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Characterisation of esterolytic activity from two wild mushroom species, *Amanita vaginata* var. *vaginata* and *Tricholoma terreum*

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ABSTRACT

Characterisation of esterase activities from the edible mushroom species, *Amanita vaginata* var. *vaginata* and *Tricholoma terreum*, were investigated. Native electrophoresis of the crude extracts prepared from both mushroom samples showed the presence of esterolytic activities. The extracts had the greatest activity in the presence of *p*-nitrophenyl butyrate (*p*NPB) as a substrate. pH and temperature optima were found to be 8.0 and 30 °C for both enzymes, respectively. V_{max} and K_m values were determined as 14.2 U/l and 71 μM for *A. vaginata* var. *vaginata* and 34.6 U/l and 9.6 μM for *T. terreum*, respectively. The pH-stability profile showed a stationary line between 3.0 and 10.0 for both enzymes. The esterolytic activities from the extracts were maintained between 10 and 40 °C for 4 h and started to decrease at 50 °C. The effects of EDTA, NaN_3, DTT and PMSF on the enzyme activity were also investigated.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) and esterases (carboxyl ester hydrolases, EC 3.1.1.1) are hydrolytic enzymes that hydrolyse ester bonds. While lipases catalyse the triacylglycerols in the lipid–water interface, esterases use soluble substrates, such as short chain carboxylic acid esters (Soliman, Knoll, Abdel-Fattah, Schimid, & Lange, 2007). These enzymes are found in animals, plants and microorganisms (Bournscheuer, 2002).

In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media. Lipases and esterases catalyse esterification, interesterification, acidolysis and alcoholysis reactions (Kawamoto, Sonomoto, & Tanaka, 1987). The synthesis of flavour esters for the food industry, modification of triglycerides for the fat and oil industry, and resolution of racemic mixtures used by the synthesis of fine chemicals for the pharmaceutical industry can be performed with these enzymes (Molinari, Brenna, Valenti, & Aragozzini, 1996).

Mushrooms are not only valuable foods but also good enzyme sources. So it is interesting to study enzyme activities from different mushroom species for identifying different enzyme characters. Esterases are valuable enzymes because they have many applications in industry. We determined that *Amanita vaginata* var. *vaginata* and *Tricholoma terreum* showed esterase activity after preliminary work investigating esterase/lipase activity of some mushroom species in our laboratories. In this paper, the characterisation of esterase activity from two mushroom species, *A. vaginata* var. *vaginata* and *T. terreum*, was reported.

2. Materials and methods

2.1. Materials and chemicals

All reagents were of analytical grade and used as obtained. Substrates were purchased from Sigma Chemical Co. (St. Louis, MO). *A. vaginata* var. *vaginata* and *T. terreum* mushroom samples were collected from the Lişer High Plateau in Macka (Trabzon, Turkey).

2.2. Enzyme extraction

Crude enzyme extracts were prepared as reported previously with slight modifications (Colak, Sahin, Yildirim, & Sesli, 2007; Kolcuoğlu, Colak, Sesli, Yildirim, & Saglam, 2007). Mushroom samples (10 g) were treated with liquid nitrogen for 15 min and the cell membranes were decomposed. The cold mushroom samples were separately homogenised in 10 ml of acetate buffer (pH 5.0), phosphate buffer (pH 7.0) and Tris–HCl buffer (pH 9.0) containing 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at 4 °C, using a blender. All of the buffers were cold and at 50 mM concentration. The homogenates were filtered and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants were treated with the same volume of cold acetone (−30 °C) and stored at 4 °C for overnight. Then the suspensions were centrifuged at 8000 rpm for 10 min at 4 °C. The pellets were

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dried and dissolved in suitable buffers. After the second centrifugation, the supernatants were used as crude enzyme extracts.

2.3. Protein determination

Protein concentrations were determined by the Lowry method, using bovine serum albumin as the standard (Lowry, Rosebrough, Farr, & Randall, 1951).

