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Partial Characterization of a Lipase from Gypsy Moth (*Lymantria dispar* L.) Larval Midgut*

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Lipase activity of the gypsy moth (*Lymantria dispar* L.) was studied by the spectrophotometric method using crude homogenate of fifth-instar larval midgut tissues as the enzyme source and p-nitrophenyl caprylate (pNPC) as substrate. A Km value of 0.310mM and a Vmax value of 1.479U/mg prot. were obtained for this substrate. Among various p-nitrophenyl esters tested, maximum activity was obtained for p-nitrophenyl caprylate and p-nitrophenyl caprate. The enzyme was most active at alkaline pH, with maximum at pH 8.2. Decreased activity was detected after preincubation in buffers of pH below 7.0 and above 8.2. The enzyme was unstable at room temperature. The enzyme was Ca^{2+} independent. Its activity was inhibited by PMSF, Fe^{2+}, Ag^{+} and Pb^{2+}, while Fe^{3+} inhibited enzyme activity by about 40%.

Key words: *Lymantria dispar* L., lipase, p-nitrophenyl esters, optimal conditions, inhibition.

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Lipases (triacylglycerol hydrolases, EC 3.1.1.3.) are a group of enzymes that successively hydrolyze triacylglycerols to di- and monoacylglycerols, with free fatty acids as coproducts (BEISSON *et al*. 2000). Lipases are generally water soluble, and catalytic reactions take place after the adsorption of enzymes onto an oil-water interface (DELLAMORA-ORTIZ *et al*. 1997; BEISSON *et al*. 2000). Additionally, lipases hydrolyze nitrophenyl esters at the oil-water interface. The released nitrophenol is water soluble and can be monitored spectrophotometrically (ZHONG & GLATZ 2006). Lipase and esterase/lipase operate on different substrates, but share structural similarity, i.e. the arrangement of three catalytic amino acid residues (the "catalytic triad"): a nucleophile (usually serine, Ser), an acid (usually aspartate, Asp), and base (histidine, His) (OLLIS *et al*. 1992; NARDINI & DIJKSTRA 1999).

Lipases are widely distributed in nature and their physical and biochemical properties, and activities, have been investigated in many species of animals (GROENER & KNAUER 1981; LAI *et al*. 1998; DEĞERLI & ALI AKPINAR 2002; OKU *et al*. 2006), plants (LAMIKANRA & WATSON 2004; ZHONG & GLATZ 2006), bacteria (SIMONS *et al*. 1996; HONG & CHANG 1998; DOSANJH & KAUR 2002; TEO *et al*. 2003; GUPTA *et al*. 2004), yeast (CRABBE *et al*. 1996) and fungi (GARCIA-LEPE *et al*. 1997; PANDEY *et al*. 1999; PERA *et al*. 2006). By using nitrophenyl esters as substrates, lipases have been investigated in bacteria (HUMBLE *et a*l. 1977; ARAGON *et al*. 2000), insects (COSTA & DA CRUZ-LANDIM 2005), and other arthropods (GIBSON & BARKER 1979; LÓPEZ-LÓPEZ *et al*. 2003).

Because of their activities in both aqueous and nonaqueous systems (ZAKS & KLIBANOV 1985), it has become evident that lipases have many applications in industry and medicine (JAEGER & REETZ 1998; SCHMID & VERGER 1998; LAYER & KELLER 2003). They also have received considerable attention in insect pest management. Considering their crucial role in digestion of food lipids and mobilization of lipids from storage tissues, insect lipases are potential targets for developing new insecticides.

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Lipases have been investigated in different insect tissues. Recent studies have focused on identification, characterization and endocrine control of lipases in the insect fat body (SUN & STEELE 2002; PATEL *et al*. 2005; AUERSWALD *et al*. 2005; PATEL *et al*. 2006; AUERSWALD & GÄDE 2006; ARRESE *et al*. 2006). The first lipase purified from an insect was a triacylglycerol-lipase from a *Manduca sexta* fat body (ARRESE & WELLS 1994). Some reports have shown lipase activity in the reproductive system of insects (VAN ANTWERPEN *et al*. 1998) and in other insect tissues (HOFFMAN 1979; SMITH *et al*. 1994; HAUNERLAND 1997; ARREGUIN-ESPINOSA *et al*. 2000). The insect midgut is an organ that allows not only modification of dietary lipids, but also the formation of tissue reserves in an organized manner (CANAVOSO *et al*. 2004). Midgut lipases have been studied in few insect species (TURUNEN & CHIPPENDALE 1977; RANA *et al*. 1997; NOR ALIZA & STANLEY 1998; RANA & STANLEY 1999; NOR ALIZA *et al*. 1999; CANAVOSO *et al*. 2004).

