J. Appl. Biol. Chem. **51**(3), 95-101 (2008)

Article

Cloning and Characterization of an Esterase from *Xanthomonas oryzae* pv. *oryzae*

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Received May 25, 2008; Accepted June 19, 2008

The gene encoding a putative esterase of *Xanthomonas oryzae* pv. *oryzae* was cloned using PCR technique. The gene was expressed with His6 tag in *E. coli*. One-step purification of the recombinant esterase with Ni-NTA resin resulted in one band by SDS-PAGE analysis. The purified enzyme showed a molecular weight of 30 kDa, as expected, therefore the enzyme was a mononer. The enzyme was the most active toward *p*-nitrophenyl (*p*-NP) acetate and *p*-NP-butyrate to a lesser extent. However, the enzyme could not hydrolyze *p*-NP-myristate, palmitate, and stearate. Therefore, the enzyme is considered as an esterase, very different from lipase. The purified esterase had optimal pH at around 8.0 and was stable in a broad range of pH values. The optimal temperature ranged from 30 to 40°C, and the residual activity observed after heat treatment at 55°C for 20 min was 72% of the initial activity. The activity was inhibited by the presence of copper and cobalt ions.

Key words: *esterase, pNP-acetate,* Xanthomonas oryzae

Esterases (EC 3.1.1.3) and lipases (EC 3.1.1.1) catalyze the hydrolysis of carboxyl esters, with or without the variable chain length of fatty acid, *i.e.*, from C2 to C18 or more. The enzymes, widely distributed in microorganisms, plants, and mammalian cells, have been purified and characterized [Janssen *et al.*, 1994; Baigori *et al.*, 1996; Hou *et al.*, 1999; Staubmann *et al.*, 1999; Fenster *et al.*, 2000; Chao *et al.*, 2003]. These enzymes belong to a superfamily, "α/β hydrolase" [Oills *et al.*, 1992; Nardini *et al.*, 1999] with their common characteristics of α/β fold, which is the central, predominantly parallel β-sheet flanked by the α-helical connections [Ollis *et al.*, 1992]. Most esterases and lipases also share a characteristic of the sequence motif, -Gly-Xaa-Ser-Xaa-Gly-, which is called the "nucleophilic elbow" [Oills *et al.*, 1992; Nardini *et al.*, 1999]. The serine residue in this motif constitutes a "catalytic triad" together with the Asp and the His residues (*i.e.*, serine-aspartic acid-histidine) in the polypeptide chain. Besides these sequences, some other sequence motifs have also been found in this superfamily [Shaw *et al.*, 2002]. The enzymes can be classified into two groups based on the sequence motif at the oxyanion hole (either GlyX or GlyGlyGlyX).

In spite of their similarities in the catalytic properties and molecular structure, esterase can be distinguished from lipase by the affinity for short chain acyl derivatives and less requirement for interfacial activation. Classification of these enzymes based on the sequence information in the public databases has been reported by Arpigny and Jaeger [1999]. Their physiological functions have been implicated in the utilization as a carbon source, pathogenicity, and detoxification [Berger *et al.*, 1998; Khalameyzer *et al.*, 1999]. In general, carboxylesterases exhibit high region- and stereo-specificities, and require no cofactor in the enzymatic reaction [Bornscheuer *et al.*, 2002].

Because of the easy availability, esterases, especially from the microbes, are of interest for the industrial application. For instance, the enzymes are very attractive biocatalysts in the applications of fine chemical synthesis, food processing, resolution of racemic mixtures of compounds in the production of pure enantiomers, ester synthesis, and transesterification [Moher *et al.*, 1989; Bornemann *et al.*, 1992; Margolin, 1993; Bornscheuer *et al.*, 2002]. For the transesterification of racemates,

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

doi:10.3839/jabc.2008.021

enantiomeric up to 98% have been achieved [Gu *et al.*, 1986; Gupta and Kazlauskas, 1993; Ozaki *et al.*, 1995].