2.4. Enzyme assay

Esterase activity was assayed by measuring spectrophotometrically the amount of released *p*-nitrophenol (*p*NP) (Lee et al., 1999). For the preparation of stock *p*-nitrophenyl butyrate (*p*NPB) solution, *p*NPB was dissolved in acetonitrile at a concentration of 10 mM. Substrate solution was prepared by mixing *p*NPB solution, ethanol and 50 mM phosphate buffer (pH 7.5) in the ratio of 1:4:95 (v/v/v), respectively. To prepare the reaction mixture, 0.6 ml of the crude enzyme extract was added to 1.8 ml of the substrate solution. After incubation of the reaction mixture at 30 °C for 20 min, the absorbance at 405 nm was measured. The amount of released *p*NP was determined for the esterolytic activity. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. One unit of enzyme activity was defined as 1 μmol of *p*NP formed per min under assay conditions.

2.5. Characteristics of the crude enzyme

2.5.1. Substrate specificity and enzyme kinetics

Stock solutions of *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl laurate (*p*NPL) and *p*-nitrophenyl palmitate (*p*NPP) were separately prepared at a concentration of 10 mM. The activity was determined as described above.

The kinetic data were plotted as reciprocals of activities versus substrate concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined as the reciprocal absolute values of the intercepts on the *x*-and *y*-axes, respectively, of the linear regression curve (Lineweaver & Burk, 1934).

2.5.2. Effect of pH on esterase activity and pH stability

The effect of pH on esterolytic activity was determined by using the following buffers (all at 50 mM): glycine–HCl buffer (pH 3.0), acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 and 7.0), Tris–HCl buffer (pH 8.0 and 9.0) and glycine–NaOH buffer (pH 10.0). The optimum pH obtained was used for determining thermal properties and other parameters.

The same buffers were used to determine pH stability of the crude extracts for esterolytic activity. The mixture (1:1) of crude extract and buffer given above was incubated for 24 h at 4 °C and the standard enzyme assay described previously was performed. The percentage residual activities were calculated by comparison with unincubated enzymes (Faiz, Colak, Saglam, Canakci, & Beldüz, 2007).

2.5.3. Effect of temperature on esterase activity and thermal stability

To determine the optimum temperature for esterolytic activity, enzymatic reactions at various temperatures over the range of 10–80 °C were performed by using the procedure described above.

In order to determine the thermal stability, aliquots of enzyme in Eppendorf tubes were incubated from 20 min to 4 h at various temperatures over 10–90 °C with 10 °C increments. After incubation, the tubes were rapidly cooled in an ice bath and then brought to room temperature. The activity was determined with the enzyme assay described previously. The percentage residual activities were calculated by comparison with unincubated enzyme (Faiz et al., 2007).

2.5.4. Effect of protein concentration on esterase activity

The hydrolysis of *p*NPB by crude extract was performed over different protein concentrations ranging from 0.005–0.7 mg/ml. Activity tests were performed under standard reaction conditions.

2.5.5. Effect of some metal ions on esterase activity

The effects of Na^+, K^+, Li^+, Ni^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Cd^{2+} and Al^{3+} on the esterolytic activity were investigated. Final concentration of each metal ion in the reaction mixture was 10 mM. The percentage remaining activities were determined by comparison with the standard assay mixture with no metal ion added (Lee et al., 1999).

2.5.6. Effect of some chemicals on esterase activity

The enzyme activities were separately monitored in the presence of Na–EDTA, sodium azide (NaN_3), PMSF and dithiothreitol (DTT) at different concentrations. The percentage remaining activities were determined by comparison with the standard assay mixture with no chemical added (Colak et al., 2005) and the I_{50} values were calculated for each chemical.

2.5.7. Native polyacrylamide gel electrophoresis

Native PAGE electrophoresis was performed by using a non-denaturating acrylamide gel of 10% (Laemmli, 1970). After electrophoresis, the gel was incubated for 30 min in a 1:1 mixture of solution A (8 mg α-naphthyl acetate dissolved in 3 ml acetone and added to 17 ml of 100 mM Tris–HCl buffer, pH 7.5) and solution B (20 mg Fast Red dissolved in 20 ml of 100 mM Tris–HCl buffer, pH 7.5) for activity staining. Appearance of red colour on the gel showed the presence of esterolytic activity (Bornscheuer et al., 1994; Faiz et al., 2007).

