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# Production and characterization of a thermostable protease produced by an asporogenous mutant of *Bacillus stearothermophilus*

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## SUMMARY

An asporogenous mutant of *Bacillus stearothermophilus* (TPM-8) which produces 4-fold higher levels of a thermostable neutral protease than does wild-type strain 308-1 was obtained by mutagenesis with ethyl methanesulfonate. The protease produced by both the mutant and wild-type strain is a metalloprotease requiring $Zn^{2+}$ and $Ca^{2+}$ for activity and thermostability, respectively. It has a temperature optimum of 80°C at pH 7.0 and is highly thermostable, retaining 60% of its activity after 60 min at 85°C. The properties of the enzyme are similar to those of thermolysin.

## INTRODUCTION

A variety of thermostable proteases is produced by the heterogeneous species *Bacillus stearothermophilus* [1,20]. The protease produced by the type strain has been compared to the metalloenzyme thermolysin [E.C.3.4.24.4] derived from *Bacillus thermoproteolyticus* [5,7]. The amino acid sequences of the two proteins show 85% homology [23], yet the *Bacillus stearothermophilus* type strain protease is much less heat stable [9,11]. Recently, other strains of *Bacillus stearothermophilus* have been isolated, including the one described in this report, which produce proteases with thermostable properties equivalent to thermolysin [14,24].

It has been demonstrated for *Bacillus* species that mutants that were sporulation-negative produced increased levels of various enzymes and insecticidal toxins [12,24]. For example, rifampin-resistant asporogenous mutants of *Bacillus cereus* produced increased levels of beta-amylase [18]. Increases in the

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production of protease and amylase by the use of asporogenous variants of *Bacillus licheniformis* and *Bacillus caldolyticus* have also been achieved [2,4,12]. This paper describes a procedure used to obtain an asporogenous mutant of *Bacillus stearothermophilus* which produces 4-fold higher levels of thermostable protease than the wild-type. Protease production by mutant TPM-8 was also compared to that of *Bacillus stearothermophilus* NRRL-3880, a strain claimed to produce industrial titers of protease [3,21]. The characterization of the enzyme and similarities to thermolysin are described.

## MATERIALS AND METHODS

### *Materials*

Sephadex-G25, Sephadex-G75, and DEAE-Sephadex were purchased from Pharmacia. Ampholine PAGPlate isoelectric focusing gels, pH range 3-5–9.5, were from LKB. Electrophoresis reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. The Bakerbond $C_{18}$ HPLC column was purchased from J.T. Baker. PM10 ultrafiltration membranes were obtained from Amicon. Carbowax Polyethylene Glycol (PEG) 8000 was purchased from Fisher Scientific. Hammerstein casein was from Chemical Dynamics Corporation. Maltodextrin 100 (M-100) was purchased from Grain Process Corporation. Thermolysin was obtained from Sigma (Protease, Type X, P 1512). All other common laboratory chemicals were obtained from Sigma or J.T. Baker. Oxidized B-chain of insulin prepared according to the method of Markussen [17] was a generous gift from Georg W. Jensen of Novo Industri A/S, Denmark. Antibodies prepared in rabbits against thermolysin (Sigma) were a generous gift from Sven Hansen of Novo Industri A/S, Denmark.

### *Growth of microorganisms*

Strain NLI-308-1 was isolated from a compost pile in Danbury, CT. The isolate was identified as *Bacillus stearothermophilus* by the National Collection of Industrial Bacteria, Aberdeen, Scotland. The strain belongs to the subgroup 3a classification of Walker and Wolf [26]. *Bacillus stearothermophilus* NRRL-3880 was obtained from the Northern Regional Research Center, Peoria, IL. All strains were maintained on slants of Luria agar (yeast extract, 5 g; bacto-tryptone, 10 g; bacto-agar, 20 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; distilled water, 1 l; pH 7.0) at 4°C.

Shake flask experiments were performed using 100 ml of medium in a 500-ml baffle flask. The basic medium used was Luria broth (yeast extract, 5 g; bacto-tryptone, 10 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; distilled water, 1 l; pH 7.0) containing either 5 or 10 g per liter of a carbon source. The carbon source for the mutation screening work was Maltodextrin 100 and was added at 5 g per liter. The flasks were inoculated with cell suspensions from overnight slant cultures incubated at 55°C. All shake flasks were grown at 55°C with agitation set at 350 RPM.