Another application of the esterase for industrial purpose could be the removal of the protecting groups as shown by the *p*-nitrobenzyl esterase from *Bacillus subtilis,* which specifically removes this residue from the antibiotic Loracarbef [Zock *et al.*, 1994]. The cooperative reaction of esterases and cellulases resulted in greater degradation of the cellulose acetate [Moriyoshi *et al.*, 2003]. The isoenzyme patterns of esterases can be used as molecular markers for the analysis of taxonomic, genetic, and evolutionary relationships between different cultivars of *Oryza sativa* [Suh *et al.*, 1997].

Despite various implications of the esterase use in the industries, only some have been purified and characterized from the microorganisms including fungi, yeast, and bacteria. In order to extend the understanding of esterase and to find a possibility of industrial applications, an esterase from *X. oryzae* was cloned based on the data in the NCBI gene bank and purified through an affinity chromatography by preparing the His-tagged recombinant protein. This paper describes biochemical properties of the purified esterase and potential application of the enzyme for the industrial use.

Materials and Methods

Bacterial strains, plasmids, and culture media. *X. oryzae* pv. *oryzae* strain (KACC 10331) was obtained from Korean Agricultural Culture Collection (KACC) and used for the genomic DNA preparation. *Escherichia coli* DH5α was used as a cloning host for the gene encoding a putative esterase of *X. oryzae*. *E. coli* BL21(DE3), which contains the gene for T7 RNA polymerase under the control of the *lac* promoter, was used for the induction and expression of the esterase gene isolated from *X. oryzae*. *E. coli* strains were maintained and grown in the Luria-Bertani (LB) medium [Sambrook *et al.*, 1989], supplemented with the appropriate antibiotic. The plasmid pGEM-T Easy (Promega, Madison, WI) was used as a cloning vector for the esterase gene. The plasmid pET-21(a) (Invitrogen, Carlsbad, CA) harboring the T7 promoter was used for the expression of esterase gene. PCR products were purified using Wizard PCR Preps DNA Purification System (Promega).

Gene cloning. *X. oryzae* cells were cultured using a medium consisting of sucrose 0.5%, yeast extract 1.0%, and peptone 0.5%. After cultivation at 30°C for 36 h, the cells were harvested and centrifuged. Subsequently, total genomic DNA was isolated using a commercial kit (Bioneer, Daejon, Korea) and stored at −20°C until used for the isolation of the putative esterase gene.

The putative gene (NCBI accession number, NC 006834; gene ID, 3265750) was amplified by PCR using a sense primer, 5'-cat atg gaa cgt atc gaa cat cgt gcc-3' (underlined seqence for *Nde*I adaptor), and an antisense primer, 5'-tcc ccg caa ggc cgc agc atg atg -3'. The amplified gene did not contain a stop codon TGA for the preparation of His6-tagged recombinant protein. The PCR product (ca. 0.8 kb) was isolated and ligated into the pGEM-T Easy vector (Promega). Competent *E. coli* DH5α cells were transformed with the ligation mixture and inoculated in the LB agar medium in the presence of ampicillin (100 μg/mL), IPTG (0.5 mM), and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 75 μg/mL). After culture at 37°C for 24 h, desired colonies were picked and inoculated in to 5 mL LB medium containing ampicillin. The cells were further cultured and harvested by centrifugation. The plasmid containing the esterase gene was isolated using a commercial kit (DyneBio, Gyeonggi, Korea). The appropriate insertion of esterase gene was confirmed by PCR and DNA sequence analysis. DNA was dideoxy-cycle-sequenced with fluorescent terminators (Big Dye, Applied Biosystems, Foster City, CA) in an Applied Biosystems ABI Prism 377 automated DNA sequencer.

Plasmid construction. The recombinant plasmid (pGEM-T Easy vector) inserted with the putative esterase gene was digested by *Nde*I and *Sal*I restriction enzymes, and the double-digested product was isolated. The isolated gene was ligated with pET-21(a), which was also digested by the same restriction enzymes. The constructed pET-21(a) vector harboring the gene was transformed into competent *E. coli* DH5α cells and cultured in LB-agar medium containing ampicillin (100 μg/mL) for 18 h. The desired colony harboring the pET-21(a) was further cultured in 5 mL LB medium containing ampicillin, followed by the isolation of pET-21(a). Insertion of the gene in the pET-21(a) was confirmed by digestion with *Nde*I and *Sal*I, followed by agarose gel analysis. The pET-21(a) ligated with the desired insert was named pET-21(a)-est.

