แหล่งใหม่ของอะไมเลสในใบกระถินอ่อน

A NOVEL SOURCE OF AMYLASE IN YOUNG *LEUCAENA* LEAVES

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บทคัดย่อ

อะไมเลสเป็นตัวเร่งปฏิกิริยาสำคัญในระบบการย่อยอาหารของมนุษย์ พืชหลายชนิดเป็นแหล่งของเอนไซม์ชนิดนี้แต่ยังไม่พบรายงานในใบกระถินเกษตร งานวิจัยนี้จึงทำการทดสอบเบื้องต้นกับใบกระถินอ่อนโดยวิธี agar well diffusion และนำมาหาสภาวะที่เหมาะสมต่อการทำงานของเอนไซม์อะไมเลสโดยการเปลี่ยนแปลงค่าพีเอช และอุณหภูมิ ผลการศึกษาพบว่าใบอ่อนของกระถินเกษตรมีเอนไซม์อะไมเลสเป็นองค์ประกอบ ของเหลวใสสีเหลือง ซึ่งได้จากการใช้เครื่องแยกกากถูกนำมาใช้เป็นตัวอย่างเอนไซม์สกัดหยาบโดยไม่มีการเจือจางแต่อย่างใด เมื่อนำไปทดสอบหากิจกรรมเอนไซม์ด้วยวิธี DNS colorimetric พบว่า อะไมเลสสามารถทำงานดีที่สุดที่ค่าพีเอช 5.8 และอุณหภูมิ 40 องศาเซลเซียส (9.54±0.17 ยูนิต/กรัม น้ำหนักใบสด) นอกจากนี้ที่อุณหภูมิ 60 องศาเซลเซียส พบว่า อะไมเลสจากใบกระถินสามารถทำงานได้ดีที่สุด ที่ค่าพีเอช 6.8 (8.24±0.00 ยูนิต/กรัม น้ำหนักใบสด) และค่าพีเอช 9.0 (8.30±0.17 ยูนิต/กรัม น้ำหนักใบสด) ส่วนที่อุณหภูมิ 70 องศาเซลเซียส พบว่า อะไมเลสจากใบกระถินสามารถทำงานได้ดีที่สุด ที่ค่าพีเอช 7.8 (8.97±0.17 ยูนิต/กรัม น้ำหนักใบสด) สิ่งที่ค้นพบยืนยันได้ว่าใบอ่อนของกระถินเกษตรซึ่งเป็นพืชหาได้ง่ายทุกพื้นที่ทั่วประเทศไทย สามารถเป็นแหล่งของอะไมเลสที่เป็นประโยชน์ด้านการเรียนการสอนทดแทนเอนไซม์ชนิดนี้ในน้ำลายมนุษย์ และเอนไซม์อะไมเลสทางการค้า

คำสำคัญ: เอนไซม์ อะไมเลสในพืช ใบกระถินอ่อน กระถินเกษตร

Abstract

Amylase is an important catalyst in the human digestive system. Plants are a source of this enzyme but there is no report of this enzyme for large *Leucaena* leaves or Krathin Kaset. The present study was divided into two parts of (1) detecting existing amylase using agar well diffusion, and (2) determining the optimal conditions of pH and temperature for enzyme activity of amylase in young large *Leucaena* leaves. Yellow clear supernatant was separated using a fruit extractor and used as a crude enzyme sample without dilution. Existing amylase in the enzyme sample was confirmed qualitatively by starch-agar well diffusion. The enzyme activity was determined, as a function of pH and temperature, using DNS colorimetric method. The optimum activity of the enzyme sample (9.54±0.17 U/g fresh weight) occurred at pH 5.8 and 40°C. Moreover, at 60°C, amylase worked best with starch solutions prepared in buffers pH 5.0 (8.24±0.00 U/g FW) and pH 9.0 (8.30±0.17 U/g FW). Similarly, enzyme activity of *Leucaena* amylase in buffer pH 7.8 (8.97±0.17 U/g FW) reached a maximum at 70°C. Large *Leucaena* leaves available anywhere in Thailand are proved as a useful teaching tool as a source of amylase from plant instead of that in human saliva, and commercial one.