Batch fermentations were carried out in 2.0-l vessels with a 1.2-l working volume (Multigen, New Brunswick Scientific). Three different fermentation media were used to compare strains for protease production. Medium I was the modified Luria broth described above containing 10 g/l Maltodextrin 100. Medium II contained the following: yeast extract, 5.0 g; bacto-tryptone, 5.0 g; M-100, 10.0 g; $(NH_4)_2SO_4$, 2.0 g; $Na_2HPO_4$, 3.4 g; $KH_2PO_4$, 1.1 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgCl_2$, 0.3 g; tap water, 1 l; pH 7.0. Medium III is the production medium for NRRL 3880 as described by Sidler and Zuber [21] and contained the following: bacto-beef extract, 3.0 g; bacto-tryptone, 2.0 g; maltose, 3.0 g; NaCl, 1.0 g; $NH_4Cl$, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; $FeCl_3$, 0.003 g; tap water, 1 l; pH 7.0. The pH was initially set at 7.0 and controlled not to fall below 6.0 using 2 M $Na_2CO_3$. The fermentations were run at 55°C, with agitation increased from an initial 500 RPM to 1000 RPM after 4 h growth.

### *Mutation*

Isolate NLI-308-1 was streaked onto skim milk agar plates (Czapek-Dox agar plus 1% skim milk, pH 7.0) and incubated 12 h at 55°C. The plates were then swabbed and the cells dispersed into 0.05 M phosphate buffer, pH 7.0. The cells were washed

once in the buffer, centrifuged (12 000 × *g*, 15 min, 5°C), the supernatant poured off, and the cells resuspended in 20 ml of buffer. The cell suspension was poured into sterile, plugged 250-ml flasks. Flasks containing cultures to be mutated had ethyl methanesulfonate [13] added to a concentration of 0.1 M, while control flasks received sterile water. The flasks were then incubated at 55°C, 250 RPM for 30 min. The cells were centrifuged at 12 000 × *g*, 15 min, 5°C after which the supernatant was poured off. The cells were resuspended in 10 ml of fresh buffer, diluted serially, plated onto skim milk agar plates, and incubated 24 h at 55°C. Colonies that appeared translucent and produced clearing zones on the skim milk agar plates were picked and restreaked onto fresh skim milk agar. Isolates that appeared sporeless after microscopic examination were evaluated in shake flasks for protease production.

*Enzyme recovery and purification*

Cell-free broth was obtained by centrifugation of cultures (described above) at 10 000 × *g* for 10 min (4°C). Thermostable protease activity from TPM-8 was purified by one of two methods. In the first method cell-free broth was concentrated two-fold by ultrafiltration (Amicon, PM10 membrane). The concentrate was applied to a Sephadex-G25 column equilibrated in 0.01 M HEPES, 2 mM $CaCl_2$, pH 7 buffer (Buffer I) as a buffer exchange step. Fractions containing protease activity were pooled, diluted 1:1 with distilled deionized water (4°C), then applied to a column of DEAE-Sephadex equilibrated in Buffer I. The protease activity, which did not bind to the column, was pooled, placed in Spectrapor dialysis tubing with MW cutoff 6 000–8 000, and covered with Carbowax Polyethylene Glycol 8000. After concentration the sample was dialyzed against Buffer I. In the second method cell-free broth was dialyzed immediately against Buffer I. The sample was applied to a Sephadex G75 column (sample volume was 2% of the column volume), then eluted with Buffer I. Fractions containing protease activity were pooled, then concentrated with Carbowax PEG 8000 as above. The molecular weight of the purified protease was obtained by SDS-polyacrylamide gel electrophoresis performed according to the method of Laemmli [15]. The p*I* of the protease was determined on Ampholine PAG-Plates, pH range 3.5–9.5, by comparison to commercially obtained p*I* standards (Pharmacia, p*I* 3–10). Duplicate gels were stained with either Coomassie Blue R or by an agarose overlay technique for protease activity. The overlay contained 1% skim milk, 1% agarose, and 100 ppm $CaCl_2$ in 0.1 M HEPES, pH 7 buffer. The gel plus overlay were incubated at 55°C for 12 h, then a solution of 10% trichloroacetic acid poured over the top. Clearing zones indicated the presence of protease activity.

