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Burkholderia lata LBBIO-BL02 lipase stable and active at simulated digestive environment

Bruno Henrique de Oliveira[1,2*], Claire Bourlieu-Lacanal[2], Jérôme Lecomte[2], Pierre Villeneuve[2], Valéria Marta Gomes do Nascimento [1,3]

[1]Universida Estadual Paulista (UNESP) – Instituto de Biociências de Rio Claro
Av. 24 A,1515. CEP: 13506-900 - Rio Claro/SP, Brasil. *E-mail: bruno@assis.unesp.br
[2]Centre de Recherche Agronomique Pour le Développement (CIRAD)/SupAgro, UMR IATE
F-34060, Montpellier, France.
[3]Universida Estadual Paulista (UNESP) - Faculdade de Ciências e Letras de Assis, Dpto. de Ciências Biológicas.
Av. Dom Antônio, 2100. CEP: 19806-900 – Assis/SP, Brasil.

ABSTRACT

Supplementation with porcine pancreatic lipase in humans with digestive disorders can be beneficial to treatment of many diseases. However, treatment with exogenous enzymes is complicated due to lipase degradation in the stomach due to acid and proteolytic activity. Our research group isolated a wild strain of Burkholderia lata (LBBIO- BL02) producer of lipase with 1137 U/mL and 2146 U/mg. The B. lata lipase was active, highly stable (>100%) and poorly degraded by pepsin at pH 2-6 (the activity at pH 4 without bile: 465 ± 26 U/mg). In conclusion, in this study were obtained results that strongly indicate that Burkholderia lata (LBBIO-BL02) lipase can be considered an efficient candidate for use as a digestive supplement without loss of the lipolytic efficiency. This enzyme remained active even after two hours of incubation with high doses of pepsin at acid pH, trypsin and bile salt.

Keywords: gastric fluid simulated, duodenal fluid simulated, bile salts, enzymatic supplement.

INTRODUCTION

Supplementation with porcine pancreatic lipase in humans with digestive disorders can be beneficial in cases of steatorrhea, maldigestion, celiac disease, cystic fibrosis, diabetes type I or II and Crohn's disease. However, treatment with exogenous enzymes as a pill or capsule is complicated due to lipase degradation in the stomach due to acid and proteolytic activity of pepsin, different regioselectivity of the human enzyme and high costs for the lipase isolation and purification. Another limitation of using pancreas extract as the enzyme source is the occurrence of gastrointestinal disorders or hyperuricemia and hyperuricosuria due to the high purine content of the product (Layer and Keller, 2003).

In this way, stable microbial lipases at acid pH, active in the presence of bile salts, regioselective and resistant to hydrolysis by digestive proteases, may represent superior therapeutic alternatives. Thus, there is great interest in therapeutic potential with lipases produced by microorganisms.

Our research group isolated a wild strain of *Burkholderia lata* (LBBIO- BL02) producer of lipase. The culture conditions for the enzyme production were studied and an economically attractive medium combined with a high enzyme activity was developed.

According to Oliveira et al. (2004), the best culture condition was composed of chicken fat, an industrial waste, and ammonium sulfate as inorganic nitrogen source, at 35 °C and pH 7.0, resulting in activity 1137 U/ml and 2146 U/mg of protein. The same authors characterized the crude enzyme and observed maximum activity at 55 °C and pH 8.0. It was observed maintenance of 100, 93 and 85% of maximum activity at 45, 50 and 60 °C respectively and 78, 82 and 100% at pH 2.2, 3 and 10, respectively. The results presented indicated potential for utilization as a digestive supplement. This study aims to increase subsidies to confirm this hypothesis by evaluating if the *B. lata* (LBBIO-BL02) lipase shows activity and stability in gastric fluid and activity in duodenal fluid, resisting acid and alkaline pH, the presence of proteases and bile salts.

MATERIALS AND METHODS

This research was developed in a collaborative project between the Laboratory of Biochemistry and Bioprocess (LBBIO) of the Department of Biological Sciences, UNESP-Brazil and the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier - France.

Enzyme production in submerged fermentation

The enzyme was produced in shake flasks of 250 mL containing 50 mL of fermentation medium kept at 35 °C and 180 rpm. The fermentation medium was composed of K_2HPO_4 (1.0 g/L), $MgSO_4.7H_2O$ (0.5 g/L), NaCl (0.38 g/L), $FeSO_4.7H_2O$ (0.01 g/L), $(NH_4)_2HPO_4$ (15.0 g/L) as N source and chicken fat (12.5 mL/L) as C source.

One-step enzyme purification

The enzyme produced in submerged fermentation was purified in an one-step process developed in our laboratory with purification factor of 12.6 and 34% of recovery. The methodology is in an application process of patent.