Keywords: enzyme, plant amylase, young *Leucaena* leaves, Krathin Kaset

Introduction

Leucaena leucacephala (Lam.) de Wit belongs to the family Fabaceae (Pooma, 2016). *Leucaena*, Krathin (in Thai) and lead tree (English) are common names. It grows rapidly by nature and several of its parts are beneficial, such as the edible leaf and seed, seed oil used as biofuel, and biomass potential (Zakayo et al., 2000; Rengsirikul et al., 2015; Devi et al. 2013). Recently, antidiabetic and antioxidant activity in *Leucaena* seed extract has been established (Chowtivannakul et al., 2016). The nutritive value of leaves has been commonly recognized as a protein abundant meal for feedstock (Chanchay & Poosaran, 2009; Aye & Adegun, 2013). *Leucaena* leaf is used as an oyster accompaniment in Surat Thani province due to the ease of plant distribution for many decades. The white-scar oyster served with *Leucaena* leaf is always a highlight dish for oyster lovers because of their uncompromising taste.

The sweet sensory experience of fresh oyster occurs as a function of amylase enzyme in young small *Leucaena* leaves (Wongchuphan et al., 2018).

Main sources of amylase include microbes (Souza et al., 2010; Chaikulsareewath & Lampluarline, 2009; Tantipaibulvut et al., 2015) and digestive juices of animals and plants (Muralikrishna & Nirmala, 2005). Amylase can be found in finger millet (Nirmala & Muralikrishna, 2003), cereal grain (Stanley et al., 2005), germinated brown rice (Thanthong, 2010), Miswak *Salvadoraperica* or Khoy (name in Thai) (Mohamed et al., 2014), Namdogmai mango (name in Thai) or *Mangiferaindica* L (Luangborisut et al., 2012), and several types of fruit in Thailand (Luangborisut & Sittikityotin, 2012). The stability of the enzyme depends on many factors including pH, temperature and reaction time course.

Leucaena has been well-known and is classified by local people in Surat Thani province into small and large ones, referred to as "Krathin Ban" and "Krathin Pun" or "Krathin Kaset", respectively. The small *Leucaena* leaf is the most widely used as an oyster accompaniment and contains amylase (Wongchuphan et al., 2018). There are no reports mentioning whether large *Leucaena* leaf also has amylase. Thus, this plant is promising as an alternative and cheap source of amylase compared to commercial amylase, especially when used as instruction media for students. The objectives of this study were to investigate amylase activity of *Leucaena* leaf, and the optimal pH and temperature of *Leucaena* amylase.

Materials and methods

Chemicals used in this work were supplied by Merck and Sigma-Aldrich. Color reagent solution was prepared by mixing 8.0 ml of warm potassium sodium tartrate solution (12.0 g of $C_4H_4O_6KNa{\cdot}4H_2O$ in 8.0 ml of 2 M sodium hydroxide solution), 20 ml of warm 96 mM of 3,5-dinitrosalicylic acid (DNS) solution, and 12.0 ml of warm distilled water.

Soluble starch supplied by Ajax Finechem (UNIVAR analytical reagent, Australia) was prepared in 1.0% (w/v) in buffer for enzyme essay.

The malt 1,4-alpha-D-glucan-glucanohydrolase (α-amylase from malt, 1:2000 IP Units) was obtained from Hi Media Laboratories Pvt. Ltd, Mumbai, India.

Preparation of enzyme sample

Naturally growing and fresh young *Leucaena* leaves were collected randomly in Khunthale, Muang, Surat Thani (Coordinates: 9°8′11″N, 99°19′13″E) during the rainy season (February – March, 2019). The young leaves with 3 pairs of pinnae (300 g) were chopped and washed with distilled water, before extraction using a fruit extractor. After centrifugation at 6,000 rpm for 10 min at room temperature, an aliquot of the yellow clear supernatant (or crude enzyme) with no dilution was obtained and used immediately for further study.

Preliminary detection of amylase in enzyme sample

Sterile starch-agar well plates (4% soluble starch and 1.5% agar in buffer at pH 5.8, 6.8 or 7.8 were prepared to evaluate the amylase activity of enzyme samples. 50 μl of 0.1% (w/v) commercial amylase solution in buffer was used as a control. Enzyme samples were original crude supernatant (S1), 10-fold diluted (S2) and 100-fold diluted (S3) supernatants. 50 μl per well of each sample was added into the starch agar wells in the same plate. All plates were incubated at 37°C for 24 h, followed by staining with 0.2% potassium iodide-iodine solution. The enzyme activity was determined by measuring the diameter of individual clear zones.