*Assay methods*

Proteolytic activity was determined by the hydrolysis of casein and subsequent reaction of trichloroacetic acid (TCA)-soluble peptides with orthophthaldialdehyde (OPA) and 2-mercaptoethanol. Reaction mixtures were comprised of 1.0 ml of 2% (w/v) Hammerstein casein and 0.5 ml of an appropriate enzyme dilution both in Buffer I. Mixtures were incubated for 30 min at 60°C, then the reaction terminated with 2.5 ml stop reagent (3.6% w/v TCA, 6.0% w/v sodium acetate, and 3.8% v/v glacial acetic acid). In control reactions the stop reagent was added prior to enzyme addition. After 20 min at 25°C the quenched reaction mixtures were centrifuged at 12 000 × *g* in a bench-top centrifuge. An aliquot (200 μl) of the supernatant was added to 3 ml of OPA reagent containing 0.05M sodium tetraborate, 1% w/v sodium dodecylsulfate, 0.8 mg/ml OPA (originally dissolved as a 40 mg/ml solution in ethanol), and 0.2% v/v 2-mercaptoethanol. After 2 min, the absorbance at 340 nm was determined. Similarly a 200 μl aliquot of a serine standard (0.2 mg/ml) was added to 3 ml of OPA reagent and the $A_{340}$ determined. Activity is expressed in KOU (kilo OPA units) where 1 KOU is defined as the amount of proteolytic enzyme which under standard conditions produces an amount of hydrolysis products, not precipitating with trichloroacetic acid, which corresponds to the formation of 1 mmol serine per min.

*Temperature and pH profiles*

Temperature profiles were performed by assaying the sample as described above except that the temperature was varied and $CaCl_2$ concentration was increased to 0.01 M.

pH profiles were derived using the synthetic substrate *N*-(3-[2-furyl]acryloyl)-glycyl-leucineamide (FAGLA) [6,8]. Enzyme was added to a solution of the substrate (1 mM) in 0.05 M Tris-Maleate, 10 mM $CaCl_2$ buffer adjusted to the appropriate pH and the reaction was monitored spectrophotometrically at 345 nm.

*Thermostability measurements*

Thermostable proteases were diluted to 0.2 or 0.4 KOU/l in either Buffer I or Buffer I with 10 mM $CaCl_2$. Samples were then incubated at the appropriate temperature and aliquots were withdrawn at 0, 15, 30, and 60 min. Aliquots were immediately assayed for protease activity as described above.

*Digestion of insulin B-chain*

Thermostable protease from TPM-8 and thermolysin ($0.675 \times 10^{-3}$ KOU) were incubated with the oxidized B-chain of insulin (0.17% w/v) in Buffer I at 60°C for 15 min. Reactions were terminated by boiling for 20 min, filtered through 0.2 $\mu$m filters, then analyzed on a Bakerbond widepore $C_{18}$ reverse-phase HPLC column. The column was equilibrated in 0.1 M ammonium sulfate, pH 3; peptides were eluted with a linear gradient 0–50% in acetonitrile.

## RESULTS

*Protease production*

A comparison of protease titers produced in shake flask cultures by 308-1 and asporogenous mutants is shown in Table 1. Numerous mutants were isolated, but only isolates found to be totally asporogenous after many generations were grown for comparison. The protease levels produced by the majority of the mutants were less than that produced by the wild type strain. Isolate TPM-8 produced almost twice the protease titer of the parent strain and was selected for further studies.

Table 1

Comparison of protease titers produced by 308-1 and its asporogenous mutants in shake flask culture

| Isolate | Protease titer (KOU/l) 12 hours | Protease titer (KOU/l) 18 hours |
|---|---|---|
| 308-1 | 60.2 | 38.7 |
| TPM-1 | 24.9 | 11.0 |
| TPM-2 | 11.1 | 8.3 |
| TPM-5 | 77.4 | 33.4 |
| TPM-6 | 52.4 | 38.7 |
| TPM-7 | 22.1 | 33.1 |
| TPM-8 | 110 | 59.2 |
| TPM-9 | 52.0 | 57.0 |
| TPM-10 | 10.0 | 8.3 |

The effect of different carbon sources (10 g/l) on protease production in shake flask by TPM-8 is shown in Table 2. The results indicate that glycerol is the best carbon source for protease production. Growth on maltose, sucrose, fructose or xylose resulted in yields comparable to that obtained by growth on maltodextrin. Glucose was a poor carbon source for enzyme production in Luria broth. The low protease titer produced on glucose was

