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Functional Proteomic Analysis of Rice Bran Esterases/Lipases and Characterization of a Novel Recombinant Esterase

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ABSTRACT: An esterase from rice (*Oryza sativa*) bran was identified on two-dimensional gel using 4-methylumbelliferyl butyrate as a substrate. The esterase cDNA (870 bp) encoded a 289 amino acid protein (designated OsEST-b) and was expressed in *Escherichia coli*. The molecular weight of recombinant OsEST-b (rOsEST-b) was 27 kDa, as measured by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Biochemical characterization demonstrated that rOsEST-b was active over a broad temperature range (optimum at 60 °C) and preferred alkaline conditions (optimum at pH 9.0). The rOsEST-b showed maximum activity toward *p*-nitrophenyl butyrate (C_4) among various *p*-nitrophenyl esters (C_4−C_{18}), indicating that rOsEST-b is an esterase for short-chain fatty acids. The kinetic parameters under optimal conditions were K_m = 27.03 μM, k_{cat} = 49 s^{-1}, and k_{cat}/K_m = 1.81 s^{-1} μM^{-1}. The activity of rOsEST-b was not influenced by ethylenediaminetetraacetic acid, suggesting that it is not a metalloenzyme. The amino acid sequence analysis revealed that OsEST-b had a conserved pentapeptide esterase/lipase motif but that the essential active site serine (GXSXG) was replaced by cysteine (C). These results suggest that OsEST-b is distinct from traditional esterases/lipases and is a novel lipolytic enzyme in rice bran.

KEYWORDS: Rice bran, lipolytic enzyme, esterase/lipase, two-dimensional gel electrophoresis

INTRODUCTION

Lipolytic enzymes, including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), catalyze both the hydrolysis and synthesis of esters and are widely distributed in nature. They are grouped into eight families[1] and have many biological functions, including hydrolysis of lipids and esters, pathogen defense, and roles in energy metabolism.[2−6] Moreover, they possess the characteristic α/β hydrolase fold in their three-dimensional structures also found in haloperoxidases, epoxide hydrolases, and dienelactone hydrolase.[7−9] Although the sequence diversity of esterases and lipases is huge, the catalytic triad (Ser-Asp-His) and pentapeptide GXSXG (where X is any amino acid) motif with serine (S) as the active site are conserved in most cases.[1,8−11] Although their molecular structures and catalytic mechanisms are very similar, lipases are distinguishable from esterase by their preferred substrates and interfacial activation/lid.[8,12,13] Another distinction is substrate specificity; esterases hydrolyze ester bonds of short-chain fatty acids with less than 10 carbons, whereas lipases hydrolyze ester bonds in long-chain fatty acids with more than 10 carbons.[10,12,13] Esterases and lipases exhibit high regio- and stereospecificities, broad substrate specificity, and the ability to remain active in organic solvents and have no cofactor requirements.[13−18] These properties are very useful in biotechnological applications, such as custom chemical synthesis in the pharmaceutical and food industries.[15,19−21]

Rice (*Oryza sativa*) is an important staple food for the majority of the world's population. Rice bran, an important and abundant byproduct, is obtained during the threshing of rice. Rice bran is a rich source of protein, vitamins, and fat[22] and is used as animal feed and fertilizer and to produce rice oil. The lipolytic enzymes of rice bran can cause rapid deterioration of rice bran oil to fatty acids and glycerol.[22,23] Although several rice bran lipases have been identified,[24−29] no rice bran lipase or esterase genes have been cloned and expressed. In this report, functional proteomics were used to analyze rice bran lipolytic enzymes and one esterase gene was isolated according to liquid chromatography/tandem mass spectrometry (LC/MS/MS) information. The esterase gene was successfully expressed in *Escherichia coli* as an active recombinant enzyme. Its biochemical properties were also characterized in this work. The results of the sequence analysis suggest that this enzyme (OsEST-b) is a new lipolytic enzyme in rice bran.

MATERIALS AND METHODS

Materials and Strains. The rice bran was obtained from a local rice mill (Tainan, Taiwan) in September. The *p*-nitrophenyl (*p*-NP) and 4-methylumbelliferyl (4-MU) esters used as substrates were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). The plasmid pET-20b(+) (Novagen, Germany) was used as both the cloning and expression vector. *E. coli* DH5α (GeneMark, Taiwan) and Tuner(DE3)pLysS (Novagen, Germany) were used as the cloning and expression hosts, respectively. All *E. coli* transformants were cultured in Luria−Bertani

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(LB) medium (1% peptone, 0.5% yeast extract, and 1% NaCl) at 37 °C. The antibiotics ampicillin and chloramphenicol were used at 100 and 34 μg/mL, respectively. The high-speed plasmid mini kit and Gel/PCR DNA fragments extraction kit were purchased from Geneaid (Geneaid Biotech, Bade City, Taiwan). Protein molecular mass markers were obtained from Fermentas (Canada).

