Research Article

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Purification and characterization of two isoforms of native α amylase from Ok-Rong mango (Mangifera indica Linn. cv. Ok-Rong)

Ok-Rong mangosundan doğal alfa amilazın iki izoformunun saflaştırılması ve karakterizasyonu (Mangifera indica Linn. cv. Ok-Rong)

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Abstract

Introduction: The tropical plant amylases involved in the fruit ripening stage is outstanding for their high activities in converting starch to sugars within a short period at high temperatures over 40°C.

Methods: The α amylase iso-enzymes from Ok-Rong mango (Mangifera indica Linn. cv. Ok-Rong) were purified in 2 steps, using 70% ammonium sulfate precipitation and affinity chromatography on a β-cyclodextrin sepharose 6B column, and characterized for biochemical properties.

Results: The enzyme was purified 105-fold with a final specific activity of 59.27 U mg⁻¹. SDS-PAGE revealed two bands of 60 and 64 kDa. pI were supposed to be 4.6 and 5.0. Those were resolved into isoforms I and II by a zymographic method. They were matched with α amylase Amy1 from Vigna mungo and α amylase-like isoform I from Theobroma cacao after LC-MS/MS analysis. Isoforms I and II exhibited maximum activity at pH 4, retained more than 50% of their activity after 1 h of incubation at pH 5–9. Two isoforms showed high activity over a wide range of temperatures at 30°C–90°C, with the highest activity at 70°C. They retained more than 50% of their activity at 30°C–40°C after 1 h of incubation. The enzymes were confirmed to be metalloenzymes by the effect of EDTA. In addition, limit-dextrin, amylopectin and soluble starch were suggested to be good substrates.

Conclusion: Two α amylase iso-enzymes were classified as members of the low-pI group of amylases with identical structure, properties and functions. They are mesophilic with high possibilities for application for many purposes.

Keywords: α Amylase; Ok Rong mango; Purification; Mesophilic; Mangifera indica.

Özet

Giriş: Meyve olgunlaştırma aşamasında yer alan tropikal bitki amilazları, 40°C'nin üzerinde yüksek sıcaklıklarda, nişastayı şekerlere kısa sürede dönüştürme yönünde yüksek aktiviteleri açısından göz çarpıktır.

Yöntemler: Ok-Rong mangosundan (Mangifera indica Linn. Cv. Ok-Rong) elde edilen alfa amilaz ikozomurlarını, 70% amonyum sülfat çökeltmesi ve β-siklodextrin sepharose 6B kolon onarımında zyomingrafik analizlerle ayrı ayrı analiz etti. Her iki izoformda da en yüksek aktivitenin pH 4'de olduğunu, pH 5–9'ta 50% den fazla azalmadığını bulduk. İki izoformda 30°C–90°C aralığında yüksek aktivite sergiliyordu. 30°C–40°C aralığında 1 saatlik süre yanıtında ise en fazla 50%uates tahmin ediliyordu. Enzimler EDTA etkisiyle metalloenzimler olarak tespit edildi. Limit-dextrin, amylopectin ve çözünümülü stokkarot gibi substratlar da destekledi.

Sonuç: İki α amilaz izoformu, aynı yapışkan struktur, özellikler ve fonksiyonlarla sınıflandırdı. Mesofiliklerdir ve birçok amaç için uygulanabilecek özelliklere sahipti.

Anahtar Kelimeler: α Amilaz; Ok Rong mangosu; Temizleme; Mesofilik; Mangifera indica.
64 kDa’lık iki bant ortaya koymuştur. Pl’nin 4,6 ve 5,0 olması gerektirirdi. Bunlar, zomografik bir yöntemle isoformları I ve II’ye dönüştürüldü. LC-MS/MS analizin- den sonra Theobroma kaka’osından Vigna mungo’dan α amilaz Amy1 ve α amilaz benzeri isoform I ile eşleştirildi. Isoformlar I ve II, pH 5’de enkübasyon 1 saat sonra aktivite- telerinin % 50’sinden fazlasını koruyarak pH 4’te maksı- mum aktivite sergiledi. İki isoform, 30–40°C’de geniş bir sıcaklık aralığında yüksek etkinlik gösterdi ve en yüksek aktivite 70°C’de gerçekleşti. 1 saat inkübasyondan sonra aktivitelerinin % 50’sinden fazlasını muha- faza ettiler. Enzimlerin, EDTA etkisi ile metaloenzimler olduğu doğrulandı. Buna ek olarak, limit-dekstrin, ami- lopektin ve çözünür nişastanın iyi substrat olduğu öne sürülmiştir.

