Concomitant Production, Partial Purification and Characterization of a Serine Protease and a Proteolysis-Resistant Metallolipase from *Bacillus pumilus* SG2

Ragupathy Sangeetha[a], Arumugam Geetha[b,*], and Irulandi Arulpandi[c]

[a] Department of Biochemistry, Vels University, Chennai – 600117, TN, India
[b] Department of Biochemistry, Bharathi Women's College, Chennai – 600108, TN, India. E-mail: geethav21@yahoo.co.in
[c] Department of Microbiology, Asan Memorial College, Chennai – 600100, TN, India

* Author for correspondence and reprint requests

Z. Naturforsch. **65c**, 61–65 (2010); received October 1/27, 2009

Our objective was to investigate the concomitant production of protease and lipase by a bacterial strain. A promising bacterial strain was isolated from a food-processing industrial effluent, which can produce both protease and lipase. The isolate was characterized by sequencing the16S rRNA gene. The PCR amplified gene was subjected to analysis by BLAST to ascertain the genetic relatedness of the isolate, *Bacillus pumilus* SG2. The enzymes were produced and subjected to purification by ammonium sulfate precipitation and dialysis followed by gel filtration chromatography; twelve-fold purity was obtained. The lipase produced was found to be proteolysis-resistant. The partially purified enzymes were characterized for their optimum pH value, temperature, response to inhibitors, surfactants and oxidants. The relative molecular weights of protease and lipase were determined as 28 kDa and 40 kDa, respectively, by zymogram studies.

Key words: Bacillus pumilus, Lipase, Protease

Introduction

Protease and lipase have a wide range of significance and are studied intensively at kinetic and molecular levels. These enzymes have innumerable industrial applications and find use in food processing, detergent, leather, dairy and baking, water treatment, and pharmaceutical industries (Rao *et al*., 1998; Sharma *et al*., 2001). Many industrial processes, like soaking of leather as a preliminary treatment in leather processing and degumming of silk threads, use both these enzymes in combination. Cheese making processes depend on enzymes, particularly proteases and lipases. Protease when added to immature cheese causes intense bitterness while lipase gives low bitterness and higher rancidity. However, lipase in combination with a protease yields good cheese flavour with low level of bitterness (Fox *et al*., 2006). The lipase and protease enzymes used in cheese ripening are obtained from different microbial sources. Sources which produce protease and lipase simultaneously can be screened to exploit and fulfill the industrial demand. The production of protease and lipase in continuous cultures of *Serratia marescens* has been reported by Henriette *et al*. (1993). However, there are no reports on purification and characterization of concomitantly produced protease and lipase.

We were hence interested in producing these enzymes from *Bacillus* sp. using a common medium and characterizing them. Since lipases are susceptible to proteolytic degradation, this study was aimed at producing a proteolysis-resistant lipase. In this investigation, we have identified a bacterium, *Bacillus pumilus* SG2, which can produce both protease and lipase and report the concomitant production, purification and characterization of both enzymes.

Material and Methods

Isolation and screening

Bacterial strains were isolated from industrial effluents and screened for production of both protease and lipase. A bacterial strain isolated from a food-processing industrial effluent was found to produce maximum amounts of protease and lipase comparatively. It was identified as *Bacillus pumilus* according to Bergy and Holt (1984) and designated as *Bacillus pumilus* SG2 in our previous study (Sangeetha *et al*., 2008).

