Brazilian Journal of Microbiology (2006) 37:307-311
ISSN 1517-8382

STUDIES ON THE STABILITY OF PROTEASE FROM *BACILLUS* SP. AND ITS COMPATIBILITY WITH COMMERCIAL DETERGENT

Wellingta Cristina Almeida do Nascimento; Meire Lelis Leal Martins*

Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brasil

Submitted: December 02, 2004; Returned to authors for corrections: February 07, 2005; Approved: May 14, 2006

ABSTRACT

Enzymes, and particularly proteases, have become an important and indispensable part of industrial processes such as laundry detergents, pharmaceuticals and food products. Detergents such as Tide®, Ariel® and Biz® contain proteolytic enzymes, most of them produced by members of the genus *Bacillus*. This paper describes the compatibility of protease produced by the thermophilic *Bacillus* sp, with commercial laundry detergent. Stability studies indicated that this enzyme retained about 95% and 74% of its maximum activity after 1h at 60ºC in the presence of glycine in combination with $MnSO_4$ and $CaCl_2$, respectively. No inhibitory effect was observed at 1.0-5.0 mM of EDTA. Triton X-100 inhibited the enzyme in all the concentrations tested. The enzyme was unstable in a 5% (v/v) concentration of peroxide solution. The protease retained more than 80% and 65% of its activity after 30 min incubation at 60ºC in the presence of Tide® and Cheer® detergents, respectively. After supplementation of $CaCl_2$ (10 mM) and glycine (1 mM), the enzyme in Tide® detergent retained more than 85% of its activity after 1h. Based on these findings, *Bacillus* sp. protease shows a good potential for application in laundry detergents.

Key words: protease, thermophilic bacterium, *Bacillus* sp., detergents

INTRODUCTION

Proteases are one of the most important groups of industrial enzymes and are used in a variety of industrial applications as laundry detergents, pharmaceuticals, leather products, as meat tenderizers, protein hydrolyzates, food products, and even in the waste processing industry (11,14). This enzyme accounts for 30% of the total worldwide production of enzymes (8).

The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* sp. (2,17). These strains are specific producers of extracellular proteases (18) and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (7).

Microbial proteases, especially from *Bacillus* sp., are the most widely exploited industrial enzymes with major applications in detergent formulations (3,4,6,10). Over the past 30 years, the importance of proteases in detergents has changed, passing from their status of minor additives to become key ingredients. The performance of protease is influenced by several factors, such as the detergent's pH, ionic strength, washing temperature, detergent composition, bleach systems and mechanical handling. Thus, the key challenge for the use of enzymes in detergent is their stability (2,12).

In this article, we examine the efficacy of protease recovered from a thermophilic *Bacillus* sp. in the presence of standard commercial detergents. We also discuss the effect of various co-factors or additives on the stability of enzyme at higher temperatures.

MATERIALS AND METHODS

Microorganism and culture conditions

The bacterial strain used in this study was a thermophilic *Bacillus* sp. previously isolated from a local soil sample (13).

*Corresponding Author. Mailing address: Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro. Av. Alberto Lamego, 2000. 28013-600, Campos dos Goytacazes, RJ, Brasil. E-mail: meire@uenf.br.

Production of the protease was carried out in a medium containing (g/L of distilled water): $MgSO_4$-0.5, K_2HPO_4-2.0, KCl-0.3, NH_4NO_3-10.0, peptone-1.0, trisodium citrate-10.0, $CaCl_2$-$2.2x10^{-3}$, ZnO-$2.5x10^{-3}$, $FeCl_3.6H_2O$-$2.7x10^{-2}$, $MnCl_2.4H_2O$-$1.0x10^{-2}$, $CuCl_2.2H_2O$-$8.5x10^{-4}$, $CoCl_2.6H_2O$-$2.4x10^{-3}$, $NiCl_3.6H_2O$-$2.5x10^{-4}$, H_3BO_3-$3.0x10^{-4}$ and Na_2MoO_4-$1.0x10^{-3}$. The pH was adjusted to 6.9-7.0 with 1.0 M NaOH and this basal medium was sterilized by autoclaving at 121ºC for 15 min. The production medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of an overnight culture and incubated at 50ºC under shaking (150 rpm) in a Thermo Forma Orbital Shaker (Ohio, USA) for 9h. Thereafter, the cell-free enzyme supernatant was obtained by centrifugation (Hermle Z 382K) at 15,500 g for 15 min at 4ºC.

