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Functional expression of keratinase (*kerA*) gene from *Bacillus licheniformis* in *Pichia pastoris*

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Abstract

A 1.2 kb DNA fragment coding for the pro-peptide and mature keratinase from *Bacillus licheniformis* PWD-1 (*kerA*) was cloned into vectors pPICZαA and pGAPZαA for extracellular expression in the methylotrophic yeast, *Pichia pastoris*. Recombinant keratinase was secreted by the pPICZαA-*kerA* transformants 24 h after methanol induction of shake-flask cultures, and reached a final yield of 124 mg l^{-1} (285 U ml^{-1}) 144 h after the induction. The recombinant keratinase was glycosylated ($\sim$ 39 kDa), and was optimal between pH 8.5–9.5 and between 55 °C–60 °C using azokeratin as substrate. The enzyme degraded bovine serum albumin, collagen, and soy protein concentrate. In conclusion, *P. pastoris* can be used as an efficient host to express keratinase for nutritional and environmental applications.

Introduction

Large amounts of feathers are constantly generated in areas of intensive poultry production and processing (Shih 1993). Although feather meal contains 96–98% crude protein, it has a limited use for animal nutrition due to its high content of keratin that is poorly digested by most proteolytic enzymes. As a result, feather meal is often pressure-cooked or chemically-treated to improve its protein digestibility. However, these treatments require considerable energy consumption and have possible detrimental effects on the nutritional quality of the final products (Moritz & Latshaw 2001). An enzyme capable of degrading keratin has been isolated and purified from *Bacillus licheniformis* PWD-1 (Lin *et al.* 1992). The gene encoding for this enzyme (*kerA*) has also been cloned and sequenced (Lin *et al.* 1995). Keratinase belongs to the subtilisin family of serine proteases and has a high homology with subtilisin Carlsberg from *Bacillus subtilis* (Evans *et al.* 2000). Pretreatment of feather meal with keratinase significantly improves its digestibility and nutritional value for broiler chickens (Williams *et al.* 1991).

Expression of keratinase in *B. licheniformis* PWD-1 is induced by adding feather powder to the medium and is affected by carbon and nitrogen sources also added in the culture, and by temperature, pH and starter condition of fermentation (Cheng *et al.* 1995). These conditions have been examined for keratinase production by *B. licheniformis* PWD-1 and *B. subtilis* FDB-29 (Wang & Shih 1999), and optimization can enhance the production of enzyme by 10-fold in *B. licheniformis* PWD-1. However, this yield is still not sufficiently high for industrial production of the enzyme.

The methylotrophic yeast *Pichia pastoris* has been successfully used for expression of heterologous proteins (Higgins & Cregg 1998, Rodriguez *et al.* 2000, Goh *et al.* 2001). This expression system uses relatively simple and inexpensive medium to produce high yields of extracellular proteins mediated by highly-inducible alcohol oxidase (AOX1) promoter or the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter. Several proteases from bacterial, fungal or mammalian origin have been successfully expressed in *P. pastoris* (Sun *et al.* 1997). It is

promising that keratinase may be used to improve the nutritional value of feather meal and to alleviate the environmental impact associated with its processing or disposal. Thus, our objective for this study was to determine if *P. pastoris* could be used to produce recombinant keratinase in an inducible or constitutive way, and to characterize the properties of the expressed protein.

