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Purification and Biochemical Analysis of Rice Bran Lipase Enzyme

Young Hee Kim*

Dept. of Bioinformatics Engineering, College of Engineering, Sangmyung University, Anseo-Dong, San 98-20, Cheonan, 330-180, Korea

Abstract

A simple procedure for the extraction of the lipolytic enzyme from rice bran has been developed. High activity of lipolytic enzyme was obtained by first defatting the rice bran to remove lipid components with various extraction conditions. Then, after five cycles of aqueous extraction, rice bran lipolytic enzyme was purified using micro- and ultrafiltration apparatus. Lipolytic enzyme activity was estimated by its hydrolytic action of tributyrin. The result indicated that the standard activity curve of butyric acid showed that the potential rice bran enzyme is a hydrolytic lipase enzyme. In addition, it showed higher lipolytic activity and specific enzyme activity with further purification by micro- and ultrafiltration. The size of rice bran lipase enzyme was identified through 15 % SDS-PAGE. The molecular weight of the rice bran lipase enzyme was 41 kDa.

Key words: lipolytic enzyme, ultrafiltration, tributylin

Introduction

Lipases are enzymes that are primarily responsible for the hydrolysis of acylglycerides. However, a number of other low- and high-molecular weight esters, thiol esters, amides, polyol/polyacid esters, etc. are accepted as substrates by this unique group of enzymes. A variety of reactions are further enlarged by the fact that lipases are capable of catalyzing the reverse reaction of synthesis just as efficiently.

Lipases therefore have a vast potential for application in a number of industries, for instance pharmaceuticals, cosmetics, leather, detergents, foods, perfumery, medical diagnostics, and other organic synthetic materials. It is important to develop the method entailed the use of cheap, readily available materials and to focus on the isolation of the lipase from a plant source, rice bran. The castor bean lipase-catalyzed hydrolysis, to manufacture fatty acids and glycerol, is already operative on a commercial scale (Stirton 1964). Enzymes, such as lipases and proteases, are extensively used for accelerating the maturation of cheese and for the production of typical flavors. During this process, there is formation of free fatty acids and soluble peptides and amino acids. Both act as flavors as well as flavor precursors (Kinsella and Hwang 1976), resulting ultimately in products of better flavor and acceptability than their untreated counterparts (Sood and Kosikowski 1979). Lipase-treated milk fats are used in the production of butter/margarine flavors (Colburn 1969; Amold et al. 1975; Gerhartz 1990). In coffee whiteners, lipases assist in imparting a rich creamy flavor (Godfrey and Reichelt 1983). Lipase is a component of a hair-waving preparation (Saphir 1967) in which it promotes penetration of the preparation.

Commercial values of agricultural commodities, such as oilseeds and cereal grains, can be affected significantly by the action of lipolytic enzymes contained in such products. A major problem in the processing of oil palm is posed by post-harvest enzymic hydrolysis of the oil, which can lead to extensive formation of free fatty acids in the oil (Desassis 1957). It is believed that the lipids in the oil palm mesocarp are protected from the lipases by the membranes of the vacuoles (Hartley 1977), and these membranes are disrupted by bruising during harvesting and handling which leads to high lipolytic activity in the oil palm during storage (Coursey 1963).

Rice bran is produced in a very large tonnage and used as animal feed after extraction of rice bran oil in many

* Corresponding author, E-mail: ykim@smu.ac.kr
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countries living on rice as Korea. Lipases play a major role in the quality of rice bran oil, an important industrial commodity in rice-producing countries. The prime drawback of rice bran as a source of oil is the presence of highly active lipolytic enzymes in the bran (Hirayama and Matsuda 1975). The lipolytic activity is observed as soon as the bran is removed from the rice, and lipolysis continues with prolonged storage (Hirayama and Matsuda 1975), yielding high levels of free fatty acids in the oil, which becomes unsuitable for processing to an edible oil. In this paper, we extracted and purified rice bran lipolytic enzyme. The biochemical activity of rice bran lipase was observed by using the aqueous extract after membrane filtration.