Extraction of Rice Bran Proteins. About 50 g of rice bran (*O. sativa*) was defatted by washing with 150 mL of *n*-hexane and stirred for 30 min for 3 times.[24] After defatting, the rice bran sample was air-dried in a fume hood until the hexane was completely removed and prepared for protein extraction. The defatted rice bran was stirred with extraction buffer containing 10 mM Tris-HCl at pH 7.5 and 1.0 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C for 12 h. The extract was passed through a swab and then centrifuged at 13 000 rpm for 10 min at 4 °C.[25]

Ammonium Sulfate Precipitation. The rice bran extract was precipitated by saturated ammonium sulfate fractionation (0−30, 30−40, 40−50, 50−60, 60−70, and 70−80%, w/v) with constant stirring at 4 °C. The precipitates in each fraction were harvested by centrifugation at 13 000 rpm for 10 min and then resuspended in 20 mM Tris-HCl buffer at pH 7.5. After desalting using a protein desalting spin column (Pierce, Rockford, IL), the lipolytic activity of each fraction were analyzed by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and a further overlay activity assay in the same gel using 4-MU butyrate as the substrate.[30−32]

Functional Proteomics: Two-Dimensional (2D) Gel Electrophoresis. After lipolytic activity analysis, the fraction of 40−50% saturated ammonium sulfate was selected for 2D gel electrophoresis using a Bio-Rad Isoelectric Focusing System (Bio-Rad Laboratories, Hercules, CA) according to the protocols of the manufacturer. The first dimension of the gel used the immobilized pH 3.0−10.0 gradient strip, and the second dimension was 12% polyacrylamide SDS gel. Lipolytic activity analysis was performed simultaneously using the same gel after SDS was removed by washing in 25% isopropanol for 15 min according to the methods described by Chuang et al.[31] and Prim et al.[32] The activity spot that exhibited blue fluorescence was cut and digested with trypsin for nanoLC/MS/MS system (QSTAR XL) analysis. The result of LC/MS/MS was further identified through the Mascot search (http://www.matrixscience.com/search_form_select.html).

RNA Extraction and cDNA Synthesis. The total RNA of rice bran was extracted using a SV total RNA isolation system (Promega, Madison, WI). The synthesis of single-strand cDNA was carried out using the CapFinder PCR library construction kit (Clontech Laboratories, Palo Alto, CA).

Cloning of Rice Bran Lipolytic Enzyme Gene and Sequence Analysis. The cDNA of rice bran lipolytic enzyme *OsEST-b* was amplified by polymerase chain reaction (PCR) using the gene-specific primers designed on the basis of the bioinformation of LC/MS/MS. The cloning sites of *Nde*I and *Xho*I (in underscore) were incorporated into the forward primer *OsEST-b*N0 (5′-ggaattc**catatg**gccacaccgcaactgct-3′) and the reverse primer *OsEST-b*C0 (5′-ccg**ctcgag**cgccgatccaaggaaacga-3′), respectively. The 870 bp *OsEST-b* was obtained through 35 cycles of PCR using denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final incubation at 72 °C for 10 min. After digestion with *Nde*I and *Xho*I (New England BioLabs, Ipswich, MA), the *OsEST-b* DNA fragment was ligated into a pET-20b(+) expression vector that was previously treated with the same restriction enzyme pair. The recombinant pET-20b_*OsEST-b* plasmid was transformed into the cloning host *E. coli* DH5α. The amino acid sequence was analyzed using the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), and the signal peptide was predicted by SignalP 3.0 in the Center for Biological Sequence Analysis (CBS) (http://www.cbs.dtu.dk/services/SignalP/).[33] The molecular mass and pI of the encoded protein were calculated by ExPASy (http://www.expasy.ch/tools/protparam.html). The active site was predicted using the pfam database (http://pfam.sanger.ac.uk/).[34]

