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Cloned *Bacillus subtilis* Alkaline Protease (*aprA*) Gene Showing High Level of Keratinolytic Activity

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ABSTRACT

The *Bacillus subtilis* alkaline protease *(aprA)* gene was previously cloned on a pUB110-derivative plasmid. High levels of expression and gene stability were demonstrated when *B. subtilis* cells were grown on the laboratory medium 2XSG. *B. subtilis* cells harboring the multicopy *aprA* gene were grown on basal medium, supplemented with 1% chicken feather as a source of energy, carbon, and nitrogen. Proteolytic and keratinolytic activities were monitored throughout the cultivation time. A high level of keratinolytic activity was obtained, and this indicates that alkaline protease is acting as a keratinase. Furthermore, considerable amounts of soluble proteins and free amino acids were obtained as a result of the enzymatic hydrolysis of feather. Biodegradation of feather waste using these cells represents an alternative way to improve the nutritional value of feather, since feather waste is currently utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Moreover, the release of free amino acids from feather and the secreted keratinase enzyme would promote industries based on feather waste.

Index Entries: *Bacillus subtilis*; alkaline protease gene; keratinolytic activity.

INTRODUCTION

Recently, our research group is focusing on the aspect of the utilization of some environmental wastes, such as chicken feather and tomato processing waste, using genetically engineered *Bacillus subtilis* strains. Although environmental wastes are found in great quantities, and many are rich in proteins and various carbon compounds, little attention is given to utilizing or recycling these wastes. Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution

and health hazards. Upon the directed utilization by genetically modified microorganisms, several valuable products are produced, such as extracellular enzymes, soluble proteins, peptides, amino acids, and other compounds, which can satisfy part of the animal feed and/or human need *(1,2)*. These products can also promote and enhance several new industries in many countries.

The *B. subtilis* alkaline protease *(aprA)* gene was cloned on a pUB110-derivative plasmid. The expression of the *aprA* gene occurred late in the stationary phase of growth, and the multicopy plasmid that carries the *aprA* gene was segregationally and structurally stable *(3)*. *B. subtilis* cells carrying the multicopy *aprA* gene were grown on basal medium supplemented with 1% whole chicken feather (nearly pure keratin protein) as a source of carbon and nitrogen. High level of keratinolytic activity was developed in the culture, and, consequently, considerable amounts of soluble proteins and amino acids were obtained as a result of the biodegradation of feather. On the other hand, the keratinase *(kerA)* gene of *B. licheniformis* PWD-1 was cloned and found to have 98 and 97% sequence homology with genes encoding subtilisin Carlsberg of *B. licheniformis* and subtilisin of *B. licheniformis* NCIB, respectively *(4)*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

B. subtilis DB100 his^- met^- (pS1) was used in this study. The pS1 plasmid (6.7 kbp) is a pUB110-derivative plasmid carrying the complete *B. subtilis (aprA)* gene, as well as a kanamycin resistance gene (selectable marker) *(3)*.

Media

Bacterial strain was activated and grown on PY medium *(5)* (Bacto peptone, 10 g; Difco [East Moseley, Surrey, UK] yeast extract, 5 g; and NaCl 5 g/L). PA medium is PY supplemented with 1.5% agar agar. Basal medium II *(6)* (NH_4Cl, 0.5 g; NaCl, 0.5 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; and yeast extract 0.1 g/L) supplemented with 1% (w/v) chicken feather, was used in the fermentation process. Kanamycin was added to a final concentration of 5 μg/mL medium.

Monitoring Proteolytic and Keratinolytic Activity

The proteolytic and keratinolytic activity was monitored throughout the growth of the bacterial strain. Cells were activated by growing them overnight on PA plates at 37°C. Fresh colonies were then transferred to 10 mL PY kanamycin medium, and the culture was allowed to grow at 37°C, with shaking, for two h. One hundred mL preautoclaved basal medium II, supplemented with 1% whole chicken feather and kanamycin

(5 μg/mL), was inoculated with 5 mL of the above culture. Cells were allowed to grow at 37°C, with shaking at 200 rpm, to the indicated time (1–5 d). At the indicated time, 1 mL culture was taken to determine bacterial growth in the form of colony-forming units, as described before *(7)*. Another 2 mL culture were taken and centrifuged at 4500*g* in a microcentrifuge for 2 min. The supernatant was then used as crude solution to determine the extracellular alkaline protease activity, as well as the keratinolytic activity.