Materials and Methods

Defatting of rice bran

1 kg of rice bran (*Oryza sativa* cv. Chuchung) was purchased from a local mill (Mokcheon, Cheonan). 10 g of milled rice bran was stirred with 30 mL of n-hexane for 30 min for defatting. At the end of stirring period, the hexane phase was decanted and 30 mL of fresh n-hexane was added to the rice bran. This procedure was repeated twice. This procedure was followed in each experiment unless mentioned otherwise.

Extraction of rice bran lipase

In order to remove the hexane completely, the defatted rice bran from above was dried in air about 1 hour and then used for extraction. For this purpose potassium phosphate buffer containing calcium chloride was used. The 50 mM phosphate buffer containing 0.5 mM calcium chloride was used to get a maximum activity in the extract (Prabhu et al. 1999). The defatted rice bran was stirred with eight times its weight of the 50 mM phosphate buffer containing 0.5 mM $CaCl_2$ (pH 7.0) at 10℃ for 30 min, after which the suspension was centrifuged for 30 min at 3,000 rpm at 4℃. The pellet was collected and resuspended in fresh phosphate buffer and this procedure was repeated six times. The supernatants were collected and pooled to give the crude lipase extract (CLE).

Lipase assay

The standard activity curve of butyric acid was required to measure the decomposed amount of tributyrin because lipase is an enzyme to catalyze the hydrolysis of tributyrin. Lipase activity was estimated by its hydrolytic action of tributyrin, which was emulsified in various emulsifying agents such as gum acacia. A 2 mL portion of 7.5% gum acacia was mixed with 10 mL of enzyme extract. To this 1 mL of tributyrin was added, and the mixture was stirred for 1 min with a magnetic needle on an orbital shaker at 30℃. Next, 0.5 mL aliquots of this reaction mixture were removed at specified intervals, and the reaction was quenched using 10 mL of methanol. This was then titrated with sodium hydroxide with phenolphthalein as an indicator. The lipase activity of the tributyrin was compared to that of natural oils such as olive oil, palm oil, and coconut oil.

Calculation of lipase activity

Lipolytic activity is expressed in terms of lipase units (U) per unit weight of rice bran (in kg). One unit is defined as the micromoles of butyric acid liberated by the hydrolysis of tributyrin at 30℃ per minute. The amount of acid released can be calculated from the difference in titer values of a sodium hydroxide solution of known molarity. Specific activity was calculated as the units per mg protein.

Purification and SDS-PAGE of lipase extract

The crude lipase extract was centrifuged at 6,000 rpm for 25 min to separate the rice bran particles from the enzyme solution. Purification step was carried out in two stages, microfiltration and ultrafiltration. The purification of the crude enzyme solution was performed by passing the solution through the 0.45 μM PVDF (polyvinyldifluoride) membranes to get a microfiltration extract (MF). The MF was initially subjected to ultrafiltration using a 100 kDa polysulfone membrane, so as to obtain a permeate containing the lipase. This permeate was then ultrafiltered through a 10 kDa polysulfone membrane, so as to obtain a purer and concentrated enzyme solution.

In order to fractionate the lipase extract from rice bran, the 60 μg of each enzyme extract CLE, MF, and UF was loaded on the 15% acrylamide gel at 10℃. 10 μL of protein standard marker (BRL) was used as standard molecular weights of known proteins. After finished SDS-PAGE (Sodium Dodesyl-PolyAcrylamide Gel Electrophoresis), the gel was soaked in 500 mL of distilled water for 30 minutes to wash the chemicals away. Then the gel was soaked in staining solution (distilled water: methanol = 1:1/ v/v, Coomassie brilliant Blue (1 g/L), acetic acid (0.7 mL/10 mL solution)). After staining, the gel was soaked in destaining solution

(distilled water: methanol: acetic acid = 5:5:1, v/v) on the shaking incubator with 35 rpm at 25℃ for one night. After destaining, the gel was dried by using the vacuum gel drying machine.

Results and Discussion

Defatting of rice bran

The defatting step is the first step of the extraction process. It was found that attempts to forego this step resulted in rather low activity yields of the lipase. Also, aqueous extraction of lipase was rendered rather difficult because of the presence of the fatty material.