Table 2

Effect of carbon source on protease production by TPM-8 in shake flask culture

| Carbon source | Protease titer[a] |
|---|---|
| Glycerol | 170 |
| Sucrose | 120 |
| Fructose | 100 |
| Maltodextrin | 100 |
| Maltose | 100 |
| Xylose | 100 |
| Starch | 70 |
| Raffinose | 30 |
| Glucose | 15 |

[a] Protease titer is expressed relative to the titer obtained on maltodextrin.

found not to be due to repression, but to low pH conditions brought on by rapid growth of the organism. When TPM-8 was grown in phosphate-buffered Luria broth with glucose, the titer was the same as that obtained on the more slowly metabolized carbon sources maltodextrin and sucrose (data not shown).

*Batch fermentation*

In batch fermentations in Medium I isolate TPM-8 gave two-fold higher levels of protease activity than in shake flask culture, the increase in activity being proportional to the increase in carbohydrate concentration, i.e. 5 g/l to 10 g/l (Fig. 1). Wild-type titer did not increase under these conditions. Thus, protease production by TPM-8 in a fermentor was almost 4-fold higher than levels produced by 308-1. The growth of TPM-8 was quite rapid and the peak titer was obtained in 7 h. A decline in protease activity was seen after the peak activity was obtained and corresponded to a rise in pH. The decrease in activity may be due to autolysis at the higher pH.

Protease production by TPM-8 was compared to that of *Bacillus stearothermophilus* NRRL-3880, a strain previously shown to produce high titers of protease [3]. Comparisons were made in three different media. As illustrated in Fig. 2, TPM-8 and NRRL-3880 produced identical titers of protease in Medium II, e.g. 60 KOU/l. When grown on Medium III, a medium optimized specifically for NRRL-3880 [21], NRRL-3880 gave titers of only 33 KOU/l. TPM-8 grew poorly on the lean medium and produced low levels of protease (data not shown). When both strains were compared in Medium I, however, TPM-8 produced 7.3-fold higher levels of protease than did NRRL-3880 (Fig. 1). The protease titer of 220 KOU/l which was obtained for TPM-8 is 3.6-fold higher than the best titer obtained for NRRL-3880 on any of the 3 media used here.

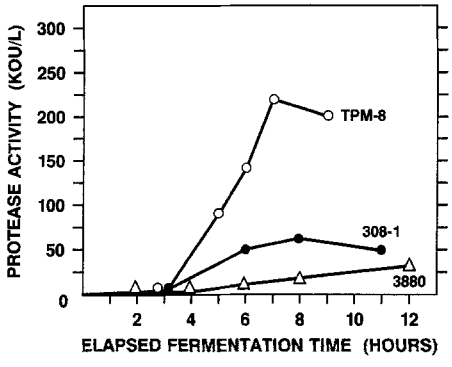


Fig. 1. Comparison of protease production by *Bacillus stearothermophilus* 308-1, NRRL-3880 and the asporogenous mutant TPM-8 in batch fermentation on Medium I. ○, TPM-8; ●, 308-1, △, NRRL-3880.

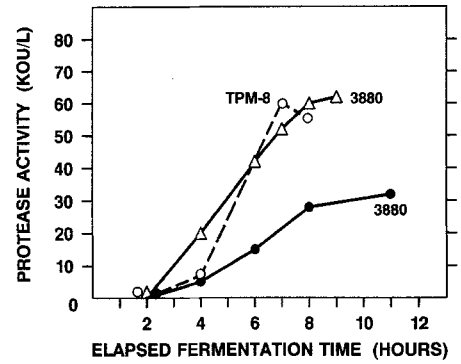


Fig. 2. Comparison of protease production by *Bacillus stearothermophilus* TPM-8 and NRRL-3880 on Medium II and III. ○, TPM-8 grown on Medium II; △, NRRL-3880 grown on Medium II; ●, NRRL-3880 grown on Medium III.

*Temperature and pH optima*

The protease from mutant TPM-8 exhibited optimal activity at 80°C and a temperature profile which was very similar to that of thermolysin (Fig. 3). The activity of TPM-8 protease was very much dependent upon the concentration of $Ca^{2+}$, espe-

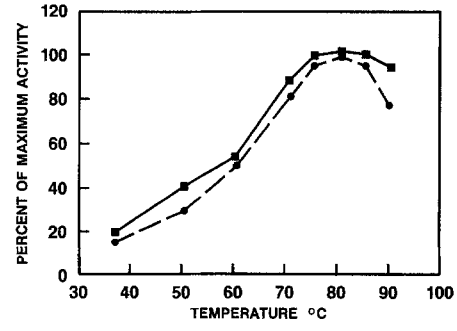


Fig. 3. Temperature profile of TPM-8 protease and thermolysin. ■, TPM-8; ●, thermolysin.

