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Research article

ACTION OF THE DIGESTIVE JUICE OF THE SNAIL *LIMICOLARIA FLAMMEA* ON RAW STARCHES.

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ABSTRACT: This study reports the action of digestive juice of snail *Limicalaria flammea* on raw starches. This juice exhibited maximum activity at pH 4.0-5.5 and was optimally active at 40-50 °C. About 82.5 % of the original amylasic activity still remained after treatment at 50 °C for 16 h. The digestive juice had a strong digesting ability towards various raw starches and efficiently hydrolyzed raw corn starch at a concentration of 1.0% and pH 5 at 40 °C in a period of 12 h. The rate of hydrolysis of the raw corn and cassava starches were 64.37 % and 42.0 % respectively while that of palmyrah palm was 29.05 %. The analysis of the hydrolyzed products of the raw starches by thin layer chromatography showed the glucose, maltosaccharide and dextrin after 12 h of hydrolysis.

Keywords: raw starch, *Limicolaria flammea*, digestive juice

INTRODUCTION

α-Amylase (E.C 3.2.1.1) is an extracellular enzyme which is widespread among higher plants, animals and microorganisms. It catalyses the hydrolysis of α-D-(1,4) glycosidic linkages in starch components and related carbohydrates. This enzyme is used in desizing fabrics, in the baking industry, pharmaceuticals and detergents (Gupta et al., 2003; Pandey et al., 2000). The α-amylases with desirable properties, for example, low pH stability, raw starch digestibility and utilization of high concentration of starch, can be very useful in related applications. Therefore, the amylases which can be able to hydrolyze the native starch are much required. The native starch being hydrolyzed at high temperatures, the thermostable enzymes were especially required in the micro-organisms. It has so far been reported that fungi and yeast, such as Aspergillus sp. (Matsubara et al., 2004), Rhizopus sp. (Morita and Fujio, 2000), and Cryptococcus sp. (lefuji et al, 1996), were good producers of raw starch digesting amylases. Different methods such as genetic engineering were used to produce efficient amylases on native starches. However, few studies were carried out on the hydrolysis of raw starch by the amylases of snail. Here we report the degradation of corn, cassava and palmyrah palm raw starches by the digestive juice of snail *Limicolaria flammea*.

MATERIALS AND METHODS

Enzymatic source

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 snails are grown in the University of Abobo-Adjame (Côte d'Ivoire).

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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).

Amylase activity and protein concentration assays

The amylase activity was assayed by measuring the reducing sugar released during the reaction 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 enzymatic reaction was then stopped by the addition of 0.3 mL of dinitrosalicylic acid solution. After 5 min heating at 100°C for the color development, the resulted samples were chilled to room temperature and then diluted with 3.0 mL distilled water. The absorbance at 540 nm was then measured. 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.

Effect of pH on enzyme activity

The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers: 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). To determine the stability of amylase, the enzyme was pre-incubated in different buffers for two hours. The residual activity was determined as described earlier.

Effect of temperature on enzyme activity and stability

The temperature optimum of the enzyme was evaluated by measuring the amylase activity at different temperatures (35-80 °C) in 0.1 M sodium acetate buffer pH 5.0. The effect of temperature on amylase stability was determined by measuring the residual activity after 80 min of pre-incubation in 0.1 M sodium acetate buffer pH 5.0 at temperatures ranging from 40 to 70°C.

Thermal inactivation

Amylase was pre-incubated at various temperatures (45°C, 50°C and 55°C) in 0.1 M sodium acetate buffer pH 5.0. At given times (15 min), aliquots of the incubation mixture were removed, brought to room temperature, and assayed for enzyme activity as described earlier

Effect of raw starch concentration on amylase activity

Effect of raw starch concentration on hydrolysis was studied by varying its concentration from 1 % to 40 % (w/v) in the reaction mixture (total volume of 10.0 mL) containing 8 U of the purified amylase by shaking at 120 rpm at 40 °C. To determine the extent of starch hydrolysis, end products were measured after 8 hours of incubation as described above.

Raw starch hydrolysis

The reaction mixture (total volume of 10.0 mL) containing 1 % of raw starch and 10 U of the purified amylase in 0.1 M sodium acetate buffer pH 5.0 was incubated by shaking at 120 rpm at 40 °C. 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 as describe above.

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.

Analysis of hydrolytic end products

The end products of starch degradation by amylases 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 et 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.

Cold conservation of Limicolaria flammea digestive juice

The enzyme is conserved at the freezer and with interval of six months time, the amylasic activity is measured, this during two years.

