Cloning and Nucleotide Sequence of the Highly Thermostable Neutral Protease Gene from *Bacillus stearothermophilus*

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The gene (*nprM*) for the highly thermostable neutral protease of *Bacillus stearothermophilus* MK232 was cloned in *Bacillus subtilis* using pTB53 as a vector. The nucleotide sequence of *nprM* and its flanking regions was determined. The DNA sequence revealed only one large open reading frame, composed of 1656 base pairs and 552 amino acid residues. A Shine–Dalgarno (SD) sequence was found 12 bases upstream from the translation start site (ATG). A possible promoter sequence (TTTTCC for the –35 region and TATTGT for the –10 region), which was nearly identical to the promoter for another thermostable neutral protease gene, *nprT*, was also found about 40 bases upstream of the SD sequence. The deduced amino acid sequence contained a signal sequence in its amino-terminal region. The sequence of the first five amino acids of purified extracellular protease completely matched residues 237–241 of the open reading frame. This suggests that the enzyme is translated as a large polypeptide containing a pre-pro structure as is known for other neutral proteases. The amino acid sequence of the extracellular form of this protease (316 amino acids, molecular mass 34266 Da) was identical to that of the thermostable neutral protease (thermolysin) from *Bacillus thermoproteolyticus* except for two amino acid substitutions (Asp37 to Asn37 and Glu119 to Gln119). The G + C content of the coding region of *nprM* was 42 mol%, while that of the third letter of the codons was lower (36 mol%). This extremely low content is an exceptional case for genes from thermophiles. When the protease genes, *nprM* and *nprT*, were cloned on pTB53 in *B. subtilis*, the expression of *nprM* was about 20 times higher than that of *nprT*. The reason for the difference between the two systems is discussed.

INTRODUCTION

Microbial metallo-(neutral)proteases (EC 3.4.24.4) from the genus *Bacillus* are widely distributed secretory enzymes, and the structural genes of several have been cloned and sequenced (Fujii *et al.*, 1983; Takagi *et al.*, 1985; Vasantha *et al.*, 1984; Yang *et al.*, 1984). These studies revealed the existence of a pre-pro structure for enzyme secretion. However, the proteases have not been well characterized enzymically. In contrast, the thermostable neutral protease from *Bacillus thermoproteolyticus* (thermolysin) has been intensively characterized (Endo, 1962; Kester & Matthews, 1977; Latt *et al.*, 1969; Morihara & Tsuzuki, 1970; Stauffer, 1971). The primary and tertiary structures, the active site and substrate-binding site have been determined (Bigbee & Dahlquist, 1974; Matthews *et al.*, 1972*a, b*; Titani *et al.*, 1972). Until recently, thermolysin was the most thermostable protease known.

We have already cloned and sequenced the gene (*nprT*) for a thermostable neutral protease, NprT, from *Bacillus stearothermophilus* CU21 (Fujii *et al.*, 1983; Takagi *et al.*, 1985). The amino acid sequence is highly homologous (85%) with that of thermolysin. However, thermolysin is more thermostable than NprT. By site-directed mutagenesis, we succeeded in enhancing the thermostability of NprT (Imanaka *et al.*, 1986). However, thermolysin was still more thermostable than the mutant form of NprT. We have independently isolated an industrially

useful strain, *B. stearothermophilus* MK232 (Kubo *et al.*, 1988), which produces a more thermostable neutral protease (NprM). When the enzymes are heated at 90 °C for 30 min, the remaining activity of NprM is about 45%, whereas that of thermolysin is about 30%. Moreover, the specific activity of NprM is 27 700 units (mg protein)⁻¹, which is higher than that [19 200 units (mg protein)⁻¹] of thermolysin by 40%.

This paper describes the cloning and expression in *Bacillus subtilis* of *nprM*, the gene for the highly thermostable neutral protease from *B. stearothermophilus* MK232, and compares the amino acid sequence deduced from the nucleotide sequence with that of thermolysin from the viewpoint of enzyme thermostability. The reason for the efficient expression of *nprM* in comparison with that of *nprT* in *B. subtilis* is also discussed.