0939–5075/2010/0100–0061 $ 06.00

16S rDNA sequencing

The molecular identification of the isolate was by sequencing the 16S rDNA. The bacterial DNA was extracted by the CTAB method (Tripathi and Rawal, 1998). The 16S rDNA gene was PCR-amplified on a thermocycler (Ericomp Delta cycler I system, Ericomp, Inc, CA, USA) using universal primers reported by Watanabe *et al.* (2001). Sequencing was done using the forward primer 5'-GCAACGCGAAGAACCTTAC-3' and the reverse primer 5'-GGTGTGTACAAGACCC-3'. The PCR mixture contained 5 μl of 10× PCR buffer, 3 μl of dNTPs mix, 1 μl each of forward and reverse primer, 1 μg of template DNA, 1 μl of *Taq* polymerase (1 U). The final reaction volume was made up to 50 μl with sterile water. The PCR was performed with an initial denaturation at 94 °C for 4 min. The subsequent 30 cycles consisted of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and chain extension at 72 °C for 2 min. The final extension was at 72 °C for 5 min. The 16S rDNA gene was sequenced using a Beckman Coulter CEQ 8000 autoanalyzer. The sequences obtained were analyzed on NCBI, BLAST database to determine the relatedness of each bacterial isolate.

Enzyme production

The production media consisted of (w/v) 0.04% $CaCl_2$, 0.02% $MgCl_2$, 1% glucose, 0.5% NaCl, 0.3% yeast extract, and 1% tributyrin (in sodium phosphate buffer, pH 9.0). Fermentation was carried out with 100 ml medium inoculated with an overnight culture (OD_{600} = 1.0) and incubated on a rotary shaker (180 rpm) for 26 h at 37 °C.

Enzyme assay

The caseinolytic activity was measured by the photometric method of Rahman *et al.* (2005). One unit (U) of protease activity is equivalent to 0.5 μg of tyrosine liberated by 1.0 ml of enzyme solution under the assay conditions. The lipase activity was assayed by the photometric method of Kordel *et al.* (1991). One unit (U) of lipase activity is equivalent to 1 μmol of *p*-nitrophenol liberated per min under the assay conditions.

Purification of enzymes

The production medium was centrifuged (at 10,000 × *g* for 20 min) and the cell-free supernatant was harvested and considered as the crude enzyme mixture. Lipase and protease were partially purified by ammonium sulfate precipitation and dialysis. The supernatant was subjected to gradient ammonium sulfate precipitation (20, 40, 60 and 80% saturation). Solid ammonium sulfate was added to the supernatant at 20% saturation and centrifuged at 20,000 × *g* and 4 °C for 10 min. Ammonium sulfate was again added to the resulting supernatant at 40% saturation and centrifuged at 20,000 × *g* and 4 °C for 10 min. This process was repeated for precipitation at 60% and 80% saturation of ammonium sulfate. The precipitates recovered by centrifugation were resuspended in a minimal amount of 0.5 M sodium phosphate buffer (pH 7.0) and the proteolytic and lipolytic activities were assayed. The fractions with maximum enzyme activities were pooled and dialysed against the same buffer extensively. The dialysate was analyzed for lipase and protease activities. The dialysate was loaded on a Sephadex G-75 column which was pre-equilibriated with 0.2 M sodium phosphate buffer. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions were collected every 5 min and analyzed for protease and lipase activities. The fractions with maximum protease and lipase activities were pooled and used for further study.

Characterization of enzymes

The optimum pH values of protease and lipase present in the partially purified enzyme mixture were studied over a range of 5.0 to 12.0 by incubating the enzyme mixture with casein for protease and tributyrin for lipase determinations. The effect of pH values on stability of the enzymes was also analyzed by pre-incubating the enzyme mixture in buffers at different pH values, 5.0 to 12.0, for 1 h at 45 ºC. The optimum temperature of both the enzymes was studied by pre-incubating the enzyme mixture at temperatures ranging from 20 ºC to 60 ºC. The stability of the enzymes was also investigated by pre-incubating the enzyme mixture at various temperatures in the range –4 ºC to 70 ºC for 1 h. The enzyme mixture was stored in 1.5-ml vials at various temperatures, 28, 37, 45, and 55 ºC, for 10 d and analyzed for protease and lipase activities on the eleventh day. The enzyme mixture was added to coagulated egg white in the ratio 1:20 and incubated at 37 ºC to

study its ability to digest natural proteins and lipids.