Expression and Purification of Recombinant Enzyme. The strain *E. coli* Tuner(DE3)pLysS was used to express recombinant OsEST-b (rOsEST-b). Cells were grown to an OD_{600} of 0.4 at 37 °C in LB broth containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). Isopropyl thio-β-D-galactoside (IPTG) was then added to a final concentration of 0.4 mM. Protein induction was carried out at a lower temperature of 25 °C for 4 h before cell harvesting. The cells were then lysed in BugBuster Protein Extraction Reagent (Novagen, Germany) according to the protocols of the manufacturer. The crude protein lysate containing soluble rOsEST-b was obtained after 10000*g* centrifugation for 15 min. The rOsEST-b was purified using BD TALON Superflow Resin designed for 6× His-tag protein affinity purification (BD Biosciences Clontech, Palo Alto, CA). After affinity purification, the partial purified recombinant enzyme was further purified using gravity-flow chromatography. The enzyme was loaded on a 1.5 × 14 cm column (Econo-Pac column, Bio-Rad Laboratories, Hercules, CA) packed with diethylaminoethyl (DEAE) Sepharose Fast Flow resin (GE Healthcare, U.K.), pre-equilibrated with 5 volumes of 50 mM sodium phosphate buffer at pH 7.0. The rOsEST-b was eluted in total 20 volumes of 50 mM sodium phosphate buffer at pH 7.0, 4 volumes for each step with a stepwise increase in NaCl (0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl). Each protein elution of 1.5 mL of buffer was collected in a separate tube. The purified recombinant enzyme was concentrated by Vivaspin 500 (5 kDa cutoff, GE Healthcare) and desalted using the protein desalting spin column (Pierce, Rockford, IL). The N-terminal amino acid sequences of the purified rOsEST-b were determined using Edman degradation. Briefly, the purified rOsEST-b was analyzed using 12% SDS−PAGE and then transferred on Immobilon-P polyvinylidene fluoride (PVDF) transfer membrane (Millipore) in a Mini Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA). After blotting, the PVDF membrane was stained with Coomassie Brilliant Blue and the target protein band was cut and sequenced using a Procise 494 protein sequencer (Applied Biosystems).

Gel Electrophoresis of Proteins and Overlay Activity Assay. SDS−PAGE (12%) was conducted following the method by Laemmli.[35] An overlay activity assay was carried out using the same gel according to the methods suggested by Chuang et al.[31] and Prim et al.,[32] with the following modifications. Briefly, after electrophoresis, SDS was removed by washing with 25% isopropanol for 15 min, after which the gel was immersed for 15 min in ddH_2O. The gel was rinsed several times in 50 mM Na phosphate buffer at pH 7.0, and then the substrate 4-MU butyrate (0.25 mM at a final concentration) was added. The blue fluorescence of the product 4-MU chromophor, detected under ultraviolet illumination, was used to estimate the lipolytic activity.

Enzyme Activity Assay and Protein Quantitation. Lipolytic activity was determined using either 4-MU butyrate or *p*-NP esters as the substrates. For colorimetric assays, the hydrolysis of *p*-NP esters was carried out at 37 °C for 3 min in 500 μL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM *p*-NP ester. The released *p*-NP was determined by measuring the absorbance at 410 nm using a Hitachi U-2010 spectrophotometer.[36] One unit of activity was defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under the aforementioned conditions. A fluorometric assay using 4-MU butyrate substrate was performed using a modulus single tube multimode reader (Promega, Madison, WI). The reaction mixture consisted of 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 10 μM 4-MU butyrate, and the purified enzyme was incubated for 2 min at 37 °C. Protein concentrations were determined by Bradford's[37] method using bovine serum albumin as the standard.

Biochemical Characterization of the Purified rOsEST-b. The optimal pH and temperature of rOsEST-b activity was determined using 4-MU butyrate as the substrate. Briefly, the activity of the enzyme over the pH range of 3.0−11.0 was measured using a 50 mM acetate buffer (pH 3.0−6.0), a 50 mM sodium phosphate buffer (pH 6.0−9.0),

