained at pH 5.0 was considered as 100%. The pH stability of the α-amylase was determined by incubating the enzyme in different buffers for 2 hours at 37°C and the residual activity was measured at pH 5.0 and 37°C.
(Relative activity : -●-; remaining activity : -o-)

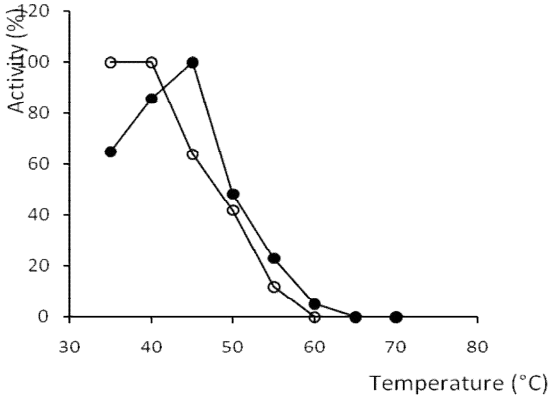


Figure 6. Effect of temperature on activity and stability of the purified α-amylase 1 The temperature profile was determined by assaying enzyme activity at various temperatures values (35 - 70° C) at pH 5. The activity of the enzyme at 45° C was taken as 100 %. The temperature stability was determined by preincubating the purified enzyme at different temperatures for 80 minutes. The residual enzyme activity was measured at 37° C and pH 5.
(Relative activity: -•-; remaining activity : -o-)

For investigation of thermal stability, the purified enzyme was incubated 1 h at temperatures ranging from 35 to 80°C (Fig.6). No loss of the activity was observed after a 1 hour incubation at 35 and 40°C. At 50°C, the enzyme retained 42% of its initial activity. However, the activity was lost at 65°C. The purified enzyme was less active under the effect of the temperature than the amylases of *Bacillus NRRLB14368 licheniformis* (81% to 90°C) (Bose and Das 1996), of *Tricholoma matsutake* (95% to 50°C) (kusuda *et al*., 2003) and of the amylases (80% to 65°C) commercial used in the oil wells (Kyaw *et al*., 2009).

Substrate specificity of the α-amylase activity

Among substrates tested the purified α-amylase was active against starch, glycogen and amylose. No activity was observed in the presence of cellulose and hemicellulose (Table 2). These results are in agreement with that of the α-amylases of *Pachyrhizus erosus* (Noman *et al*., 2006) and *Chromohalobacter sp TVS P101* (Prakash *et al*., 2010) indicating the specificity for the α-anomeric α (1-4) linkage.

Table 2. Substrate specificity of the purified α-amylase

substrates	hydrolysis
Starch	+
Glycogen	+
Cellulose	-
hemicellulose	-
amylose	+

(+): hydrolyzed (-): non hydrolyzed

Effect of metal ions and reagents on the α-amylase activity

Although Na^+ did not affect the enzyme activity, Mg^{2+}, Zn^{2+}, Ca^{2+} had little effect on enzyme activity (Table 3). Inhibition by Hg^{2+} and Cu^{2+} on A1 activity was also noticed with the α-amylases of *Streptococcus bovis 148* (Satoh *et al*., 1997) and *Bacillus Halodurans MSZ-5* (Murakami *et al*., 2008). Mn^{2+} greatly increased the enzyme activity. Observation that ion Hg^{2+} inhibited the enzyme suggested that the thiol group was essential for the α-amylase activity (webb, 1966; Yang and Liu 2004) as confirmed by PCMB inhibition. Ion inhibitions are reliable to interactions between the ion and enzyme active site functional groups (Nirmala and Muralikrishna, 2003) or the competition for the calcium ion site on the enzyme (Shaw and Ou-Lee 1984). The chelating agent EDTA did not affect the enzyme activity indicating that α-amylase A1 was not a metalloenzyme (Kiran Chandra 2008). The severe inhibition of SDS (about 96%) confirmed the destruction of α-amylase protein structures. The increase of activity when mercaptoéthanol was tested is in agreement with its thiol groups protection rules.

Table 3. Effects of various metal ions and reagents on α-amylase activity

	Conc. (mM)	Relative activity (%)
Reference	0	100
Na^+	1	102
Sr^{2+}	1	135,38
Mg^{2+}	1	83,92
Ca^{2+}	1	116,08
Ba^{2+}	1	123,98
Zn^{2+}	1	96,75
Cu^{2+}	1	2,63
Mn^{2+}	1	241,23
Ni2+	1	80,99
Hg2+	1	23,21
DTNB	1	142,11
5PCMB	1	51,99
SDS	1	3,30
Mercaptoethanol	1	143,27
EDTA[a]	1	100,2

The activity of the α-amylase was determined by incubating the enzyme in the presence of various metal ions (1 mM) for 10 min at 37°C and pH 5

Kinetic parameters of the purified α-amylase

The α -amylase hydrolyzed corn starch with a V_{max} and apparent K_M values of 8 mg/min and 3.57 mg/ml respectively. This K_M value is low when comparing to that of *Eleusine coracana* (5,9-14,3mg/ml) (Nirmala and Muralikrishna, 2003) α -amylase but it remained in the range (0,35 to 11,66mg/ml) of most amylases (Najafi and Kembhavi, 2005).

