Production and Characterization of Keratinase of a Feather-degrading Bacillus licheniformis PWD-1

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The keratinase produced by Bacillus licheniformis PWD-1 was induced by feather powder. Maximal enzyme production could be achieved by culturing in a medium containing 1% hammer-milled feather powder (100 mesh) at 45°C for 30 h. Maximal growth of PWD-1 was achieved at 50°C, and maximal enzyme induction was at 45°C. The molecular mass and isoelectric point of this enzyme was 31.4 kDa and 8.5, respectively. This enzyme is pH 5 stable. The optimal reaction pHs for feather powder and casein were 8.5 and 10.5 to 11.5, respectively. The optimal reaction temperature was 50°C to 55°C. The relative activity of this enzyme toward casein, feather powder, keratin, elastin, and collagen was 100: 52:41:18:7, and 100:56:32:3 for Suc-AAPL-pNA, Suc-AAPF-pNA, Suc-AAPM-pNA, and Suc-AAAV-pNA (Suc, succinyl; pNA, p-nitrophenylalanilide).

Keratinase proteins are insoluble and resistant to degradation by common proteolytic enzymes because of its extensive cross-linking by disulfide bonds, hydrogen bonding, and hydrophobic interaction. Despite the unusual stability of keratinase proteins, several microbial keratinolytic proteases have been reported, such as the keratinase of Trichophyton mentagrophytes,11) proteinase K of Tritrichrum album,21) the alkaline proteinase of alkalophilic Streptomyces,31) the thermostable alkaline protease of Bacillus sp. No. AH-101,51) and the alkaline elastase of alkalophilic Bacillus Ya-B.5,6) A feather-degrading bacterium, Bacillus licheniformis PWD-1, was enriched from a poultry waste digestor by Williams et al.3) The keratinase produced by this strain was purified from the 4-day-culture medium with hammer-milled chicken feathers as the main nutrient.6) The purified keratinase hydrolyzed a broad range of substrates and was shown to be a useful enzyme for promoting the hydrolysis of feather keratin9) and improving the digestibility of feather meal.9) However, the enzyme productivity of the published method of strain PWD-1 was low.7) In this study, we have increased about 40-fold the enzyme productivity by modifying the cultivation method. Under this production method, the industrial application of this enzyme was becoming more feasible. Further characterization of this enzyme was also presented.

Materials and Methods

Materials. Bovine serum albumin, collagen, gelatin, elastin, subtilisin Carlsberg, Suc-Ala-Ala-Pro-Phc-pNA (Suc-AAPF-pNA; Suc, succinyl; pNA, p-nitrophenylalanilide), Suc-Ala-Ala-Pro-Met-pNA (Suc-AAAPM-pNA), Suc-Ala-Ala-Pro-Leu-pNA (Suc-AAPL-pNA), and Suc-Ala-Ala-Ala-Val-Ala-pNA (Suc-AAA-pNA) were purchased from Sigma Chemical (U.S.A.). Casein was from Merck Darmstadt (Germany). Feather meal from Itochu Feed Mills (Japan). Keratin powder from Tokyo Kasai (Japan) and Nacalai Chemical (Japan). Alkaline elastase Ya-B was prepared as previously reported.36)

Microorganism and culture conditions. Bacillus licheniformis PWD-1 was obtained from Dr. J.C.H. Shih of North Carolina State University (Raleigh, U.S.A.). This strain was generally maintained on basal medium (0.5 g of NH₄Cl, 0.5 g of NaCl, 0.3 g of K₂HPO₄, 0.4 g of KH₂PO₄, 0.1 g of MgCl₂·6H₂O, and 0.1 g of yeast extract per liter of distilled water) containing 1% of hammer-milled feather powder (100 mesh) and 2% Difco Bacto agar.

Enzyme production. For seed culture, strain PWD-1 was grown for 24 h at 45°C in a 250-ml shaking flask containing 50 ml of the basal medium containing 1% commercial feather meal. This seed culture was then inoculated to make 2% in 2.5 liters of the basal medium containing 1% chicken feather powder in a 5-liter mini-jar fermentor (Mitsuwa Rikagaku Kogyo Co., Osaka, Japan). Fermentation was done at 45°C with 500-rpm agitation and 0.5 vol/vol per min aeration. After 30 h, the culture was cooled rapidly to 4°C, and the cells were then removed by centrifugation.