Materials and methods

DNA amplification and cloning

The full sequence of *kerA* gene from *B. licheniformis* PWD-1 (signal peptide, pro-peptide, and mature protein) was cloned into the plasmid PLB29 (Lin *et al.* 1997). The sequence containing the pro-peptide and mature protein was amplified by PCR using PLB29 as the template. Primer ker1 [forward: 302–321]: 5′-GGTACCGCTCAACCGGCGAAAAATGT-3′ was designed to remove the native signal peptide sequence in the gene and to generate a *Kpn*I site. Primer ker3 [reverse: 1440–1455]: 5′-GCTCTAGATCATGGAACGGATTCA-3′ was designed to generate a *Xba*I site at the 3′ end of the amplified sequence. The thermal program used to amplify the sequence included 1 cycle at 94 °C (2.5 min), 30 cycles of [94 °C (0.65 min), 60 °C (0.85 min), and 72 °C (2 min)], and 1 cycle at 72 °C (10 min). The PCR reaction mixture contained 55 pmol of each primer, 6 units of Taq DNA polymerase, 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, and 200 μM each dNTPs (Promega, Madison, WI) in 100 μl total volume. PCR reaction was carried out in a Gene Amp PCR system 2400 (Perkin Elmer, Norwalk, CT). Both primers were synthesized at the Cornell University Oligonucleotide Synthesis Facility. Amplified PCR products were resolved by 0.7% low melting agarose (Gibco BRL, Grand Island, NY) gel electrophoresis. A gel slice containing the expected band (1.16 Kb) was excised and DNA was eluted using QIAEX II Kit (Qiagen, Valencia, CA). The PCR product was cloned into pGEM-T vector (Promega) according to the manufacturer instructions and transformed into INVαF′ (Invitrogen, Carlsbad, CA) to screen for positive colonies. The isolated fragment was inserted into pPICZαA or pGAPZαA (Invitrogen) at the *Kpn*I and *Not*I sites in frame with the alpha factor lead sequence in the vector. The construct was transformed into INVαF′ that was plated on LB medium containing 25 μg zeocin ml^{-1} to select positive colonies to prepare DNA for yeast transformation.

Yeast transformation and expression

Pichia pastoris strain X33 (Invitrogen) was grown in yeast extract/peptone/dextrose medium (YPD) and prepared for transformation according to the manufacturer instructions. Plasmid DNA was linearized using *Pme*I (pPICZαA) or *Bsp*HI (pGAPZαA) prior to transformation into *Pichia* by electroporation (1.5 kV, 50 μF, 186 Ω. ECM 600 Electro Cell Manipulator, Genetronics, BTX Instrument Division, San Diego, CA). After incubation for 2 h at 30 °C in 1 M sorbitol without agitation, cells were plated in YPDS-zeocin agar medium (100 μg zeocin ml^{-1}) to screen integration of the transformed gene into the host chromosomal DNA. After 48 h, pPICZαA-*kerA* transformants were inoculated in 9 ml buffered media with 0.5% (v/v) glycerol (BMGY) and grown for 36 h, pelleted (3500 × *g*, 10 min) and then resuspended in 9 ml buffered media with 0.7% (v/v) methanol (BMMY) to induce protein expression. Cultures were induced every 24 h with 0.7% (v/v) methanol. To produce a large amount of enzyme for purification, colonies were grown in 100 ml BMGY up to turbidity ($OD_{600\ nm}$) of 25 (=18.5 g dry wt l^{-1}). Cells were then pelleted and resuspended in BMMY medium to a turbidity of 1 (0.74 g dry wt l^{-1}). Cultures were inoculated every 24 h with 0.7% (v/v) methanol. Samples were collected for cell density and activity determination at different time points during the 144 h culture. The pGAPZαA-*kerA* transformants were grown in YPD broth and samples were collected for cell density and activity during the incubation period of 72 h. Cell density was based on turbidity measurements at 600 nm.

Keratinase activity assay

After removal of cells by centrifugation, keratinase activity was determined in the supernatant using the methodology described by Lin *et al.* (1992). Briefly, 5 mg of azokeratin were added to a 1.5 ml centrifuge tube along with 900 μl of 50 mM Tris/HCl, 1 mM $CaCl_2$ (pH 8.5), the mixture was agitated until the azokeratin was completely suspended. Then, 0.1 ml of appropriately diluted enzyme was added, and the mixture was incubated for 30 min in a 60 °C water bath. The reaction was terminated by adding 0.2 ml

