BMB
reports

Purification and characterization of hepatic lipase from *Todarodes pacificus*

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Lipase was purified from squid (*Todarodes pacificus*) liver in an attempt to investigate the possibility of applying the enzyme for biotechnological applications. Crude extract of squid liver was initially fractionated by the batch type ion exchange chromatography. The fraction containing lipase activity was further purified with an octyl-Sepharose column. Finally, lipase was purified by eluting active protein from a non-dissociating polyacrylamide gel after zymographic analysis. Molecular weight of the purified enzyme was determined to be 27 kDa by SDS-polyacrylamide gel electrophoresis. The enzyme showed the highest activity at a temperature range of 35-40°C and at pH 8.0. The activity was almost completely inhibited at 1 mM concentration of Hg^{2+} or Cu^{2+} ion. Partial amino acid sequence of the enzyme was also determined. [BMB reports 2008; 41(3): 254-258]

INTRODUCTION

Lipase (EC 3.1.1.3) is a group of enzymes characterized by the ability to hydrolyze triacylglycerol (TAG) at oil-water interface. It has received much attention as key enzymes in biotechnological applications, such as food processing, detergent, and biomedical sciences (1-3). Moreover, lipase immobilized on a solid support has been proved to be an attractive biointerface for biodiesel production (4, 5) or biosensor to detect serum TAG level (6, 7).

Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Although the extracellular lipases of microbial origin represent the most widely used class of enzymes in biotechnology, there are numerous reports regarding applications of animal lipases. Crude preparation of porcine pancreatic lipase has been used for the biotransformation of organic compounds or therapy of pancreatic diseases (8, 9). Crude lipase obtained from the intestinal tract of rainbow trout (*Oncorhynchus mykiss*) was proved to be useful for lipolysis of oils (10).

There have been efforts to find lipases to meet the requirements for a given application because characteristics of the purified lipases are variable depending on their sources. However, relatively a few number of lipases has been identified or purified from marine organisms (11-16) In this paper, we report the purification and characterization of hepatic lipase from squid (*Todarodes pacificus*).

RESULTS AND DISCUSSIONS

Assay methods

We used three different assay methods to identify and quantify lipase activity. The colorimetric method was routinely used for initial estimation of lipase activity throughout the purification steps because of its high sensitivity and conveniency. Major drawback of this method is the error caused by nonspecific reactions of 2,3-dimercapto-1-propanol tributyrate (DMPTB) and 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) with various enzymes and chemicals present in tissue extracts (17). Although the problem of nonspecific reactions could be partially resolved by adding PMSF to the crude extract and by using a blank that did not contain DMPTB (17, 18), the colorimetric assay method may not be sufficiently reliable when used for the discrimination of lipase from other esterases.

More specifically, hydrolytic activity at oil-water interface, which is the characteristics of lipases, could be determined by the pH-stat method where the activity is measured on an emulsion of TAG by neutralizing the released fatty acids with NaOH in order to maintain the pH at a constant end point value. But special equipment is required for the pH-stat method. We devised a simple protocol to test if the purified enzyme is active at oil-water interface. Reaction mixture containing the enzyme and oil emulsion was incubated with stirring at 37°C and pH change was monitored for 1 min. As the reaction rate (ΔpH/sec) estimated by this method was well correlated with the amount of *Candida rugosa* (*C. rugosa*) lipase (Fig. 1), it was possible to determine that each fraction had the characteristics of lipase or not.

Zymographic technique is an efficient tool for distinguishing isoenzymes or detecting enzyme activity in crude extracts. Lipase activity can be stained on a gel with α-naphthyl acetate and Fast Red TR. Several different activity bands were ob-

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Received 10 August 2007, Accepted 5 October 2007