Enzyme activity determination

Amylase activity was assayed according to the procedure of Bernfield (1955) with minor modification. Soluble starch solution (1.0% (w/v), 1 ml) in 0.3 ml of the working buffer (50 mM) was incubated with enzyme sample (0.1 ml) at 40°C for 3 min. After mixing well, the reaction was stopped by addition of 0.6 ml DNS color reagent and boiled for 10 min. After letting to cool, 10 ml of water was then added to each sample and the sample blank. Absorbance was measured at 540 nm using a UV-Vis spectrophotometer (Lambda-365 PerkinElmer, USA). All measurements were carried out in triplicate (n=3). Glucose was used as a standard. The enzyme activity was calculated as activity unit/g fresh weight (U/g FW). One unit of enzyme activity was defined as 1.0 mg glucose equivalent released from starch in 3 min at defined pH and temperature. Two factors; pH and temperature, were varied.

Effect of pH

pH of working buffers was varied from 4.0 to 9.0. The 50 mM buffers used were acetate buffer at pH 4.0 and 5.0, phosphate buffer at pH 5.8, 6.8 and 7.8, and Tris-HCl buffer at pH 9.0. Enzyme activity was monitored with the DNS assay.

Effect of temperature

To investigate the effects of temperature on enzyme activity, a constant amount of enzyme at optimum pH was used as mentioned above. Reaction temperature was varied from 40°C to 70°C for 3 min reaction each time.

Results and discussion

A crude extract was obtained about 20-30 ml per one hundred grams of fresh *Leucaena* leaves. The results from starch-agar well diffusion of enzyme sample revealed that young *Leucaena* leaf contains amylase. Starch digestion in the presence of *Leucaena amylase* designated as S1, S2 and S3, and commercial amylase labeled as a control is shown in Figure 1.

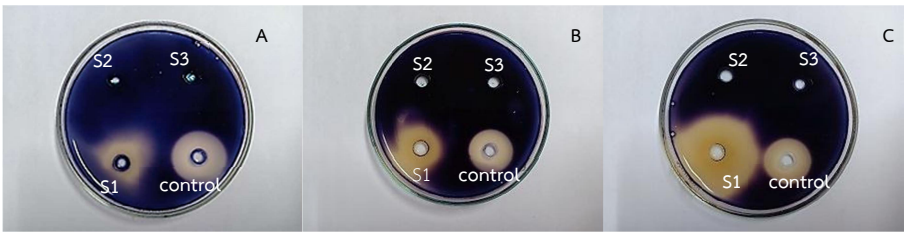

Figure 1 Starch agar plates showing starch digestion of *Leucaena* amylase and commercial amylase (control) in a buffer (A) pH 5.8 (B) pH 6.8 (C) pH 7.8. Enzyme concentration was S1 (no dilution), S2 (10-fold) and S3 (100-fold dilution).

The clear zone diameters of *Leucaena enzyme* samples and the commercial control are shown in Table 1. Only undiluted *Leucaena* amylase caused starch digestion, with a clear zone ranging from 2.3 to 3.6 cm diameter in different pH buffers. A clear zone was not observed with 10- and 100-fold dilutions. Hence, less amount of the enzyme was not very active or denatured during the experiment as 24 h incubation is quite a long time. Consequently, dilution of *Leucaena* enzyme was not recommended.

Table 1 Clear zone of *Leucaena* enzyme samples and commercial amylase.

Enzyme samples	Clear zone diameter (cm)		
	pH 5.8	pH 6.8	pH 7.8
Commercial amylase	2.5	2.3	2.3
S1	2.3	2.6	3.6
S2	0	0	0
S3	0	0	0

The preliminary test of amylase enzyme suggests fresh large *Leucaena* leaves contain amylase active over a wide range of pH. Different enzyme activity at different experimental pH values was inferred from different diameters of the clear zone.

The effect of pH on amylase activity of the enzyme sample at 40°C is shown in Figure 2. It was found that *Leucaena* amylase was active over a broad pH range between 4.0 and 9.0, while the maximum activity was found at pH 5.8 (9.54±0.17 U/g FW). Thus, pH 5.8 was selected for further study in the effect of temperature on *Leucaena* amylase. *Leucaena* amylase can work best at near neutral pH, similarly to bacterial amylase at pH 6.5 (Chaikulsareewath & Lampluarline, 2009).

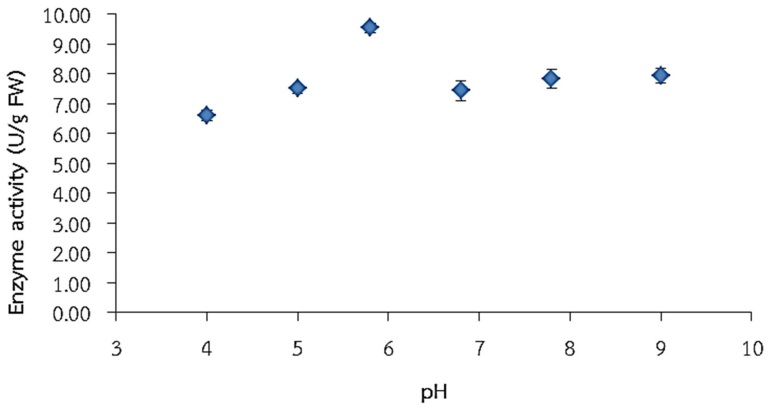

Figure 2 Effect of pH on enzyme activity of *Leucaena* amylase (n=3) at reaction temperature = 40°C. The error bars were standard deviation (S.D.).