METHODS

Bacterial strains, plasmids, phages and culture conditions. The *Bacillus* strains used were *B. stearothermophilus* MK232 [Cm^r *nprM*⁺ (structural gene of neutral protease)] (Kubo *et al.*, 1988) and *B. subtilis* MT-2 (*trpC2 leuC7 hsdR hsdM Npr*⁻) (Fujii *et al.*, 1983). *Escherichia coli* K12 JM103 [$\Delta(lac-pro)$ *thi strA endA sbcB hsdR*] was the host strain for phages M13mp10 and M13mp11. Plasmids used were pTB53 (Km^r Tc^r) (Imanaka *et al.*, 1981a; Imanaka *et al.*, 1984) and pNP22 [Km^r Tc^r *nprT*⁺ (structural gene of neutral protease from *B. stearothermophilus* CU21)] (Takagi *et al.*, 1985). Bacteria were grown in L broth, on L agar or LC agar (L agar plus 1% casein) (Fujii *et al.*, 1983). The antibiotics used were kanamycin and tetracycline (each 5 mg ml⁻¹) for both *B. stearothermophilus* and *B. subtilis*.

Isolation of plasmid and chromosomal DNA, restriction enzyme treatment, ligation of DNA and transformation. Plasmid DNA was prepared by either the rapid alkaline extraction method or CsCl/ethidium bromide equilibrium density gradient centrifugation as described by Imanaka *et al.* (1982). Chromosomal DNA was prepared as described by Imanaka *et al.* (1981b). Treatment of DNA with restriction enzymes and ligation of DNA with T4 DNA ligase were done as recommended by the manufacturer. For transformation of *B. subtilis* with plasmid DNA, competent cells were prepared as described by Imanaka *et al.* (1981a).

Gel electrophoresis for DNA analysis and isolation. For the analysis of DNA, agarose or polyacrylamide gel electrophoresis was done under standard conditions (Maniatis *et al.*, 1982). Recovery of DNA from either low-melting point agarose or polyacrylamide gels was done by the standard method (Maniatis *et al.*, 1982).

DNA sequencing analysis. DNA sequencing was done by the dideoxy method with *E. coli* JM103 and M13 phages (Messing, 1983). Specific fragments were subcloned and a set of exonucleaseIII-mung bean nuclease-Klenow fragment generated deletions starting from *HpaI* and *PvuII* restriction sites were constructed using a deletion kit from Takara Shuzo. Both strands were sequenced and the sequence was generated across the restriction sites defining the ends of subcloned fragments.

Detection of protease-producing colonies and assay of protease activity. Protease-producing colonies on plates were detected, and protease activity was assayed for casein hydrolytic activity as described by Fujii *et al.* (1983). We defined 1 unit of protease as the quantity required to increase the absorbance at 275 nm by the equivalent of 1 µg tyrosine min⁻¹ at 37 °C.

Purification of extracellular protease. The culture broth supernatant was treated by ultrafiltration [through UF-10ps and UF-100ps, filters (Tosoh, Japan)] to remove smaller (molecular mass <10 kDa) and larger (>100 kDa) proteins, respectively; the resulting protein was precipitated by adding ethanol to a final concentration of 60% (v/v). After freeze-drying, the precipitate was dissolved in about 10 ml buffer solution (50 mM-Tris/HCl, pH 7.5, 5 mM-CaCl₂). The solution was subjected sequentially to Toyopearl HW-50 and TSK gel G-2000 SW HPLC column chromatography. The enzyme fractions for neutral protease were collected and immediately inactivated to minimize self-digestion by adding trichloroacetic acid to a final concentration of 2% (w/v). The protein was precipitated, isolated by centrifugation (15 000 g, 30 min) and dissolved in 0.8 ml 98% (v/v) formic acid. After desalting, the sample was subjected to TSK gel phenyl 5 PW RP HPLC column chromatography (Tosoh); the peak fractions were collected and then freeze-dried. This sample was used for the determination of the amino acid sequence.

Protein analysis by SDS-PAGE. This was done as described by Matsumura *et al.* (1984).

Amino-terminal amino acid sequence. This was determined by Edman degradation as described by Takagi *et al.* (1985).