Expression and preparation of cell-free extract. The pET-21(a)-est was transformed into competent *E. coli* BL21(DE3) cells, and the cells were inoculated in LB-agar medium containing ampicillin (100 μg/mL). After culture for 18 h, the desired colony harboring the pET-21(a)-est was picked and further cultured in 5 mL LB medium supplemented with ampicillin. The incubation was continued with shaking at 30°C until the A_{600nm} reached 0.5, and the cells were inoculated in to 200 mL of the same LB medium. After further incubation for 4 h, the induction was carried out by adding IPTG to a final concentration of 0.5 mM. An additional culture was

carried out for 12 h with slow shaking (rpm 50) at 28°C. The cells were harvested by centrifugation and washed once in buffer A (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole). The washed cells were suspended in buffer A, disrupted by sonication on ice, and then centrifuged at 10,000 g for 10 min. The cell-free extract obtained by centrifugation was used for the protein purification by affinity chromatography.

Purification of the His6-tagged protein. The crude extract suspended in 50 mL of the buffer A was loaded slowly in to a HisÞBind Resin column (1×10 cm) previously charged with Ni(II) ion and equilibrated with the buffer A. After the column was washed with buffer B (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 80 mM imidazol), elution was performed with an elution buffer (50 mM Tris-HCl pH 7.9, 500 mM NaCl, and 50-1000 mM imidazole linear gradient) at a flow rate of 0.5 mL/min. The fraction containing the putative esterase was monitored by assaying the esterase activity using *p*-NP-acetate as a substrate. The fractions containing the His6-tagged esterase were then collected and dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 20% glycerol, and 2 mM EDTA). The active fraction obtained was used for the analysis of SDS-PAGE and esterase assay for characterization.

Enzyme assay. The enzyme activity was measured with *p*-NP-acetate as a substrate by the method of Wood *et al.*, [1995]. One milliliter of the assay reaction mixture contained 2 mM *p*-NP acetate, 50 mM Tris-HCl (pH 7.5), and suitable amount of enzyme preparation. The enzymatic reaction was performed for 20 min at 37°C and the released *p*-nitrophenol content was determined by a spectrophotometric method at 405 nm. One unit of the esterase activity was defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol per min under the standard assay conditions. The molar absorptivity of *p*-nitrophenol is 18,389 M^{-1} cm^{-1} at 405 nm.

The active fractions hydrolyzing *p*-NP-acetate were also assayed for their catalytic potential against other substrates with different chain length such as *p*-NP butyrate, decanoate, myristate, palmitate, and stearate (each 1 mM) under the standard assay condition.

Effect of pH on the activity and pH stability. The effect of different pH on the enzyme activity was determined using *p*-NP-acetate. The enzymatic reactions were performed at pH 4.0-5.5 (50 mM citrate phosphate buffer), pH 5.5-7.0 (50 mM potassium phosphate buffer), and pH 7.0-9.0 (50 mM Tris-HCl buffer), and the esterase activity was determined as described above. For the test of pH stability, the enzyme fraction (50 μL) was distributed into different buffers (200 μL) showing different pH values ranging from 3.0 to 10.0: citrate phosphate (pH 3.0 to 5.0), potassium phosphate (pH 6.0 to 7.0), and Tris-HCl buffer (pH 8.0 to 10.0). After keeping for 1 h at room temperature, the aliquots were removed, and the esterase activity was measured under the standard assay condition. Since carboxyl ester bonds can be hydrolyzed, to some extent, at acidic or basic condition, control experiments were performed using the enzyme-free reaction mixture, and the values obtained were subtracted from each experiment.