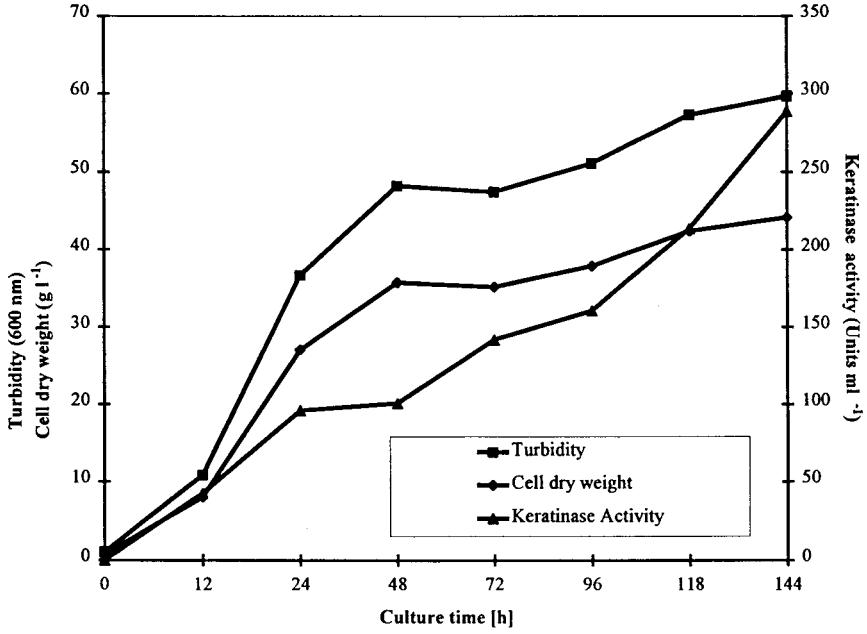

Fig. 1. Time-course of keratinase expression in *P. pastoris* transformed with pPICZαA-*kerA*. Cell density was based on turbidity measurements at 600 nm. Keratinase activity was measured using azokeratin as substrate in 50 mM Tris/HCl/1 mM $CaCl_2$, pH 8.5 and 60 °C. One unit of keratinase activity is defined as the amount of enzyme that causes an increase in the A_{450} of 0.01, against the control, after 30 min in the test reaction.

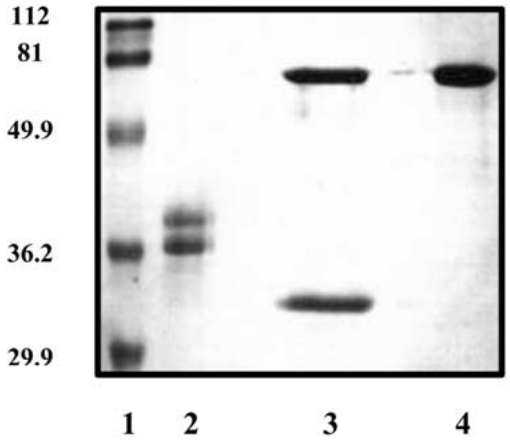

Fig. 2. A representative gel (SDS-PAGE, $n = 3$) to show the molecular mass of the partially purified keratinase expressed by *P. pastoris* transformed with pPICZαA-*kerA* before and after deglycosylation. Lane 1, pre-stained marker (Bio-Rad, low range, values on the left are molecular mass in kDa); lane 2, glycosylated keratinase; lane 3, deglycosylated keratinase; lane 4, EndoHf.