Solvents such as petroleum ether, n-hexane, and n-heptane were tested, but n-hexane was appeared to give the better results (Anita et al. 1999). We repeated the defatting step by the addition of fresh n-hexane twice. We compared this process to addition of fresh n-hexane once. The repeated defatting step was more efficient for removing fatty materials when compared to the addition of fresh n-hexane once (Anita et al. 1999). As a result of high volitility of n-hexane, it helps oil recovery easier. The effect of lipase activity was also observed. When 10 g of rice bran was incubated in 30 mL of n-hexane at 10℃ and 30℃, no deleterious effect was observed at 10℃, at least up to 30 min when compared to dwindling result within 10 min at 30℃.

Effect of substrate

In order to test the biochemical activity of rice bran lipase, the hydrolytic action of tributyrin was estimated for the its reaction rate. Lipolytic activity is expressed in terms of lipase units (U) per unit weight of rice bran (in Kg). One unit is defined as the micromoles of butyric acid liberated by the hydrolysis of tributyrin. The amount of acid released was calculated from the difference in titer values of a sodium hydroxide solution of known molarity (Table 1). It was found that the amount of sodium hydroxide used was proportional to that of liberated tributyric acid (Figure 1). The comparison result of lipolytic activity was summarized in Table 2. It was clear that the rate of hydrolysis was highest in the case of tributyrin. Among the oils, it showed that olive oil was hydrolyzed faster than the other oils. Other study on pancreatic lipase have yielded similar results, namely, that shorter chains are hydrolyzed by the lipase enzyme faster than longer chains. In addition, oleyl chains have been reported to be released slightly faster than other long chain-fatty acids (Aizono et al. 1973; Shastry and Rao 1971; Noda and Kobayashi 1968).

Table 1. The amount of NaOH by the amount of butyric acid (mg/mL) in titration.

Conc. of butyric acid (mg/mL)	Amount of NaOH (mL)	Conc. of bytyric acid (mg/mL)	Amount of NaOH (mL)
1	5	6	30
2	10	7	35
3	15	8	40
4	20	9	45
5	25	10	50

Table 2. Hydrolytic activity of rice bran lipase on different substrates.

Substrate	Initial reaction rate (mol acid/ min)
Tributyrin	1.20
Olive oil	0.28
Palm oil	0.14
Coconut oil	0.08

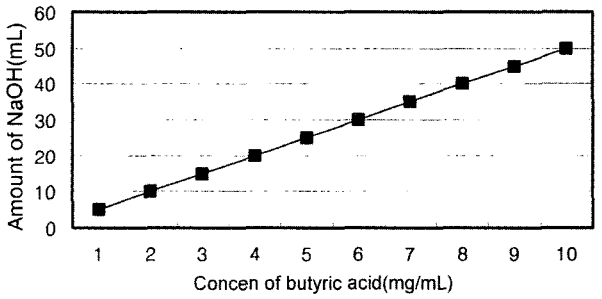

Figure 1. The standard activity curve of butyric acid. The used amount of NaOH (mL) by the concentration of liberated butyric acid (mg/mL) in titration.

Aqueous extraction and membrane filtration of the rice bran lipase

Defatted rice bran, obtained by the procedure optimized above, was stirred with potassium phosphate buffer to extract the lipolytic enzyme. The lipolytic enzyme was extracted with phosphate buffer in the range of pH 5-8 at 10℃. Though the pH of the buffer was varied, the ionic strength of the phosphate buffer was always maintained constant at 50 mM. Also, all of the buffers contained 0.5 mM of $CaCl_2$. Maximum activity of the lipolytic enzyme was observed at pH 7-7.5. These results are in agreement with those reported by Shastry and Rao (1971).

In order to purify the rice bran lipase enzyme, membrane

Table 3. Membrane filtration of the aqueous extract of rice bran lipase. CLE (Crude Lipase Extract, it contains all extract from 1st CLE to 6th CLE), MF (MicroFiltration extract, PVDF membrane), UF (UltraFiltration extract, 100 kDa membrane, and 10 kDa retentate).