Effect of temperature and thermal stability. To determine the effect of temperature on the esterase activity, the enzymatic reaction was performed on a water bath at different temperatures ranging from 20 to 60°C, and the esterase activity was determined under the standard assay condition using *p*-NP-acetate as the substrate. For the test of heat stability, the enzyme preparations were loaded in to 1.5 mL tubes and incubated in a water bath at 55 or 70°C. The tubes were removed at 5 min intervals, chilled on ice, and centrifuged briefly. The residual activities were measured under standard assay conditions as described above.

Other analytical methods. Protein concentration was determined by the method of Bradford [1976] using bovine serum albumin as a standard protein. SDS-PAGE was performed according to the method of Laemmli [1970] using 5% stacking gel and 12.5% resolving gel, followed by the staining of the proteins with Coomassie Brilliant Blue R-250.

Results and Discussion

Expression and purification of putatiave esterase. To obtain a large amount of the enzyme from *X. oryzae*, attempt was made to express the His6-tagged esterase in *E. coli* BL21(DE3) harboring the pET-vector. However, routine culture of the cell at 37°C and rpm 200 resulted in the formation of an inclusion body, thus it was difficult to obtain a soluble enzyme having the esterase activity. Formation of the insoluble enzyme was suggested based on the very low esterase activity and the absence of the expected protein band in SDS-PAGE of the crude extract prepared by mechanical disruption, sonication, commercial cell lysis kit, or combination of the methods (detailed not shown). To circumvent the problem, the cells were cultured under the low growth condition by keeping the incubation temperature at 28°C and lowering the agitation rate to 50 rpm. Under this condition, the gene was overexpressed (Fig. 1. lane 3), as expected, and the formation of the inclusion body was significantly decreased as revealed by SDS-PAGE analysis and the esterase activity assay (data not shown).

Purification of the recombinant enzyme was facilitated

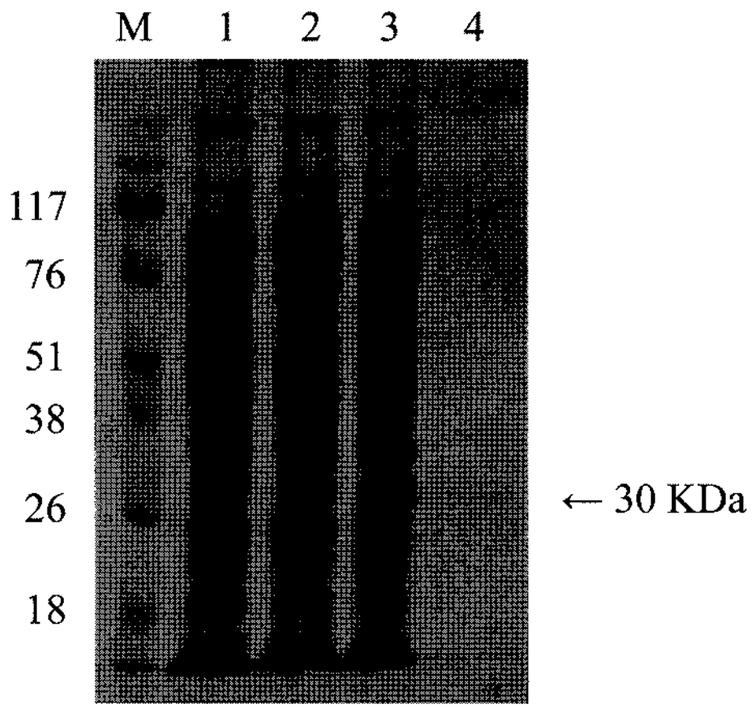

Fig. 1. SDS-PAGE analysis of the esterase originated from *X. oryzae*. Lane M, molecular mass markers; 1, crude extract from *E. coli* BL21(DE3) not harboring plasmid; 2, crude extract from *E. coli* BL21(DE3) harboring pET-21(a), which did not contain insert (*i.e.*, vector control); 3, crude extract from *E. coli* BL21(DE3) transformed with pET-21(a)-est; 4, the His6-tagged esterase purified by affinity chromatography using the Ni-charged His·Bind Resin.