Sonuç: İki alpha amilaz izo-enzimi, düşük plaseboylu ami- laz grubuna benzer yapı, özellikler ve fonksiyonlarla sınıflandırıldı. Birçok amaçla uygulama imkanı yüksek mezofiliklerdir.

Anahtar Kelimeler: α amilaz; Ok Rong mango; saflaştırma, mezofilik, Mangifera indica.

Introduction

α Amylase (α-1,4-gluco-oligosaccharide, EC3.2.1.1) is a hydrolyzing enzyme that randomly cleaves an internal α-1,4-glycosidic linkage in starch and glycogen to produce malto-oligosaccharide and glucose [1]. This enzyme family is widely applied in many purposes including health promotions and industries, such as paper, sugar, textile, ethanol, etc., and it currently accounts for approximately 30% of the world’s industrial enzyme production [2]. Commonly available ones on the market are those from microorganisms, but there are limitations due to their specificities, activities and stabilities. Thus, novel amyl-ases, especially from nature, is important. Those with high activity and stability have been sought out for many years. Recently, the enzymes involved in the fruit ripening stage is outstanding. The predominant carbohydrate in the raw stage of fruit is starch, which is replaced to a large extent by sugars, and α amylases are responsible for this process. Plant and fruit amylases are attractive because of their high activities in converting starch to sugars within a short period [3]. This always occurs at high temperatures over 40°C.

Mango (Mangifera indica Linn.) is a famous tropical fruit, commonly consumed as a dessert. Mango fruit mainly contains water and carbohydrates, with little protein and fat content. In the unripe stage, when carbohydrates is abundant, the carbohydrates are then replaced with sugars such as sucrose, glucose and fructose [4]. For starch content, amylase and amylpectin are the major ones in the fruit. Amylose is a glucose polymer linked by α (1–4) gly- cosidic bonds in linear structure. Amylopectin is a glucose polymer, as well, but linked by 94%–96% α (1–4) bonds in the linear portion and 4%6% α (1–6) bonds in the branch structure [5]. Ok-Rong mango (M. indica Linn. cv. Ok-Rong) is a native Thai mango commonly grown in all areas of Thai-land (local name; Mamuang Ok-Rong). It is a large green tree that grows up to 20 m tall. This mango is edible, both in the green and ripe stage. The ripening one is defined by a soft texture, a yellow peel and unique sweet taste. For Ok-Rong mango, the plastid α amylase gene from Ok-Rong mango has been clarified to be a 594-amino acid residue sequence [6]. Three dimensional structure and catalytic site were computationally predicted. In this study, we intended to further study the α amylase iso-enzymes. They were puri- fied by affinity chromatography using β-cyclodextrin, and characterized for its biochemical properties.

Materials and methods

Plant materials

Ok-Rong mangos (Mangifera indica Linn. cv. Ok-Rong) in the ripening stage were collected from Sri Sa Ket province (North eastern part of Thailand). The pulps were stored at −80°C before use.

Purification of Ok-Rong mango α amylase

Sample preparation and all purification steps were carried out at 4°C. Mangos were peeled, sliced into small piece. Mangos pulps was homogenized using homogenizer. Large particles were removed by many layers of gauze. After being filtered, samples were centrifuged at 10,000 g for 15 min. The supernatant was ‘crude extract’ for next step purification and kept at −70°C until used. Protein contents were estimated by the Bradford method [7] using bovine serum albumin as the standard.