3. Result and discussion

In the present study, esterase potentials of two mushroom species were evaluated. Native PAGE and activity staining indicated that *A. vaginata* var. *vaginata* and *T. terreum* have esterolytic activity (Fig. 1a). The R_f values for each mushroom were calculated. They were found as 0.47 and 0.61 for *A. vaginata* var. *vaginata*, 0.42 and 0.47 for *T. terreum*. This result showed that each crude extract has at least two enzymes showing esterolytic activity. The existence of more than two isoenzymes having esterolytic activity has been reported for different organisms like *Bacillus subtilis* (Eggert et al., 2002), EM2L8 (Park et al., 2007) and *Lycoperdon perlatum* (Colak, Camedan, Faiz, Sesli, & Kolcuoglu, in press).

3.1. Substrate specificity and enzyme kinetics

Substrate specificity of the each crude enzyme was tested toward several *p*-nitrophenyl esters having different chain lengths and percentage relative activities were calculated (Table 1). The highest activity for both crude extracts was obtained toward *p*NPB, whereas no activity or very slight activity was obtained toward *p*NPP. When *p*NPL was used, the relative activity decreased 62% for *T. terreum* but no activity was observed for *A. vaginata* var. *vaginata*. A similar result was obtained for a thermostable esterase from *Geobacillus thermoleovorans* YN cloned and characterised. Substrate specificity was initially tested toward several *p*-nitrophenyl esters. The highest activity was obtained toward *p*-nitrophenyl acetate, and also PNP-butyrate was hydrolysed quite well, whereas *p*-nitrophenol esters with longer chain lengths were converted only slightly or not at all. This confirms the assumption that the enzyme is an esterase rather than a lipase (Soliman et al., 2007). These results showed that esterolytic enzymes in the crude extracts prepared from *T. terreum* and *A. vaginata* var. *vaginata* could be true esterases.

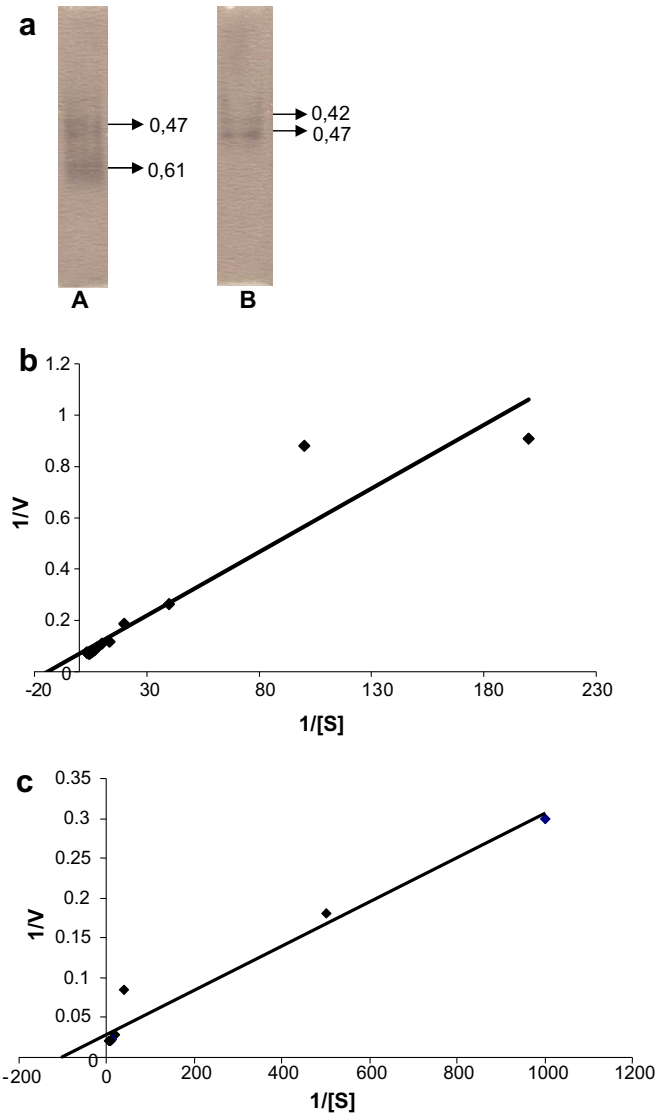

Fig. 1. (a) Native PAGE profiles for crude extracts of *A. vaginata* var. *vaginata* (A) and *T. terreum* (B). Lineweaver–Burk plots of *A. vaginata* var. *vaginata* esterase (b) and *T. terreum* esterase (c) toward *p*-nitrophenyl butyrate as a substrate at 30 °C.