Table 1. Substrate specificity of an esterase from *X. oryzae*, assayed using different substrates. The enzymatic reaction was performed under standard assay condition using different substrates. The enzyme activity hydrolyzing *p*-NP-acetate was considered as 100%, which was equivalent to 92.8 units/mg protein. Each value represents the average of three experiments

Substrate	Relative activity (%)
p-NP-acetate	100
p-NP-butyrate	38
p-NP-decanoate	4
p-NP-myristate	<1
p-NP-palmitate	<1
p-NP-stearate	<1

by the presence of six histidine residues. Figure 1 shows SDS-PAGE analysis of the active fraction obtained from the affinity chromatography. The electrophoresis performed in the presence of the reducing agent, β-mercaptoethanol, showed one band with a molecular mass of about 30 kDa.

Substrate specificity. To assess the substrate specificity of the enzyme, a series of fatty acid *p*-nitrophenyl esters with different chain lengths, from C2 to C18, were tested as substrates under the standard assay condition (Table 1). Maximum activity was observed from the assay using *p*-NP-acetate and the enzyme hydrolyzed *p*-NP-butyrate to the extent of 38%. However, the enzyme was almost inactive toward the substrates having carbon chain length higher than 10.

This profile is a characteristic of an esterase activity, *i.e.*, an enzyme only active on short-chain fatty acid esters as compared to lipases, which are also active on long-chain insoluble fatty acid esters [Rhee *et al.*, 2005]. Therefore, the purified enzyme in this study was classified as an esterase and did not show the characteristics of the lipase.

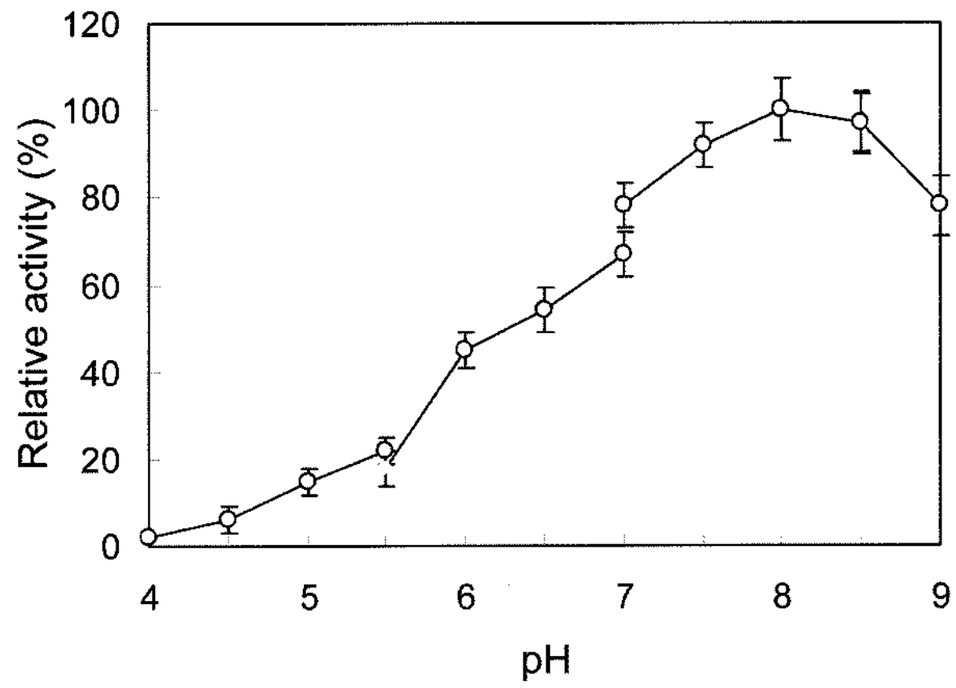

Fig. 2. pH effect on the esterase activity of *X. oryzae*. The reaction mixture consisted of 2 mM *p*-NP acetate, 50 mM sodium acetate buffer (pH 4.0-6.0), sodium phosphate buffer (pH 6.0-7.0), and Tris-HCl (pH 7.0-9.0). Maximum activity was considered as 100% activity, equivalent to 102.4 units/mg protein. Each point represents the average of three experiments.

pH optimum and pH stability. The effect of pH on the esterase activity was determined by assaying the rate of *p*-NP-acetate hydrolysis at different pH values ranging from 4.0 to 9.0 (Fig. 2). Maximum activity was observed around pH 8.0, and, generally, higher activities occurred at the alkaline pH rather than at the acidic pH. At pH 6.0, only 44% of maximum activity was observed, and the enzyme showed almost no hydrolysis of the substrate at pH 4.0. It was difficult to assay the enzyme activity at pH higher than 10 with the substrate due to the significant chemical hydrolysis of *p*-NP-acetate.