and a 50 mM borate buffer (pH 9.0−11.0) at 40 °C. Enzyme activities were also assayed at various temperatures (25−90 °C) at pH 9.0 in sodium phosphate buffer. To analyze thermal stability, the enzyme was incubated at various temperatures over the range of 37−90 °C for 15 min, and residual activity was determined at 55 °C and pH 9.0 using 4-MU butyrate as the substrate. Kinetic analyses of the activity of purified rOsEST-b toward the substrate *p*-NP butyrate was carried out under optimum pH and temperature conditions. For the substrate specificities assay, the *p*-NP ester of various chain-length fatty acids (*p*-NP butyrate, C_4; *p*-NP caprylate, C_8; *p*-NP caprate, C_{10}; *p*-NP myristate, C_{14}; *p*-NP palmitate, C_{16}; and *p*-NP strearate, C_{18}) were determined by measuring the amount of *p*-nitrophenol released. To determine the sensitive to various metal ions (NaCl, KCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, and $ZnCl_2$), 5 mM of the tested metal ion was added to the enzyme reaction mixture of 50 mM Tris-HCl (pH 9.0) and then assayed for enzyme activity after preincubation at 30 °C for 30 min. The effect of chemical reagents and inhibitors on enzyme activity were measured by incubating the enzyme for 30 min at 30 °C in 50 mM Tris-HCl (pH 9.0), including EDTA, Triton X-100, Tween 80, SDS, dithiotheretiol (DTT), phenylmethanesulfony fluoride (PMSF), and iodoactamide. After incubation, the remaining activity was determined at 55 °C by adding *p*-NP butyrate as the substrate to the final reaction volume of 500 μL. The enzyme activity in the absence of metal ions or reagents was defined as 100%.

RESULTS AND DISCUSSION

Functional Proteomic Analyses of Rice Bran Lipolytic Enzymes. To clone the functional lipolytic enzyme gene from rice bran, functional proteomic approaches were used. The extract from defatted rice bran was precipitated by saturated ammonium sulfate fractionation (described in the Materials and Methods) and then resuspended in 20 mM Tris-HCl buffer (pH 7.5). The proteins were isolated by 12% SDS−PAGE, and then the lipolytic activity of each fraction was analyzed by the overlay activity assay in the same gel using 4-MU butyrate as the substrate. The result showed that the fraction from 40−50% saturated ammonium sulfate had the highest lipolytic activity, as revealed by the blue fluorescence of the product 4-MU (data not shown). As a result, this fraction was used for 2D gel electrophoresis analysis. As showed in Figure 1, there were at least two spots that exhibited lipolytic activity (blue fluorescence) on the 2D gel using 4-MU butyrate as a substrate, suggesting that rice bran contained several esterases or lipases. The spot that showed the highest activity was excised (indicated by an arrow in Figure 1), and the peptide mass fingerprint of the trypsin-digested activity spot was analyzed by LC/MS/MS and identified by Mascot search. In all, six peptides were obtained (Table 1 and Figure 2), and the Mascot search revealed that this activity spot shared the highest identity to a putative rice protein (accession number NP_001043244), which contains a conserved region associated with esterase/lipase activity (Figure 3). Specific primers were designed on the basis of the coding region sequence of Os01g0531500 (encoding NP_001043244). The 870 bp DNA fragment was amplified by PCR; this fragment was named *OsEST-b*. The plasmid pET-20b_*OsEST-b* containing the *OsEST-b* gene was confirmed to have the correct sequence by DNA sequencing.

Using ExPASy, the molecular mass of OsEST-b was predicted to be 31 kDa, with a predicted pI of 6.42. In addition, the active site was predicted with the pfam database;[34] the putative catalytic triad is composed of Cys^{154}, Asp^{202}, and His^{237} (Figure 3). The essential active site was cysteine (C^{154}), which substituted for serine (S), and appeared to be different from the general esterase/lipase motif GXSXG. A nucleophile−His−acid catalytic triad was highly conserved, and only the histidine in the triad was invariant in α/β hydrolase fold enzymes.[9] The cysteine in the active site could act as a nucleophile-like serine in the α/β hydrolase fold enzymes.[9]

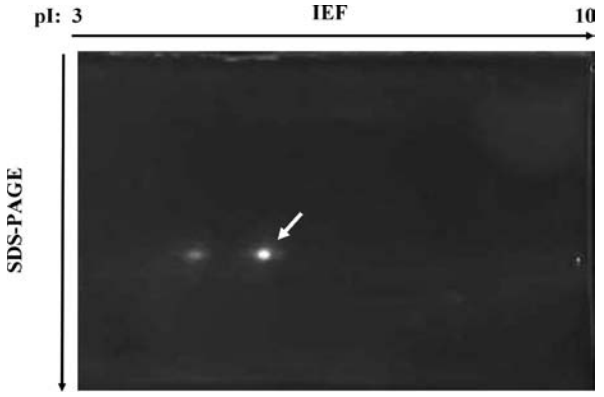

Figure 1. Two-dimensional electrophoresis analysis of rice bran extracts after ammonium sulfate precipitation. The fraction obtained from 40−50% ammonium sulfate saturation was separated using 2D gel on an immobilized pH gradient strip (pH 3.0−10.0) and 12% SDS−PAGE. After electrophoresis, lipolytic activity was analyzed using 4-MU butyrate as a substrate. The lipolytic activity spot that revealed the highest activity with blue fluorescence is indicated by an arrow.