Ammonium sulfate fractionation

Protein was precipitated at 70% saturation of ammonium sulfate, centrifugated, dissolved and dialyzed against 50 mM sodium acetate buffer, pH 6.0, containing 5 mM CaCl₂, to remove the ammonium sulfate salt.
Epoxy-activated Sepharose 6B affinity chromatography

The samples were applied to an affinity column containing epoxy-activated Sepharose 6B beads conjugated with β-cyclodextrin according to the method of Vretblad [8]. The sample (20 mL) was loaded onto the column (1.6 cm × 20 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 5 mM CaCl₂. The column was washed with 20 mM sodium acetate buffer, pH 6.0, containing 25 mM CaCl₂ and 200 mM NaCl. The proteins were eluted with 10 mg/mL β-cyclodextrin in washing buffer. The active fractions were pooled, dialyzed against 50 mM sodium acetate buffer, pH 6.0, containing 5 mM CaCl₂, and concentrated by SpeedVac concentrator.

Electrophoresis and pI determination

SDS-PAGE

The proteins samples were separated on 13% acrylamide gel in a discontinuous buffer system as described by Laemmli [9] and visualized with Coomassie blue and silver staining.

2D-PAGE

Samples were separated on a non-linear pH 3–10 strip for isoelectric focusing (IEF), and then subjected to a second dimension (SDS-PAGE). The protein spots were analyzed using ImageMaster 2D Platinum Software (GE Healthcare, Germany) for their MW and pI.

Mass spectrometry

Protein bands were identified using LC-MS/MS technique. The single bands were individually cut and treated by in-gel digestion technique [10] before being subjected to a nanoliquid chromatography system (EASY-nLC II, Bruker), coupled to a QTOF mass spectrometer (MicrOTOFQ-II, Bruker) and equipped with an ESI nano-sprayer under service from the Khon Kaen University Research Instrument Center, Thailand. The peptide mass fingerprint was analyzed using information from the NCBI database and the MASCOT MS/MS Ion Search Program (www.matrixscience.com) with these searching parameters: (1) enzyme: trypsin; (2) carbamidomethyl (C) as the fixed modification and oxidation (M) as the variable modification; (3) a peptide charge state of +1, +2, and +3; and (4) instrument type: ESI-QUAD-TOF.

α Amylase activity assay

For assay in solution, method was modified from Bernfeld [11]. One hundred microliters sample was added to 250 μL of 2% soluble starch in 0.1 M sodium acetate buffer, pH 6, containing 50 mM CaCl₂, incubated at 37°C for 10 min. The reaction was terminated with 250 μL of 3,5-dinitrosalicylic acid reagent in boiling water for 5 min. After cooling, the mixture was diluted with DDW, and determined for its absorbance at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar in 1 min under the defined conditions.

For the zymographic method, the enzyme activity was detected on 13% SDS-PAGE containing soluble starch as substrate. Samples were loaded on the gel, run at 150 V for 1 h, refolded in 1% Triton-X 100 for 1 h. Gel was immersed in 1% starch for 1 h and washed with DDW for 2 min. The gel was stained with iodine solution for 5 min. The excess iodine was washed off with cold DDW. Gel was soaked in 1% acetic acid and observed for activity bands. Intensity of the clear zone was analyzed using ImageJ software (http://imagej.nih.gov/ij/links.html).

Characterization

Effect of pH and temperature on α amylase activity

pH effects were probed over the range of 2–11 using 0.1 M of the appropriate buffers containing 50 mM CaCl₂. The buffers were glycine-hydrochloric buffer (for pH 2.0), citrate-phosphate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0) and carbonate-bicarbonate buffer (pH 9.0–11.0). pH stability was assessed by pre-incubating the enzyme at a different pH for 1 h at 25°C and subjected to a zymographic method. The effects of temperature were examined in the range from 30°C to 90°C. The thermal stability was investigated by pre-incubating enzyme solution at a range from 30°C to 90°C, for 1 h. Experiments were performed in triplicate.