Enzyme prep	Volume (mL)	Absorbance at 280 nm	Conc. of protein (mg/mL)	Total protein (mg)	Enzyme activity (U/mL)	Specific activity (U/mg)
CLE	250	0.603	880	2120000	3.6×10^{-7}	4.0×10^{-9}
MF	150	0.561	820	123000	6.0×10^{-7}	7.3×10^{-8}
UF	120	0.560	810	97200	7.5×10^{-7}	9.2×10^{-8}

filtration step was carried out in two stages, microfiltration and ultrafiltration. The protein distribution obtained during this purification process is shown in Table 3. Enzyme activity in the MF extract was increased from 3.6×10^{-7} to 6.0×10^{-7} U/mL. Generally amount of total protein (mg) was decreased. However, enzyme activity (U/mL) and specific activity (U/mg) were increased. Enzyme activity in the MF extract was increased only marginally from 3.6×10^{-7} U/mL to 6.0×10^{-7} U/mL. But specific activity in the MF extract was increased from 4.0×10^{-9} (U/mg) to 7.3×10^{-8} U/mg. An 18-fold increase in specific activity of the MF extract was obtained by microfiltration using the PVDF membrane. On further purification, enzyme activity in the 10 kDa retentate in the UF extract went up to 7.5×10^{-7} U/mL, while the specific activity went up to 9.2×10^{-8} U/mg. An 23 fold increase in specific activity of the UF extract was obtained by ultrafiltration using 10 kDa retentate. Therefore, the series of purification steps enables a doubling of enzyme activity and further increased for the specific activity, with concomitant removal of almost three-forth of nonspecific proteins. The volume of the enzyme solution was also more than halved, allowing for a much more concentrated and pure enzyme. The above results were sufficient because the value itself of specific activity was high comparatively, which showed a similar result compared to other study (Anita et al. 1999). Because the main objective was to obtain high yields of the pure lipase, further purification of the bran lipase by microfiltration through a 0.45 μM PVDF membrane, followed by a 2-step ultrafiltration through polysulfone membranes of molecular weight cutoff 100 and 10 kDa, resulted in improved activity and specific activity. The stability was also enhanced significantly at each step.

Analysis of lipase protein

When the purified enzyme was subjected to disc electrophoresis in 15% SDS-Polyacrylamide gel at pH 4.5, it migrated as a defined band, as shown in Figure 2. And it was confirmed that the molecular weight of the rice bran lipase was 41 kDa. And also it was reported that the

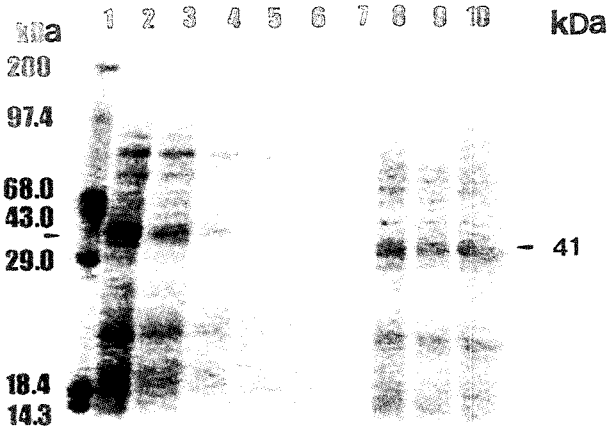

Figure 2. The purified lipase enzymes loaded on the 15% SDS-PAGE. Lane 1: protein standard marker; BRL, lane 2: crude lipase extract 1; CLE 1, lane 3: CLE 2, lane 4: CLE 3, lane 5: CLE 4, lane 6: CLE 5, lane 7: CLE 6, lane 8: mixed CLE; from 1st CLE to 6th CLE, lane 9: MF extract; microfiltration extract; PVDF membrane, lane 10: UF extract: ultrafiltration extract, 100 kDa membrane, 10 kDa retentate.

homogeneity of the purified enzyme preparation was appeared to the homogeneous by criterion of CM-sephadex C-50 column chromatography (Funatsu et al. 1971). Although it was assumed our proteins was homogeneous, there was a dark band. The dark band above 41 kDa lipase band was maybe inferred as dimer forms of lipase protein because the size of the dark band was about 80 kDa (Figure 2). We also noticed that the concentration of lipase protein became weak gradually from 1st crude extract (lane 2 of Figure 2) to 6th crude extract (lane 7 of Figure 2). This might be the reason that the total protein content was decreased as we subjected the protein extract to the column (Table 3). However it helped that unnecessary bands were somewhat disappeared.

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