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Thermoactivity and effects of organic solvents on digestive lipase from hepatopancreas of the green crab

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ABSTRACT

Unlike classical digestive lipases, the crab digestive lipase (CDL) displayed its maximal activity at a high temperature. The CDL activity's optimal temperature, when using emulsified or monomolecular film as substrate, was 60 °C. To our knowledge, this is the first report of an animal digestive lipase having such an optimal temperature. The maximum activity of CDL appeared at pH 8. Lipase activity was compatible with the presence of organic solvents, except for butanol. Furthermore, the hydrolysis was found to be specifically dependent on the presence of Ca^{2+} ions, since no significant CDL activity was detected in the presence of ion chelators such as EDTA. Nevertheless, the CDL does not require Ca^{2+} to trigger the hydrolysis of tributyrin emulsion. Interestingly, Zn^{2+} and Cu^{2+} ions acted as strong inhibitors of CDL activity when using tributyrin as substrate. Lipase stability in the presence of organic solvents, as well as at high temperatures, makes it a good candidate for application in non-aqueous catalysis.

1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) constitute a group of enzymes defined as carboxylesterases that catalyse the hydrolysis (and synthesis) of long-chain acyl-glycerols at the lipid–water interface.

Lipases, have been widely used for biotechnological applications in detergents, and dairy and textile industries, production of surfactants and oil processing. In recent years, lipases have received considerable attention with regard to the preparation of enantiomerically pure pharmaceuticals, since they have a number of unique characteristics: substrate specificity, regiospecificity and chiral selectivity (Kim, Park, Lee, & Oh, 1988). Given that such reactions are sometimes performed most efficiently at elevated temperatures and in organic solvents, converging attempts have been made to find thermostable lipases which would have advantages over labile enzymes in such applications (Kim, Kim, Lee, Park, & Oh, 2000). In recent years, a number of thermophilic microorganisms producing thermoactive lipases and esterases have been isolated and characterised (Dharmsthiti, & Luchai, 1999; Imamura & Kitaura, 2000; Kim, Sung, Kim, & Oh, 1994; Lee et al., 1999; Markossian, Becker, Markl, & Antranikian, 2000; Nawani, Dosanjh, & Kaur, 1998; Schmidt-Dannert, Sztajer, Stocklein, Menge, & Schmid, 1994). Thermophilic lipases show higher thermostability, higher activity at elevated temperatures, and often show more resistance to chemical denaturation, making them ideal tools for industrial and chemical processes where relatively high reaction temperatures and /or organic solvents are used. As each industrial application may require specific properties of the biocatalysts, there is still an interest in finding new lipases that could create novel applications.

The presence of a lipolytic activity and partial characterisation of a lipase in marine organisms have been reported in few studies. The presence of lipase activity was detected in the gastric juice of Homarus americanus (crustacean) (Brockerhoff, Hoyle, & Hwang, 1970). Recently, we have purified crab digestive lipase (CDL) from crab hepatopancreas and some of its catalytic properties were determined (Cherif et al., 2007). Pure CDL, has a molecular mass of 65 kDa. No similarity was found between the N-terminal amino acid residues of CDL and those of known other digestive lipases. CDL is a serine enzyme; it does not need colipase to express its full activity. The aim of this study was to check some other biochemical properties of CDL. We studied the catalytic properties and the stability of CDL which may be of interest in industrial or biotechnological applications. The parameters tested were pH, temperature, and influence of effectors, such as metal ions and organic solvents. Furthermore, the long-term stability of CDL was studied over a period of several months.

Abbreviations: CDL, crab digestive lipase; TC_4, tributyrin; PC, phosphatidylcholine.

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2. Materials and methods

2.1. Lipids

The 2-3-*sn*-dicaprin was prepared from tricaprin (Sigma Chemical Co. St-Louis, USA) by stereospecific enzymatic hydrolysis of the sn-1 ester bond, as described previously (Rogalska, Nury, Douchet, & Verger, 1995). The collapse pressure of 2,3-*sn*- dicaprin is 40 mN/m (Rogalska et al., 1995). Phosphatidylcholine was from Sigma Chemical (St. Louis, USA). Tributyrin (99%, puriss) was from Fluka (Buchs, Switzerland).

2.2. Animals

Crabs (*Carcinus mediterraneus*) were collected alive from the area of Sidi-Mansour (Sfax, Tunisia). A stock of hepatopancreas, removed immediately after dissection of the crabs, was stored at −20 °C. Comparable results were obtained when hepatopancreases were used immediately after dissection or after storage (data not shown).