Activity and stability in simulated gastric fluid

The lipase activity was evaluated in simulated gastric fluid (SGF): NaCl (75 mmol/L), soybean lecithin (17 mmol/L), $CaCl_2$ (0.15 mmol/L), glycerol (120 mmol/L), HCl (pH 4.0), triolein (113 mmol/L) and pepsin (Sigma, from porcine gastric mucosa, 700 U/mg) from 1000 to 8000 U/mL. The lipase (0.5 mg/mL) was incubated for 2 h at 250 rpm and 37 °C, samples were taken at 5, 30, 60 and 120 min. The fatty acids releases were evaluated by HPTLC.

To test *B. lata* resistance to pepsin, the lipase was incubated with a solution containing pepsin (4.0 mg/mL). The pH was adjusted to 6, 5, 4, 3 and 2 with HCl 0.1 N. Residual lipase activity was measured at various times (5, 30, 60 and 120 min). Assays were performed in triplicate. One unit of lipase activity was defined as the releases of 1 μmol/min of free fatty acid per mL of enzyme at pH 8.0 and 55 °C.

Activity in simulated duodenal fluid

The lipase activity was also evaluated in simulated duodenal fluid (SDF): soybean lecithin (4 mmol/L), glycerol (60 mmol/L), triolein (56 mmol/L), trypsin from 94 to 376 U/mL, sodium taurodeoxycholate (NaTDC) from 4 to 16 mmol/L. The lipase (0.5 mg/mL) was incubated for 2 h at 250 rpm and 37 °C, samples were taken at 5, 30, 60 and 120 min. The fatty acids releases were evaluated by HPTLC. Assays were performed in triplicate.

Quantification of free fatty acids by high performance thin layer chromatography (HPTLC)

After hydrolysis, 200 μL of the reaction medium were extracted in 80 μL HCl 0.1 N, 1.5 mL Chloroform:Methanol (3:2) and 48 μL NaCl 0.73%. The organic phase (0.5 μL) was

applied (5 mm band) on a HPTLC silica gel Plate 60 (Merk, Darmstadt, Germany) using an ATS4 automatic sampler (Camag, Switzerland). The plate was then developed with hexane:diethyl ether:formic acid (75:25:1; v/v/v). Plates were revealed by dipping the plate into a copper sulphate:phosphoric acid:methanol:water (50:40:25:390, v/v/v/v) solution and heating at 180 °C for 7 min. TLC bands were quantified using a TLC Scanner 3 (Camag Switzerland) at 350 nm. Oleic acid (from 0.1 to 3.5 μg/mL) was used as a standard.

RESULTS AND DISCUSSION

For an enzymatic supplement be used as a digestive assistant, the enzyme should be resistant to low pH and to action of proteases and bile salts. Thus, the effects of these parameters on the activity and stability of the lipase produced by *B. lata* (LBBIO-BL02) were evaluated. The *B. lata* lipase shows high stability, greater than 99%, at all tested pH's (2-10), placing it as highly active to postprandial pH levels of the stomach and intestine (duodenum).

The activity and stability of *B. lata* lipase to pepsin, first protease found by oral enzymes, is a very important assay because many lipases can be completely inactivated by digestive proteases. The *B. lata* lipase showed good activity at the presence of pepsin at pH 4 and 37 °C even with high concentration of pepsin. The enzyme was 50% active only when pepsin activity was higher than 4000 U/mL. Using 1000 and 2000 U/mL of pepsin the lipase maintained 83 and 75% of activity after 120 min incubation. In healthy adult human the pepsin activity is about 600 U/mL (Henderson et al., 2001). These results suggest that, *in vivo* and during feeding, the *B. lata* lipase is active at high proportions (more than 80%) even in the presence of gastric pepsin. The time the feeding tends to remain in the stomach is highly variable depending on a variety of factors, including the amount of feed, the amount of fat, and also the acidity of the stomach. However, on average, all food must have left the stomach within 2 to 4 hours (Rao et al., 2011).

Regarding the stability, the *B. lata* lipase shows no degradation by pepsin (2800 U/mL) at any pH tested (2-6). The enzyme shows activation after been incubated at acid pH and there is no difference between control and pepsin assays. In vivo and during a meal, the *B. lata* lipase is likely to be completely safe from both acid and pepsin inactivation (Figure 1).

The effects of pancreatic protease trypsin in the *B. lata* lipase activity were also evaluated. There was no influence of trypsin in the lipolytic activity at pH 6.0 and 37 °C, even with trypsin values of 376 U/mL. In a healthy adult human trypsin activity is close to 100 U/mL (Moreno et al., 2006). Thus, the *B. lata* lipase proved active and resistant to proteases (pepsin and trypsin) at pH 4-6.