Enzymes and chemicals. Restriction endonucleases and M13 sequencing kit were purchased from Toyobo. T4 ligase, bacterial phosphatase and low-melting-point agarose were from Takara. Kanamycin and tetracycline were from Sigma. [α -³²P]dCTP was from Amersham. All other chemicals were from Wako.

Analysis of amino acid sequence homology. Amino acid sequence homology was analysed with an NEC PC-9801 computer (Nippon Electric) and 'GENIAS' system (Mitsui).

Fig. 1. Structure of plasmid pTZ232 and its derivatives. Black bars indicate the DNA from *B. stearothermophilus* MK232. The neutral protease gene is contained in a black bar representing the 5.5 MDa *Pst*I fragment of pTZ232. Broken parallel lines indicate deletion of the corresponding fragments. Cleavage sites of *Bam*HI, *Eco*RI, *Hind*III, *Hpa*I, *Pst*I, *Pvu*II and *Sal*I are indicated by B, E, H, Hp, P, Pv and S, respectively. The position and direction of transcription of the *nprM* gene are indicated by an arrow.

RESULTS

Cloning of *nprM* in *B. subtilis*

Chromosomal DNA (about 5 µg) from *B. stearothermophilus* MK232 was digested with *Pst*I and ligated with a *Pst*I digest (about 2 µg) of pTB53 in a total volume of 50 µl. The ligation mixture was used to transform *B. subtilis* MT-2, and about 10^5 transformants were obtained on LC agar containing 5 µg kanamycin ml⁻¹. Of these, eight colonies could form haloes. All were also resistant to tetracycline. Plasmid DNA prepared from each isolate (Km^r Tc^r Npr⁺) was used to transform strain MT-2. All the transformants exhibited the phenotype Km^r Tc^r Npr⁺.

To confirm the cloning of the thermostable neutral protease gene, extracellular protease was assayed in these transformants. The protease was inhibited by EDTA but not by phenylmethylsulphonyl fluoride (data not shown), indicating that the enzyme is a neutral-(metallo)protease. The pH optimum of the protease was between 6.5 and 7.5 (data not shown). Furthermore, protease activity was retained after heat treatment at 65 °C for 15 min, indicating that the protease was thermostable. These results imply that the thermostable neutral protease gene (*nprM*) from *B. stearothermophilus* MK232 was cloned in *B. subtilis*.

One of the recombinant plasmids carrying *nprM* was designated pTZ232. pTZ232 contained a 5.5 MDa insert of *B. stearothermophilus* MK232 DNA. The restriction map of pTZ232 is shown in Fig. 1. When the 1.2 MDa *Sal*I-*Pst*I fragment was deleted, protease activity was not lost. However, deletion of either the 2.0 MDa *Bam*HI-*Pst*I fragment or the 1.5 MDa *Hpa*I-*Pst*I fragment from pTZ232 resulted in loss of protease activity. It is concluded therefore that the *nprM* gene is located around the 2.0 MDa *Hind*III-*Pvu*II fragment of pTZ232.

Nucleotide sequence of *nprM*

The nucleotide sequence of the *nprM* gene and its flanking regions was determined (Fig. 2). The sequence revealed only one large open reading frame, composed of 1656 nucleotides (552 amino acid residues). A Shine-Dalgarno (SD) sequence (GAAAAGG) was found 12 bases upstream from a likely translation start site (ATG) (Moran *et al.*, 1982). A putative promoter

-100
 CATGCATAGGAAAATGTGAAAAAACCGTAGGGAATTATCAACTATATCACTCTATTTTCCCAATACAAATA
 -35 region

CTGTAATAATATTGTGTTAATATTCTAATAACAAAGAATAAAGGAGGAGGATGAAAAATGAAAAGGAATAATTGGAAA
 -10 region SD

+1
 ATGAAAAGGAAAAATGAAAATGAAATTACGATCGTTGGTGTTCGACGAGGACTAGCGGCCCAAGTATTTTACCT
 MetLysArgLysMetLysMetLysLeuArgSerPheGlyValAlaAlaGlyLeuAlaAlaGlnValPheLeuPro
 1 25