The effect of inhibitors like phenylmethane sulfonyl fluoride (PMSF), EDTA, phenanthroline, dithiothreitol, iodoacetate at 10 mM concentration in dimethyl sulfoxide (DMSO) was studied by pre-incubating the enzyme mixture with the inhibitors for 30 min at 37 ºC. The effect of additives like SDS, Triton X-100, H_2O_2, and sodium hypochlorite on the activity of protease and lipase was studied at a content of 2% v/v or w/v. The residual enzyme activity after an incubation period of 20 min was determined.

The purity of the enzyme mixture was determined by SDS-PAGE using 12.5% polyacrylamide gel according to the method of Laemmli (1970). The enzyme mixture was subjected to activity staining on zymograms for confirmation and to determine their molecular weights. Electrophoresis was performed on 0.1% copolymerized gelatin as substrate according to De Azeredo *et al.* (2004) to study the protease activity. The lipase activity staining was performed according to the method of Singh *et al.* (2006).

Results and Discussion

Concomitant production of protease and lipase and the role of protease in the extracellular synthesis of lipase have been studied in *Acinetobacter calcoaceticus* and *Pseudomonas* sp. (Cordenons *et al.*, 1996; Christen and Marshall, 1984). Proteases have been reported to affect the concomitant production of lipase either by promoting the processing of extracellular lipase or degrading it. Our study emphasizes the concomitant production of protease and a proteolysis-resistant lipase. There are no reports on investigations of concomitant production and simultaneous existence of both lipase and protease produced by *Bacillus* species. Studies on proteolysis-resistant lipase have been performed by investigating the resistance of lipase to commercial proteases like proteinase K and savinase (Zhang *et al.*, 2008). Hence we attempted to study the concomitant production of a protease and a proteolysis-resistant lipase using *Bacillus* sp.

The 1.4-kb 16S rRNA gene was PCR-amplified in order to characterize the isolate, *Bacillus pumilus* SG2. BLAST analysis indicated that SG2 (Genbank: GQ398413) was closely related to *B. pumilus* Dwi strain and *B. pumilus* HBP 8 strain (100% homology).

The enzymes were produced on a common production medium under culture conditions which were optimized earlier (data not shown). The specific activity of protease was 20 U/mg and that of lipase was 18 U/mg in the cell-free supernatant and was considered as control (100% yields). The proteolytic and lipolytic activities in the purified enzyme mixture were 240 U/mg and 214 U/mg, respectively. The enzymes were purified twelve-fold and the purification by ammonium sulfate precipitation, dialysis and gel filtration chromatography had removed other protein contaminants. This has been confirmed by investigating the purity of the enzyme mixture by SDS-PAGE. The purified enzyme mixture exhibited two distinct bands on the SDS-PAGE gel. The zymogram analyses confirmed the presence of protease as clear zones of proteolysis against a blue background and lipase was confirmed by the presence of a brownish yellow band against a pink background. The molecular weight of protease was found to be 28 kDa and that of lipase was 40 kDa using protein molecular weight markers (Sigma) (Fig. 1).

Protease and lipase produced by *Bacillus pumilus* SG2 exhibited maximum activity at alkaline pH, and it is significant that alkaline enzymes have great industrial potential. The maximum ac-

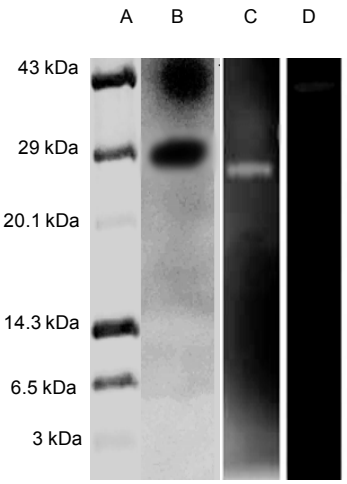

Fig. 1. SDS-PAGE and zymogram study of partially purified enzymes. Lane A, gel stained with coomassie blue exhibiting markers; lane B, purified enzymes; lane C, zymogram demonstrating proteolytic activity; lane D, zymogram demonstrating lipolytic activity.