Enzyme assay

The activity of protease was assessed in triplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.2% (w/v) azocasein in 0.1 M Tris/HCl buffer (pH 8.0) at 60ºC for 10 min. The 1 mL reaction was terminated by the addition of 0.5 mL of 15% trichloroacetic acid and then centrifuged at 20,600 g for 5 min, after cooling. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 420 nm equal to 1.0 in 60 minutes (9). Protein was measured by Lowry's method modified by Peterson (15).

Effect of $CaCl_2$, $MnSO_4$ and glycine on protease stability

The enzyme preparation was incubated in the presence of $CaCl_2$ (10 mM), $MnSO_4$ (10 mM), $CaCl_2$ (10 mM) + glycine (1 mM) and $MnSO_4$ (10 mM) + glycine (1 mM) at 60ºC. The residual activity (%) was measured at various intervals of time under standard assay conditions.

Effect of some surfactants and oxidizing agents on protease activity

The enzyme was preincubated with Triton X-100 (1-5% v/v), sodium dodecyl sulfate (0.1-0.5%), hydrogen peroxide (2.5 and 5.0%) and ethylenediaminetetraacetic acid (1, 2, 5 and 10 mM) at 60ºC for 15 and 30 minutes and protease activity was measured under standard conditions.

Compatibility with various commercial detergents

The ammonium sulfate-precipitated enzyme preparation was used for the detergent compatibility studies. The cells and residues were removed from the culture broth by centrifugation and concentrated by precipitating the enzyme with ammonium sulfate (60% saturation). The precipitate was dissolved and dialyzed overnight against 10 mM Tris/HCl buffer, pH 8.0. The dialyzed enzyme was then concentrated by lyophilization and used for subsequent studies.

The detergent brands used were Ariel®, Biz®, Cheer®, Tide®, Campeiro® and Omo®. They were diluted in double distilled water to a final concentration of 7 $mg.mL^{-1}$ to simulate washing conditions. The enzyme in the detergent was deactivated by heating at 100ºC for 10 min. After that, a protease concentration of 0.55 $mg.mL^{-1}$ was added in solution and incubated at 60ºC for 60 min. Aliquots (0.5 mL) were taken at different time intervals and the residual activity determined at 60ºC and compared with the control sample incubated at 60ºC without any detergent (1, 16).

Effect of $CaCl_2$ and glycine on protease stability in commercial detergent

The detergents were diluted in double distilled water to a final concentration of 7 $mg.mL^{-1}$. A protease concentration of 0.55 $mg.mL^{-1}$ was incubated at 60ºC in detergent in the presence of $CaCl_2$ (10 mM), glycine (1 mM), $CaCl_2$ (10 mM) + glycine (1 mM) at 60ºC. At 10-min intervals, a sample (0.5 mL) was removed and the residual activity determined at 60ºC and compared with the control sample incubated at 60ºC with no detergent.

RESULTS AND DISCUSSION

Effect of $CaCl_2$, $MnSO_4$ and glycine on protease stability

Fig. 1 illustrates the thermostability of protease activity from *Bacillus* sp. in the presence of Ca^{2+}, Mn^{2+} at 60ºC. The results are expressed as percentage of residual activity, taking into account the activity determined with the non-treated enzyme samples.