Enzyme purification. Culture fluid was concentrated with a spiral cartrige concentrator (Amicon, U.S.A.). The molecular weight cutoff value of the membrane was 10,000. Ammonium sulfate was added to the supernatant to 80% saturation. The resulting precipitate was collected by centrifugation and was dissolved in a minimal volume of 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM CaCl₂ (buffer A), and dialyzed overnight against the same buffer. The enzyme solution was passed through a Fractogel DEAE-650 column, that had been equilibrated with buffer A. The colorless flowthrough fraction was put on a Fractogel CM-650 column equilibrated with buffer A. After being washed with buffer A, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer B. Finally, the enzyme solution was put on a Toyopearl HW50 column to remove NaCl and peptides, formed probably by enzyme autodigestion. The purified enzyme was homogeneous, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). About 68 mg of purified enzyme was obtained from 1 liter of culture broth of PWD-1.

Enzyme activity assays. Keratinolytic activity was estimated by incubating enzyme with 10 mg of feather powder in 1 ml of buffer A, with vigorous shaking for 1 h at 45°C. The reaction was stopped by adding 2 ml of TCA solution (0.11 M trichloroacetic acid, 0.22 M CH₃COONa, 0.33 M CH₃COOH). After 30 min of incubation at 30°C, the substrate was removed by centrifugation and the absorption of the supernatant was measured at 275 nm. Caseinolytic activity was estimated by incubating the enzyme with 5% casein in 1 ml of buffer A for 10 min at 30°C. The reaction was stopped by adding 2 ml of TCA solution. After 30 min of incubation at 30°C, the precipitate was removed by centrifugation and the absorption of the supernatant was measured at 275 nm. Units of keratinolytic and caseinolytic activities were both expressed as μmol of tyrosine released.
per minute.

**Protein measurement.** Protein was measured by the method of Lowry et al.\(^{10}\) with bovine serum albumin as a standard.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE).** The method was done on 10% acrylamide slabs using a modified Laemmli buffer system.\(^ {11}\) Coomassie brilliant blue R-250 was used for staining. The molecular weight markers used was prestained SDS–PAGE standards (low range, Bio-Rad Laboratories, U.S.A.).

**Polyacrylamide gel iso-electrofocusing.** Iso-electrofocusing was done with a Bio-Rad Model 111 mini IEF cell on 5% polyacrylamide slabs containing 2% Ampholyte with a pH range of 3.0–10.0. The pH gradient was identified by the migration of the pI markers (IEF Standards, Bio-Rad Laboratories, U.S.A.).

**Results**

**Effects of feathers on keratinase production**

Table I summarizes the effects of feather powder and other protein supplements on cell growth and enzyme production. The feather powder greatly increased production of keratinase. The highest enzyme activity was obtained with 1% feather powder supplement. Higher feather concentrations increased the viscosity of culture medium and thus reduced the oxygen supply. The feathers were milled to average size of 100 mesh, which was suitable for enzyme induction. Over-milled feathers increased the viscosity of the culture medium. On the contrary, the under-milled feathers reduced the hydrolytic efficiency. Both resulted in low enzyme production and cell growth. Keratin powders from two different makers were poor substrates for enzyme induction. The molecular structure of these commercial keratin powders, which might be important for enzyme induction, might have been destroyed during their preparation.

The influence of the addition of various carbon and nitrogen sources to the basal medium with or without feather powder supplement are shown in Table II. Again, the feather powder increased the production of keratinase. In the absence of feather powder, only soybean meal and trypotent induced significant amount of enzyme activity. These two nutrients exerted a positive cumulative influence on enzyme production in the presence of feather powder. Galactose and molasses increased the enzyme induction in the feather powder medium, but not in the absence of feather powder. The addition of glucose, sucrose, and malt extract interfered with the enzyme induction by feather powder.

**Effects of culture temperature on the production of keratinase**

As shown in Fig. 1, maximum growth of *B. licheniformis* PWD-1 was obtained at culturing temperature of 50°C, which was the same as those reported by Williams et al.\(^ {7}\) Maximum enzyme induction, however, was obtained at 45°C. The enzyme induction decreased drastically at culture temperatures higher than 50°C. In our modified culture method, both starter culture and main culture were done at 45°C.