Table 1. Protein Identification of Lipolytic Enzyme from Rice Bran Using Mascot Search

peptide sequence	position	mass (Da)	Mascot score
DDTTFDAYVVGK	54−65	1329.60	70
NHAVHISQIGEGYR	86−99	1579.78	61
ALIPDLYR	100−107	959.54	33
VALDVAEAQHLMEGLDWPGAVK	110−131	2348.18	48
AQAPIQAHFGELDSFVGFADVTAAK	190−214	2589.28	49
SSGVPHEVHIYPGCSHAFMNTSPEAVK	222−248	2954.33	38

MATPQLLLRRAFSSSFLSSPFRRPPLHPARSFVPPRAAMASSAAPFHMVQ
IQRDDTTFDAYVVGKENAPGIVVLQEWWGVDYEIKNHAVHISQIGEGYR*A*
*LIPDLYR*GKVALDVAEAQHLMEGLDWPGAVKDIQASVKWLKANGSPKVGV
TGYCMGGALSIASGVSVPEVDAVVAFYGTPPSELADASKAQAPIQAHFGE
LDSFVGFADVTAAKSLEEKLKSSGVPHEVHIYPGCSHAFMNTSPEAVKRR
KEMGLTDENQAAIDLAWSRFSTWMGRFLGSA

Figure 2. Peptide mass fingerprinting of the lipolytic enzyme from rice bran. After 2D gel electrophoresis, the peptide mass fingerprint of the trypsin digested activity spot was analyzed by LC/MS/MS and further identified by Mascot search. Peptide sequences are presented as underlined and in italics.

Expression and Purification of the Recombinant Enzyme. rOsEST-b was produced using the pET-20b(+) expression system under the conditions described in the Materials and Methods. The recombinant enzyme was purified using BD TALON Superflow Resin and then gravity-flow chromatography with DEAE Sepharose Fast Flow resin. The rOsEST-b was eluted for each step with a stepwise of 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl in 50 mM sodium phosphate buffer at pH 7.0. Each fraction was analyzed by SDS−PAGE and the overlay activity assay in the same gel.

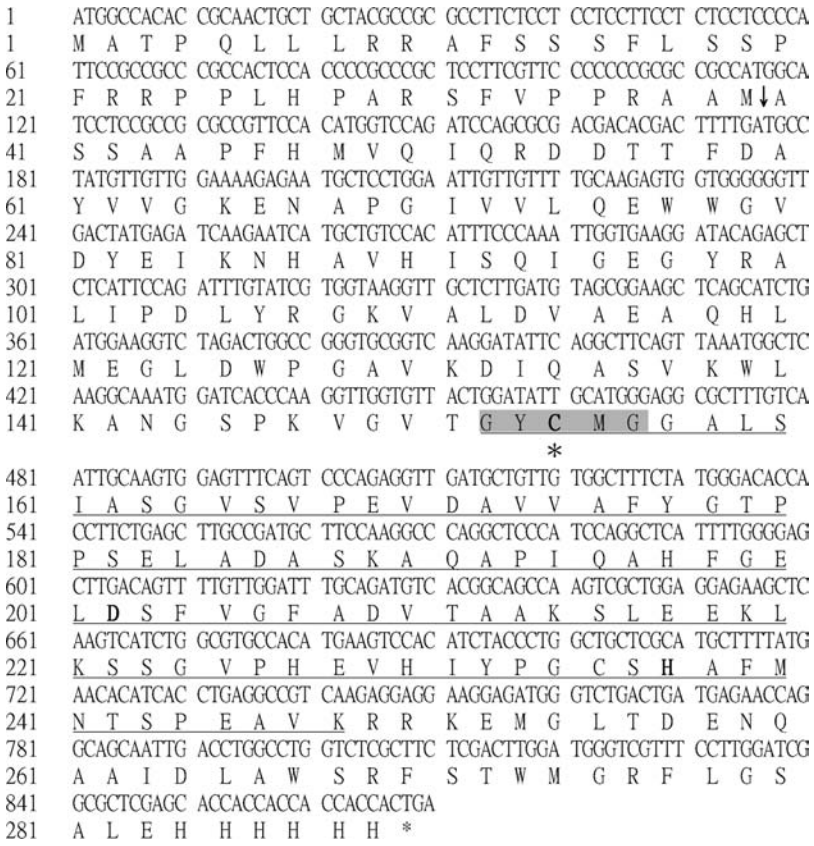

Figure 3. Deduced amino acid sequence of rOsEST-b. The single arrow indicates the cleavage site for the signal peptides in transformed *E. coli*. The putative catalytic triad is composed of Cys[154], Asp[202], and His[237], as indicated by bold letters. The predicted active site is marked with a star symbol, and the putative conserved motif is indicated by a gray block. The putative region of the esterase/lipase positions 152−248 is underlined.