Proteolytic Activity

Proteolytic activity was measured according to the method of Cliffe and Law *(8)*, using hide powder azure (HPA). One unit of enzyme is the amount of enzyme that develops a change of absorbance against control reaction, at 595 nm/30 min at 37°C. Additionally, the proteolytic activity was determined according to the method of Lin et al. *(9)*, using skim milk agar plate.

Keratinolytic Activity

Keratinolytic activity was determined based on the free amino (NH_2 groups) that were released as a result of the biodegradation of feather by bacterial cells. Free amino groups were determined as described earlier *(9)*, and a standard curve for leucine (0.01–0.1 μmol) was established. Additionally, the physical appearance of chicken feather in the culture was observed.

Analysis of Soluble Proteins by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell-free supernatant of the feather culture was precipitated with solid ammonium sulfate to reach 70% saturation, and was kept on an ice bath for 2 h. The mixture was centrifuged at 7000*g* for 30 min at 4°C. The pellet was suspended in a small volume of Tris-HCl buffer, pH 8.0, and dialyzed overnight against the same buffer. A second precipitation by acetone (3 vol) was carried out, and the precipitate was collected by centrifugation at 3500*g* for 5 min, using microcentrifuge. The pellet was directly suspended in a small volume of sample application buffer (SAB) and applied to 10% SDS-polyacrylamide gel. The SDS-PAGE was carried out according to the method of Laemmli *(10)*.

Amino Acid Analysis

Amino acids were analyzed using Beckman 119 CL amino acid analyzer, (Beckman Instruments, Palo Alto, CA), using standard amino acids. The analysis was performed at the Central Laboratory, Faculty of Agriculture, University of Alexandria.

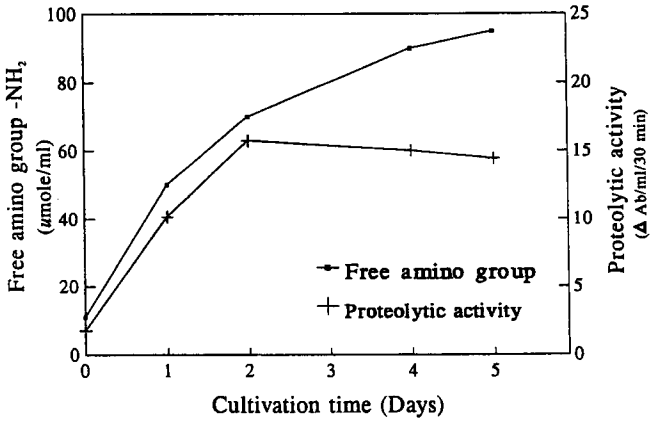

Fig. 1. Monitoring proteolytic and keratinolytic activities of the bacterial strain throughout the cultivation time. Activated *B. subtilis* DB100 (pS1) cells were grown on basal medium II, supplemented with 1% whole chicken feather and 5 μg/mL kanamycin, to the indicated time. Proteolytic activity (+) is expressed as the change in absorbance at 595 nm, of the released blue color from HPA, per mL supernatant per 30 min at 37°C. Free amino groups, expressed as μmol of leucine per mL cell-free supernatant, represent the keratinolytic activity of the alkaline protease.