100
 TACAATCGGCTGGCTTCATCGGAAACACGTTACATGGAACCAACAATTTCAAACCCCTCAATTCAATCTCCGGTGAAT
 TyrAsnArgLeuAlaSerThrGluHisValThrTrpAsnGlnGlnPheGlnThrProGlnPheIleSerGlyAsp
 50

200
 CTGCTGAAAGTGAATGGCAATCCCCAGAAGAACTCGTCTATCAATATGTGAAAAAAACGAAAACAAGTTTAAA
 ArgLeuLysValAsnGlyThrSerProGluGluLeuValTyrGlnTyrValGluLysAsnGluAsnLysPheLys
 75

300
 TTTCATGAAAACGCTAAGGATACTCTACAATTGAAGAAAAGAAAATGATAACCTTGGTTTACGTTTATGCGC
 PheHisGluAsnAlaLysAspThrLeuGlnLeuLysGluLysLysAsnAspAsnLeuGlyPheThrPheMetArg
 100

400
 TTCCAACAAACGTATAAAGGGATTCTGTGTGTTGGACAGGTAGTAACTGCGCACGTGAAAGATGGCAGCCGTGACG
 PheGlnGlnThrTyrLysGlyIleProValPheGlyGlnValValThrAlaHisValLysAspGlySerLeuThr
 125

500
 GCGCTATCAGGGACACTGATTCCAATTCCGAATTGGACACGAAAGGATCCTTAATAAGCGGGAAGAAATGAGT
 AlaLeuSerGlyThrArgIleProIleProAsnLeuAspThrLysGlySerLeuLysSerGlyLysLysLeuSer
 150

600
 GAGAAACAAAGCGGTGACATGCTGAAAAAGATTTAGTGCCAAATGTAACAAAGGAAGTACCGGAATATGAACAG
 GluLysGlnAlaArgAspIleAlaGluLysAspLeuValAlaAsnValThrLysGluValProGluTyrGluGln
 175

700
 GGAAAAGACACCGAGTTTGTGTGTTATGTCAATGGGACGAGGCTTCTTTAGCGTACGTTGTCAATTTAAACTTT
 GlyLysAspThrGluPheValValTyrValAsnGlyAspGluAlaSerLeuAlaTyrValValAsnLeuAsnPhe
 200

800
 TTAACCTCTGAACAGGAAACTGGCTGTATATCATTGATCCCGTAGACGGAAAAATTTTAAATAAATTTAACCAA
 LeuThrProGluProGlyAsnTrpLeuTyrIleIleAspAlaValAspGlyLysIleLeuAsnLysPheAsnGln
 225

900
 CTTGACGCCGCAAAACCAGGTGATGTGAAGTCGATAACAGGAACATCAACTGTCGGAGTGGGAAAGAGGACTACTT
 LeuAspAlaAlaLysProGlyAspValLysSerIleThrGlyThrSerThrValGlyValGlyArgGlyValLeu
 → → → → →
 250

1000
 GGTGATCAAAAAATATTAATACAACCTACTCTAGCTACTACTATTTACAAAGATAATACGCGTGAAATGGGATT
 GlyAspGlnLysAsnIleAsnThrThrTyrSerThrTyrTyrTyrLeuGlnAspAsnThrArgGlyAsnGlyIle
 275

1100
 TTCACTGATGATGCGAAATACCGTACGACATTGCGGGGAAGCTTATGGGCAGATGCAGATAACCAATTTTTCGG
 PheThrTyrAspAlaLysTyrArgThrThrLeuProGlySerLeuTrpAlaAspAlaAspAsnGlnPhePheAla
 300

1200
 AGCTATGATGCTCCAGCGGTGATGCTCAATTATTACGCTGGTGTGACATATGACTACTATAAAAAATGTTCTATAAC
 SerTyrAspAlaProAlaValAspAlaHisTyrTyrAlaGlyValThrTyrAspTyrTyrLysAsnValHisAsn
 325

1000
CGTCTCAGTTACGAACGAAATAATGCAGCTATTAATCATCCGTTTCATTATAGCCAAAGGCTATAATAACGCATT
ArgLeuSerTyrAspGlyAsnAsnAlaAlaIleArgSerSerValHisTyrSerGlnGlyTyrAsnAsnAlaPhe
350