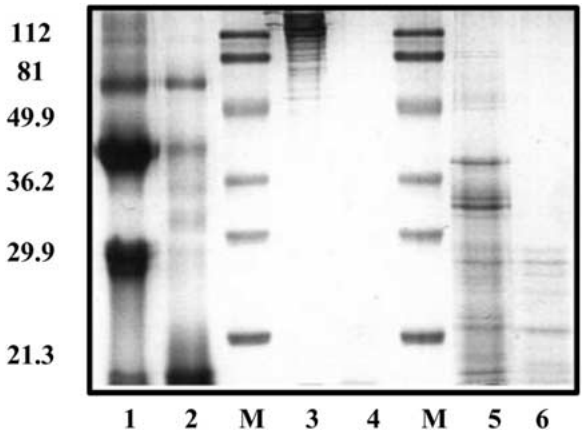

Fig. 3. A representative gel ($n = 3$) to show hydrolysis of bovine serum albumin (BSA), collagen and soy protein concentrate (SPC) by keratinase expressed in *P. pastoris* transformed with pPICZαA-*kerA*. M, pre-stained marker (Bio-Rad, low range as in Figure 2). Lane 1, BSA; lane 2, BSA + keratinase; lane 3, collagen; lane 4, collagen + keratinase; lane 5, SPC; lane 6, SPC + keratinase. Values on the left are molecular masses of the standard (kDa).

10% (w/v) trichloroacetic acid. The mixture was centrifuged at 13 000 × g for 10 min and filtered through a 0.45 μm syringe filter (Pall Corporation, Ann Arbor, MI) and A_{450} of the filtrate then measured. A control was done incubating 5 mg of azokeratin in 900 μl of buffer without the enzyme and stopping the reaction with 0.2 ml of 10% (w/v) trichloroacetic acid. One unit of keratinase activity is defined as an increase in the A_{450} of 0.01 after 30 min in the test reaction as compared to the control reaction.

SDS-PAGE

Samples were subjected to SDS-PAGE [13% (w/v) acrylamide] using a Mini-Protean II Cell (Bio-Rad Laboratories, Hercules, CA) (Laemmli 1970). Protein was stained with Coomassie Brilliant Blue R-250, using the pre-stained molecular weight marker (Bio-Rad).

Protein purification

Ammonium sulfate was added to the culture supernatant at 55 and 100% saturation. The resulting precipitate after centrifugation of the 100% saturation solution at 25 000 $\times$ *g* for 60 min was resuspended in 10 mM MES pH 5.8 and dialyzed extensively against the same buffer. The dialyzed enzyme solution was passed through a carboxymethyl cellulose column, and eluted by using a linear gradient of 0 to 0.5 M NaCl in 10 mM MES, pH 5.8. As a final step, the enzyme solution was dialyzed against 10 mM MES, pH 5.8 to remove the excess of NaCl. Protein concentration was determined using the method of Lowry.

Determination of optimal pH and temperature

Optimal pH was determined at 60 °C using 3.5 μg of the partially purified recombinant keratinase with the following buffers: 50 mM citrate/1 mM $CaCl_2$ (pH 6), 50 mM Tris/HCl/1 mM $CaCl_2$ (pH 7.5, 8.5, 9.5), and 50 mM Tris/NaOH/1 mM $CaCl_2$ (pH 10.5). The percentage of activity remaining was calculated at each one of the different pH tested using the highest value of activity obtained (289 units ml^{-1}, pH 8.5) as 100%. Temperature optimum was determined in 50 mM Tris/HCl/1 mM $CaCl_2$ (pH 8.5) using 2 μg keratinase and 1 mg BSA ml^{-1} as substrate.

Deglycosylation of keratinase

Endoglycosidase H*f* (EndoH*f*, 0.07 IUB units) was incubated with 3.5 μg of keratinase for 2 h at 37 °C according to the manufacturer instructions (New England Biolabs, Beverly, MA). The deglycosylated proteins were analyzed by SDS-PAGE as described above.

Hydrolysis of different sources of proteins by recombinant keratinase

The effectiveness of the recombinant keratinase in hydrolyzing BSA, collagen, and soy protein concentrate was estimated. To a final digestion volume of 250 μl, 1.15 μg keratinase was added. The concentrations (mg ml^{-1}) of BSA, collagen, and soy protein concentrate were 1, 0.155 and 0.265, respectively. The hydrolysis was carried out in 50 mM Tris/HCl/1 mM $CaCl_2$, pH 8.5 at 60 °C for 2 h. A Fifteen μl of the BSA digestion and 30 μl of the collagen or the soy protein concentrate digestion were loaded and run on SDS-PAGE [13% (w/v) acrylamide] against controls digested without keratinase.