Keywords: Lipase, Liver, Purification, Squid, *Todarodes pacificus*

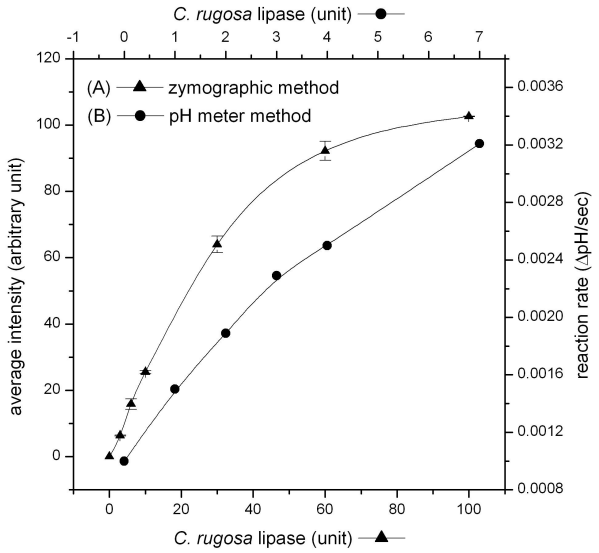

Fig. 1. Standard curves of two lipase assay methods; (A) zymographic (▲) and (B) pH meter method (●).

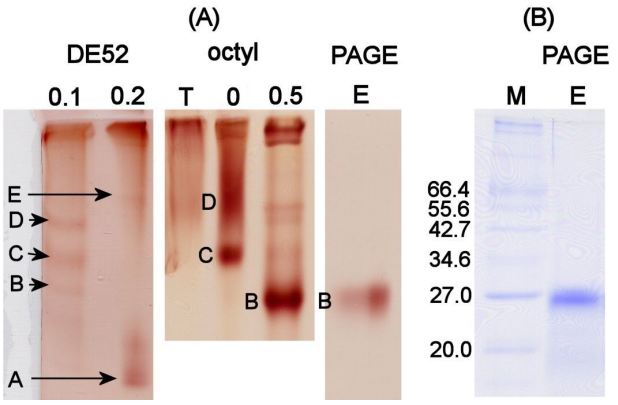

Fig. 2. Electrophoretic analysis of the *Todarodes pacificus* lipase. (A) Zymographic analysis of fractions: 0.1 M (lane 0.1) and 0.2 M NaCl fraction (lane 0.2) of DE-52 chromatography (left); Triton X-100 (lane T), 0 M (lane 0) and 0.5 M $(NH_4)_2SO_4$ fraction (lane 0.5) of octyl-Sepharose chromatography (middle); the protein prepared by electroelution of active band from polyacrylamide gel after zymographic analysis (lane E) (right). (B) SDS-PAGE analysis of the *Todarodes pacificus* lipase: molecular weight markers in kDa unit (lane M) and the protein prepared by electroelution of active band from polyacrylamide gel after zymographic analysis (lane E).

served in the zymogram of squid liver extract. Those bands might represent lipase isoenzymes or nonspecific esterases because α-naphthyl acetate is not a specific substrate. Nevertheless, by the zymographic method, we were able to trace each activity band during purification courses (Fig. 2) and to match a specific band with the hydrolytic activity at oil-water interface. Quantitative determination of a specific activity was also possible by estimating its band intensity and comparing with a standard curve prepared with *C. rugosa* lipase (Fig. 1).

Lipase purification

In a previous work, acetone powder method was used to remove fat from the liver tissue (16). Although this method was successful in obtaining clarified extract, lipase activity per gram of liver tissue was only 25 % of that observed in the crude extract prepared by homogenizing liver in 50 mM Tris-HCl, pH 7.0. We also tried ammonium sulfate fractionation of the crude extract. Lipase activity was mainly associated with 20-50 % ammonium sulfate fraction but the recovery yield was not better than the acetone powder method.

Therefore, the crude extract was directly subjected to a batch type ion-exchange chromatography. The crude extract was mixed with DE-52 anion exchange resin and oil emulsion and unadsorbed proteins were removed from the resin by decanting. After repeated washing, the resin was packed into a column. Adsorbed proteins were fractionated with a stepwise NaCl gradient. As a result, initial fractionation as well as clarification of the extract was achieved. Activity was found in both of 0.1 and 0.2 M fraction when estimated by the colorimetric method. In contrast, hydrolytic activity at oil-water interface was detected only in the 0.1 M fraction. Zymographic analysis showed that the two fractions contain different activity bands; bands B through D in the 0.1 M fraction and bands A and E in the 0.2 M fraction (Fig. 2).