1100
TGGAAACGGTTCGCAAAATGGTGTATGGCGATGGTGATGGTCAAACATTTATTCCTTTCTGGTGGTATTGATGTG
TrpAsnGlySerGlnMetValTyrGlyAspGlyAspGlyGlnThrPheIleProLeuSerGlyGlyIleAspVal
375

1200
GTCGCTCATGAGTTAACGCATGCCGTAACCGATTATACAGCCGGACTCATTATCAAACGAATCTGGTGCAATT
ValAlaHisGluLeuThrHisAlaValThrAspTyrThrAlaGlyLeuIleTyrGlnAsnGluSerGlyAlaIle
400

AATGAGGCAATATCTGATATTTTTGGAACTTAGTCGAATTTTACGCTAAACAAATCCAGATTGGGAAATTGGA
AsnGluAlaIleSerAspIlePheGlyThrLeuValGluPheTyrAlaAsnLysAsnProAspTrpGluIleGly
425

1300
GAGGATGTGTATACACCTGGTATTTCAGGGGATTCTGCTCCGTTCTGATGTCGGATCCGGCAAAGTATGGTGATCC
GluAspValTyrThrProGlyIleSerGlyAspSerLeuArgSerMetSerAspProAlaLysTyrGlyAspPro
450

1400
GATCACTATTCAAACGCGTATACAGGCACGCAAGATAATGCCGGGGTTCATATCAATAGCAGGAATTATCAACAA
AspHisTyrSerLysArgTyrThrGlyThrGlnAspAsnGlyGlyValHisIleAsnSerGlyIleIleAsnLys
475

GCCGCTTATTTGATTAGCCAAGGCCTACGCATTACCGTGTGAGTGTGTGCGGAATCGGACGCGATAAAATGGGG
AlaAlaTyrLeuIleSerGlnGlyGlyThrHisTyrGlyValSerValValGlyIleGlyArgAspLysLeuGly
500

AAAAATTTCTATCGTGCATTAACGCAATATTAACACCAACGTCCTCACTTTAGCCAACTTCGTGCTGCCGCTGTT
LysIlePheTyrArgAlaLeuThrGlnTyrLeuThrProThrSerAsnPheSerGlnLeuArgAlaAlaAlaVal
525

1600
CAATCAGCCACTGACTTGTACGGTTCGACAAGCCAGGAAGTCGCTTCTGTGAAGCAGGCCCTTTGATGCCGTAGG
GlnSerAlaThrAspLeuTyrGlySerThrSerGlnGluValAlaSerValLysGlnAlaPheAspAlaValGly
550

1700
GTGAAATAAAAGTGGTATCTCATCAGTGGGGGATTTTTCCTCCACTGATGTTTGTGTTGTGATCAATGATGTCAG
ValLys***

Fig. 2. Nucleotide sequence and amino acid sequence of the neutral protease gene *nprM*. The nucleotide sequence is counted from the first base of the translation start site as +1. The amino acid sequence is shown beneath the nucleotide sequence. The amino-terminal amino acid sequence of extracellular protease, determined by Edman degradation, is specified below the amino acid sequence by arrows. The first amino acid of translation (Met) is counted as 1. The putative Shine-Dalgarno (SD) sequence (nucleotide -18 to -12) and promoter region (-35 and -10 regions) are shown by solid lines below the nucleotide sequence. Asterisks (***) indicate a stop codon. A putative transcriptional terminator containing an inverted repeat is shown by arrows (→ ←) below the nucleotide sequence. Amino acid substitutions in comparison with thermolysin (Titani *et al.*, 1972) are indicated by broken lines.

sequence (-35 region: TTTTCC; -10 region: TATTGT), which was nearly identical to the known promoter (-35 region: TTTTCC; -10 region: TATTTT) for another thermostable neutral protease gene, *nprT* (Takagi *et al.*, 1985), was also found about 40 bases upstream of the SD sequence. Amino-terminal amino acids deduced from the nucleotide sequence exhibited a typical signal sequence with basic amino acids (Lys, Arg), followed by a hydrophobic core (Holland *et al.*, 1986; Perlman & Halvorson, 1983). A typical terminator sequence was also found immediately downstream from the open reading frame.