2.3. Preparation of hepatopancreas extract

Extraction buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8) was added to the hepatopancreas sample in the proportion of 5 ml per 1 g of fresh tissue. The mixture was then stirred with a magnetic bar during 15 min at 4 °C. The extract was centrifuged for 15 min at 15,000 rpm and the clear supernatant was collected to measure the lipase, and the phospholipase, activities.

2.4. Lipase activity determination

2.4.1. pH-stat method: hydrolysis of emulsified substrate

The lipase activity was assayed by measuring the free fatty acids released from mechanically stirred emulsions of triacylglycerols, using 0.1 N NaOH with a pH-Stat (Metrohm, Switzerland). The kinetic assay was performed, under optimal condidions, at pH 8 and 60 °C, using 0.25 ml of TC4 (Sigma) in 30 ml of 2.5 mM Tris–HCl, 150 mM NaCl and 0.5 mM sodium deoxycholate (NaDC)) or 1 ml of olive oil (Sfax-huile, Tunisia), in 30 ml of 2.5 mM Tris–HCl, 150 mM NaCl, in the absence of Gum Arabic and emulsified mechanically by stirring (Tiss, Carriere, & Verger, 2001). One lipase unit corresponds to 1 μmol of fatty acid released per minute.

2.4.2. Baro–Stat method: hydrolysis of monolayer substrate

Measurements were performed with KSV-2000 Baro–Stat equipment (KSV-Helsinki). The principle of the method was described previously (Verger & de Haas, 1973). It involves the use of a "zero-order" trough with two compartments, a reaction compartment and a reservoir compartment, which were connected to each other by a small surface channel. The aqueous subphase of the reaction compartment was thermostatted with an immersed glass coil and was agitated with two magnetic stirrers at 250 rpm. The reactions were performed at variable temperatures (20–65 °C). The reaction compartment had a surface of 100 cm^2 and a volume of 130 ml. The reservoir compartment was 147 mm wide and 249 mm long. The aqueous subphase was composed of 10 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

The enzyme solution was injected into the subphase of the reaction compartment only when the lipid film covered both compartments. A mobile barrier, automatically driven by the Baro–Stat, moved back and forth over the reservoir to keep the surface pressure (π) constant, thus compensating for the substrate molecules that were removed from the film by enzyme hydrolysis. The surface pressure was measured on the reservoir compartment with a Wilhelmy plate (perimeter 3.94 cm) attached to an electromicrobalance, which was connected in turn to a microprocessor programmed to regulate the mobile barrier movement. As shown previously (Verger & de Haas, 1973), the sensitivity of the Wilhelmy plate was estimated to be 0.2 mN m^{-1}.

2.5. Phospholipase activity determination

The phospholipase activity was measured titrametrically at pH 8 and 50 °C with a pH-Stat, under the standard assay conditions described previously (Cherif et al., unpublished data), using phosphatidylcholine emulsion as substrate in the presence of 5 mM NaTDC and 10 mM $CaCl_2$. One unit of phospholipase activity was defined as 1 μmol of fatty acid released per minute.

2.6. Lipase

Crab digestive lipase (CDL) was purified to homogeneity at our laboratory as previously described (Cherif et al., 2007).

2.7. Determination of protein concentration

Protein concentrations were determined as described in previous work (Cherif et al., 2007), using BSA as reference.

2.8. Effect of temperature and pH on activity and stability of CDL

The thermal profile of enzyme activity was measured at temperatures between 0 and 70 °C. The incubation was performed in a temperature-controlled cuvette holder. In order to determine the thermal stability of CDL, aliquots of the pure enzyme solution were incubated successively for 5, 10, 20, 40, and 60 min at 0 °C, 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The thermal stability was also checked, under the same conditions, in the presence of PC (10 mg/ml), used as a substrate analogue. Immediately after incubation, enzyme activity was measured as described above. The long-term stability was determined over a period of 120 days. Samples were stored at 5 °C (refrigerator) and at room temperature. Activity in subsamples was measured at intervals. The pH optima for CDL was determined using the following buffers: 50 mM sodium acetate buffer (pH 4–6), 50 mM potassium phosphate buffer (pH 6–8), 50 mM Tris–HCl buffer (pH 7–9), and 50 mM glycine-NaOH buffer (pH 8–11). Enzyme reactions in each buffer were carried out at 60 °C and their residual activities were measured periodically. In order to determine the pH stability, pre-incubation was performed in each buffer, at room temperature, for 15 min. The pH of each buffer was adjusted at 60 °C, the enzyme reaction temperature.