Optimum activity of the esterase at alkaline pH was similar to those of some microbial and plant esterases. For instance, pH optima ranging from 8.0 to 9.5 were reported for *A. fatua* [Mohamed *et al.*, 2000], and tomato [Stuhlfelder *et al.*, 2002].

Figure 3 shows the effect of pH on the enzyme stability. The residual activities after 1 h treatment at different pH values were determined and plotted against the pH values. The esterase was found to be considerably stable at a broad range of pH values, and the enzyme retained over 90% of the maximum activity at alkaline pH ranging from 7.0 to 9.0. The enzyme also retained considerable activity at acidic pH, and the residual activity was 64% of the maximum activity at pH 4.0. This high stability at different pH values makes the esterase of *X. oryzae* valuable for industrial application at broad range of pH.

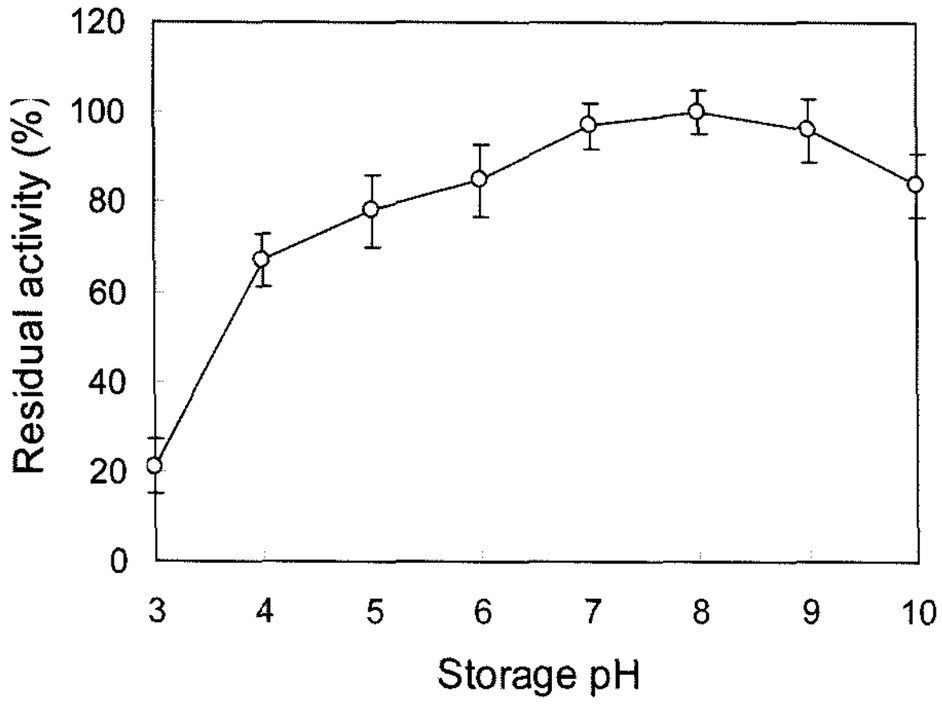

Fig. 3. pH stability of the purified esterase. The esterase preparation was suspended in different buffers having pH ranges from 3.0 to 10.0; 50 mM sodium acetate buffer for pH 3.0-5.0, sodium phosphate buffer for pH 6.0-7.0, and Tris-HCl buffer for pH 8.0-10.0. After 1 h standing, an aliquot was withdrawn, and the esterase activity was measured at pH 8.0 under the standard assay condition. Maximum activity, at pH 8.0, was considered as 100% activity equivalent to 94.7 units/mg protein. Each point represents the average of three experiments.