RESULTS AND DISCUSSION

Monitoring Proteolytic and Keratinolytic Activity of *aprA* Gene

Earlier the author reported the expression of the cloned *aprA* gene throughout the growth of *B. subtilis* DB100 (pS1) strain in 2×SG (sporulation medium) (*3*). This medium was used to give a short exponential phase and an extended stationary phase. Expression of the *aprA* gene started late, after 2 h from the end of the exponential phase (*3*). Proteolytic and keratinolytic activity of the alkaline protease was monitored throughout the growth of the same strain that harbors the *aprA* gene, on basal medium II, supplemented with 1% whole feather. Proteolytic activity, as measured rapidly and accurately by the synthetic substrate HPA, increased as the cultivation time was increased, up to d 2, then remained constant (Fig. 1). Data of skim milk agar plate gave the same pattern (not shown). On the other hand, keratinolytic activity, as determined by the free amino group method, using standard curve for leucine, increased gradually as the cultivation time was increased up to d 5, when it reached about 95 μmol free ($-NH_2$ group) per mL culture (Fig. 1).

The physical appearance of chicken feather in the culture was observed each day and compared to the control (d 0), in order to evaluate the level of keratinolytic activity of the culture (Fig. 2). Most of the feather

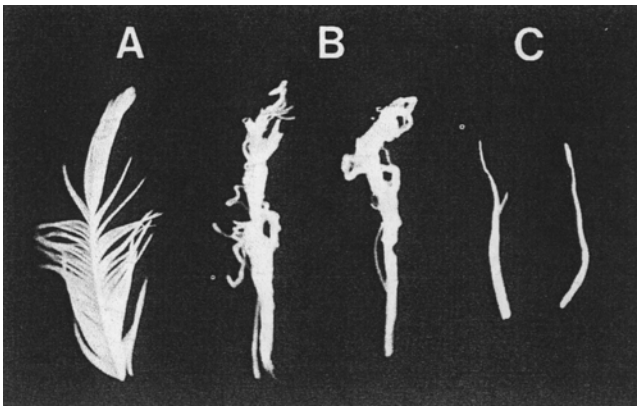

Fig. 2. Keratinolytic activity of the alkaline protease enzyme secreted by *B. subtilis* DB100 (pS1) cells in a culture containing 1% whole feather. A–C represent feather samples of the above culture after 0, 1, and 2 d after inoculation, respectively.

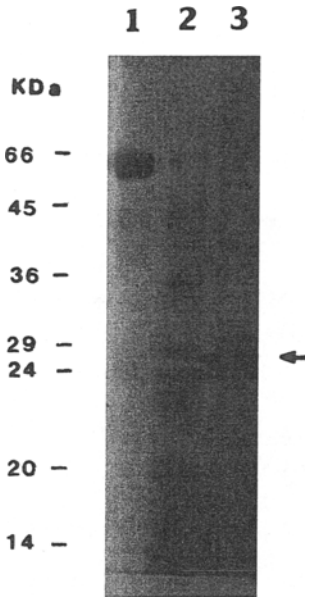

Fig. 3. SDS-polyacrylamide gel to analyze possible soluble proteins resulting from the biodegradation of whole feather at d 4. Preparation of samples and gel condition was described in Materials and Methods. Lanes 1, 2, and 3 represent BSA, mol wt marker, and 100 μg of soluble proteins sample, respectively. Arrow points at the location of alkaline protease enzyme (about 27 kDa protein). Note that considerable amounts of small mol wt proteins appear at the tail of the gel.