tivity of protease was observed at pH 10.0 and that of lipase was at pH 9.0. However, both enzymes had optimal activity in the pH range 8.0 to 11.0. The enzymes were found to be stable between pH 7.0 to 11.0, and nearly 28% reduction in activities at pH 6.0 and 12.0 and a drastic reduction by 82% at pH 5.0 was observed. The temperature at which both enzymes exhibited maximum activity was 45 ºC. The stability of both enzymes was appreciable at a temperature range 30 ºC to 55 ºC. There was a 30% loss in lipolytic activity and a 46% loss in proteolytic activity at 60 ºC. Both enzymes were unstable at –4 ºC and 70 ºC. The stability of the enzymes in a wide range of temperatures contributes to the exploitation of these enzymes for various industrial purposes.

Enzymes can be classified based on their response to various inhibitors. The protease activity was inhibited by 80% by PMSF while no reduction in activity of protease was observed with other inhibitors, thus indicating the presence of serine at or near the active site of the protease enzyme. The activity of lipase was reduced by 70% by dithiothreitol, 72% by iodoacetate, 52% by phenanthroline, a classical metalloprotease inhibitor, and 36% by EDTA. This indicates the presence of cysteine at or near the active site of the lipase enzyme. Thus the protease produced by *Bacillus pumilus* SG2 was a serine protease and the lipase was a cysteine lipase which was metal-dependent. A lipase from a *Bacillus* sp. was found to be metal-dependent, *i.e.* its activity was enhanced in the presence of metals, while EDTA did not affect the enzyme activity and thus was not a metalloenzyme (Sharma *et al.*, 2002). Hadj *et al.* (2007) have purified a serine protease from *B. licheniformis* which is calcium-dependent.

The protease and lipase of *Bacillus pumilus* SG 2 were stable in the presence of surfactants and oxidants and this is an important prerequisite for these enzymes to be perfect detergent additives. The enzyme activities were not influenced by SDS, Triton X-100 and H_2O_2 but the addition of sodium hypochlorite to the enzyme mixture reduced the activity of protease by 30% and that of lipase by 36% (Table I). Olsen (2000) has patented a solution containing protease and lipase which can be used for cleaning purposes. The desired properties in a detergent enzyme are good temperature stability, stability at pH 9.0–10.5, and relative non-dependence on metal ions, along with compatibility with detergent components, such as surface-active reagents, builders, and bleaching agents as reported by Jasvir *et al.* (1999).

Table I. Effect of inhibitors, surfactants, and oxidants on the activity of protease and lipase.

Reagent	Activity of protease (%)	Activity of lipase (%)
None	100	100
Inhibitors		
PMSF	20	97
Dithiothreitol	98	30
Iodoacetate	99	28
EDTA	94	64
Phenanthroline	97	48
Surfactants		
SDS	96	97
Triton X-100	96	98
Oxidants		
H_2O_2	91	90
Sodium hypochlorite	70	64

The enzyme activity in the absence of any reagent is considered to be 100%.

The purified enzyme mixture was stored for 10 d at various temperatures to analyze the stability of lipase in the presence of protease during shelf-life. After 10 d, the specific activity of protease was determined as 238 U/mg and that of lipase was 209 U/mg, *i.e.* enzyme activity was not lost. Thus lipase proved to be proteolysis-resistant. The enzyme mixture was added to coagulated egg white and the insoluble form was converted to a completely soluble form within 8 h.

Thus a protease and a proteolysis-resistant lipase were concomitantly produced using *Bacillus pumilus* SG2. The enzymes in combination are stable and can be exploited by many industries like bakery, dairy, and detergents.