Fig. 3. Computer search for homologous regions of the amino acid sequence. The neutral proteases of *B. stearothermophilus* MK232 and *B. stearothermophilus* CU21 (Takagi *et al.*, 1985) are compared along the entire coding region. Open and solid bars in both axes correspond to the pre-pro and extracellular regions of protease, respectively. Sequence number is shown on both axes. The minimum length of the homologous region was taken as three amino acids.

Amino acid sequence of the extracellular neutral protease

The extracellular form of the protease was purified from the culture broth of *B. stearothermophilus* MK232, and the amino-terminal amino acid sequence was determined through five cycles of Edman degradation using an automatic sequencer. The first five amino acids were Ile-Thr-Gly-Thr-Ser. The sequence completely matched that deduced from the nucleotide sequence starting at ATA (+709 to +711, Ile237 in Fig. 2, at position 1 as extracellular form). The corresponding amino acids are indicated with arrows in Fig. 2. The molecular mass of the extracellular protease calculated from the amino acid sequence (316 amino acids, mol. mass 34266 Da) was nearly the same as the known value for the enzyme (34 kDa) (Kubo *et al.*, 1988). These results indicate that the open reading frame is translated as a large polypeptide containing a pre-pro structure, as is the case for other neutral proteases (Takagi *et al.*, 1985; Vasantha *et al.*, 1984).

The amino acid sequence of the polypeptide was compared with the published sequences of other thermostable neutral proteases (Takagi *et al.*, 1985; Titani *et al.*, 1972). The sequence of the extracellular form of this protease was identical to that of the thermostable neutral protease (thermolysin) from *B. thermoproteolyticus* except for the two amino acid substitutions [Asp37 to Asn37 (corresponding to Asn273 in Fig. 2), Glu119 to Gln119 (corresponding to Gln355 in Fig. 2)]. The extracellular proteases from *B. stearothermophilus* strains MK232 and CU21 show a very high degree of homology (85% of the amino acids are identical) whereas the pre-pro regions are less closely related (40% amino acid identity) (Fig. 3).

Expression of nprM and nprT in B. subtilis

The promoter sequences of *nprT* and *nprM* may be similar as mentioned above. Nevertheless, strain MK232 containing *nprM* produced much more protease than did strain CU21 containing *nprT*. To investigate the difference in enzyme productivity on an equal basis, the two protease genes were subcloned in *B. subtilis*, by using the low-copy-number vector plasmid pTB53 as shown schematically in Fig. 4. The recombinant plasmids containing *nprM* and *nprT* were

Fig. 4. Construction of pTNM53 and pTNT53. Thick lines in pTZ232 and pNP22 represent *B. stearothermophilus* MK232 DNA and *B. stearothermophilus* CU21 DNA, respectively. Km^r and Tc^r indicate kanamycin- and tetracycline-resistance genes, respectively.

Fig. 5. Time course of neutral protease production and cell growth for *B. subtilis* MT-2 carrying either pTNM53 or pTNT53. Cells were grown at 37 °C in L broth containing 5 μg tetracycline ml^{-1} on a rotary shaker. The culture supernatants were used for enzyme assay (●). Bacterial growth (■) was measured as the optical density at 660 nm.

designated pTNM53 and pTNT53, respectively. *B. subtilis*(pTNM53) produced about 20 times more protease in 24 h than did the strain carrying pTNT53, although both strains exhibited the same growth pattern (Fig. 5).