RESULTS

Effect of pH and temperature

Limicolaria flammea digestive juice exhibited maximum activity at pH 4.0-5.5 and was optimally active at 40-50 °C. La caractérisation du suc digestif de l'escargot *Limicolaria flammea* a montré que l'activité amylasique est maximale à des pHs acides et à des températures de 40°C à 50°C. Leurs températures optimales sont inférieures à celle de l'alpha amylase de *Bacillus sp. PN5* (90°C) (Saxena et al, 2000) mais supérieures à celle de l'alpha amylase de *Bacillus sp L1711* (35°C) (Bernhardsdotter et al.,2005).

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Thermal inactivation

This study showed that no loss of the activity was observed after 16 hour of incubation at 37 and 40 °C. At 50°C, the digestive juice retained 82.5 % of its initial amylasic activity (Figure 1). It is more stable at the temperature of 50 °C than α-amylase (70 %) of *Bacillus amyloliquefaciens* (Demirkan et al.,2005) and α-amylase (72 %) of *Bacillus subtilis* (Asgher et al.,2007). However, this activity is less stable than α-amylase of *Bacillus cohnii US147* which keeps 100 % of its activity at 70 °C after 1 hour of incubation (Ghorbel et al., 2009) and the amylase of *Bacillus Stearothermophilus* which loses only 8.0 % of its activity at the temperature of 100 °C (Chakraborty et al., 2000).

Effect of raw starch concentration on the activity of the digestive juice

The percentages of hydrolysis of cassava and corn native starches decreased considerably from 37.0 to 13.0 % and 60.0 to 29.0 % respectively when the concentrations of these starches increase from 1.0 to 12.0 %. That of palmyrah palm raw starch decrease from 25.0 to 4.0 % in the same range of starch concentration (1.0-12.0 %). The hydrolysis extents of corn and cassava native starches vary less from 14.0 to 40 %. The effect of the raw starch concentration on the activity of the digestive juice of snail *Limicolaria flammea* showed that the increasing of starch quantity reduced the rate of hydrolysis. This phenomenon was also observed by certain authors. Thus, the hydrolysis extent rate of the native starch of potato by the glucoamylase of the yeast *Aureobasidium pullulans* decreased from 85,6 to 60.0 % when the starch concentration increases from 1.0 to 8.0 % (Li et al., 2007).

Raw starch hydrolysis by the digestive juice

The digestive juice of snail *Limicolaria flammea* was used to hydrolyze the native corn, cassava and palmyrah palm starches during 12 hours.After three hours of reaction, the three studied native starches were hydrolyzed with respective percentages of 25.8 %, 11.0 % and 4.82 % (Table 1). At twelve hours of incubation, these rates of hydrolysis are 64.37 % for the starch of corn, 42.0 % for the starch of the manioc and 29.05 % for the starch of the palmyrah palm.

Table 1: Hydrolysis rate of raw starches by the digestive juice of the snail *Limicolaria flammea*

Starches	Hydrolysis rate (%)		
	3 hours	6hours	12hours
Corn	25,8	59,66	64,37
Cassava	11	34,24	42
Palmyrah palm	4,82	23,6	29,05

(Raw corn, cassava or palmyrah palm starch at 1%) in 0.1 M acetate buffer pH 5. Reaction mixtures (10.0 mL) contained 10.0U of digestive juice and substrate raw starches, incubated at 40 ◦C for 12 h. shaking speed 120 rpm.

The action of digestive juice on the raw corn starch gives the highest percentage of hydrolysis compared to the native starches of cassava and palmyrah palm after 12 hours of incubation. For the same reaction time, the α - amylase of *Bacillus sp Yx-1* hydrolyzes the raw starch of potato with a percentage (63.2 %) very close to that of the snail *Limicolaria flammea* (Liu et Xu, 2008). On the other hand, the α-amylase of *Bacillus sp I-3* has a percentage of hydrolysis of the native starch of potato superior (90.0 %) to that of the snail *Limicolaria flammea* in only six hours of reaction (Goyal et al, 2005). For sufficiently long times of incubation, certain amylases have poor yield of conversion of the native starch such as the α-amylase (Amy II) of ground worm *Eisenia foetida* which hydrolyses the raw starches of corn and cassava respectively with 2.0 % and 1.22 % after 72 hours of incubation (Ueda et al., 2008). The susceptibility of the starch grains to be degraded by the enzymes depends on several factors. Crystallinity, the shape and the size of the starch grain as well as the enzymatic source are abundantly cited in the literature like factors of control of the enzymatic hydrolysis (Goyal et al., 2005 ; Noda *et al* ; 2008 ; Liu et Xu ; 2008). The enzymatic conversion of these starches is thus related to their physicochemical properties and that of the digestive juice of snail *Limicolaria flammea*. This hydrolysis showed that the variability of the starch grain size could influence the enzymatic attack considerably. Indeed, the starch of large dimension are less hydrolyzed than those which have a small size (Nidhi et al., 2005; Noda et al., 2005; 2008). This could be an explanation to the weak hydrolysis of the palmyrah palm starch which have large size with a lengthened form.