The gypsy moth, *Lymantria dispar* L., is a highly polyphagous insect and one of the major defoliators of hardwood trees in Europe, north-west Africa, Asia and North America. Its host range is estimated to be over 500 plant species, among which oaks are ranked as most favored (LANCE 1983). There has been considerable interest in the investigation of midgut enzymes since larval midgut is a possible target for insect control, as a primary interface between insect and environment. Many studies have focused on digestive enzyme activities in the gypsy moth (VALAITIS 1995; PERIĆ-MATARUGA *et al*. 1997; LAZAREVIĆ *et al*. 1998; LEE *et al*. 1998; VLAHOVIĆ 2003; LAZAREVIĆ *et al*. 2004), but data on lipase activity are lacking. Midgut lipases are essential for lipid digestion and it is important to determine their catalytical properties and parameters such as specificity, thermal stability and pH optima. The aim of the present study was to determine optimal conditions for enzyme activity *in vitro*, and to examine some properties of gypsy moth midgut lipase.

Material and Methods

Insect rearing

Egg masses of the gypsy moth were collected from the oak forest of Bogovadja (70 km southwest of Belgrade, Serbia). During the winter period, egg masses were kept at 4°C until May, when they were transferred to a constant temperature of 23°C to hatch. The caterpillars were reared at constant temperatures of 23°C, a photoperiod of 12L:12D, and fed an artificial high wheat germ (HWG) diet (O'DELL *et al*. 1985).

Preparation of crude midgut homogenates

The caterpillars were always sacrificed in the morning (9h a.m.), three days after molting into the fifth instar. Their midguts were dissected on ice and washed several times with an ice-cold physiological saline solution (0.15% NaCl). A pool of 50 midguts was homogenized on ice, in saline solution (1:10 wet wt/vol) for 30s, and the homogenate was centrifuged at 10,000g, for 20 min at 4°C. The supernatant (crude midgut extract) was aliquoted and kept at -20°C until use. Protein concentration was estimated by the BRADFORD (1976) method with bovine serum albumin as a standard.

Enzyme activity assay and substrate specificity

Enzyme activity was assayed according to ARREGUIN-ESPINOSA *et al*. (2000), with slight modification. Six various chromogenic substrates, p-nitrophenyl derivatives of fatty acid esters, were tested: p-nitrophenyl caprylate, p-nitrophenyl caprate, p-nitrophenyl laurate, p-nitrophenyl myristate, p-nitrophenyl palmitate and p-nitrophenyl stearate. The number of C atoms in their fatty acid residues is 8, 10, 12, 14, 16 and 18, respectively. The substrate solutions were prepared by mixing and heating in 4% Triton X-100 solution for a few minutes. The reaction mixture contained Tris/HCl buffer (50mM; pH 8.2), substrate (0.25mM, final concentration), and enzyme extract. The reaction was started by addition of the substrate, and the cuvettes containing the reaction mixture were immediately transferred into a spectrophotometer. Enzyme activity was measured at 37°C, by continuous three minute monitoring of the release of p-nitrophenol at 410nm. One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol of p-nitrophenol per minute under the given assay conditions. p-Nitrophenyl caprylate (pNPC) was used for further characterization of the enzyme.

Kinetic study

The effect of different concentrations of pNPC (ranging from 0.05 to 0.4mM, final concentration) was tested at 37°C and pH 8.2 (50mM Tris/HCl buffer). A Lineweaver-Burke plot was constructed to calculate the Michaelis-Menten constant (Km) and the maximum activity rate (Vmax).

Effects of pH on enzyme activity and stability

The effect of pH on enzyme activity was determined by performing an activity assay in 50mM buffers within the pH ranges: 2.2, 2.8 (Gly/HCl buffer), 3.2-6.2 (sodium citrate buffer), 6.5, 7.0