Table 1. Purification of lipase from *Todarodes pacificus*. One unit hydrolyzes 1.0 microequivalent of fatty acid from a triglyceride in 1 hr at pH 7.7 and 37°C

Step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Yield (%)	Purification fold
Crude extract	90	810	9	100	1
DE-52	14.4	330	23	40.7	2.6
Octyl-Sepharose	1.35	180	133	22.2	15
PAGE	0.0044	12.6	2,840	1.6	316

Consequently, the 0.1 M fraction was further purified by the octyl-Sepharose chromatography. Hydrophobic interaction chromatography takes advantage of the characteristics of lipases to become adsorbed on any hydrophobic interface via interfacial activation. Because of its low resolution, however, applications have been mostly limited to the purification of secreted microbial lipases from culture media (19-22). When the 0.1 M NaCl fraction of DE-52 chromatography was purified with an octyl-Sepharose column, hydrolytic activity at oil-water interface was successfully separated from other activity bands. It was recovered in the 0.5 M ammonium sulfate fraction which was shown to contain the activity band B in the zymographic analysis (Fig. 2). The bands C and D were detected in the 0 M ammonium sulfate fraction. At this stage, lipase was

separated from other activity bands although still contaminated with other proteins as judged by SDS-PAGE (data not shown).

As a final step to purify the enzyme to homogeneity, the 0.5 M ammonium sulfate fraction was resolved on a non-denaturing polyacrylamide gel and stained by the zymographic method. The protein contained in the activity band was recovered by the electro-elution method. The purified protein appeared as a single band both in zymographic analysis and SDS-PAGE (Fig. 2). Efficiency of each purification step is summarized in Table 1. The electrophoretic purification step was highly efficient in separating lipase from contaminating proteins though the yield was low.

Characterization

Molecular weight of the lipase purified from Japanese flying squid (*Todarodes pacificus*) liver was estimated to be 27 kDa by SDS-PAGE (Fig. 2). Amino-terminal amino acid sequence of the enzyme was GKFCCAPEKFSANTM. Internal sequences determined after tryptic digestion were YTNLLTSGNR, DDPSVFVL, LNDATMTTLA and ENLDASWLK. We could not find any protein sequence in PDB showing significant homology with those sequences. Hepatic lipase of neon flying squid (*Ommastrephes bartramii*) was partially purified and characterized (16). They reported that the enzyme had a molecular weight of 33 kDa when estimated by SDS-PAGE. Due to the lack of sequence data, it is not clear yet weather the two enzymes are homologous proteins or not.

Activity of the purified enzyme was optimal at temperature between 35 and 40°C and at pH 8.0 (data not shown). When the enzyme was incubated for 20 min at different temperatures, its activity maintained 100 % until 40°C but decreased sharply at higher temperatures (data not shown).

Effect of metal ions on the activity of the *Todarodes pacificus* lipase was investigated. The lipase activity in the presence of a metal ion was compared with the control including no metal ion whose activity was taken as 100 %. Relative activities at 1 mM of Cu^{2+}, Hg^{2+}, Pb^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+} and Li^{+} were 0, 4.4, 24.4, 36.2, 49.1, 64.2, 90.0 and 98.2 % respectively. Strong inhibition was observed with heavy metals such as $Cu^{2+,}$ Hg^{2+}, Pb^{2+}, Co^{2+} and Cd^{2+}. In general, lipase activity has been known to be inhibited by heavy metals like Co^{2+}, Hg^{2+}, Cu^{2+} and Sn^{2+} (23-26). The metal ions may have variable effects on oil-water interface through interaction with free fatty acids (24). Mercury ion (Hg^{2+}) is known to bind to thiol groups of proteins, forming stable complexes (24).