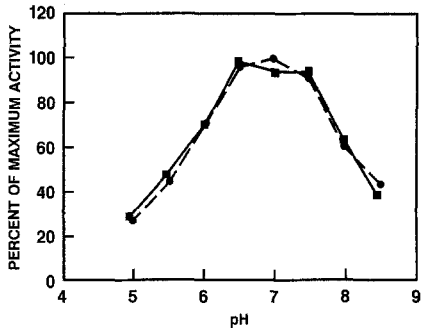


Fig. 4. pH profile of TPM-8 protease and thermolysin. ■, TPM-8; ●, thermolysin.

cially at high temperatures. For example, with 2 mM rather than 10 mM $CaCl_2$ the temperature optimum of TPM-8 protease was shifted to a lower temperature of 70°C (data not shown). This requirement of the protease for $Ca^{2+}$ was also reflected in a dramatic decrease in activity when chelating agents such as EDTA were used or in the presence of phosphate buffers which precipitate $Ca^{2+}$ (data not shown). The optimal pH for TPM-8 protease activity was 7, identical to that of thermolysin, as shown in Fig. 4.

*Thermostability*

Thermostability tests were performed at either 80°C or 85°C in the presence of 2 or 10 mM $Ca^{2+}$. As shown in Fig. 5A, with either 2 mM or 10 mM $CaCl_2$ TPM-8 protease retained at leat 80% of its activity after 60 min at 80°C. At 85°C in the presence of 10 mM $CaCl_2$ TPM-8 protease lost ~ 40% of its activity in 60 min (Fig. 5B). Lowering the $CaCl_2$ concentration to 2 mM resulted in a loss of 93% activity after 60 min. When the temperature was increased to 90°C (10 mM $CaCl_2$), total activity was lost within 15 min (data not shown). In all cases TPM-8 protease exhibited thermostability behavior similar to thermolysin.

*Metal requirement*

Thermolysin is known to be a zinc-containing metalloprotease [16]. Loss of zinc results in loss of activity. In order to determine the requirement of TPM-8 protease for zinc, the protease was treated

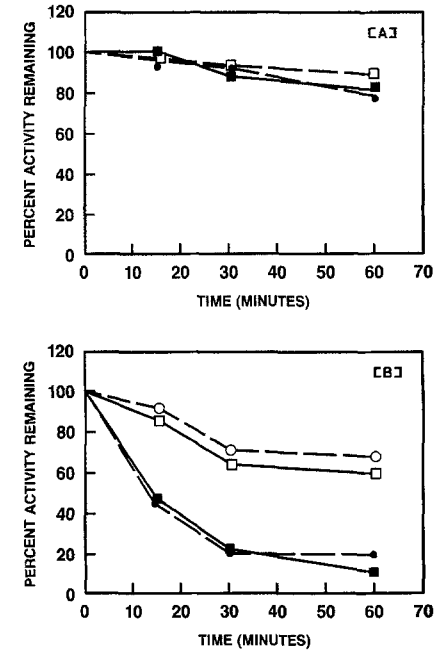


Fig. 5. Thermostability of TPM-8 protease and thermolysin at 80°C [A] and 85°C [B]. ■, TPM-8 + 2 mM $CaCl_2$; □, TPM-8 + 10 mM $CaCl_2$; ●, thermolysin + 2 mM $CaCl_2$; ○, thermolysin + 10 mM $CaCl_2$.

with 1,10-phenanthroline as shown in Fig. 6. Loss of activity occurred with increasing concentrations of the chelating agent, 0% activity remaining with 1 mM 1,10-phenanthroline present. When zinc acetate was added to TPM-8 protease inactivated by 1 mM 1,10-phenanthroline, up to 98% of the original activity was restored (Table 3). These data indicate that TPM-8 protease, like thermolysin, is a zinc-containing metalloprotease.