The thermostability profile indicated that the enzyme retained about 80% of its activity after 30 min at 60ºC. The separate addition of $CaCl_2$ and $MnSO_4$ improved the enzyme's thermostability. The protease retained more than 90% and 45% of its maximum activity after 1 and 2h, respectively, at 60ºC in the presence of $MnSO_4$. In the presence of $CaCl_2$, the

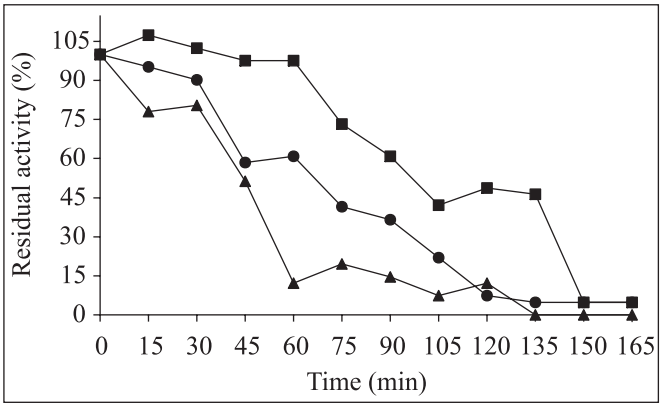

Figure 1. Thermostability of protease produced by *Bacillus* sp. at 60ºC, in presence of $MnSO_4$ (■), $CaCl_2$ (●) and control (▲). 100% of enzyme activity =1.76 U/mgProtein.

residual activity of the enzyme was about 60% after 1h at 60ºC (Fig. 1).

The addition of glycine, separately and in combination with $MnSO_4$ or $CaCl_2$, also improved the thermostability of the protease, as indicated in Figs. 2(a) and 2(b), respectively. The enzyme retained about 95% and 73% of its maximum activity after 1h at 60ºC in the presence of glycine in combination with $MnSO_4$ and $CaCl_2$, respectively. These results are similar to the findings of Banerjee *et al*. (1), who reported that $CaCl_2$ and glycine separately and in combination improved the thermostability of protease from *Bacillus brevis*.

Effect of inhibitors and some surfactants

The enzyme solution was mixed with EDTA, a metalloprotease inhibitor, and pre-incubated at 60ºC for 15 and 30 minutes, and then protease activity was determined as described earlier. The results were recorded as the percentage of residual activity calculated with reference to activity controls incubated in the absence of this compound (Table 1).

No inhibitory effect was observed at 1, 2, 5 and 10 mM of EDTA. However, with 10 mM the enzyme retained about 84% of its activity when incubated for 30 min. This finding was similar to that of Tunga *et al*. (19), who reported that the protease from *Aspergillus parasiticus* was not inhibited by 5.0 mM EDTA. In *Bacillus licheniformis* ATCC 21415 (12), the protease was inhibited by 20 mM EDTA and lost about 62% of its original activity. These authors attributed this result to chelation of calcium ions, which are necessary for enzyme activation or participate in the enzyme molecule. As for the effects of surfactants, in the presence of Triton X-100, the enzyme incubated for 30 min was inhibited in all the concentrations tested, and this inhibition was more pronounced at 3 and 4%. In addition, the protease did not display good stability in a 5% (w/v) concentration of peroxide solution for 30 min at 60ºC. In the presence of 0.1% SDS (w/v), the enzyme retained 87% activity at 60ºC for 15 min. However, in concentrations higher than 0.3%, the enzyme was completely inhibited.