Effect of raw starch concentration on the activity of the purified α-amylase

Maximum hydrolysis rates at 26%, 11% and 4% were obtained respectively for corn, cassava and palmyrah palm at 1% w/v starch concentration.

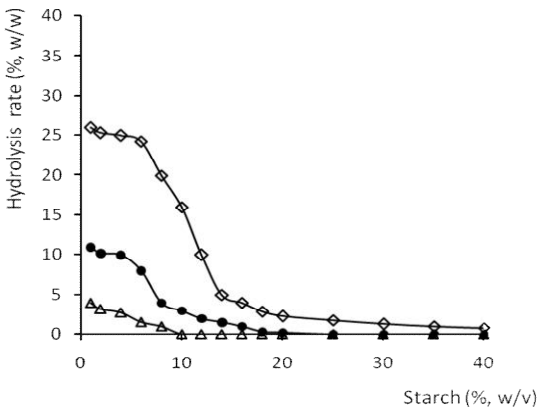
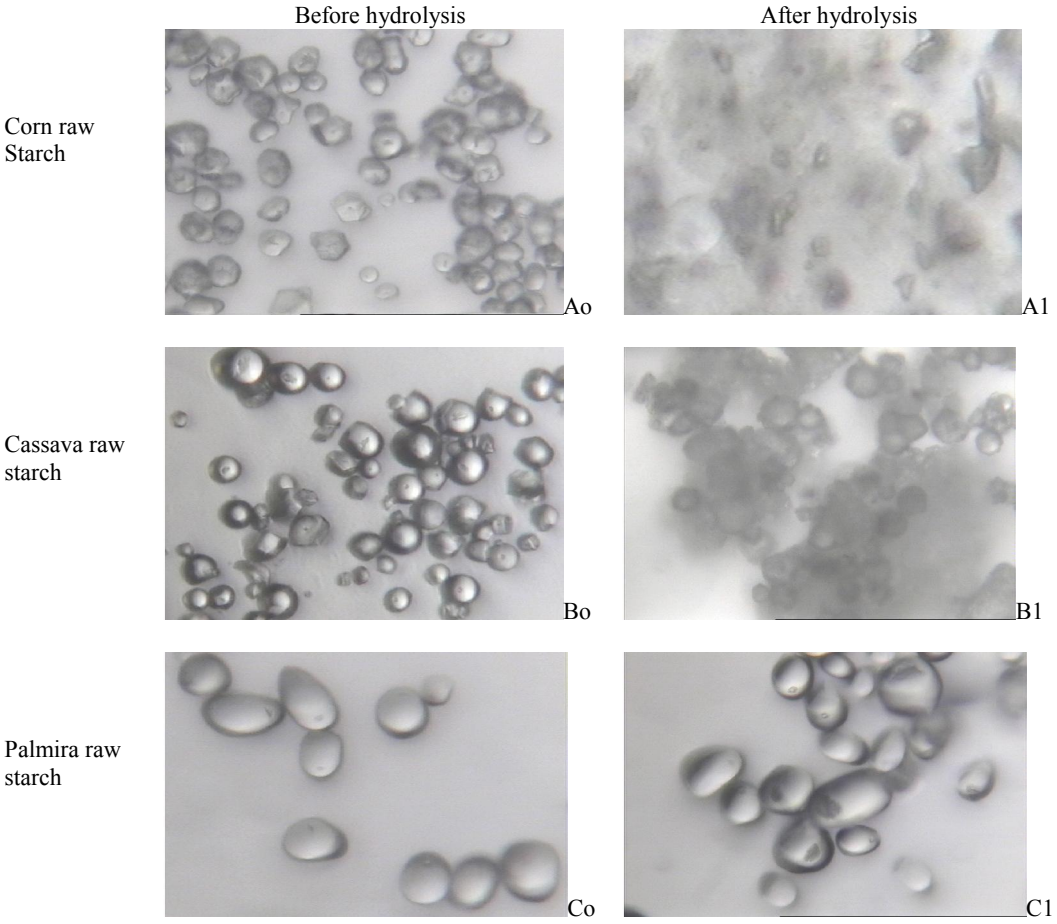


Figure 7. Effects of starch concentrations on hydrolysis extent Temperature 37°C, buffer 0,1 M acetate buffer pH 5, shaking speed 110 rpm, enzyme concentration 15 U/ml, reaction time 6h
(corn : -◊-, cassava :-•-, palmyrah palm : Δ)