Results

Expression of kerA *in* P. pastoris

Both of the pPICZαA-*kerA* and the pGAPZαA-*kerA* transformants were able to express recombinant keratinase. However, yeast transformation efficiency and protein expression were lower for the constitutive expression vector pGAPZαA than the inducible expression vector pPICZαA. Keratinase activity was detected in the culture supernatant of the pPICZαA-*kerA* transformants 24 h after methanol induction (Figure 1). The activity increased steadily up to 144 h after the induction, to a final value of 285 Units ml^{-1} corresponding to 124 mg recombinant keratinase l^{-1}. In contrast, no keratinase protein expression was detected in transformants with only the coding region of mature keratinase, without its pro-peptide, inserted in the expression vector pPICZαA (data not shown).

Properties of the recombinant keratinase

The recombinant enzyme was glycosylated, and showed two bands of approximately 39 kDa (Figure 2). After deglycosylation of the expressed enzyme with EndoH*f*, it displayed a single band of approx. 31 kDa. The recombinant keratinase effectively degraded not only its substrate azokeratin, but also bovine serum albumin, collagen, and soy protein concentrate (Figure 3). Addition of 1 mM $CaCl_2$ to the assay buffer caused a 3-fold increase in the efficiency of azokeratin degradation by the recombinant keratinase (data not shown). Optimal temperature for bovine serum albumin or azokeratin hydrolysis was between 60–65 °C, with fairly high activity remaining at 50 or 75 °C (Figure 4). Optimal pH was between 8.5 and 9.5, with 83 and 71% of activity remaining at pH 7.5 and 10, respectively.

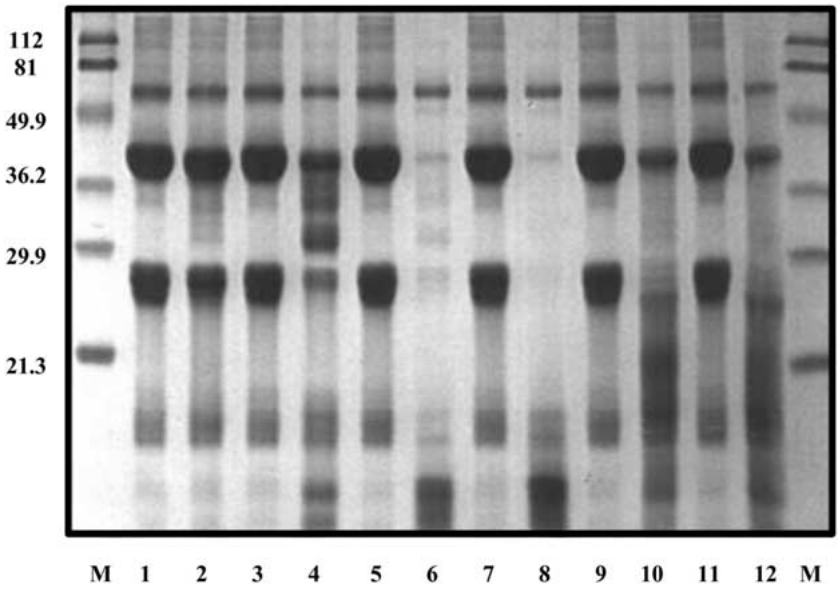

Fig. 4. A representative gel ($n = 3$) to show optimal temperature for the hydrolysis of bovine serum albumin (BSA) by keratinase expressed in *P. pastoris* transformed with pPICZαA-*kerA*. After being dissolved (1 mg ml^{-1}) in 50 mM Tris/HCl/1 mM $CaCl_2$, pH 8.5, BSA was digested by 2 μg of keratinase for 30 min at different temperatures. M, pre-stained marker (Bio-Rad, low range as in Figure 2). Lanes 1, 3, 5, 7, 9 and 11, negative controls (without keratinase) at 4, 37, 50, 60, 75 and 85 °C, respectively; lanes 2, 4, 6, 8, 10, and 12, incubated with keratinase at 4, 37, 50, 60, 75 and 85 °C, respectively. Values on the left are molecular masses of the standard (kDa).