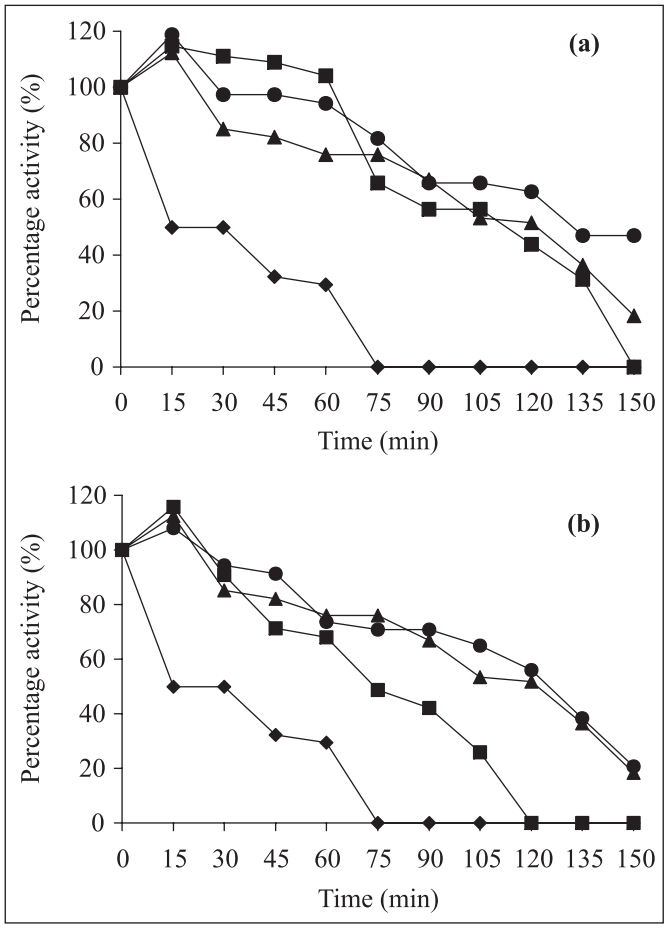

Figure 2. Thermostability of protease produced by *Bacillus* sp. at 60ºC , **(a)** in presence of $MnCl_2$ (■), glycine (▲), glycine + $MnSO_4$ (●) and control (◆), **(b)** in presence of $CaCl_2$ (■), glycine (▲), glycine + $CaCl_2$ (●) and control (◆). 100% of enzyme activity = 2.7 U/mgProtein.

Table 1. Effect of various inhibitors and surfactants on protease activity

Residual protease activity (%)		
Inhibitors/Surfactants	Time of incubation	
	15	30
Control	100	100
EDTA 1mM	100	106
EDTA 2mM	107	106
EDTA 5mM	104	105
EDTA 10mM	94	84
Triton X-100 1%	121	47
Triton X-100 2%	118	47
Triton X-100 3%	93	5
Triton X-100 4%	32	3
Triton X-100 5%	56	53
H_2O_2 2.5%	95	0
H_2O_2 5.0%	86	10
SDS 0.1%	87	26
SDS 0.2%	36	3
SDS 0.3%	4	0
SDS 0.4%	0	0
SDS 0.5%	0	0

The activity is expressed as a percentage of the activity level in the absence of inhibitors or surfactants (100% of enzyme activity = 4.47 U/mgProtein).

Compatibility with various commercial detergents

Protease from thermophilic *Bacillus* sp. retained more than 80% and 65% of its activity after 30 min incubation at 60ºC in the presence of the detergent brands Tide® and Cheer®, respectively (Fig. 3). After supplementation of Ca^{+2} and glycine, the enzyme in the Tide detergent was stable for 30 min at 60ºC, retaining more than 85% of its activity after 1h (Fig. 4). All proteases are stabilized in the presence of a certain level of free Ca^{+2}. Therefore, 100-1000 ppm of Ca^{+2} is normally added to liquid detergents that contain protease. Other agents such as glycine will partly inhibit the activity of proteases (e.g., by binding on the active side). However, the protease will recover its full activity when the detergent is diluted in the washing process (6). The protease from *Bacillus brevis* showed compatibility at 60ºC with commercial detergents such as Ariel®, Surf Excels®, Surf Ultra® and Rin® in the presence of Ca^{2+} and glycine. This enzyme retained more than 50% activity with most of the detergents tested, even after 3h incubation at 60ºC (1). Bhosale *et al.* (5) reported that protease preparation from *Conidiobolus coronatus* showed compatibility at 50ºC, in the presence of 25 mM $CaCl_2$ in a variety of commercial detergents. This enzyme retained 16% activity in Revel®, 11.4% activity in Ariel® and 6.6% activity in Wheel®.