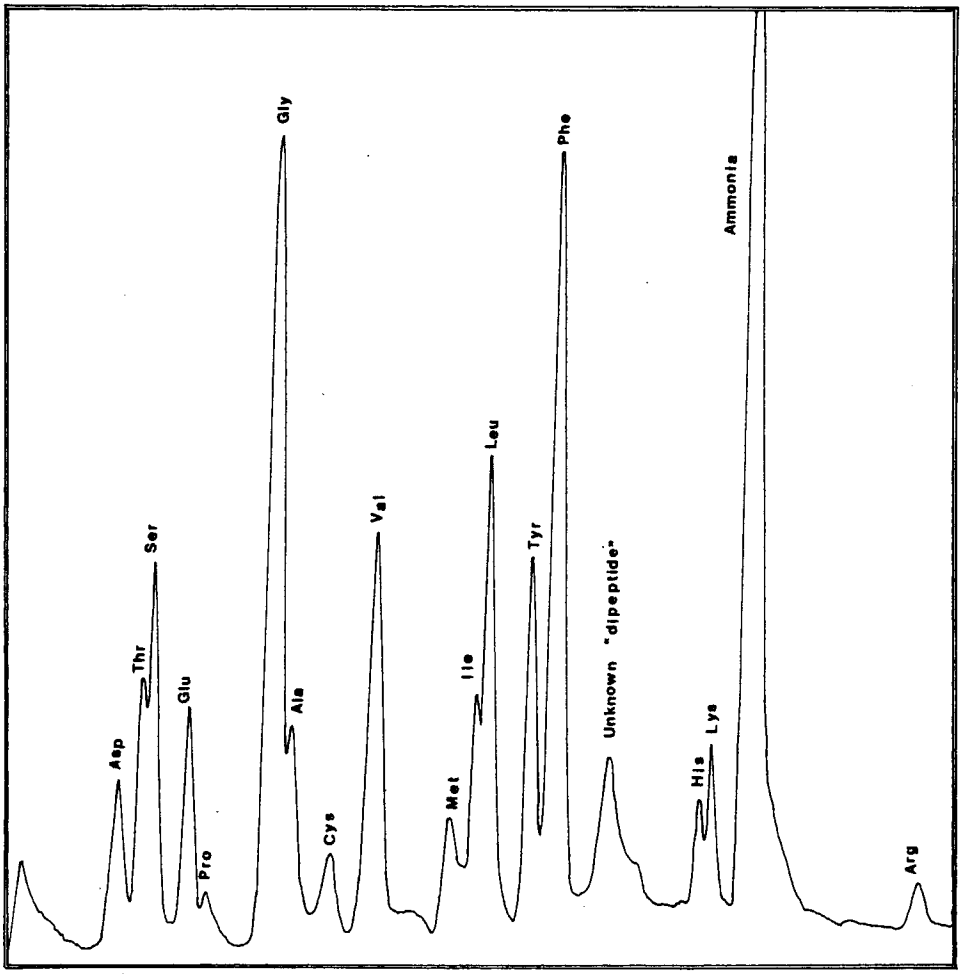

Fig. 4. Amino acid analysis of cell-free and protein-free sample derived from d 4. Analysis was carried out as described in the text, using amino acid analyzer. Several amino acids, such as gly, phe, leu, val, ser, and tyr, resulted from the utilization process.

was degraded after 48 h except the feather shaft, which was completely degraded after 72 h of cultivation.

Alkaline Protease is Acting as a Keratinase

Data shown above would indicate that the cloned *aprA* gene encoded a protease that has a high level of keratinolytic activity. Several *Bacillus* serine proteases have been cloned, sequenced, and characterized *(4,11–13)*. Members of the subtilisin family of serine proteases share extensive sequence homology. The keratinase (*kerA*) gene of *B. licheniformis* shares 98 and 97% sequence homology with genes encoding subtilisin Carlsberg of *B. licheniformis* and subtilisin of *B. licheniformis* NCIB *(4)*. Moreover, the alkaline protease of *Bacillus* sp AH101, which shows high hydrolyzing

activity against keratin, has extensive sequence homology with subtilisin BPN^-, subtilisin Carlsberg 221 and Ya-B alkaline protease *(14)*. Additionally, the activity of the keratinase enzyme secreted by *B. licheniformis* PWD1 was inhibited by PMSF, which inhibits serine proteases *(4)*. The alkaline protease enzyme (in this study) is also inhibited by PMSF (not shown).

On the other hand, considerable amounts of soluble proteins and free amino acids were obtained as a result of the enzymatic hydrolysis of feather (Figs. 3 and 4). Biodegradation of feather waste using *B. subtilis* DB100 (pS1) strain represents an alternative way to improve the nutritional value of feather, since feather waste is currently utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Moreover, the release of several amino acids from feather, as well as secreted keratinase, would promote industries based on feather waste.

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