Effect of metal ions and chemical reagents on α amylase activity

The effects of various metal ions, inhibitors, and detergents were examined by pre-incubating the enzyme with 10 mM of each of the following metals: NaCl, CaCl₂, KCl,
ZnSO₄, and HgCl₂. Other reagent tests included 10 mM EDTA, 10 mM β-mercaptoethanol, and 1% Triton X-100. Samples were incubated at 25°C for 1 h. The remaining activity was determined by a zymographic method. Experiments were performed in triplicate.

Substrate specificity

The substrate specificity was investigated by incubating the enzyme solution with 1% (w/v) soluble starch, amyllopectin, limit-dextrin, β-cyclodextrin and glucose using the zymographic method. Experiments were performed in triplicate.

Molecular weight and pI determination

Two isoforms of α amylase were successfully separated on a 2D-PAGE profile. After being analyzed with ImageMaster 2D Platinum Software, the upper band weighed 64 kDa and had a pl of 5.0. The lower band was found to be a 60 kDa protein with a pl of 4.6 (Figure 3).

Results

Purification of α amylases

From the β-cyclodextrin affinity chromatography profile, a single peak of protein with α amylase activity was obtained (Figure 1). The peak was in fractions 21–24. The α amylase from Ok-Rong mango was purified 105-fold with a 4.67% recovery yield and 59.27 U mg⁻¹ of specific activity (Table 1). After being checked by SDS-PAGE and the zymographic method, the α amylase solution exhibited two protein bands with amylase activity toward starch (Figure 2).

Protein identification

The two protein bands were separately cut for mass spectrometry analysis by LC-MS/MS. Peptide mass...
fingerprint analyzes of these isoforms were matched with Amy1, the $\alpha$ amylase from *Vigna mungo* (for the upper band), and the $\alpha$ amylase-like isoform I from *Theobroma cacao* (for the lower band). These are secreted amylases classified as members of Family 1 of $\alpha$ amylases (Table 2).
Enzyme characterization

Effect of pH and temperature on α amylase activity

The effect of pH was examined at 37°C using pH values ranging from 2 to 11. The maximum activity of the two isoforms was observed at pH 4 (Figure 4A). To probe the pH stability, the enzyme solution was pre-incubated with different buffers for 1 h. The residual relative activity was higher than 50% at pH 5–9 after pre-incubation and had a decrease of 80% by pH 11 (Figure 4B). To study the effect of temperature, the α amylase activities of two isoforms were determined at different temperatures ranging from 30°C to 90°C. The α amylase activity remained higher than 80% at 40°C–70°C. The optimal temperature was 70°C (Figure 5A). For temperature stability, α amylases activities were studied by pre-incubating the enzyme at different temperatures for 1 h. The α amylase activity remained higher than 80% over the range of 30°C–40°C (Figure 5B).

Effect of metal ions and reagents on α amylase activity

Zinc ion did not affect α amylase activity, whereas Hg²⁺ completely inhibited the enzyme activity (Table 3). β-Mercaptoethanol had some effect on the activity. The enzyme solution retained more than 80% of its activity.
Table 3: Effect of various metal ions on the purified amylases from Ok-Rong mango.

| Treatment   | Isoform I | Isoform II |
|-------------|-----------|------------|
| EDTA        | 0 ± 0.0   | 0 ± 0.0    |
| Triton-X 100| 103 ± 2.5 | 109 ± 0.6  |
| β-mercaptoethanol | 84 ± 3.9 | 88 ± 0.9   |
| Hg²⁺        | 0 ± 0.0   | 0 ± 0.0    |
| Na⁺         | 94 ± 1.3  | 102 ± 6.0  |
| Co²⁺        | 84 ± 4.2  | 94 ± 0.5   |
| Zn²⁺        | 99 ± 2.6  | 97 ± 2.5   |
| Ca²⁺        | 106 ± 6.5 | 107 ± 2.4  |
| Non         | 100       | 100        |

Values are expressed as the mean ± standard error of three replicates.