Table 4. Hydrolysis rate of raw starches by purified raw starch-degrading amylase of *L. flammea*

Time (Hrs)	3			6			12		
Starch	Palmirah	cassava	corn	Palmir	cassava	corn	Palmira	cassava	corn
Hydrolysis (%, w/w)	0	0	0,8	4	14,56	29,0	6	17	38

(raw corn, cassava or palmyrah palm starch at 1%) in 0.1 M acetate buffer pH 5. Reaction mixtures (5 ml) contained 1ml of purified enzyme and substrate raw starches, incubated at 40 ◦C for 12 h



Photography: Scanning optical micrographs (x 400) of native and enzyme-treated starches. (Ao, Bo, Co) Native starch granules; (A1, B1, C1) starch granules hydrolyzed by α-amylase for 12 h.

The results in Fig.7 indeed demonstrate that more reducing sugars were released from corn starch than from cassava starch or palmyrah palm starch by the purified α-amylase when increasing the concentration from 1 to 40% (w/v). From 1 to 6% (w/v) the hydrolysis extent of corn starch by the amylase was about 26 % then it decreased sharpely to 5.0% when the concentration of corn starch was increased from 6% to 14.0% within 6 h reaction. The hydrolysis extent decreased slightly from 5.0% to 1% when the concentration of cassava starch increased from 14.0% to 40.0% (w/v). Same hydrolytic profiles were observed with cassava or palmyrah palm starches. The rate of hydrolysis of corn starch by the α-amylase is lower than that (53%) reached in eight hours by the amylase of Bacillus *sp YX-1*(Liu and XU., 2008), however the rate of the A1 amylase is better than that (14%) reached in 24 hours by the amylase of *Thermobifida fusca NTU22* (Yang and Liu., 2004) for corn raw starch hydrolysis.

Purified α-amylase adsorption on raw starches

Among the raw starches, the adsorption rate was highest in the case of corn starch (53%) followed by cassava starch (51%) while adsorption was lower for the palmyrah palm starch (38%). These adsorption rates are smaller than the rate of *Bacillus amyloliquefaciens* (81%) amylase on soluble starch (Gangadharan *et al*., 2009) and high than *Bacillus substilis IF 012114* on corn starch (10%) (Mitsuiki *et al*., 2005). Regarding our results this indicates a strong correlation between the adsorption and rate of hydrolysis even if some amylases hydrolyzing their substrates without adsorption are reported (Hamilton *et al*., 1998).

Raw starch hydrolysis by the purified α-amylase

The optical microscopy scanning of the raw starch kernels showed small, round and rough granules for corn (photo Ao), round and smooth with many sizes granules for cassava (photo Bo) and the palmyrah palm showed big, oval and very smooth granules (photo Co). After the enzymatic treatment for 12 hours, the progress of degradation was not homogenous (Table 4). The hydrolysis rate increased more during 0 to 6 hours of reaction than after (6 to 12 hours). Thus, starches were rapidly digested with enzyme at the first stage of hydrolysis. Similar observations on hydrolysis have been reported in barley and quinoa starches (Tang *et al*., 2002a,b; Vasanthan and Bhatty,1996). Different rates in the early and the later stages of

Figure 8. Thin-layer chromatography analysis of the main hydrolysis products of corn raw starch by the purified a-amylase

1. Starch hydrolyzed by the α–amylase, 2. maltose, 3. glucose.

hydrolysis can be understood by considering that the enzymes initially attack the more amorphous regions of the starch granules, whereas the less accessible crystalline regions are hydrolyzed at a slower rate. Optical microscopy scanning showed that most of the corn starch granules lost their structure and shell residues resulting from total digesting of the inner parts of granules (photo A1). The cassava starch granules were partially destroyed and their surfaces were rough. Both entire and disrupted granules were observed in the same sample (photo B1). The surfaces of the bigger palmyrah palm starch granules remained smooth and some granules displayed cavities near the hylum (photo C1). These results confirm that the raw starch hydrolysis depends not only on the enzyme physico-chemical properties but also on the type of the starch granule (Yetti *et al*., 2000). The monosaccharides and oligosaccharides were detected in the hydrolysate (Fig.8) of the three samples of starch by the purified α-amylase. The hydrolysis of starches yielded glucose, maltose, and more DP oligosaccharides as end products. This result is in conformity with that of amylases of *Bacillus licheniformis NCIB 6346* on the soluble starch (Morgan and Priest, 1981), of *Eisenia foetida* on the crude starch of rice (Ueda *et al*, 2008). However, the amylases of *Bacillus halodurans LBK 34* released in majority only the maltohexose (Hashim *et al*, 2005) and those of *Bacillus sp Kms-1378* gave maltooligosaccharides (Higarashi *et al*., 1998). The amylase of *Tricholoma matsutake* did not release glucose after hydrolysis of amylose (Kusuda *et al*, 2003).

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