Another important characteristic of *B. lata* lipase is the activation of this enzyme in the presence of high concentrations of bile salt NaTDC, the enzyme was 3.5 times more active at 16 mmol/L of NaTDC, four times the standard concentration of the human organism (Moreno et al., 2006). Studies of microbial lipases for the treatment of exocrine insufficiency are often limited by the inhibition caused by bile salts (Svendsen et al., 2008), at this sense, the *B. lata* lipase shows a great advantage.

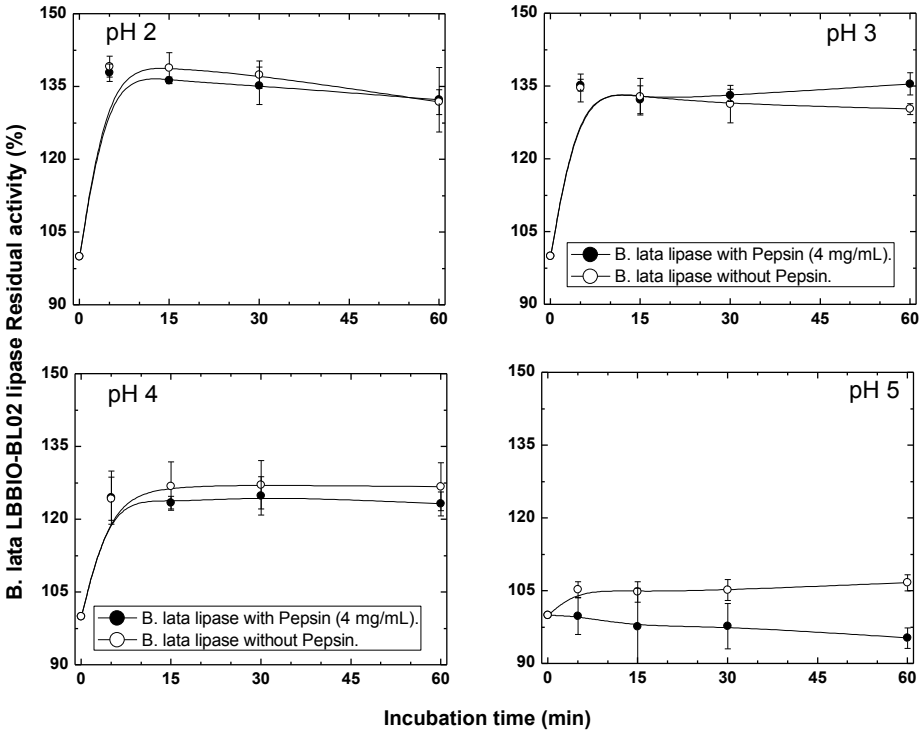

Fig. 1: Time-course stability of *B. lata* (LBBIO-BL02) lipase (3 mg/mL) at various pH levels during TAG lipolysis with and without pepsin (4 mg/mL). The experiments were performed without bile. After the incubation the residual lipase activity was measured at pH 8.0 and 55 °C. The activities were compared with the lipase without prior incubation (1907 U/mg). Values are mean ± SD (n = 3).

CONCLUSIONS

In conclusion, in this study were obtained results that strongly indicate that *Burkholderia lata* (LBBIO-BL02) lipase can be considered an efficient candidate for use as a digestive supplement without loss of the lipolytic efficiency. This enzyme remained active even after two hours of incubation with high doses of pepsin at acid pHs, trypsin and bile salt.

REFERENCES

Henderson TR, Hamosh M, Armand M, Mehta NR, Hamosh P. 2001. Gastric proteolysis in preterm infants fed mother's milk or formula. Adv Exp Med Biol 501:403-8.

Layer P, Keller J. 2003. Lipase supplementation therapy: standards, alternatives, and perspectives. Pancreas, 26:1-7.

Moreno MPdlC, Oth M, Deferme S, Lammert F, Tack J, Dressman J, Augustijns P. 2006. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. J Pharm Pharmacol, 58:1079–1089.

Oliveira BH, Santos RÉ, Loiola LEA, Nascimento VMG. 2015. Overproduction and properties of lipase by a wild strain of *Burkholderia lata* LBBIO-BL02 using chicken fat. Ann Microbiol, 65: 865-877.

Svendsen A, Skjoet M, Yaver D, Christensen LLH, Larsen SE, Lundin N, Lamsa M, Gregory PC. 2008. New lipase, useful for manufacturing a pharmaceutical composition for treating digestive disorders, pancreatic exocrine insufficiency, pancreatitis, cystic fibrosis, or diabetes type I or II. Patent Number: WO2008079685-A2.

Rao SSC, Camilleri M, Hasler WL, Maurer AH, Parkman HP, Saad R, Scott MS, Simrn M, Soffer E, Szarka L. 2011. Evaluation of gastrointestinal transit in clinical practice: position paper of the American and European Neurogastroenterology and Motility Societies. Neurogastroenterology Motil, 23:8–23.

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