Table 4: Substrate specificity of the amylases from Ok-Rong mango.

| Substrate        | Relative activity (%) |
|------------------|-----------------------|
| Limit-dextrin    | 100                   |
| Amylopectin      | 84 ± 1.1              |
| Starch           | 82 ± 2.0              |
| Glycogen         | 20 ± 1.7              |
| Maltose          | 21 ± 2.8              |
| β-cyclodextrin   | 0 ± 0.0               |

Values are expressed as the mean ± standard error of three replicates.

Both isoforms of α amylase were completely inhibited by 10 mM EDTA.

Substrate specificity

Limit-dextrin was relatively found to be the best substrate (Table 4). Amylopectin and soluble starch were good substrates for α amylase from Ok-Rong mango.

Discussion

The finding for novel enzyme still be necessary in the field of enzyme technology for the best one. Plant is one of the most common sources for amylase enzyme studies due to its important role for basic metabolism including rapid degradation of starch during germination and fruit ripening. Mangos, *M. indica*, normally accumulated starch in pulp as the main storage. Recently, there are one report described that the saliva amylase was able to degraded mango starch. It showed obvious pits and cracks due to the action of amylase in the stomach under the observation by optical microscopy [12]. For amylases in mango fruits, not only pulp, mango peel also exhibited α amylase activity during ripening stage [13]. However, strains and varieties vary their basic biochemical properties. For Alphonso mango, the hydrolase including α amylase showed relatively high activity and gradually increased with maximum levels of activity around climacteric stage [14]. It was observed to be increased in the middle stage of ripening process in Ashwina hybrid mango, as well [15]. This related with an increase in amylase activity only on the 6th day after harvesting in Haden Mango [16]. The α-type amylase has been detected to bind and degrade starch granules in the early stage of ripening, but it degrade soluble starch for all the period of ripening stage in Keitt mangos [17]. Whereas the β-type amylase exhibited significantly higher activity than the α-type for the early stage just only for starch granule degradation. For application in post-harvesting technology, mango have to be carefully harvested and kept to prevent from any texture damages. Those including spongy tissues in Tommy Atkins mango resulted in three times amylase activity lower than that of healthy pulp [18] and the remaining of unhydrolyzed starch. Our previous study revealed that amylase in Ok-Rong mango pulp showed significantly high activity among Thai fruits during ripening stage (data not shown). Therefore, this study started from the purification of amylase using β-cyclodextrin affinity chromatography. This column was successful for purifying many plant α amylases in just one step [19, 20]. Bands from SDS-PAGE and zymographic analysis were 60 and 64 kDa with apparent α amylase activity. For plant amylases, masses in range 45–70 kDa with pl 4.5–5.5 are quite common (Table 5). They were classified into the low pl group α amylases [26].

These two isozymes in this study were identified as members of Family 1 of α amylases. Those are found in all plants especially cereal grain endosperm [27] with varieties structure and properties (isozymes) even in one individual organisms. There are five α amylase isozymes in apple [28], two isozymes in root beet [29], and two isozymes in azuki bean [25]. Pea (*Pisum sativum*) seedlings contained two to seven isozymes [30, 31]. For Keitt mango, at least three bands of amylolytic activity were observed. They became gradually higher in fruit extract at 3 and 5 days after harvesting [17].

Many α amylases in plants showed activity toward starch over a broad range of temperatures, 40°C–70°C (Table 5). The medium optimal temperature, at 70°C, also found for two α amylase isozymes from azuki bean including two isozymes of this report. These enzymes can be
α Amylases from Ok-Rong mango (M. indica) were purified by β-cyclodextrin affinity chromatography for the first time with a final specific activity of 59.27 U mg⁻¹. The two isoforms of α amylase from Ok-Rong mango were characterized. Both were major enzymes and classified as members of the low-pl group of amylases with identical structure, properties and functions. They are mesophilic. It is highly possible that these enzymes could be applied for many purposes, including industries where a variety of enzymatic reactions is needed.

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