Discussion

It is clear from the present study that *P. pastoris* can be used as a host to express keratinase from *B. licheniformis* PWD-1. Of the two expression systems used, the inducible AOX1 promoter drives a higher yield of the enzyme than the constitutive GAP promoter. The yield of keratinase obtained from the *P. pastoris* transformants of pPICZαA-*kerA* is higher than that reported by Lin *et al.* (1997) and Evans *et al.* (2000) from *B. licheniformis* PWD-1 and *B. subtilis* FDB-29. As optimization of the fermentation conditions for these bacterial systems can enhance the keratinase yield (Wang & Shih 1999), it should be possible to improve the expression efficiency of the *P. pastoris* system for a large scale of keratinase production, using some of the well-established protocols (Thorpe *et al.* 1999). Biochemical properties of the recombinant keratinase expressed by *P. pastoris* are similar to those of the thermophilic protease with alkaline optimal pH and a wide range of protein substrates like keratin, bovine serum albumin, collagen, or soy protein concentrates (Cheng *et al*. 1995, Evans *et al*. 2000, Lin *et al*. 1992). Comparatively, the expressed keratinase is more effective in hydrolyzing bovine serum albumin than soy protein concentrate. There are small differences in pH and temperature optimum between the recombinant keratinase expressed by *P. pastoris* and by *B. licheniformis* PWD-1 (Lin *et al.* 1992), probably due to differences in glycosylation capacity of the expression hosts and(or) the assay conditions.

Inclusion of 1 mM $CaCl_2$ in the assay buffer improved significantly the efficiency of azokeratin degradation by the expressed keratinase. Ca^{2+} plays an important role in stabilizing several members of the subtilisin protein family including subtilisin Carlsberg, subtilisin BPN or Proteinase K through two different Ca^{2+}-binding sites in these proteins (Pantoliano *et al.* 1988). A positive effect of Ca^{2+} on enzyme activity and stability at elevated temperatures has also been reported for the extracellular keratinase produced by thermophilic actinomycete *Thermoactinomyces candidus* (Ignatova *et al.* 1999).

Previously, *kerA* has been expressed in bacteria that do not glycosylate the proteins (Evans *et al*. 2000, Lin *et al*. 1992). The amino acid sequence of keratinase from *B. licheniformis* PWD-1 has 5 potential *N*-glycosylation sites, and *P. pastoris* is able to add *O*- and *N*-linked carbohydrate moieties to secreted proteins (Bretthauer & Castellino 1999). Therefore, it is not surprising that the molecular weight of our recombinant keratinase is greater than that expressed in *Bacillus*. Nevertheless, the deglycosylated keratinases from all of these systems share a similar size (31 kDa)

(Evans *et al*. 2000, Lin *et al*. 1992). In addition, protein expression in yeast systems can be enhanced by engineering additional glycosylation sites near the *N*-termini of the target protein (Sagt *et al*. 2000). As keratinase has several amino acids close to the *N*-termini that can be used to introduce new potential glycosylation sites, it may be another simple way to improve keratinase expression in *P. pastoris*.

In conclusion, the *B. licheniformis* PWD-1 keratinase is expressed and secreted by *P. pastoris* in an inducible system. The recombinant enzyme is a glycosylated protein of approximately 39 kDa and shares similar biochemical properties to the native bacterial enzyme. Using this expression system, acceptable yields of recombinant keratinase may be obtained upon optimization of growth and expression conditions. The enzyme can be used to improve the nutritional values of feather meal as an economical protein source.

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