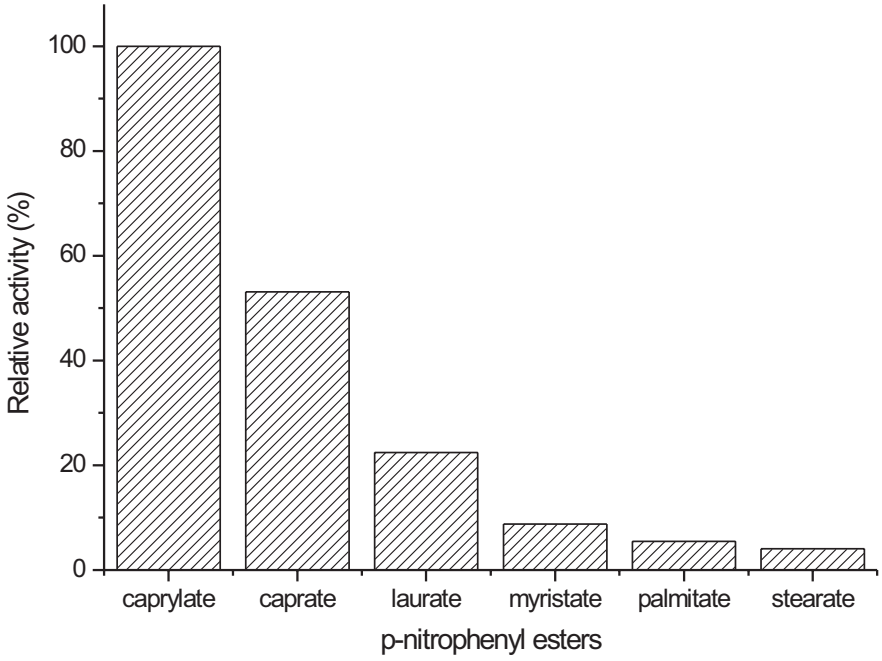

Fig. 1. Substrate specificity of gypsy moth midgut lipase expressed as percentage of maximum activity obtained with p-nitrophenyl caprylate.

(sodium phosphate buffer), 7.6-8.8 (Tris/HCl buffer) and 9.0-11.0 (Gly/NaOH buffer), at a temperature of 37°C. In order to analyze the pH stability, enzyme samples were preincubated for one hour in the above-mentioned buffers. Their residual activities were assayed at pH 8.2 (50mM Tris/HCl buffer) and expressed as a percentage of maximal activity.

Effect of temperature on enzyme stability

To determine thermal stability, the enzyme solution was preincubated in 50mM Tris/HCl buffer (pH 8.2) for one hour at different temperatures within the range of 20°C to 70°C. The remaining enzyme activity was determined and expressed as a percentage of maximal activity.

Effects of metal ions and chemical reagents on enzyme activity

The effects of metal ions and chemical reagents on enzyme activity were investigated through the addition of various metal salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $Cd(NO_3)_2$, $MnCl_2$, $FeCl_3$, $FeSO_4$, $CuSO_4$, $AgNO_3$, $Pb(NO_3)_2$) and, PMSF and EDTA. The enzyme was preincubated for 30 min in a solution of 1mM compound in Tris/HCl buffer (pH 8.2). The enzyme activity was calculated relative to the activity of the enzyme preincubated without any additional compound.

Results and Discusion

The activity of gypsy moth midgut lipase was determined using p-nitrophenyl esters as substrates. We tested six chromogenic substrates (p-nitrophenyl derivatives of fatty acid esters, C8-C18) for determination of substrate specificity. The enzyme was more active on shorter chain fatty acid esters containing C8 (caprylate), C10 (caprate) and C12 (laurate), with maximum activity for the C8 fatty acid ester (Fig. 1). Enzymatic activity on longer chain fatty acid esters was below 10% of maximum activity. Due to the fact that the enzyme showed maximum activity on p-nitrophenyl caprylate (pNPC), we chose this substrate for further investigations. pNPC has been previously used as a substrate in the characterization of esterase (CHOI *et al*. 2004), lipase (ARAGON *et al*. 2000; BENDICHO *et al*. 2001; ROSETTO *et al*. 2003), but also for the assessment of esterase/lipase activity (COSTA & DA CRUZ-LANDIM 2005). There has been some debate as to whether enzyme activity measured with pNPC reflects a true lipase or esterase. In a study on midgut esterases in *Periplaneta americana,* HIPPS and NELSON (1974) point out that a distinction between esterases and lipases based on substrate hydrolysis is not always clear-cut. Recently, it has been shown that esterases act on water soluble substrates with a preference for short fatty acid chains, while true lipases work at the water-lipid interface and their major substrates are long chain triacylglycerols (JAEGER *et al*. 1994; ARPIGNY & JAEGER 1999).