**Effects of medium pH on the production of keratinase**

The effects of the initial pH of the medium on the production of keratinase were examined using the basal medium containing 1% feather powder. The highest enzyme

**Table II. Effects of Various Carbon and Nitrogen Sources on Keratinase Production by B. licheniformis PWD-1**

Cultivations were done as described in Table I.

<table>
<thead>
<tr>
<th>Addition (1%)</th>
<th>Enzyme activity (units/ml)</th>
<th>Cell growth (CFU/ml, 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Feather</td>
<td>– Feather</td>
</tr>
<tr>
<td>None</td>
<td>25.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>31.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Lactose</td>
<td>26.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Molasses</td>
<td>31.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Malt extract</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>35.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>28.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Tryptone</td>
<td>28.5</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Keratin (NC), Nacalai Chemical; Keratin (TK), Tokyo Kasei.

**Fig. 1. Effects of Culture Temperature on the Production of Keratinase.**

Cultivation was done at the designated temperature for 30h in a medium containing 1% feather powder in basal medium. Enzyme activity was assayed with casein as the substrate.
production was obtained with a starting pH of 8.7 (Fig. 2). When the initial pHs were between 5.0 and 10.0, the final pHs of the medium after 30 h of cultivation were from 5.0 to 9.0.

Effects of starter culture condition
The starter culture condition had profound effects on the production of keratinase. Following the procedure of Lin et al., B. licheniformis PWD-1 was incubated in 10 ml culture medium containing 1% hammer-milled chicken feather powder in basal medium at 50°C for 4 days and then transferred to a 1-liter culture of the same medium and cultured at 50°C for another 4 days. As shown in Fig. 3A, maximum enzyme activity was about 3.5 units/ml obtained after 48 h of cultivation. The low enzyme productivity might be due to poor cell growth and viability in the starter culture. When the hammer-milled chicken feather powder used in the starter culture was replaced with the commercial feather meal, PWD-1 grew to a cell density higher than 10⁹ CFU/ml within 24 h in the starter cultivation. The commercial feather meal contains fish meal and other nutrients in addition to feathers, and is much easier to hydrolyze. Our modified culture method is to culture PWD-1 in a starter culture medium containing 1% commercial feather meal in basal medium at 45°C for 24 h, and then 2% transferred to the main culture medium containing 1% feather powder (100 mesh) in basal medium at 45°C for 30 h. As shown in Fig. 3B, the enzyme was secreted at the late logarithmic phase and reached a maximum level of 25 units per milliliter after 30 h of cultivation. The pH of the culture medium was changed from 7.8 to about 9.

Characterization of keratinase
The apparent molecular mass of the purified keratinase was estimated to be 31.4 kDa as measured by SDS–PAGE. The isoelectric point was estimated to be 8.5 by isoelectric focusing. The optimal reaction pHs and temperatures measured with casein, feather powder, and Suc-AAPF-pNA as the assay substrates were 10.5–11.5, 8.5 and 8.5–9.0, and 55, 50, and 50°C, respectively. The optimal pH and optimal temperature of the same enzyme measured by Lin et al., using the azokeratin hydrolysis assay were 7.5 and 50°C, respectively. This enzyme was quite stable in the pH range of 5 to 12 and maintained more than 80% activity after 1 h at 30°C. Thermal stability was investigated by incubating the enzyme in buffer A at a designated temperature for 1 h.

Fig. 3. Growth and Keratinase Production in Cultures of B. licheniformis PWD-1 Using Feather Powder (A) or Feather meal (B) as the Major Nutrient in the Starter Culture.
(A): PWD-1 was grown for 96 h at 50°C in 10 ml of culture medium containing 1% hammer-milled feather powder in basal medium, and then 2% transferred to 100 ml of medium containing 1% of hammer-milled feather powder in basal medium in a 500-ml shaking flask. Cultivation was done at 50°C. (B): PWD-1 was grown for 24 h at 45°C in 10 ml of culture medium containing 1% commercial feather meal in basal medium, and then 2% transferred to 100 ml of medium containing 1% hammer milled feather powder in basal medium in 500 ml shaking flask. Cultivation was done at 45°C. Enzyme activity was assayed with casein as the substrate.