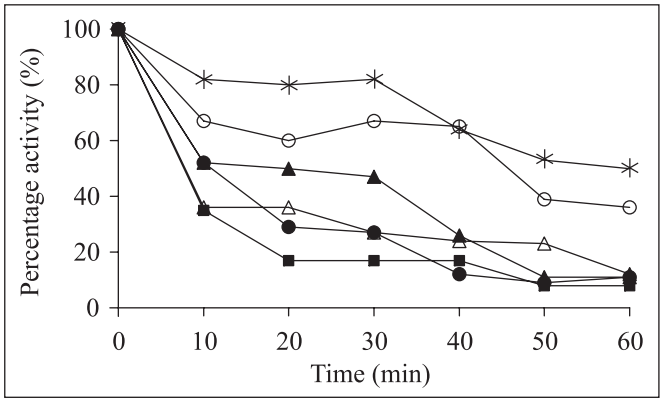

Figure 3. Compatibility of protease activity from *Bacillus* sp. with commercial detergents (■ Ariel®, ▲ Biz®, ○ Cheer®, ✳ Tide®, ● Campeiro®, △ Omo®). The activity is expressed as a percentage of the activity level in the absence of detergents (100% of enzyme activity =11.85 U/mgProtein).

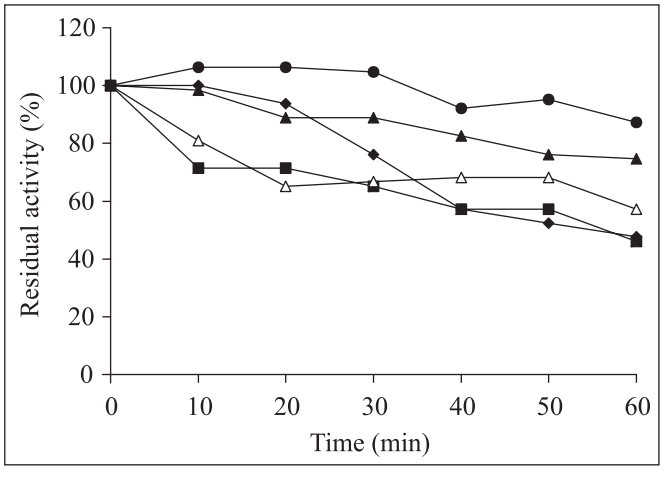

Figure 4. Compatibility of protease from *Bacillus* sp. with Tide®. (◆ control, ■ enzyme + detergent, △ enzyme + glycine + detergent, ▲ enzyme + $CaCl_2$ + detergent, ● enzyme + glycine + $CaCl_2$ + detergent). 100% of enzyme activity =11.31 U/mgProtein.

ACKNOWLEDGMENTS

The authors thank the FAPERJ for financial support and the CNPq for providing a scholarship to posgraduate student W.C.A. Nascimento.

RESUMO

Estudos sobre a estabilidade de uma protease de *Bacillus* sp. e sua compatibilidade com detergentes comerciais