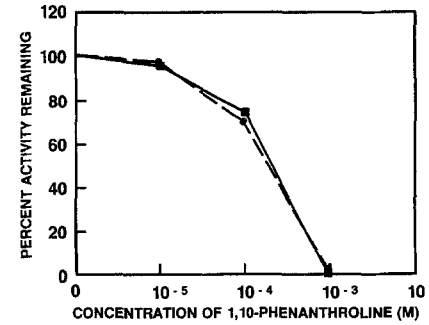


Fig. 6. Effect of 1,10-phenanthroline on thermostable protease activity. ■, TPM-8; ●, thermolysin.

Table 3

Reconstitution of 1,10-phenanthroline-treated TPM-8 protease with zinc acetate

| Zinc acetate (mM) | Percent of original activity |
|---|---|
| 0 | 10[a] |
| 0.1 | 70 |
| 0.25 | 93 |
| 0.5 | 91 |
| 0.75 | 98 |
| 1.0 | 86 |
| 2.0 | 23 |

[a] Value shown is the average from 2 experiments.

*Molecular weight and pI*

Thermostable protease from TPM-8 was purified by chromatography on either an anion exchange resin (DEAE-Sephadex) or a gel filtration resin (Sephadex-G75, see Table 4). The ion-exchange method resulted in a 6.9-fold purification with 100% recovery of activity, yielding one major band on SDS-PAGE of molecular weight 14 000. When the protease was purified by gel filtration, activity eluted from the Sephadex-G75 column with apparent molecular weight of 35 000–39 000. The degree of purification was 3.0-fold and 86% of the activity was recoverd. On SDS-PAGE one major band of molecular weight 39 000 was detected. These results indicate that the TPM-8 protease which has a molecular weight of ~ 37 000 in native form undergoes autolysis, generating a low molecular weight (~ 14 000) product. When a commercial preparation of thermolysin (Sigma) was overloaded on SDS-PAGE, both a major band of molecular weight 39 000 and a minor band of 14 000 were seen as well.

Sephadex-G75-purified TPM-8 protease was applied to isoelectric focusing gels for p*I* determination. With either a Coomassie Blue protein stain or a protease activity stain, one band was detected which corresponded to a p*I* value of ~5.0. A commercial sample of thermolysin behaved identically to TPM-8 protease on the IEF plate, exhibiting an apparent p*I* of 5.0.

*Immunological characteristics*

Antibodies prepared against thermolysin (Sigma) were used to test the antigenic properties of TPM-8 protease. Ouchterlony double diffusion tests [18] were performed with thermolysin antibody in the central well and thermolysin or Sephadex-G75-pu-

Table 4

Purification of TPM-8 protease

| Step | | Volume (ml) | Activity (KOU/l) | Total recovery (%) | Specific activity (KOU/g) | Fold purification |
|---|---|---|---|---|---|---|
| *Scheme 1* | | | | | | |
| 1. | Concentrated cell-free broth | 50 | 110 | 100 | ~68 | 1.0 |
| 2. | Sephadex-G25 | 146 | 47 | 124 | N.D.[a] | N.D. |
| 3 a. | DEAE-Sephadex | 380 | 16 | 107 | 470 | 6.9 |
| b. | PEG-concentrate | 38 | 152 | 104 | 470 | 6.9 |
| *Scheme 2* | | | | | | |
| 1. | Dialyzed cell-free broth | 1.4 | 62.0 | 100 | ~68 | 1.0 |
| 2. | Sephadex-G75 | 9.5 | 7.9 | 86 | 201 | 3.0 |

[a] N.D. = not determined.

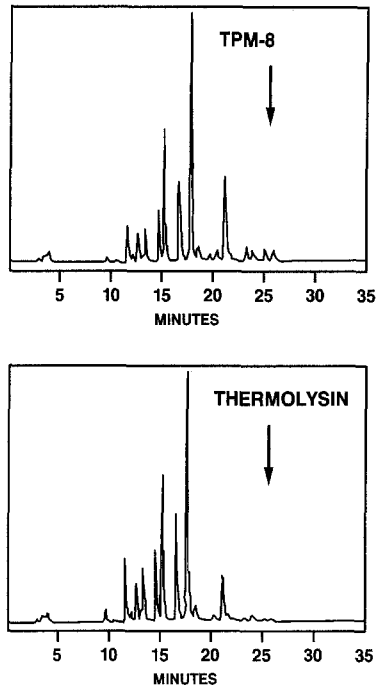


Fig. 7. HPLC chromatograms of peptides derived from the digestion of oxidized insulin B-chain with TPM-8 protease and thermolysin. The position where intact insulin B-chain elutes is indicated by an arrow.

rified TPM-8 protease in the outer wells. A line of identity was detected between the thermolysin and TPM-8 protease-containing wells, indicating that the two proteins share common antigenic determinants (data not shown).