Bergy N. R. K. and Holt J. G. (1984), Systematic Bacteriology, Vol. 1–4. Lippincott William and Wilkins, Baltimore, USA.

Christen G. L. and Marshall R. T. (1984), Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. J. Dairy Sci. **97**, 1680–1687.

Cordenons R. G., Kok R., Hellingwerf K. J., and Nudel C. (1996), Effect of nitrogen sources on the regulation of extracellular lipase production in *Acinetobacter calcoaceticus* strains. Biotechnol. Lett. **18**, 633–638.

De Azeredo L. A. I., Freire D. M. G., Soares R. M. A., Leite S. G. F., and Coelho R. R. R. (2004), Production and partial characterization of proteases from *Streptomyces* sp. isolated from Brazilian cerrado soil. Enzyme Microbiol. Technol. **34**, 354–358.

Fox P. F., Law J., McSweeney P. L. H., and Wallace J. (2006), The biochemistry of cheese ripening. In: Cheese: Chemistry, Physics and Microbiology (Fox P. F., ed.). Aspen Publishers, Frederick, MD, pp. 389–438.

Hadj A., Agrebi R., Frikhac B.-G., Kamoun A.-S., Kanoun S., and Nasri M. (2007), Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. Enzyme Microbiol. Technol. **40**, 515–523.

Henriette C., Zinebi S., Aumaitre M. F., Petitdemange E., and Petitdemange H. (1993), Protease and lipase production by a strain of *Serratia marcescens* (532 S). J. Ind. Microbiol. **12**, 129–135.

Jasvir S., Gill N., Devasahayam G., and Sahoo D. K. (1999), Studies on alkaline protease produced by *Bacillus* sp. NG312. Appl. Biochem. Biotechnol. **76**, 57–63.

Kordel M., Hofmann B., Schomburg C., and Schmid R. D. (1991), Extracellular lipase of *Pseudomonas* sp. strain ATCC 21808: purification, characterization, crystallization and preliminary X-ray diffraction data. J. Bacteriol. **173**, 4836–4841.

Laemmli U. K. (1970), Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature (London) **277**, 680–685.

Olsen H. S. (2000), Cleaning-in-place of soiled process equipment, esp. in dairy or slaughter house – by circulating a solution containing a protease and a lipase. US 6071356.

Rahman R. N. Z. A., Geok L. P., and Basri M. (2005), Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa*. Biores. Technol. **96**, 429–436.

Rao M., Tanksale A. M., Ghatge M. S., and Deshpande V. S. (1998), Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. **68**, 597–635.

Sangeetha R., Geetha A., and Arulpandi I. (2008), Optimisation of protease and lipase production by *Bacillus pumilis* SG 2 isolated from an industrial effluent. The Internet J. Microbiol. **5**, 2.

Sharma R., Chisti Y., and Banerjee U. C. (2001), Production, purification, characterization, and applications of lipases. Biotechnol. Adv. **19**, 627–662.

Sharma R., Soni S. K., Vohra R. M., Gupta L. K., and Gupta J. K. (2002), Purification and characterisation of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1. Process Biochem. **37**, 1075–1084.

Singh R., Gupta N., Goswami V. K., and Gupta R. (2006), A simple activity staining protocol for lipases and esterases. Appl. Microbiol. Biotechnol. **70**, 679–682.

Tripathi G. and Rawal S. K. (1998), Simple and efficient protocol for isolation of high molecular weight DNA from *Streptomyces aureofaciens*. Biotechnol. Tech. **12**, 629–631.

Watanabe K., Kodama Y., and Harayama S. (2001). Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J. Microbiol. Methods **44**, 253–262.

Zhang Y., Menga K., Wang Y., Luo H., Yang P., Shi P., Wu N., Fan Y., Li J., and Yao B. (2008), A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae* k11. Enzyme Microbiol. Technol. **42**, 346–352.