The highest degree of purity was attained in the fraction eluted with 0.1−0.2 M NaCl (data not shown). Therefore, the purified enzyme was pooled from these fractions and concentrated. The purified rOsEST-b was found to be highly homogeneous by 12% SDS−PAGE. The overlay activity assay of rOsEST-b is shown in Figure 4. Although no signal peptide at the N terminus of OsEST-b was predicted by the SignalP 3.0 program,[33] the molecular weight of rOsEST-b was estimated to be about 27 kDa by SDS−PAGE, which was less than the theoretical molecular weight (31 kDa) predicted by ExPASy. This indicated that the rOsEST-b possibly had a signal peptide and was recognized in the *E. coli* expression system. The same cases have been found in previous studies.[38,39] The N-terminal amino acid sequence of purified rOsEST-b was directly determined by Edman degradation as ASSAAPFHM, which was different from the original predicted N-terminal sequence of MATPQLLLR. This result suggested that rOsEST-b contained a signal peptide that was recognized and cleaved between Met[39] and Ala[40] in the *E. coli* expression system (Figure 3).

Effect of the Temperature and pH on the Purified rOsEST-b. Purified rOsEST-b showed optimum activity at 60 °C and maintained higher activity (>50% relative activity) at temperatures ranging from 30−70 °C (Figure 5A), suggesting that rOsEST-b could adapt to a broader temperature range for its hydrolysis reaction. Additionally, the activity was observed between pH 8.0 and 10.0 with greater than 47% relative activity. The optimal pH of rOsEST-b was determined at 9.0. However, no activity was detected over pH 3.0−6.0 (Figure 5B), suggesting that rOsEST-b activity is optimal in alkaline conditions, a characteristic that could be applied to the detergent industry.[40,41] The thermal stability of rOsEST-b was analyzed by preincubation at various temperatures (37−90 °C) before assay at optimal reaction conditions (55 °C and pH 9.0). It was found that the thermal stability was relatively stable below 60 °C (87% of residue activity) but that rOsEST-b lost its stability rapidly at temperatures exceeding 60 °C (Figure 6).

Kinetic Parameter and Substrate Specificity of the Purified rOsEST-b. Kinetic analyses of rOsEST-b using *p*-NP butyrate (C_4) as the substrate and the kinetic parameter, such as K_m (27.03 μM), k_{cat} (49 s^{-1}), and k_{cat}/K_m (1.81 s^{-1} μM^{-1}), were calculated. To investigate the substrate specificity of rOsEST-b, the hydrolyzing activities of purified enzyme toward *p*-NP esters of various carbon chain lengths was determined at pH 9.0 and 55 °C. Substrates included butyrate (C_4), caprylate (C_8), caprate (C_{10}), myristate (C_{14}), palmitate (C_{16}), and strearate (C_{18}). As shown in Table 2, rOsEST-b showed efficient hydrolyzation toward short-chain fatty acids (C_4−C_{10}). Among these short-chain fatty acids, rOsEST-b had maximal activity toward *p*-NP butyrate (C_4). On the other hand, its activity decreased rapidly toward *p*-NP myristate (C_{14}) and palmitate (C_{16}), and no activity was detected toward *p*-NP strearate (C_{18}). These results suggest that OsEST-b is an esterase for short-chain fatty acids.[10,42,43]

Effect of Metal Ions and Chemical Reagents on the Purified rOsEST-b. The effects of metal ions and chemical reagents on the activities of rOsEST-b are presented in Table 3. In comparison to the control (without metal ions or reagents), monocations (Na^+ and K^+) had no influence on enzyme activity. The activity of the rOsEST-b enzyme was slightly inhibited by Ca^{2+}, Mn^{2+}, and Mg^{2+}, whereas the activity was significantly decreased by