2.9. Effects of metal ions and organic solvents

In order to determine the effects of various metal ions and organic solvents on lipase activity, the activity was assayed at 60 °C after pre-incubation of the purified CDL with each compound for 15 min at room temperature. Lipase assays were performed in the presence of divalent ions (Zn^{2+}, Cu^{2+},Ca^{2+} or Mg^{2+}). The effects of organic solvents on enzyme activity were determined in the presence of increasing concentrations of acetone, methanol, ethanol, 2-propanol and butanol.

3. Results and discussion

3.1. Lipolytic activity of the crab hepatopancreas

The lipase activity level measured in the crab hepatopancreas

extract was found to be 50 U/g of fresh digestive tissue, using TC_4 as substrate. Furthermore, phospholipase activity was detected in the crab hepatopancreas, using phosphatidylcholine emulsion, and it was found to be around 2 U/g of fresh hepatopancreas. In contrast to mammalian pancreatic lipase-related protein 2, having both lipase and phospholipase activities (Fauvel et al., 1981), the purified CDL did not present any phospholipase activity. Crab hepatopancreas is equipped with separated lipase and phospholipase enzymes assuming the hydrolysis of dietary lipids.

3.2. Annual distribution of the CDL activity levels

Crabs used in this study were collected in all months during the year. We were interested in checking the yearly distribution of the lipase activity in relation to the average temperature measured during each month. The maximum lipase activity was measured during the summer (from June to October); the level reached 20 U/g of corporal weight. In winter, the lowest temperatures were recorded and the lipase activity decreased significantly (5–10 U/g of corporal weight) (data not shown).

3.3. Effects of temperature on activity and stability of CDL

Lipase activity was tested at temperatures ranging from 0 to 70 °C, using tributyrin or olive oil emulsions as substrate. In contrast to all known animal digestive lipases, which display a maximal activity around 37 °C, the activity of CDL increased linearly from 20 °C to 60 °C. The reaction rate increased significantly (2.5-fold) when the temperature varied from 37 °C to 60 °C and reached its maximum value at 60 °C (Fig. 1A). Lipase activity of juvenile *Cherax quadricarinatus* digestive gland, using β-naphthyl caprylate as substrate, was optimum between 35 °C and 45 °C and decreased above 50 °C (Lopez-Lopez, Nolasco, & Vega-Villasante, 2003). A similar response for lipase activity was found by Biesot, and Capuzzo, (1990), in stage I of the lobster *Homarus americanus*, with triolein as substrate, peaking at 45 °C and decreasing above 50 °C. Ramana-Rao and Surendranath (1991) found optimal lipase activity at 37 °C in *Metapenaeus monoceros*. The thermostability of CDL alone was also determined by measuring the residual activity after incubation of the pure enzyme at various temperatures. CDL retained 80% of its maximum activity after incubation for 30 min at 50 °C (data not shown). When the enzyme was incubated at 60 °C, it was completely inactivated after 15 min (Fig. 2). To explain how the CDL can efficiently hydrolyse its substrate at 60 °C, and how the kinetic of hydrolysis remains linear during 5 min (Fig. 1B), the CDL was incubated at 60 °C in the presence of an analogous substrate, phosphatidylcholine (PC). Our results, presented in Fig. 2, clearly show that the presence of PC significantly increases the thermostability of the CDL. To confirm this result about the optimal reaction temperature of CDL, we used another system, the monomolecular film technique. Fig. 3 shows that CDL was able to efficiently hydrolyse a film of 2,3-*sn*-dicaprin maintained at 35 mN/m. As obtained with emulsified substrate, the maximal CDL activity was measured at 60 °C with dicaprin film. Comparable results have been obtained with many enzymes, where the presence of the substrate increases their thermostability (Verger, 1984).