Table III. Hydrolysis of Various Proteins with Keratinase and Other Microbial Proteases
The enzyme was incubated with 20 mg substrate (feather powder, keratin, elastin, or collagen) in 1 ml of buffer with shaking for 1 h at 37°C. Absorption at 275 nm of supernatant was measured after TCA precipitation as described in Materials and Methods. The caseinolytic activity was measured as described in Materials and Methods. The following buffer systems were used: buffer A for keratinase, Tris–HCl buffer (50 mM, pH 9) for subtilisin, and carbonate buffer (50 mM, pH 10.5) for alkaline elastase. Data were expressed as a ratio of specific activity (A₄₅₅/mg protein).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Feather/ Casein</th>
<th>Keratin/ Casein</th>
<th>Elastin/ Casein</th>
<th>Collagen/ Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinase</td>
<td>0.52</td>
<td>0.41</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>Subtilisin Carlsberg</td>
<td>0.17</td>
<td>0.25</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>Alkaline elastase</td>
<td>0.12</td>
<td>0.23</td>
<td>0.58</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Keratin: Tokyo Kasei.
* Type VIII, purchased from Sigma without further purification.
The enzyme was stable up to 40°C and completely lost the activity above 65°C.
In comparison with alkaline elastase5,6 and subtilisin Carlsberg, the keratinase had higher feather powder and keratin hydrolyzing activities. Table III shows the hydrolyzing activity toward keratin, elastin, and collagen in relation to casein. The relative activity of the keratinase toward feather powder and casein was about 3 and 4 times higher than those of subtilisin Carlsberg and alkaline elastase.

The relative activity of keratinase and other two subtilisins toward four synthetic peptide substrates are shown in Table IV. The relative activities of keratinase toward these synthetic peptides were quite similar to those of subtilisin Carlsberg, but different with those of alkaline elastase. Suc-AAPL-pNA was the best substrate for keratinase and subtilisin Carlsberg, and Suc-AAVA-pNA was very poor substrate for these two enzymes.

Discussion

The synthesis of extracellular enzymes in B. subtilis may be constitutive or partially inducible.12 Peptides or proteins have been reported to induce proteinase synthesis in a number of microorganisms.13,14 Our studies on keratinase synthesis by B. licheniformis PWD-1 indicated that the keratinase synthesis is inducible. Feather powder, milled to a suitable size, was the best inducer for the keratinase synthesis of PWD-1. Soybean meal can also serve as a specific inducer, thereby exerting a cumulative positive influence with feathers on enzyme production (Table II). A large effect of the starter culture condition was found. Although we do not have a satisfactory explanation for the observed increase of keratinase production caused by changing the feather powder into commercial feather meal in the starter culture medium, it could be a direct effect of improvement of the cell growth during the starter cultivation.

In this study, casein was generally used as a substrate for the activity assay in estimating the enzyme productivity or in monitoring activity during enzyme purification. The caseinolytic activity assay is a standard method for protease activity, and it provides accurate results for these purposes in comparison with keratinolytic activity assay using insoluble keratin or feather powder. The protease productivity of strain PWD-1 estimated by caseinolytic activity assay or by keratinolytic activity assay are always well correlated. Since the activity assay methods are different, it is difficult to compare the enzyme productivity of our result with that of Lin et al.34 directly from the activity level in fermentation broth. However, Lin et al. purified 1.5 mg of enzyme from 1 liter of culture broth. In our case, about 68 mg of enzyme was purified from 1 liter of culture broth. The major differences between our cultivation procedure and that of Lin et al. are cultivation temperature (45°C vs. 50°C), the major nutrient in the starter culture medium (feather meal vs. feather powder), and the aeration rate. Lin et al. cultivated the PWD-1 in a 3-liter shaking flask containing 1.0 liter of medium with shaking at 120 rpm. Our aeration conditions were 500 rpm and 0.5 vol/vol per min for a mini-jar fermentor or 100 ml medium in a 500-ml shaking flask with shaking at 160 rpm. Obviously our aeration rate was much higher than that in Lin’s procedure.

Table V summarizes the general properties of the keratinase together with those of subtilisins. Their optimal reaction pHs and temperature estimated with casein as the substrate are quite similar. The molecular weight of keratinase by SDS–PAGE was 31,400, which is higher than those of the other three enzymes. However, the molecular weight of keratinase was estimated by SDS–PAGE, while the other three were calculated from the amino acid sequences. Although the substrate specificity toward several peptide substrates of the PWD-1 keratinase (Table IV) are similar to those of other subtilisins, its hydrolysis ability toward feather powder is significantly higher (Table III).

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References

Keratinase of *Bacillus licheniformis*