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

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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 60°C in the presence of glycine in combination with MnSO₄ and CaCl₂, 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 60°C in the presence of Tide® and Cheer® detergents, respectively. After supplementation of CaCl₂ (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).

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Production of the protease was carried out in a medium containing (g/L of distilled water): MgSO₄·0.5, K₂HPO₄·2.0, KCl-0.3, NH₄NO₃·10.0, peptone·1.0, trisodium citrate·10.0, CaCl₂·2.2x10⁻³, ZnO·2.5x10⁻³, FeCl₃·6H₂O·2.7x10⁻², MnCl₂·4H₂O·1.0x10⁻², CuCl₂·2H₂O·8.5x10⁻⁴, CoCl₂·6H₂O·2.4x10⁻³, NiCl₂·6H₂O·2.5x10⁻⁴, H₂BO₃·3.0x10⁻⁴ and Na₂MoO₄·1.0x10⁻³. The pH was adjusted to 6.9-7.0 with 1.0 M NaOH and this basal medium was sterilized by autoclaving at 121°C 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 50°C 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 4°C.

**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 60°C 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 CaCl₂, MnSO₄ and glycine on protease stability**

The enzyme preparation was incubated in the presence of CaCl₂ (10 mM), MnSO₄ (10 mM), CaCl₂ (10 mM) + glycine (1 mM) and MnSO₄ (10 mM) + glycine (1 mM) at 60°C. 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 60°C 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 deactivate by heating at 100°C for 10 min. After that, a protease concentration of 0.55 mg.mL⁻¹ was added in solution and incubated at 60°C for 60 min. Aliquots (0.5 mL) were taken at different time intervals and the residual activity determined at 60°C and compared with the control sample incubated at 60°C without any detergent (1, 16).

**Effect of CaCl₂ 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 60°C in detergent in the presence of CaCl₂ (10 mM), glycine (1 mM), CaCl₂ (10 mM) + glycine (1 mM) at 60°C. At 10-min intervals, a sample (0.5 mL) was removed and the residual activity determined at 60°C and compared with the control sample incubated at 60°C with no detergent.

**RESULTS AND DISCUSSION**

**Effect of CaCl₂, MnSO₄ and glycine on protease stability**

Fig. 1 illustrates the thermostability of protease activity from *Bacillus* sp. in the presence of Ca²⁺, Mn²⁺ at 60°C. 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 60°C. The separate addition of CaCl₂ and MnSO₄ improved the enzyme’s thermostability. The protease retained more than 90% and 45% of its maximum activity after 1 and 2h, respectively, at 60°C in the presence of MnSO₄. In the presence of CaCl₂, the

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Figure 1. Thermostability of protease produced by *Bacillus* sp. at 60°C, in presence of MnSO₄ (■), CaCl₂ (●) 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 MnSO₄ or CaCl₂, 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 MnSO₄ and CaCl₂, respectively. These results are similar to the findings of Banerjee et al. (1), who reported that CaCl₂ 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.

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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 Ca²⁺ 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 Ca²⁺. Therefore, 100-1000 ppm of Ca²⁺ 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 60°C with commercial detergents such as Ariel®, Surf Excels®, Surf Ultra®, and Rin® in the presence of Ca²⁺ and glycine. This enzyme retained more than 50% activity with most of the detergents tested, even after 3h incubation at 60°C (1). Bhosale et al. (5) reported that protease preparation from Conidiobolus coronatus showed compatibility at 50°C, in the presence of 25 mM CaCl₂ in a variety of commercial detergents. This enzyme retained 16% activity in Revel®, 11.4% activity in Ariel®, and 6.6% activity in Wheel®.

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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® contêm 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 micrororganismo 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 60°C na presença de glicina em combinação com MnSO₄ e CaCl₂, 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 CaCl₂ (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