3.4. Effects of pH on activity and stability of CDL

The influence of pH on the CDL activity was tested, using emulsions of short-chain (TC_4) or long-chain (olive oil) triacylglycerols as substrates. The optimal pH of pure CDL activity, using TC_4 or olive oil emulsions as substrates, was 8 (data not shown). This result recalls that observed in the case of pancreatic lipases in the absence of colipase, showing an optimal activity expressed at

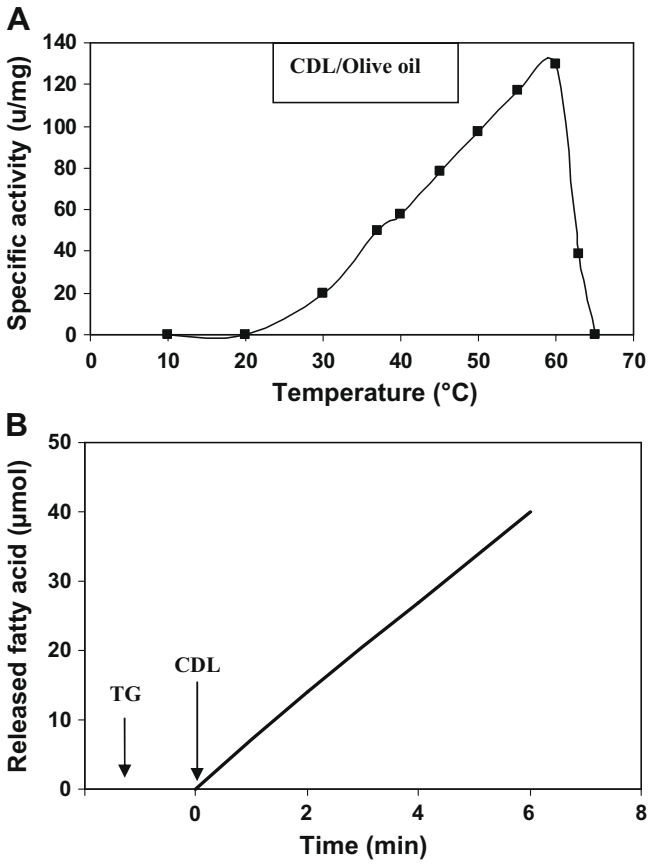

Fig. 1. (A) Effects of temperature on the CDL activity using pH-stat method. The activity was measured at pH 8 at various temperatures using olive oil emulsion as substrate. (B) Kinetic of olive oil emulsions hydrolysis by CDL (7U). Lipolytic activity was followed at pH 8 and 60 °C.

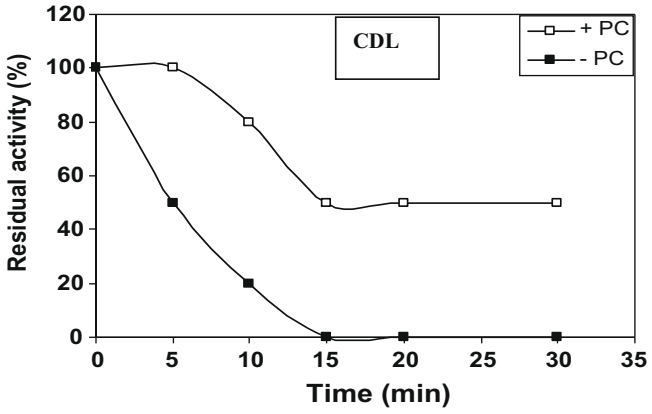

Fig. 2. Effect of temperature on the CDL stability. The enzyme was incubated at 60 °C in the absence and presence of phosphatidylcholine (PC) (10 mg/ml). The activity was tested at pH 8 and 60 °C using olive oil emulsion as substrate.

alkaline pH values (Verger, 1984). Furthermore, some studies of the effect of pH on lipase or esterase activity in crustaceans have been reported (Brockerhoff et al., 1970) for lipase activity, using triolein and tributyrin as substrate in gastric juice of adult *Homarus americanus*. Tietz, and Shuey, (1993) reported that optimum pH for lipase ranged between pH 7.4 and 10, depending on the type of substrate and buffer used. Figueiredo, Kricker, and Anderson, (2001) found that lipase activity in adult *Cherax quadricarinatus* gastric fluid was highest at pH 8, but they did not find lipase activ-