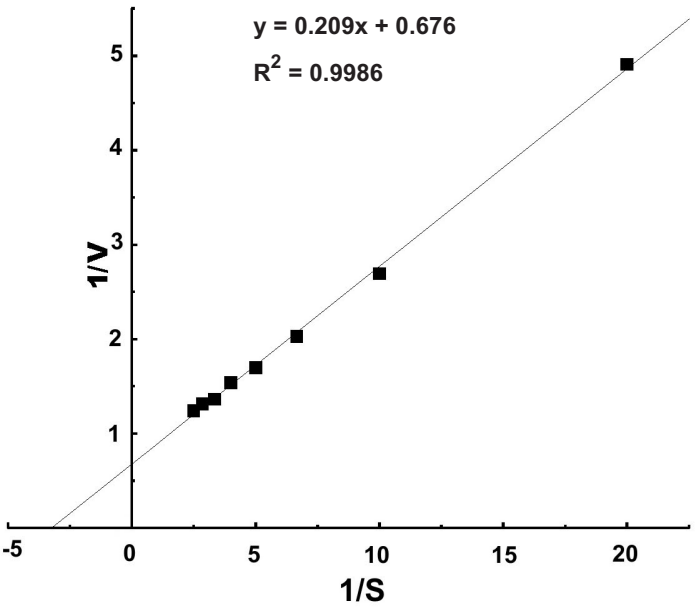

Fig. 2. A Lineweaver-Burke plot of the hydrolysis of pNPC by gypsy moth midgut lipase. V – lipase activity as U/mg prot., S – substrate concentration as mM.

In this experiment we showed that the crude protein extract splits the caprate and laurate substrates, although not as well as the caprylate substrate, which suggests the existence of true lipase in crude homogenate.

The substrate affinity of the crude protein extract was measured by altering the concentration of pNPC from 0.05mM to 0.4mM. Figure 2 depicts the results represented on a Lineweaver-Burke plot. The Km was calculated as 0.310mM, and the Vmax was 1.479U/mg of protein. These values were higher than values obtained for gypsy moth midgut esterase with 1-naphtyl acetate as substrate (KAPIN & AHMAD 1980). The high Vmax for gypsy moth midgut lipase may indicate high substrate turnover, and the relatively high pNPC Km for gypsy moth midgut lipase could be the consequence of an unpurified enzyme.

In terms of the effect of pH on gypsy moth lipase activity, the enzyme showed maximal activity at pH 8.2, with a decline at pH higher than 8.8 (Fig. 3A). At pH lower than 6.5 the enzyme was almost inactive. CANAVOSO & WELLS (2000) have shown that in the *Manduca sexta* (Lepidoptera) larval midgut hydrolysis of triacylglycerol to glycerol and fatty acids is also favored by alkaline pH. In *Spilosoma obliqua* larvae (Lepidoptera), gut lipase shows maximum activity at pH 8.0 (ANWAR & SALEEMUDDIN 1997), while the gut lipase of *Drosophila melanogaster* (Diptera) has shown maximum activity at pH 7 (SMITH *et al*. 1994). In most insect species the pH of midgut lumen ranges from 6-7.5 (HOUSE 1974). In the gypsy moth the pH of empty midgut lumen is 10.77. The pH decreases by about 1.2 pH units, from anterior to posterior midgut, after ingestion of artificial diet (KEATING *et al*. 1990). After ingestion of oak leaves, the pH of gypsy moth midgut lumen has been measured to be within the range of 8.0-8.6 (APPEL & MAINES 1995), which is in agreement with the alkaline pH optimum of gypsy moth midgut lipase (Fig. 3A). Unlike the results obtained for lipase, gypsy moth midgut esterase shows maximal activity between pH 7.5 and 7.7 (KAPIN & AHMAD 1980). Figure 3B shows enzymatic activity after the preincubation of crude extracts in buffers of various pH. There was a complete loss of activity at pH below 5.0, and a decline of activity at pH higher than 8.2. Temperature stability studies indicated a progressive loss of activity with an increase of preincubation temperature (Fig. 4). Approximately a 10% loss in activity occurred when the enzyme was kept at 20°C for one hour and a 40% loss was noted at 40°C. The enzyme was almost inactive after preincubation at 70°C, which is considered to be due to protein denaturation. In determination of lipase stability in *Cephaloleia presignis* (Coleoptera) haemolymph, a decrease in enzyme activity has been shown at pH values above 8 and a temperature above 40°C (ARREGUIN-ESPINOSA *et al*. 2000).