Optimum temperature and thermal stability. The temperature dependence for the esterase activity was measured with *p*-NP-acetate as a substrate at pH 8.0 (Fig. 4). The esterase hydrolyzed *p*-NP-acetate at a broad range of temperature, with the maximum activity at 35°C. The hydrolytic activity of the esterase decreased gradually when the incubation temperature decreased from the optimum temperature. However, the enzyme still showed a higher activity at 20°C, with 74% of maximum activity. The activity rapidly decreased when the incubation temperature was raised up to 50°C. The optimum temperature of the esterase was close to those reported for several other esterases of *Micrococcus* sp. ATCC8459 and tomato (40°C) [Bhowmik and Marth, 1990; Stuhlfelder *et al*., 2002], *Lactobacillus casei* sub. and *L. fermentum* (30-35°C) [Lee and Lee, 1990; Gobbetti *et al*., 1997].

Thermal stability of the esterase was determined by retaining the enzyme preparation at 55 and 70°C for a given time and by assaying residual activity of the heat-treated esterase (Fig. 5). After 20 min of heat treatment at 55 and 70°C, the residual activities were 72 and 56% of the initial activity, respectively; 30 min of heat treatment brought about a considerable decrease in the residual activities, resulting in 46 and 23% of the initial activity, respectively.

Effect of cations on the esterase activity. The effects of various divalent cations at 0.2 and 1.0 mM on the esterase activity were tested using *p*-NP-acetate as the substrate (Table 2). All examined cations, except Ca^{2+},

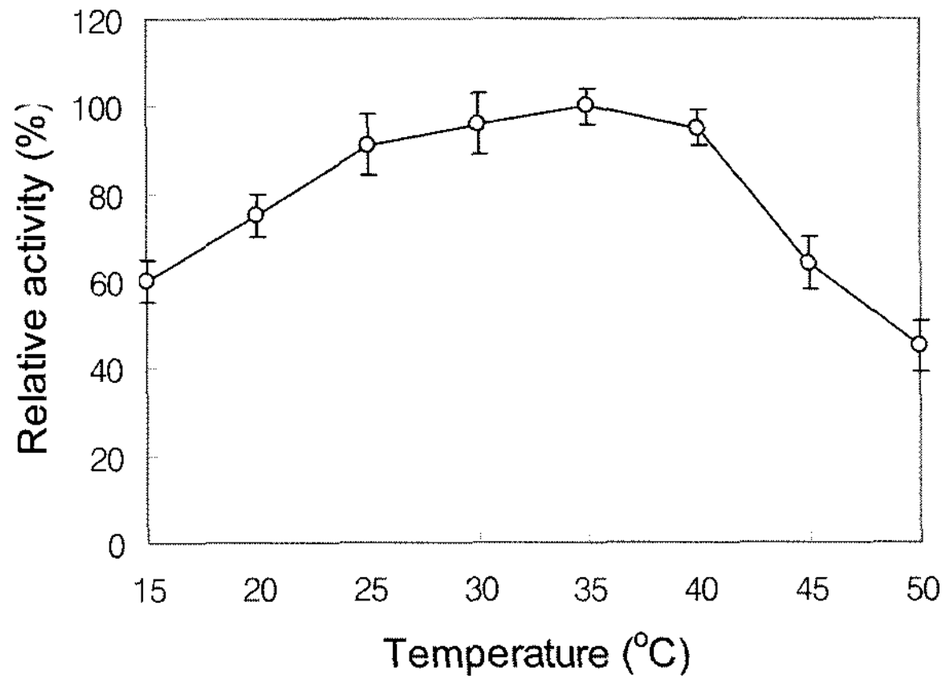

Fig. 4. Effect of temperature on the esterase activity of *X. oryzae*. The esterase activity was measured at different temperatures under the standard assay condition. The maximum activity at 35°C was considered as 100% activity, which was equivalent to 91.2 units/mg protein. Each point represents the average of three experiments.