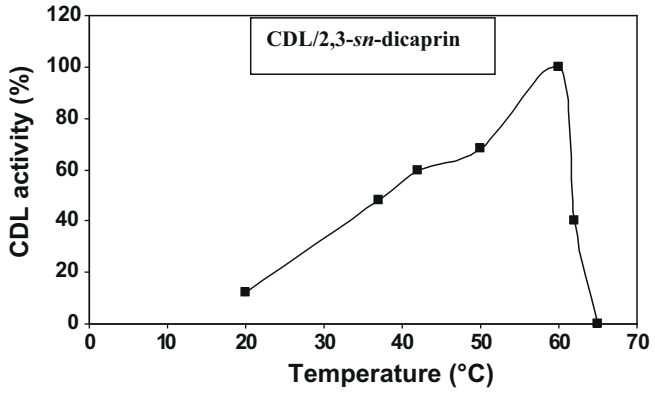

Fig. 3. Effect of temperature on the CDL activity using Baro–Stat method (monomolecular film technique). The CDL activity was tested at pH 8 at various temperatures, using 2,3-*sn*-dicaprin as substrate maintained at 35 mN/m. The kinetic recording was performed with a "zero-order" trough (volume, 130 ml; surface, 100 cm^2). Buffer: 10 mM Tris–HCl, pH 8, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

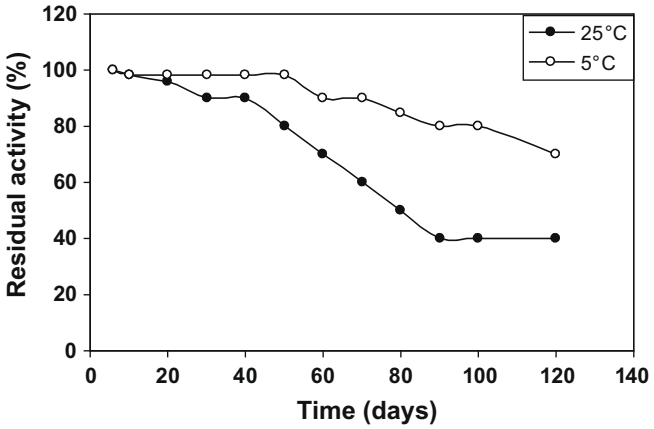

Fig. 4. Long-term stability of CDL stored in the refrigerator (5 °C) and at room temperature (25 °C). Assays were carried out under standard conditions as described in the text.

ity in the hepatopancreas. This absence of lipase in the hepatopancreas suggests synthesis in the form of a proenzyme; however, the molt stage was not considered in that study (Figueiredo et al., 2001). In contrast, we found lipase activity in juvenile hepatopancreas extract. The source of the enzyme could explain why our results differed from those of other authors, as could the physiological condition of the animal. Interestingly, CDL was found to be most stable at higher pH values, and maintains 65% of its activity after 4 h of incubation at pH 10 and loses its activity at pH values lower than 4 (data not shown). It is noteworthy that, so far, only a few microbial lipases, like that of *Humicola lanuginose* (Bool, & Huge-Jensen, 1988), in addition to a lipase purified from the scorpion (Zouari, Miled, Cherif, Mejdoub, & Gargouri, 2005) have shown such a resistance to high pH values.

3.5. Long-term stability

In the course of the long-term stability experiment, the activity of CDL, which was stored at room temperature, decreased within the first day towards 95% of initial activity (Fig. 4). During the first week, activity did not drop below 90% of initial values. Later on, a continuous decrease was evident towards 40% of initial activity after 90 days. In contrast, the samples stored in the refrigerator maintained more than 70% of initial activity after 90 days. In conclusion, CDL activity remained surprisingly stable up to 120 days, although CDL was not maintained in a stability-enhancing medium, e.g. supplements of Ca^{2+}, glycerol, or ammonium sulphate, but just in plain de-mineralised water.

3.6. Effects of organic solvents

Organic solvents can be advantageous in various industrial enzymatic processes; e.g. the reaction media used in biocatalytic esterification and trans-esterification contain less than 1% water. The use of organic solvents can increase the solubility of non-polar substrates, increase the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination (Heitmann, 1994). Organic solvents affect the performance of the enzymes in different ways. Lipases are diverse in their sensitivity to solvents, but there is general agreement that polar water-miscible solvents are more destabilizing than are water immiscible solvents (Nawani et al., 1998). In this study, the crab digestive lipase showed high stability in the presence of water-miscible organic solvents, since it retained almost 100% activity after exposure, for 1 h at 25 °C, to 40% methanol, 40% ethanol, 40% 2-propanol or 40% acetone (Table 1). Addition of 40% methanol to the pure CDL caused a 10% immediate increase of the lipolytic activity in comparison to the control. Similar results have been obtained for *Bacillus thermocatenulatus* lipases (Schmidt-Dannert et al., 1994; Schmidt-Dannert, Rua, Atomi, & Schmidt, 1996), which increased activity in the presence of 30% methanol, 30% ethanol or 30% acetone.