*Insulin digestion pattern*

In order to determine whether TPM-8 protease and thermolysin have similar substrate specificities, we compared the products generated by hydrolysis of the oxidized B-chain of insulin. The results shown in Fig. 7 indicate that virtually the same peptides were generated by both enzymes. Thus, TPM-8 protease and thermolysin have similar specificities for peptide bond hydrolysis.

## DISCUSSION

Mutation of the wild type strain of *Bacillus stearothermophilus* 308-1 to asporogeny resulted in a population of hypo- and hyperproducers for protease. The best hyperproducing, asporogenous mutant (TPM-8) gave protease yields in shake flasks that were double those of wild-type. Furthermore, for TPM-8 in fermentor culture protease titers increased proportionally with carbohydrate concentration, whereas for the wild-type strain they did not.

The protease titers produced by TPM-8 were compared to those obtained with *Bacillus stearothermophilus* NRRL-3880, which has been shown to produce high levels of thermostable protease [3,21]. While the best protease titers obtained with NRRL-3880 were identical to those produced by wild-type 308-1, they were 3.6-fold less than those produced by mutant TPM-8 in batch fermentations on optimized media. These data indicate that the isolation of asporogenous mutants can be a useful technique to increase protease titers in strains which already produce high levels of extracellular enzyme.

The protease from TPM-8 was identical to thermolysin in all aspects that were tested, including temperature and pH optima, p*I*, molecular weight, and mode of proteolytic attack. Both are metalloenzymes requiring zinc for activity and calcium for thermostability.

In Table 5 we compare the thermostable metalloprotease from *Bacillus stearothermophilus* strain TPM-8 with various other metalloproteases produced by members of the heterogeneous species *Bacillus stearothermophilus* and *Bacillus thermoproteolyticus*. Included is the protease produced by *Bacillus stearothermophilus* type strain ATCC 12980 (CU21). In general, the proteases from strains KP 1236, MK 232, and TPM-8 possess thermostability properties which are more like those of thermolysin than those of the type strain protease. Other strains of *Bacillus stearothermophilus* have been isolated by Feder et al. [7] and Taki et al. [24] which produce enzymes with thermolysin-like properties or which are immunologically identical to thermolysin. Using antibodies made against purified thermolysin, we have found a reaction of identity between the *Bacillus stearothermophilus* TPM-8 protease and thermolysin. In conclusion, we have isolated a strain of *Bacillus stearothermophilus*

Table 5

Comparison of thermostable proteases produced by different strains of *Bacillus stearothermophilus*

| Enzyme source | M.W. (Kd) | p*I* | pH optimum | Temp optimum | Thermo-stability[a] | Ref. |
|---|---|---|---|---|---|---|
| *Bacillus stearothermophilus* TPM-8 | 39.0 | 5.0 | 7.0 | 80°C | 60%/60′/85°C 10mM $Ca^{2+}$ | This paper |
| *Bacillus stearothermophilus* CU21 [ATCC 12980]-type strain | 36.0 | NA[b] | 7.0 | NA | 50%/30′/75°C | 10,11 |
| *Bacillus stearothermophilus* NCIB 8924 | 34.5 | NA | 7.4 | 65°C | 40%/15′/75°C 10mM $Ca^{2+}$ | 21,22 |
| *Bacillus stearothermophilus* NRRL-3880 | 35.0 | NA | 7.2 | 70°C | 60%/15′/75°C 10mM $Ca^{2+}$ | 3,21,22 |
| *Bacillus stearothermophilus* KP 1236 | 33.0 | 7.5 | 7.0 | 80°C | 75%/10′/85°C 2mM $Ca^{2+}$ | 24 |
| *Bacillus stearothermophilus* MK 232 | 34.0 | NA | 7.5 | 70°C | 45%/30′/90°C | 14 |
| *Bacillus thermoproteolyticus* | 34.5 | 5.0 | 7.0 | 80°C | 60%/60′/85°C 10mM $Ca^{2+}$ | This paper, 7 |

[a] Percent remaining activity after incubation at specified time (min) and temperature.
[b] NA = Information not available.

which produces a metalloprotease with thermostability equivalent to thermolysin and which through mutation to asporogeny has become a hyperproducing strain.

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