As enzimas, principalmente as proteases, têm uma participação importante e indispensável em muitos processos industriais tais como na indústria farmacêutica, de alimentos e de detergentes. Alguns detergentes como Tide®, Ariel® e Biz® contem enzimas proteolíticas em sua formulação, sendo a maioria produzida por bactérias do gênero *Bacillus* sp. Neste artigo, foi avaliada a compatibilidade de uma protease produzida por um microrganismo termofílico, *Bacillus* sp., com alguns detergentes comerciais. Estudos sobre a estabilidade mostraram que a enzima reteve cerca de 95% e 74% de sua máxima atividade após 1h a 60ºC na presença de glicina em combinação com $MnSO_4$ e $CaCl_2$ respectivamente. A enzima não foi inibida em presença de 1.0 - 5.0 mM de EDTA. A adição de Triton X-100 inibiu a atividade enzimática em todas as concentrações estudadas. A enzima não foi estável na presença de uma solução a 5% (v/v) de peróxido de hidrogênio. A protease manteve mais de 80% e 65% de atividade após 30 min de incubação na presença dos detergentes Tide® e Cheer® respectivamente. Após adição de $CaCl_2$ (10 mM) e glicina (1 mM), a enzima manteve mais de 85% de atividade após 1 hora de incubação em presença do detergente Tide®. Considerando estas propriedades, a protease de *Bacillus* sp., pode ser útil na indústria de detergentes.

Palavras-chave: protease, bacteria termofílica, *Bacillus* sp., detergentes

REFERENCES

1. Banerjee, U.C.; Sani, R.K.; Azmi, W.; Soni, R. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Proc. Biochem.*, 35, 213-219, 1999.
2. Beg, Q.K.; Gupta, R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb. Technol.*, 32, 294-304, 2003.
3. Beg, Q.K.; Sahai,V.; Gupta, R. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Proc. Biochem.*, 39, 203-209, 2003.
4. Beg, Q.K.; Saxena, R.K.; Gupta, R. De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Proc. Biochem.*, 37, 1103-1109, 2003.
5. Bhosale, S.H.; Rao, M.B.; Deshpande, V.V.; Srinivasan, M.C. Thermostability of high activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20). *Enzyme Microb. Technol.*, 17, 136-139, 1995.
6. Godfrey, T.; West, S. Industrial Enzimology. 2nd Ed. Stockton Press Ed. US and Canadá, 609 p.1996.
7. Han, X.Q.; Damodaran, S. Isolation, identification and fermentation of *Bacillus* species producing a detergent-stable endopeptidase. *J. Agric. Food Chem*., 45, 4191-4195, 1997.
8. Horikoshi K. Alkalophiles - from an industrial point of view. *FEMS Microbiol. Rev*., 18, 259-270, 1996.
9. Janssen, P.H.; Peek, K.; Morgan, H.W. Effect of culture conditions on the production of a extracellular proteinase by *Thermus* sp. Rt41A. *Appl. Microbiol. Biotechnol*., 41, 400-406, 1994.
10. Johnvesly, B.; Naik, G.R. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Proc. Biochem*., 37, 139-144, 2001.
11. Joo, H.S.; Chang, C.S. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Proc. Biochem.*, 40, 1263-1270, 2005.
12. Mabrouk, S.S.; Hashem, A.M.; El-shayed, N.M.A.; Ismail, M.S.; Abdel-Fattah, A.F. Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. *Biores. Technol*., 69, 155-159, 1999.
13. Nunes, A.S.; Martins, M.L.L. Isolation, properties and kinetics of growth of a thermophilic *Bacillus*. *Braz. J. Microbiol*., 32, 271-275, 2001.
14. Pastor, M.D.; Lorda, G.S.; Balatti, A. Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration rates. *Braz. J. Microbiol*., 32, 1-8, 2001.
15. Petterson, G.L. A simplification of the protein assay method of Lowry *et al*. which is more generally applicable. *Analytical Biochem*., 83, 346-356, 1977.
16. Phadatare, S.U.; Deshpande, V.V.; Srinivasan M.C. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents. *Enzyme Microb. Technol.*, 15,72-6, 1993.
17. Priest, F.G. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev*., 41, 711-753, 1977.
18. Singh, J.; Batra, N.; Sobti, C.R. Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Proc. Biochem*., 36, 781-785, 2001.
19. Tunga, R.; Shrivastava, B.; Benerjee, R. Purification and Characterization of a Protease from Solid State Cultures of *Aspergillus parasiticus*. *Proc. Biochem.*, 38, 1553-1558, 2003.