Table 1
Substrate specificities of *A. vaginata* var. *vaginata* and *T. terreum* esterases towards *p*-nitrophenyl esters.

Substrate	Relative activity (%)	
	A. vaginata var. *vaginata*	*T. terreum*
p-Nitrophenyl acetate ($C_8H_7NO_4$)	10.03	2.53
p-Nitrophenyl butyrate ($C_{10}H_{11}NO_4$)	100	100
p-Nitrophenyl laurate ($C_{18}H_{27}NO_4$)	0	62
p-Nitrophenyl palmitate ($C_{22}H_{35}NO_4$)	0	0.19

Michaelis–Menten constants (K_m) and maximum reaction velocities (V_{max}) were determined from the Lineweaver–Burk plots (Fig. 1b and Fig. 1c) as 71 μM and 14.2 U/l, respectively, for *A. vaginata* var. *vaginata*, and 9.6 μM and 34.6 U/, respectively, for *T. terreum*,. The calculated K_m and V_{max} values of some esterolytic enzymes characterised toward *p*NPB were 6.74 mM and 5350 μmol/min.mg for *G. thermoleovorans* (Soliman et al., 2007), 4 mM and 10 μmol/min.ml for *Bacillus coagulans* BTS-3 (Pahujani, Kanwar, Chauhan, & Gupta, 2008), and 1 mM and 63.7 U/mg for *E. coli* (Kim et al., 2006), respectively.

3.2. Effect of pH on esterase activity and stability

The effect of pH on esterase activity and pH stability was tested using *p*-nitrophenyl butyrate. As shown in Fig. 2a, the pH optima of both enzymes were found to be 8.0. The residual activities were determined after 24 h of incubation at various pH values. The pH-stability profiles for both species showed a horizontal line ranging from pH 3.0–10.0 (Fig. 2b). It appears that the esterases of both species are quite stable between these pH values. The stability of the enzymes in acidic, neutral and basic pHs is important in terms of industrial applications. Similar results were reported earlier for esterases from *G. thermoleovorans* YN (Soliman et al., 2007), *Mucor* sp. (Abbas, Hiol, Deyris, & Comeau, 2002) and *Cucurbita pepo* (EII) (Fahmy et al., 2007).

3.3. Effect of temperature on esterase activity and thermal stability

Temperature-activity graphics are shown in Fig. 3a for *A. vaginata* var. *vaginata* and *T. terreum*. Esterases of both crude extracts were most active at temperatures between 30 and 40 °C but activity strongly decreased above 50 °C for *A. vaginata* var. *vaginata* and 60 °C for *T. terreum*. Esterases with optimum temperatures of 30-40 °C were also reported for *C. pepo* Elc and EII (Fahmy et al., 2007), *P. fluorescens* (Khalameyzer, Fischer, Bornscheuer, & Altenbuchner, 1999) and *S. simulans* (Sayari, Agrebi, Jaoua, & Gargouri, 2001). Fig. 3b and c show the loss of activity of the enzymes incubated for different lengths of time at various temperatures. The enzymes remained stable at temperatures 30 and 40 °C when