Lipases can be classified in terms of specificity toward the position of the acyl group of TAG into two groups: 1,3-specific and nonspecific. The substrate in the colorimetric assay method, DMPTB (2, 3-dimercapto-1-propanol tributyrate), is a TAG analogue which has two thioester groups in 2- and 3-position. The 1,3-specific lipase can hydrolyze one of the two thioester groups, whereas nonspecific ones hydrolyze two groups. Therefore, an increase in absorbance should be observed when nonspecific lipase is added to the reaction mixture containing 1,3-specific lipase. We could not observe any significant increase in absorbance when *C. rugosa* lipase, which is nonspecific, was added to the reaction mixture containing the purified lipase (Fig. 3). This result indicates that the purified enzyme is nonspecific.

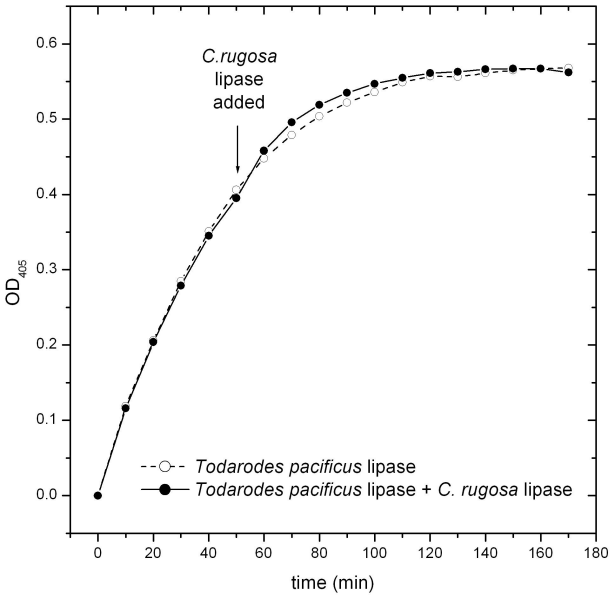

Fig. 3. Positional specificity of the *Todarodes pacificus* lipase.

MATERIALS AND METHODS

Assay methods

Lipase activity was routinely determined by the colorimetric assay method (18). Reaction mixtures containing 0.2 mM 2,3-dimercapto-1-propanol tributyrate (DMPTB), 0.8 mM 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 1 mM EDTA, 0.05 % Triton X-100, 50 mM Tris-Cl, pH 7.5 and enzyme were prepared in a 96-well microplate. The plate was incubated for 30-60 min at 37°C and absorbance of each well at 405 nm was measured by using a microplate reader.

Lipase and other esterase activities were identified and estimated by the zymographic method (27). Proteins were resolved on a 10 % non-denaturing polyacrylamide gel. Zymogram was prepared by immersing the gel in a staining solution (0.2 M Tris-Cl, pH 7.5, 0.25 % sodium deoxycholate, 0.02 % α-naphthyl acetate and 1 mg/ml Fast Red TR) for 1 h at 37°C. Intensity of the resulting red band was estimated and converted to lipase activity by using a standard curve prepared with known amount of *Candida rugosa* (*C. rugosa*) lipase (Sigma-Aldrich).

Hydrolytic activity at oil-water interface was determined by monitoring pH change. A reaction mixture containing 20 µl of 10x TK buffer (10 mM Tris, pH 7.5, 200 mM KCl), 125 µl of oil emulsion, and 55 µl of H_2O was prepared in a microplate

well. For the preparation of oil emulsion, 2.5 ml olive oil, 1 g Arabic gum and 45 ml H_2O were combined and sonicated. The final volume was adjusted to 50 ml with H_2O after setting the pH to 7.5 with 50 mM NaOH. The microplate well containing the reaction mixture was placed in a 37°C incubator and a pH meter microelectrode was immersed in the mixture. The reaction mixture was stirred with a magnetic stirrer. Sample (50 μl) was added to the mixture and pH was recorded for 1 min at an interval of 10 sec. Reaction rates (ΔpH/sec) were obtained from the graph of pH change plotted against time.