The enzyme was preincubated for 30 minutes in the presence of various compounds (1mM, final concentration) in order to determine the effects of chemical reagents and metal ions. The remaining enzymatic activity was measured with the enzyme-assay method described in the materials and meth-

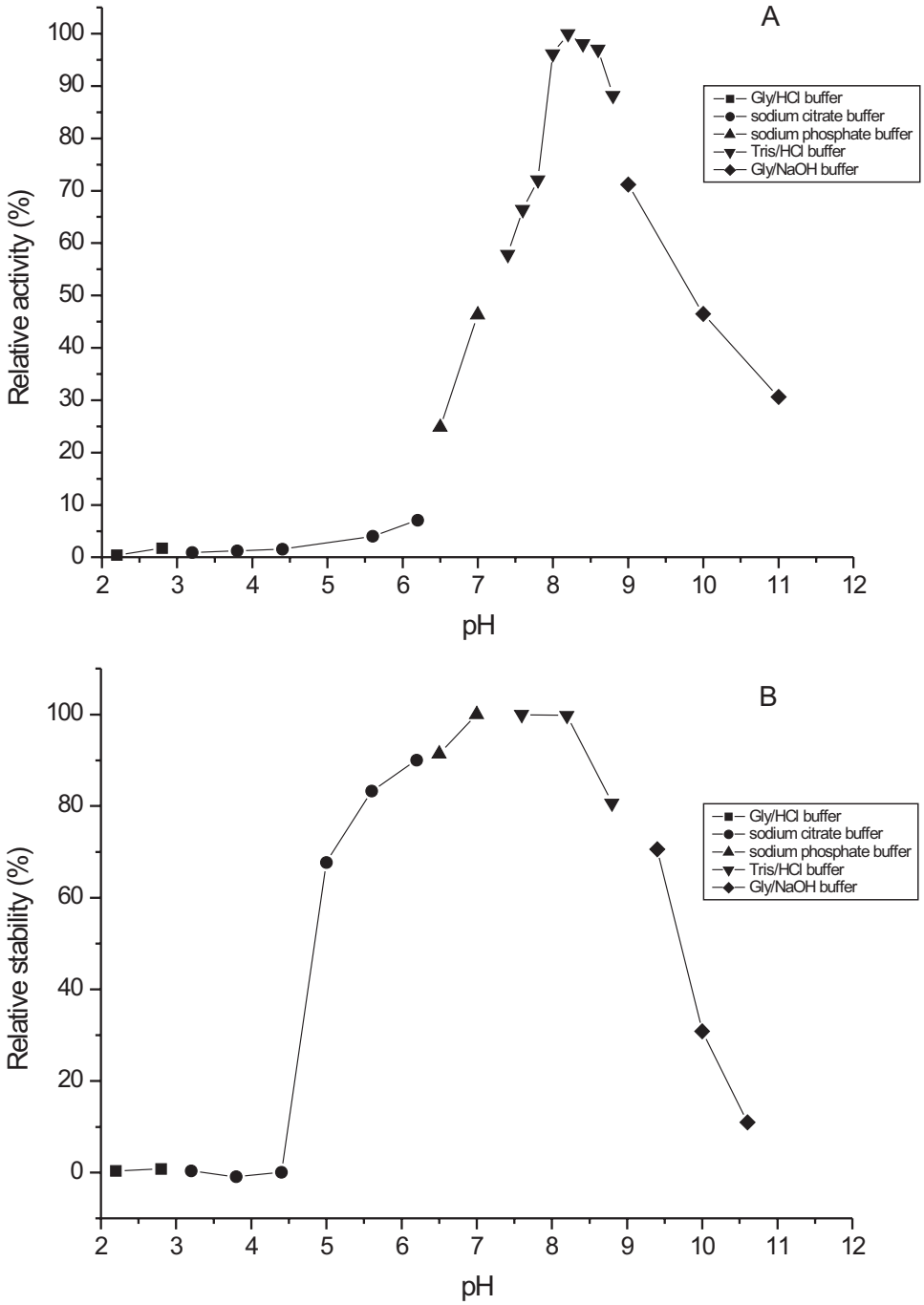

Fig. 3. Effect of pH on the activity of lipase from crude homogenates of gypsy moth larval midgut expressed as a percentage of maximum activity (A) – Effect of pH on enzyme stability expressed as a percentage of residual activity after preincubation at indicated pHs (B).