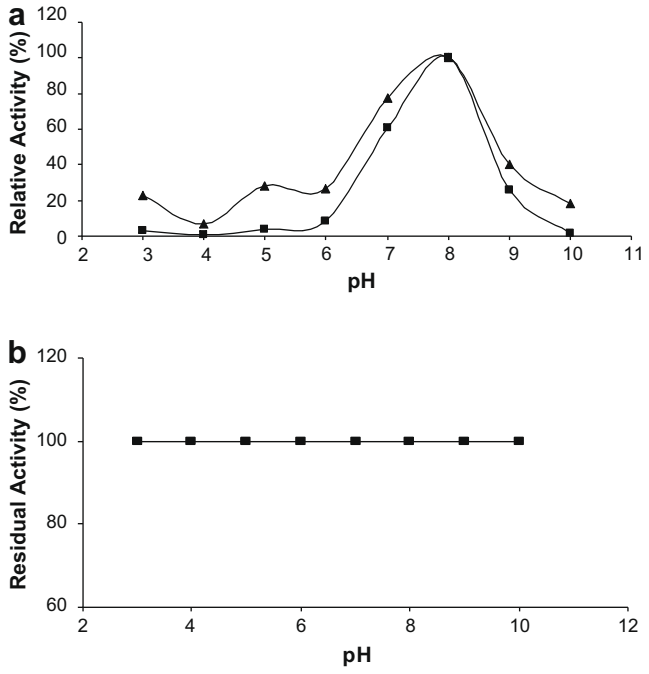

Fig. 2. (a) Effect of pH on esterolytic activity of *A. vaginata* var. *vaginata* (▲) and *T. terreum* (■). Assays were performed in 50 mM of different buffer systems at indicated pH; glycine–HCl buffer (pH 3.0), acetate buffer (pH 4.0 and pH 5.0), phosphate buffer (pH 6.0 and pH 7.0), Tris–HCl buffer (pH 8.0 and pH 9.0) and glycine–NaOH buffer (pH 10.0) at 30 °C. *p*-Nitrophenyl butyrate used as a substrate. (b) pH stability profile of esterolytic activities of *A. vaginata* var. *vaginata* and *T. terreum*. Residual activity was determined after 24 h incubation at indicated pH at 4 °C. The activity assays were carried out under standard conditions (pH 8.0 Tris–HCl buffer at 30 °C).