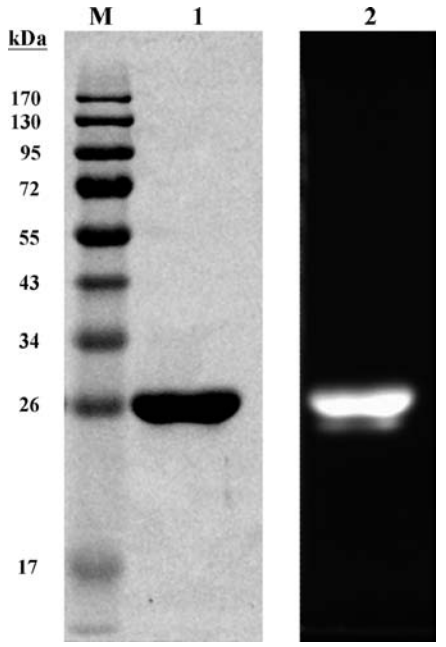

Figure 4. SDS−PAGE analysis of purified rOsEST-b stained with Coomassie Brilliant Blue R-250 (lane 1) and overlay activity assay of the lipolytic activity using 4-MU butyrate as the substrate (lane 2). Lane M is a PageRuler prestained protein marker (Fermentas).

5 mM Zn^{2+} (loss of 37% relative activity). For metagenomic lipase lipG, divalent metal ions, particularly Ca^{2+} or Mn^{2+}, are necessary for the catalytic activity of the enzyme.[44] Zhang et al.[42] indicated that the manganese ions might be a significant cofactor in the esterase action of the metagenomic esterase EstAS. The stabilization of some lipases by calcium has also been demonstrated in previous studies.[45−47] In contrast to these studies, OsEST-b did not require metal ions for hydrolysis; moreover, the activity of rOsEST-b was not affected by the presence of EDTA (5−10 mM). These results suggest that rOsEST-b is not a metalloenzyme requiring divalent cations as cofactors.[13,42,48]

Esterase activity was significantly reduced by 1 mM SDS and completely inhibited when the concentration reached 5 mM. Other chemical reagents, such as DTT (1 mM), Triton X-100 (0.1%), and Tween 80 (0.1%), also reduced the activity of the enzyme by 52, 17, and 26%, respectively. According to sequence analysis using the pfam database, the putative active site of the OsEST-b is cysteine and not traditional serine. To study the role of cysteine, the serine protease inhibitor PMSF and the alkylating agent iodoacetamide were used in this work. As expected, rOsEST-b activity was not affected by 1 mM PMSF because the putative active site of OsEST-b was cysteine. Interestingly, the enzyme activity was not influenced by iodoacetamide, which served as a thiol-blocking reagent, suggesting that OsEST-b may possess some protective conformation that helped avoid the inhibitory effect of iodoactamide.[42,47−50] A site-directed mutagenesis study will be conducted to confirm the function of Cys^{154}.

In conclusion, we have successfully cloned and identified an esterase, OsEST-b, from rice bran using functional proteomic techniques. The recombinant OsEST-b was expressed in *E. coli*, and many aspects of enzymatic activity were determined. It showed high activity toward short-chain fatty acids but greatly diminished activity toward long-chain fatty acids. Its activity was not affected by EDTA, suggesting that OsEST-b is not a