ods and compared with the activity of the enzyme preincubated without any additional compound. Enzymatic activity was strongly inhibited by Fe^{2+}, Ag^{+}, Pb^{2+} and PMSF, but was not significantly affected by Na^{+}, K^{+}, Mg^{2+}, Mn^{2+} and EDTA. Fe^{3+} inhibited enzymatic activity by about 40% (Table 1). A serine-modifying reagent, PMSF, almost completely inhibited enzymatic activity, suggesting the presence of serine in the active site of the enzyme. Lipases typically have a Ser-Asp-His catalytic triad where serine is found within the middle of the conserved consensus GXSXG motif in the active site (BRENNER 1988; DRABLOS & PETERSEN 1997). The metal chelator, EDTA, did not show a strong inhibitory effect on enzyme activity, suggesting that it is not a metalloenzyme. Therefore,

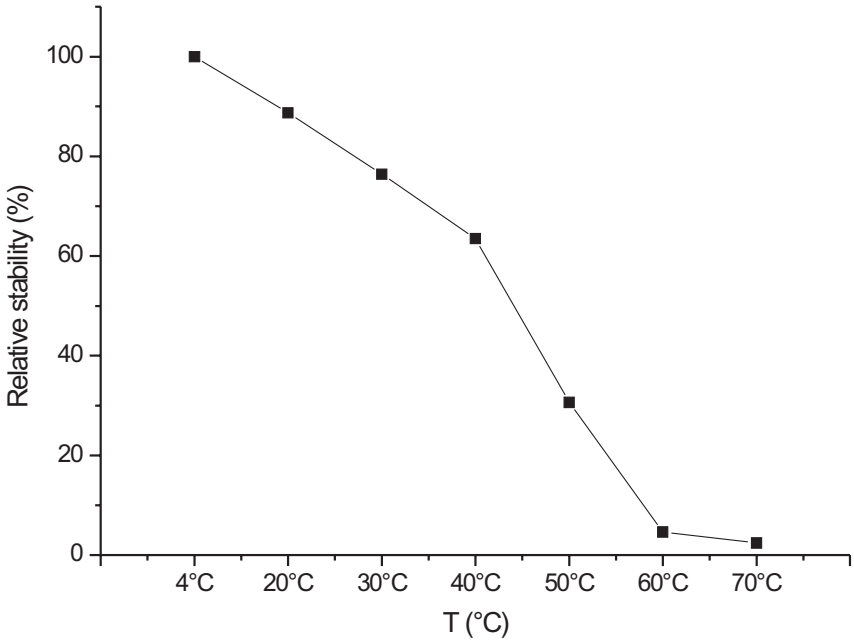

Fig. 4. Effect of temperature on enzyme stability of gypsy moth midgut lipase expressed as percentage of remaining activity after preincubation at different temperatures.

Table 1

Effects of chemical reagents and metal ions on lipase activity. Enzymatic activity was measured after preincubation of crude extracts with various compounds at a final concentration of 1mM. Relative activities were calculated as a percentage of the activity in the absence of any compound

Compound	Relative activity (%)
-	100.00
Na^{+}	94.82
K^{+}	84.69
Ca^{2+}	96.71
Mg^{2+}	98.09
Cd^{2+}	101.67
Mn^{2+}	95.83
Fe^{2+}	25.54
Fe^{3+}	58.56
Cu^{2+}	78.41
Ag^{+}	7.57
Pb^{2+}	6.22
PMSF	9.10
EDTA	93.60

the lack of an *in vitro* inhibitory effect of Cd^{2+} (Table 1) is also expected, taking into account that Cd^{2+} affects enzyme activities through replacing divalent ions in their active sites (JACOBSON & TURNER 1980; STOHS & BAGCHI 1995). Previous investigations have shown that cadmium had an *in vivo* inhibitory effect on the activity of gypsy moth midgut esterase using o-nitrophenyl butirate as substrate (unpublished data). We showed that Ca^{2+} insignificantly decreased enzyme activity, revealing that gypsy moth midgut lipase is Ca-independent. It has been shown that phospholipases of *Nicrophorus marginatus* (Coleoptera), (RANA *et al*. 1997) and *Cochliomyia hominivorax* (Diptera), (NOR ALIZA *et al*. 1999) are Ca-dependent, while the phospholipase *of Aedes aegypti* (Diptera) is Ca-independent (NOR ALIZA & STANLEY 1998).

To our knowledge, this is the first report on gypsy moth midgut lipase activity. In this study we described the optimal conditions necessary for determination of enzyme activity *in vitro*, and also, some biochemical characteristics of the enzyme. Future investigations will be focused on the purification of the enzyme and further investigations of its properties.

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