3.7. Effects of metal ions on CDL activity

No significant CDL activity was detected on olive oil in the presence of 5 mM chelator ions, such as ethylene diamine tetraacetic acid (EDTA) (Fig. 5). Furthermore, the EDTA was also found to be a powerful inhibitor when added during the time course of olive oil hydrolysis by CDL (data not shown). The EDTA has thus chelated bivalent ions that are necessary for the CDL to express its activity. To identify the nature of these ions, increasing concentrations of bivalent ions (Ca^{2+}, Mg^{2+}, Cu^{2+} or Zn^{2+}) were added to the lipolysis medium containing EDTA. The addition of Mg^{2+}, Cu^{2+} or Zn^{2+} ions failed to restore the CDL activity. Meanwhile, when adding increasing concentrations of Ca^{2+}, the CDL activity was progressively restored to reach its maximal value at 8 mM Ca^{2+} (Fig. 5). In contrast to what has been observed with olive oil, used as substrate, no bivalent ions were required to trigger the hydrolysis of TC_4 emulsion by CDL, since the activity was not affected by the presence of EDTA. Interestingly, the CDL was fully inhibited by the adding of Cu^{2+} or Zn^{2+} ions to the hydrolysis medium, whereas Ca^{2+} and Mg^{2+} ions had no effect on CDL activity (data not shown). These results recall those obtained for crayfish lipase which is fully inhibited by the presence of Cu^{2+} or Zn^{2+} using α-naphthyl esters as substrate, (Benzonana, & Desnuelle, 1968). Evidently, as has been

Table 1
Stability of crab digestive lipase in organic solvents.

Organic solvent	Relative activity% (0 h)	Relative activity% (1 h)	Relative activity% (2 h)
Control	100 ± 3.7	100 ± 6.1	100 ± 1.6
Acetone	94 ± 1.3	92 ± 13.2	80 ± 2.1
Methanol	102 ± 1.2	110 ± 9.7	85 ± 2.6
Ethanol	92 ± 3.2	96 ± 1.9	80 ± 4.3
2-Propanol	98 ± 4.7	100 ± 2.4	82 ± 2.5
Butanol	0	0	0

Pure crab digestive lipase was incubated in each organic solvent (40%) at 30 °C for 1 and 2 h. Values represent the means of three replicates.

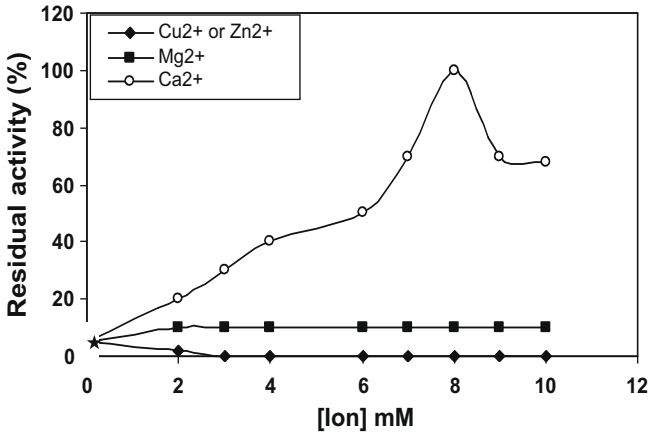

Fig. 5. Effects of increasing Ca^{2+}, Mg^{2+}, Cu^{2+} or Zn^{2+} ion concentrations on the CDL residual activity using olive oil emulsion as substrate. CDL activity was measured at 60 °C and pH 8. The star indicates the lipase activity measured in the absence of $CaCl_2$ and in the presence of 5 mM EDTA or EGTA.

reported for porcine pancreatic lipase (Verger, 1984), CDL activity is dependent on Ca^{2+} ions only when long-chain triacylglycerols are used as substrate.

4. Conclusion

Described herein is the characterisation of a lipase from crab hepatopancreas. This lipase has several advantageous features for industrial applications. The thermostable, lipid-hydrolysing enzymes may be applied to treat lipid-rich industrial effluents, to produce inter-esterification substances in the food industry, or to synthesise useful chemical compounds. Stability of CDL in the presence of organic solvents, and its tolerance to high temperatures, makes it a good candidate for application in non-aqueous biocatalysis.

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