The effect of temperature on amylase activity at pH 5.8 is shown in Figure 3. In the range of studied temperature, we found that 40°C was the optimal temperature for *Leucaena* enzyme activity (9.54±0.17 U/g FW). However, increasing temperature

slightly decreased enzyme activity. Crude amylase enzyme produced from *Bacillus* sp. was reported previously to be active maximally at 50°C (Chaikulsareewath & Lampluarline, 2009) and at 70°C (Sawangsri et al., 2011).

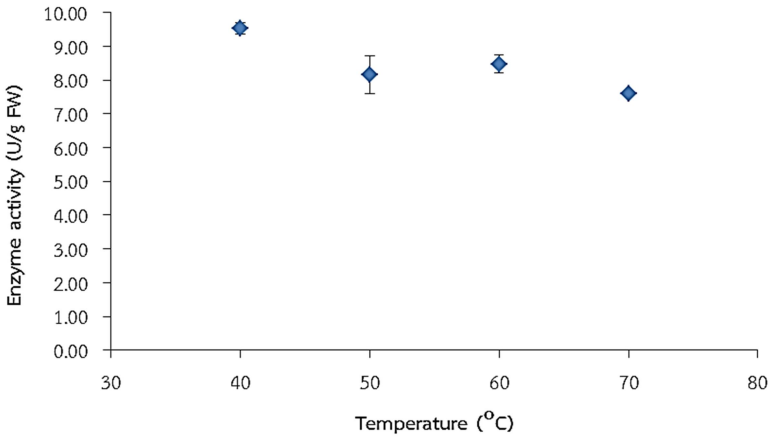

Figure 3 Effect of temperature on enzyme activity of *Leucaena* amylase (n=3) at pH 5.8

According to the results of Figure 2, enzyme activities at pH 5.0, 6.8, 7.8 and 9.0 were also high. The amylase activity at these pH values was then determined at different temperatures. The results as shown in Table 2 revealing that at temperatures above 50°C (60, 70 and 80°C), amylase exhibited different activity when pH was varied. Moreover, amylase was completely inactivated at 80°C at pH 6.8 and 9.0.

Table 2 Effect of temperature on enzyme activity of *Leucaena* amylase at pH 5.0, 6.8, 7.8 and 9.0

pH	Enzyme activity (U/g FW)				
	40°C	50°C	60°C	70°C	80°C
5.0	7.50±0.15	7.22±0.04	7.48±0.68	7.93±0.22	6.97±0.15
6.8	7.43±0.34	7.78±0.10	8.24±0.00	5.94±0.23	-
7.8	7.83±0.30	7.83±0.10	7.02±0.10	8.97±0.17	6.53±0.16
9.0	7.92±0.24	7.82±0.21	8.30±0.17	8.00±0.19	-

Remarks All values were shown as mean ± S.D.

For the crude enzyme, the enzyme activity measured in the buffer pH 5.8 suggests that *Leucaena* enzyme is similar to the human amylase as the optimum temperature around 37°C. However, using pH 5.0 and 7.8 could be preferable in order to take advantage of the high thermal stability of *Leucaena* amylase at 70°C. Thermostable amylase produced from a soil bacterium has the highest activity at 60°C (Rattanasuk, 2018) which is comparable to *Leucaena* amylase working under pH 6.8 and 9.0. Thus, *Leucaena* could be used for simple experiments of this enzyme instead of commercial amylase that is quite expensive. Moreover, *Leucaena* would be a plant source of amylase enzyme for high school students and undergraduates learning about the digestive enzyme instead of that in human saliva, because of its easy availability. Moreover, in the industrial scales, amylase has been definitely needed in different industrial production processes, for example, food, pharmaceutical and clinical applications (Wang et al., 2019; Souza & Magalhães, 2010).

Conclusions

It could be concluded that large *Leucaena* or Krathin Kaset leaves could be considered as a valuable source of plant amylase. This enzyme is thermostable and active over a wide range of pH, thus it can be applied in several applications.

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