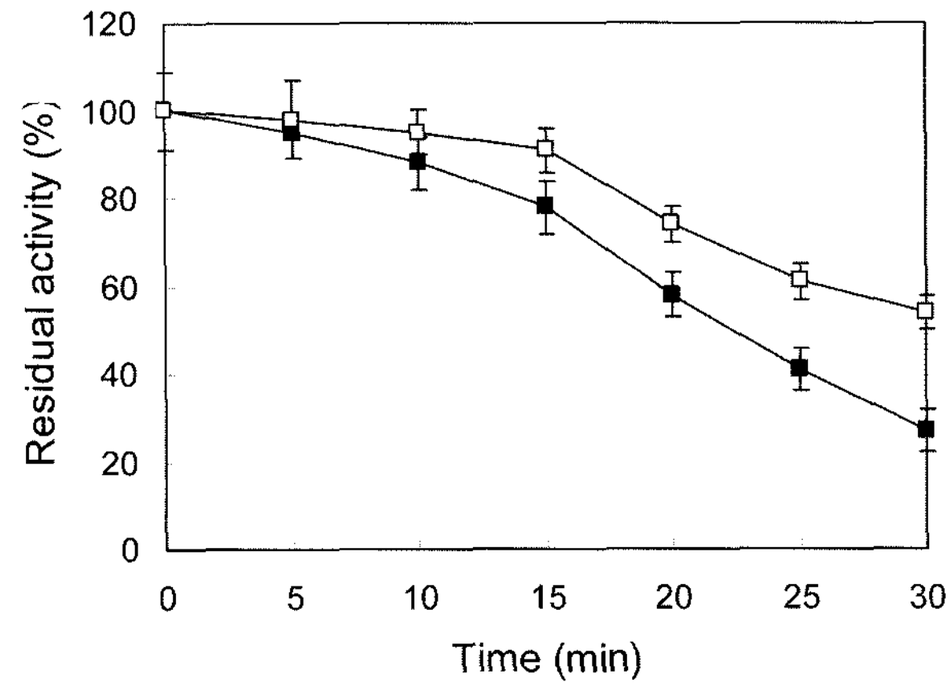

Fig. 5. Heat stability of the esterase from *X. oryzae*. The enzyme preparations were heat-treated at 55 (-□-) or 70°C (-■-), and each aliquot was withdrawn at an interval of 5 min. The residual activity was then assayed under the standard assay condition. The initial activity, without heat treatment, was considered as 100% activity, which was equivalent to 117.4 units/mg protein. Each point represents the average of three experiments.

showed inhibitory effects on the esterase activity. Inhibitory effect of the copper ion was the highest. Additions of 0.2 and 1 mM copper ion markedly decreased the enzyme activity, to the extent of 45 and 21% activities, compared to that of the control. The enzyme activity was also significantly inhibited by the presence of Co^{2+} ion, respectively showing 51 and 39% activities of the control at the same concentration. However, Ca^{2+} slightly stimulated the enzyme activity, and 131% of the control activity was obtained by addition of 1.0 mM Ca^{2+} ion. The stimulatory effect of Ca^{2+} could be attributed to the better alignment of the enzyme on the

Table 2. Effect of divalent cations on the activity of esterase. The enzyme activity was tested in the presence of various divalent cations using *p*-NP acetate as a substrate, and the activity in the absence of cation was taken as the control (l00% activity), equivalent to 104.7 units/mg protein. Each value represents the average of three experiments

Compound	Concentration (mM)	Relative activity (%)
Control		100
Cu^{2+}	0.2	45
	1.0	21
Co^{2+}	0.2	51
	1.0	39
Mg^{2+}	0.2	97
	1.0	94
Mn^{2+}	0.2	64
	1.0	45
Zn^{2+}	0.2	91
	1.0	87
Ca^{2+}	0.2	102
	1.0	131

substrate molecule [Lee and Lee, 1990]. *A. futua* esterase also showed similar stimulatory effect by Ca^{2+} [Mohamed *et al.*, 2000].

Conclusion. A putative esterase from *X. oryzae* was cloned and characterized. Because the higher activity towards short chain acyl derivatives is a prominent characteristic of the esterase, the enzyme was considered as an esterase rather than a lipase. For the application of the enzyme in the biotechnological field, investigation on the substrate specificity towards natural carboxyl esters should be further processed.

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