DISCUSSION

The thermostability of protease NprM is higher than that of thermolysin by about 10%, and the specific activity of NprM is about 40% higher than that of thermolysin. To compare the amino acid sequences, we cloned the *nprM* gene in *B. subtilis* and determined the complete nucleotide sequence. The amino acid sequence of NprM deduced from the nucleotide sequence

Fig. 6. Three-dimensional structure of thermolysin (Matthews *et al.*, 1972*a*). Open circles, α -carbon positions. The zinc atom is drawn stippled with its three protein ligands shown diagrammatically as broken lines. Four calcium atoms are shown as solid circles. Amino acid substitutions are shown by arrows.

was identical to that of thermolysin except for two amino acid substitutions (Asp37 to Asn37 and Glu119 to Gln119, for the extracellular form). Both substitutions are the addition of an amino group to an acidic amino acid, resulting in an uncharged polar amino acid. The two substitutions are located in Domain A (Ile1–Gly136) and not in Domain B (Ile156–Lys316) of the enzyme (Fig. 6), and might contribute to the effective electrostatic balance in Domain A without any significant change of three-dimensional protein structure. In other words, Asn37 and Gln119 might reduce the repulsion with negatively charged amino acids in the three-dimensional vicinity, Asp32 and Asp72, respectively (Fig. 6). Moreover, the substituted amino acids, Asn37 and Gln119, might also increase the hydrogen bonding with neighbouring amino acids. Thus, Asn37 is surrounded by Asn19, Asn21, Asn33, Thr34, Arg35, Asn96 and Asn97 (Fig. 6), all of which are potentially able to form hydrogen bonds. Likewise, Gln119 is surrounded by Arg101, Ser102, Ser103, Asn116, Ser118 and Met120 (Fig. 6). According to the statistical data on enzyme

thermostability mentioned by Argos *et al.* (1979), the amino acid substitution Asp to Asn could enhance thermostability. This is one example where amino acid substitutions which might increase hydrogen bonding or give a good electrostatic balance were generated in nature and result in enhanced thermostability and specific activity of the enzyme.

We have also succeeded in enhancing the thermostability of the neutral protease NprT by replacing Gly144 with Ala144 (Imanaka *et al.*, 1986). The amino acid substitution was made to increase the internal hydrophobicity and stabilize an α -helix for strong packing. Therefore, many factors such as the hydrophobicity and charge of amino acids and their hydrogen bonding, in addition to changes in secondary and tertiary structures of an enzyme, should be considered in the enhancement of thermostability.

The G + C content of thermophilic bacterial genes is in general higher than that of mesophilic genes (Kagawa *et al.*, 1984; Nakajima *et al.*, 1985; Takagi *et al.*, 1985; Winter *et al.*, 1983). In our previous work, the G + C content of the coding region for the *nprT* gene was 58 mol%, while that of the third letter of the codons was higher (72 mol%) (Takagi *et al.*, 1985). Surprisingly, however, the G + C content of the coding region for the *nprM* gene was 42 mol%, while that of the third letter of the codon was lower (36 mol%). These values are very similar to or a little lower than those for the neutral proteases from *B. amyloliquefaciens* (46 and 49 mol%, respectively: Vasantha *et al.*, 1984) and *B. subtilis* (44 and 42 mol%, respectively: Yang *et al.*, 1984). Although the amino acid sequence homology between the two extracellular proteases from *B. stearothermophilus* is very high (Fig. 3), the G + C contents of *nprM* and *nprT* are quite different from each other. The difference between the two genes suggests that they may possibly have different evolutionary origins.

There exists a direct perfect repeat sequence (at positions -23 to -7, and -3 to 14) in the translation start site region. Therefore, the direct repeat might play an important role in the regulation of gene expression. Since a typical SD sequence is located at positions -18 to -12, the same sequence (at positions 3 to 9) might be used as another SD sequence. In this case, it is most likely that Met7 in Fig. 2 is used as a translation start codon. Even if the two sets of translation start sites functioned in reality, no difference would be found in the extracellular protease.

We compared the expression of the *nprM* gene with that of the *nprT* gene in *B. subtilis*. In spite of being cloned in the same low-copy-number vector plasmid (pTB53), the productivity of NprM was about 20 times higher than that of NprT. The difference in the enzyme productivity might be explained by the following arguments. (1) Since the signal sequence and the following pro-region of NprM are relatively different from those of NprT as mentioned above (see Fig. 3), the secretion efficiencies of the two enzymes might be different. (2) Since the G + C contents of both genes are quite different, the codon usage might affect the gene expression in *B. subtilis*. (3) Since the two tandem SD sequences and the corresponding initiation codons are found, translation efficiency might be increased. Mechanisms for the high productivity of NprM are now under investigation.

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