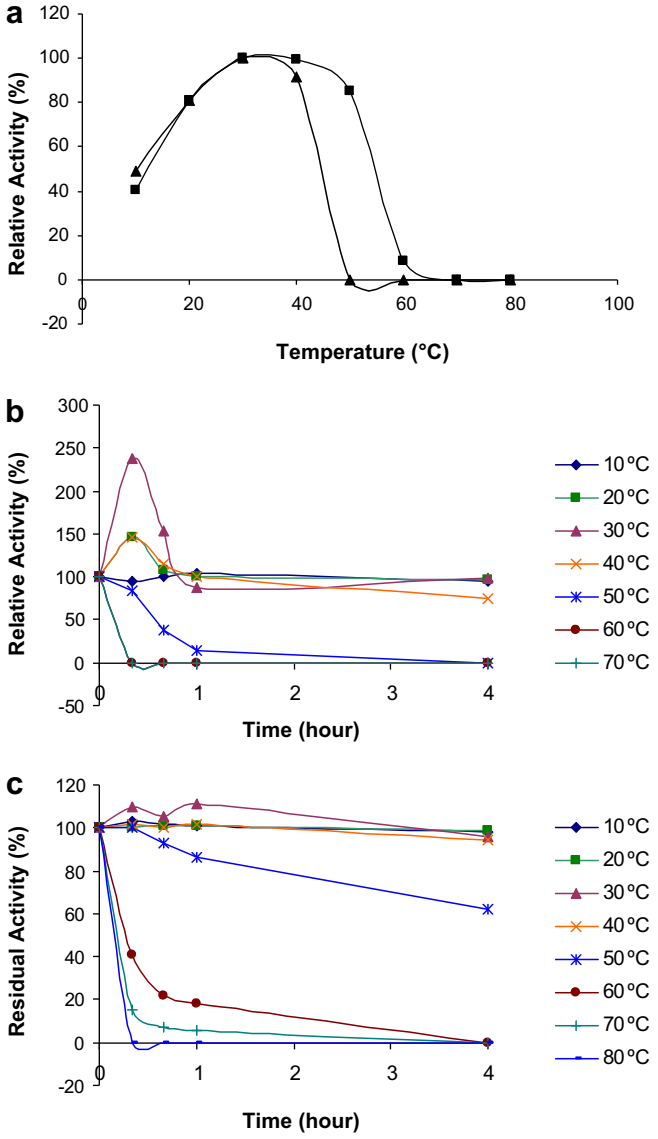

Fig. 3. (a) Effect of temperature on esterolytic activity of *A. vaginata* var. *vaginata* (▲) and *T. terreum* (■). Reactions were carried out by using *p*-nitrophenyl butyrate as a substrate in 50 mM Tris–HCl buffer, pH 8.0, at different temperatures from 30–80 °C. Thermal stability profile for esterolytic activity of *A. vaginata* var. *vaginata* (b) and *T. terreum* (c). Crude extracts were incubated from 20 min to 4 h at various temperatures over 10–70 °C with 10 °C increments. The percentage residual activities were calculated by comparing with unincubated enzyme.

incubated for 4 h. However, they are unstable at temperatures above 50 °C. The activities were fully lost between 70 and 80 °C. Interestingly the activity of esterase of *A. vaginata* var. *vaginata* at 30 °C for 20 min increased 2.5-fold when compared with the initial activity. Esterase activity for both crude extracts was maximal and fairly stable between 30 and 40 °C.

3.4. Effect of protein concentration on esterase activity

The protein concentrations in the crude extracts of *A. vaginata* var. *vaginata* and *T. terreum* were found to be 1.8 mg/ml and 4.2 mg/ml, respectively. The effect of protein contents on the *p*NPB esterase activity were investigated by varying protein concentrations between 0.01 and 0.7 mg/ml. The optimum protein concentrations were found to be 0.35 mg/ml for *A. vaginata* var. *vaginata* and 0.5 mg/ml for *T. terreum*. These protein contents were used for the kinetic studies.

Table 2
Effect of various metal ions on *A. vaginata* var. *vaginata* and *T. terreum* esterolytic activities.

Metal ion	Residual activity (%)	
	A. vaginata var. *vaginata*	*T. terreum*
None	100	100
K^+	100	100
Na^+	88	99
Li^+	80	103
Ni^{2+}	67	86
Mn^{2+}	89	63
Cu^{2+}	82	81
Cd^{2+}	77	36
Co^{2+}	84	27
Ca^{2+}	75	85
Al^{3+}	100	68

Table 3
I_{50} values of some chemicals for esterolytic activity.

Chemicals	I_{50} for *A. vaginata* var. *vaginata* (mM)	I_{50} for *T. terreum* (mM)
Na-EDTA	47.7	44.5
NaN_3	6.6	27.6
DTT	0.7	0.7
PMSF	2.8	0.9

3.5. Effect of some metal ions and chemicals on esterase activity

Metal ions have an important role on enzyme activity and structure for binding the amino acid residues in specific sites (Bock, Katz, Markham, & Glusker, 1999; Ditusa, Christensen, McCall, Fierke, & Toone, 2001). The effects of Na^+, K^+, Li^+, Ni^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Cd^{2+} and Al^{3+} on esterolytic activities of *A. vaginata* var. *vaginata* and *T. terreum* are shown in Table 2. Activity was inhibited in the presence of Na^+, Li^+, Ni^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, $Ca^{2+}$and Cd^{2+} for *A. vaginata* var. *vaginata* and Ni^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Cd^{2+} and Al^{3+} for *T. terreum*. All inhibitions occurred to different degrees but not completely. Similar results were obtained with the esterolytic enzyme of *Anoxybacillus gonensis* A4 (Faiz et al., 2007).

I_{50} values of Na-EDTA, NaN_3, PMSF and DTT for esterolytic activity are shown in Table 3. I_{50} values of NaN_3, PMSF and DTT were found to be very low concentrations when compared to I_{50} values of Na-EDTA. The inhibition of esterolytic activity in the presence of Na-EDTA and NaN_3 can be attributed to their metal-chelating effect. DTT, a reducing agent of disulfide bridges, also inhibited the enzyme activity. It could be thought from this result that –SH groups were important for enzyme activity. The inhibition of enzyme activity with PMSF interacting selectively and irreversibly with the serine hydroxyl groups can be attributed to the presence of serine residues in the active site (Faiz et al., 2007).

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