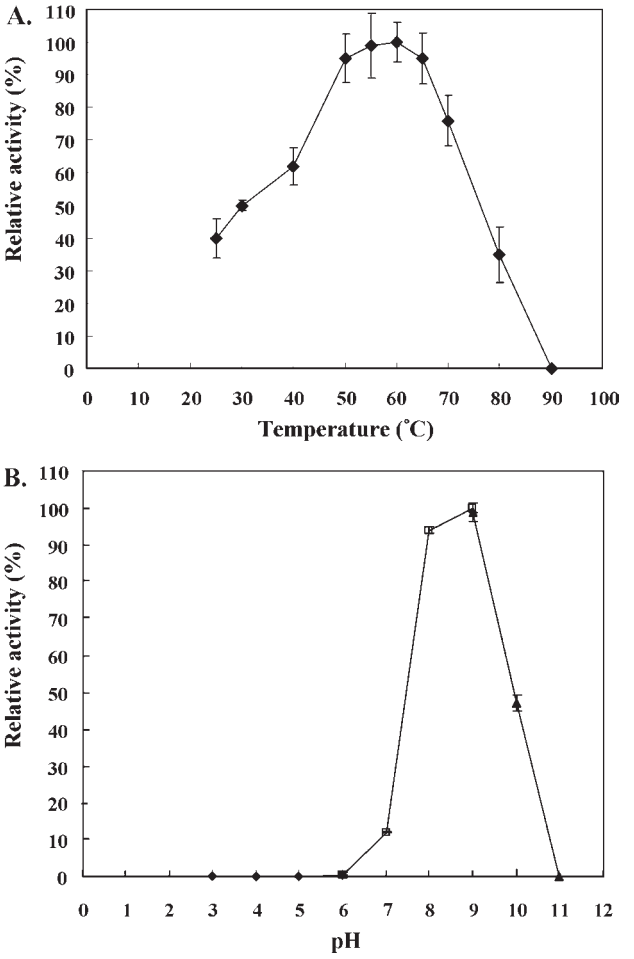

Figure 5. (A) Optimum temperature and (B) pH profiles of the purified rOsEST-b. (A) Enzyme activity was measured using 4-MU butyrate as a substrate at different temperatures (25−90 °C) and pH 9.0 (sodium phosphate buffer). Activity at 60 °C corresponds to 100%. (B) Activity of the enzyme from pH 3.0−11.0 was measured using 50 mM acetate buffer (pH 3.0−6.0, ◆), 50 mM sodium phosphate buffer (pH 6.0−9.0, □), and 50 mM borate buffer (pH 9.0−11.0, ▲) at 40 °C. Activity at pH 9.0 corresponds to 100%.

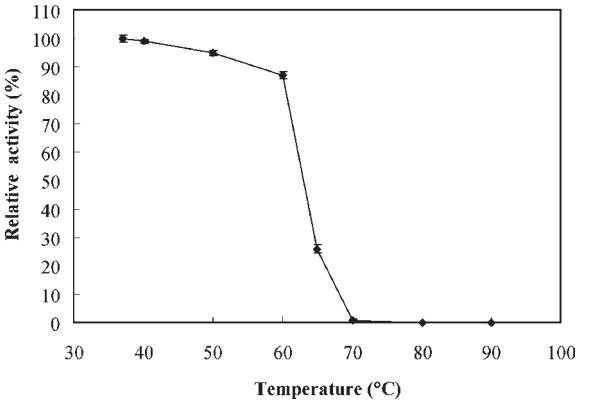

Figure 6. Thermal stability of the purified rOsEST-b. After 15 min of incubation at different temperatures, the residual activities were measured by a modulus single tube multimode reader using 4-MU butyrate as a substrate at 55 °C and pH 9.0. Activity at 37 °C corresponds to 100%.

Table 2. Substrate Specificities of the Purified rOsEST-b with the *p*-NP Ester of Various Chain-Length Fatty Acids

substrate	specific activity[a] (units/mg)
p-NP butyrate (C_4)	93.67 ± 0.28 (100)[b]
p-NP caprylate (C_8)	76.50 ± 1.23 (81)
p-NP caprate (C_{10})	55.67 ± 0.29 (59)
p-NP myristate (C_{14})	1.09 ± 0.01 (1)
p-NP palmitate (C_{16})	0.55 ± 0.01 (0.5)
p-NP strearate (C_{18})	ND[c]

[a] Unit definition: 1 unit of activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of *p*-NP ester per minute at 55 °C and pH 9.0. Values are the mean ± standard deviation (SD) from three independent experiments. [b] Relative activities (percent) of each enzyme are given in parentheses, with the highest activity denoted as 100%. [c] ND = not detectable.

Table 3. Effects of Metal Ions, Chemical Reagents, and Inhibitors on the Activity of the Purified rOsEST-b[a]

	relative activity (%)
control	100
metal ions (5 mM)	
Na^+	97
K^+	100
Ca^{2+}	89
Mn^{2+}	85
Mg^{2+}	87
Zn^{2+}	63
chemical reagents	
EDTA (5 mM)	102
DTT (1 mM)	48
Triton X-100 (0.1%)	83
Tween 80 (0.1%)	74
SDS (1 mM)	69
SDS (5 mM)	0
inhibitors	
PMSF (1 mM)	100
iodoacetamide (1 mM)	92

[a] Activity without metal ions and reagents was defined as 100%.

metalloenzyme. Meanwhile, OsEST-b presented a broader temperature range and preferred alkaline conditions at its hydrolysis reaction. Hence, it could be used in biotechnological and detergent applications. The predicted active site cysteine was found to be different from the traditional active site serine of esterase/lipase. Furthermore, the enzyme activity was not affected by iodoactamide, suggesting that it may possess some conformation that could protect the active site. The real function of cysteine will be further investigated. On the basis of these results, we suggest that OsEST-b is different from traditional esterases/lipases and may possess a unique function in rice bran. The biological function of the novel enzyme will be investigated in future studies.

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