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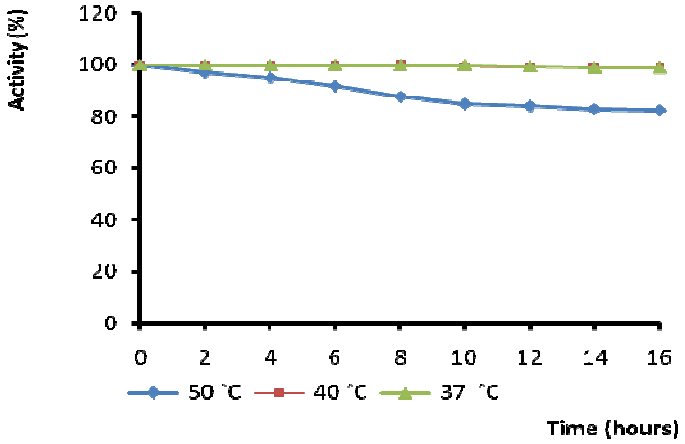

Figure 1: Thermal inactivation of the amylasic activity of the digestive juice of the snail *Limicolaria flammea* at 37°C, 40°C and 50°C.

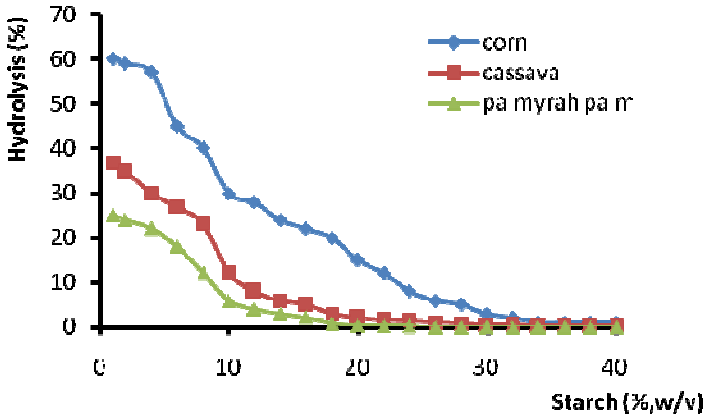

Figure 2: Effects of starch concentrations on hydrolysis extent
Temperature 40 °C, buffer 0,1 M acetate buffer pH 5, shaking speed 120 rpm, enzyme concentration 8 U/ml, reaction time 8 h

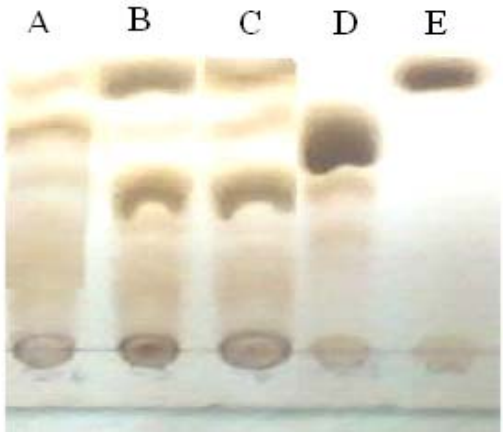

Figure 3: Thin-layer chromatography analysis of the main hydrolysis products of palmyrah palm, corn and cassava raw starches by the digestive juice of snail *Limicolaria flammea*.

A: palmyrah palm starch hydrolyzed; B: corn starch hydrolyzed; C: cassava starch hydrolyzed; D: maltose; E: glucose.

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Analysis of ends products of hydrolysis

The analysis of the end products of hydrolysis of the native corn, cassava, and palmyrah palm starches revealed the presence of glucose, maltose and the oligomaltoses (figure 3). The release of glucose during the hydrolysis of the starches by the digestive juice could be due to the action of an exo-amylase of the digestive juice and /or to that of the α-glucosidase which activity was detected in the digestive juice (no shown) of the snail *Limicolaria flammea*.

Cold conservation

The conservation by congelation at -18 °C of the digestive juice of snail *Limicolaria flammea* during two years does not modify the catalytic activities of amylase (Table 2).

Table 2: Effect of congelation at -18°C on the amylasic activities of the digestive juice of snail *Limicolaria flammea*.

Activity	Time of congelation (mois)				
	0	**6**	**12**	**18**	**24**
Digestive juice (%)	100	100	100	100	100

CONCLUSION

The digestive juice of snail Limicolaria flammea showed a raw starch digesting activity. This activity is stable at the temperature of 40 °C which is that of the conversion of the native corn, cassava and palmyrah palm starches. The rates of hydrolysis obtained are 64.37 % for corn, 42.0 % for cassava and 29.05 % for palmyrah palm after 12 hours of incubation.

The digestive juice has an interesting property which is that to be able to conserve the enzymatic activities after congelation for two years.

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