Lipase purification

Japanese flying squids (*Todarodes pacificus*) were caught in the East Sea of Korea. Fresh squid liver was mixed with four volumes of 50 mM Tris-Cl, pH 7.0, 0.2 mg/ml phenylmethylsulfonyl fluoride (PMSF) and completely homogenized. The homogenate was centrifuged at 15,000 g for 20 min. The supernatant was filtered through a piece of gauze and further clarified by centrifugation at 60,000 g for 30 min.

The crude extract was mixed with equal volume of DE-52 (Whatman) slurry equilibrated with 50 mM Tris-Cl, pH 8.0 and the mixture was swirled for 1 h at 4°C to allow proteins adsorbed to the ion exchange resin. The supernatant was discarded by decanting. The resin was washed twice by gently mixing with 5 volumes of 50 mM Tris-Cl, pH 8.0 and discarding the supernatant after the resin settled. Resulting slurry was poured into a column and washed with 50 mM Tris-Cl, pH 8.0 until absorbance of the eluate at 280 nm reached a baseline. Adsorbed proteins were eluted with a stepwise gradient of 0.1 M, 0.2 M, 0.5 M, and 1 M NaCl in 50 mM Tris-Cl, pH 8.0.

Ammonium sulfate solution (3.0 M) was added to the 0.1 M NaCl fraction of the DE-52 chromatography to make a final concentration of 1.0 M ammonium sulfate. The sample was applied to a 1.1 × 6 cm octyl-Sepharose (GE Healthcare) column equilibrated with 1 M ammonium sulfate, 50 mM Tris-Cl, pH 7.5. The column was washed with 1 M ammonium sulfate, 50 mM Tris-Cl, pH 7.5 and eluted with 0.5 M and 0 M ammonium sulfate in 50 mM Tris-Cl, pH 7.5. The column was finally washed with 0.1 % Triton X-100 in 50 mM Tris-Cl, pH 7.5.

Active fraction of the octyl-Sepharose chromatography was concentrated and applied to a preparative non-denaturing polyacrylamide gel. Lipase activity was identified by the zymographic analysis. Lipase protein was recovered from a gel slice containing the active band by the electroelution in a dialysis bag filled with 25 mM Tris base, 250 mM glycine, pH 8.3. The electroelution was performed in a cold chamber at 50 V for 1 h.

Characterization

Molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12 % gel with Tricine buffer system. Broad range protein molecular weight maker was purchased from New England Biolabs (Ipswich). The gel was stained with Coomassie Brilliant Blue R250.

Amino terminal sequence was determined at Korea Basic Science Institute (KBSI) by the Edman degradation method. Internal sequences were determined at KBSI by ESI-MS (electrospray ionization-mass spectrometer) method after in-gel trypsin digestion. The sequences were used to search homologous lipases or esterases in Protein Data Bank (PDB) by the BLAST search method.

The colorimetric assay method was used for the determination of optimum temperature, thermal stability and optimum pH. In pH range of 4.13-7.06, citrate-phosphate buffer was used instead of Tris-Cl buffer to prepare the reaction mixture. As the hydrolysis rate of TAG is dependent on pH, control experiments were carried out at each pH with reaction mixtures that did not contain enzyme.

Effect of metal ions on the activity of the purified enzyme was analyzed by the colorimetric assay method. Final concentration of the metal ions in the reaction mixture was adjusted to 1 mM and EDTA was omitted from the reaction mixture.

Positional specificity of the purified enzyme was tested by the method of Farias *et al*. (28). Two reaction mixtures containing the purified enzyme were prepared according to the standard colorimetric assay method except that concentrations of DMPTB and DTNB were lowered to 0.05 and 0.2 mM respectively. Sufficient amount (10 units) of *C. rugosa* lipase was added to one of the two reaction mixtures at a time indicated by the arrow and the two reaction mixtures were further incubated until the absorbance reached a plateau.

Acknowledgements

This work was supported by "Regional Technology Innovation Program (grant no. RTI05-01-02)" of the Ministry of Commerce Industry and Energy (MOCIE) and by the Korea Science and Engineering Foundation (KOSEF) through the East Coastal Marine Bioresources Research Center at the Kangnung National University, Gangneung, Korea.

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