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Addition of a methyl group changes both the catalytic velocity and thermostability of the neutral protease from *Bacillus stearothermophilus*

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Specific activity was compared between wild-type (WT) neutral protease from *Bacillus stearothermophilus* and mutant protease (M1; Gly144 replaced by Ala144) with enhanced thermostability. When casein was used as a substrate, M1 showed 1.5-times higher specific activity than that of WT. In contrast, the specific activities of M1 for soluble reduced lysozyme and insulin B chain were lower than those of WT by 17.2 and 13.2 %, respectively. After digestion of the insulin A chain by these enzymes, the peptide products were purified and the N-terminal amino acid sequences were determined. WT enzyme cleaved insulin A chain at three sites, whereas no digestion was observed with M1. Using Z-Gly-Leu-NH_2 as a substrate, the kinetic parameters were determined. The K_m values are nearly equal for both enzymes, whereas the k_{cat} of M1 (240 min^{-1}) was much smaller compared to the WT (830 min^{-1}). The data indicate that the mutation (addition of a methyl group) exerts an effect by changing both the catalytic velocity and thermostability.

Protease, neutral; Amino acid substitution; Catalytic velocity

1. INTRODUCTION

Site-directed mutagenesis has made it possible to introduce amino acid substitutions at specific positions. However, the criteria for the rational alteration of enzyme functions remain ambiguous due to the limited information available on the relationship between enzyme structure and function. Neutral and alkaline proteases from *Bacillus* sp. have been intensively studied in order to clarify the structure of proteases [1-4]. We have cloned and sequenced the thermostable neutral protease gene (*nprT*) from *B. stearothermophilus* [5,6] and studied the role of the pre-pro structure [7]. One genetically engineered mutant enzyme (M1) with enhanced thermostability was obtained by substitution of the glycine at position 144 with alanine. The amino acid is located in an α-helix but does not function directly as the catalytic site (fig.1) [8]. However, the effect of this mutation on enzyme activity is unknown. Here, we compared the specific activity and kinetic parameters (K_m and k_{cat}) between wild-type (WT) and M1 proteases. The effect of single amino acid substitution on enzyme activity is also discussed.

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Abbreviations: WT, wild-type neutral protease; M1, mutant neutral protease with substitution of Gly144 by Ala; Z-Gly-Leu-NH_2, 2-(benzyloxycarbonyl)-L-Gly-L-Leu-NH_2

2. MATERIALS AND METHODS

B. subtilis MT-2 (*trpC2 leuC7 hsrM hsmM* Npr^-) was used as a host [5]. Recombinant plasmids pNP22-3 (Km^r *nprT*$^+$ (structural gene of wild-type neutral protease)) and pNP22-3-m1 (Km^r *nprT*$^+$*m1* (mutation m1, Gly 144 → Ala)) were used for production of wild-type (WT) and mutant (M1) enzymes, respectively [8].

Bacteria carrying recombinant plasmid were grown in YPC medium containing 5 μg/ml of kanamycin [5]. Protease was

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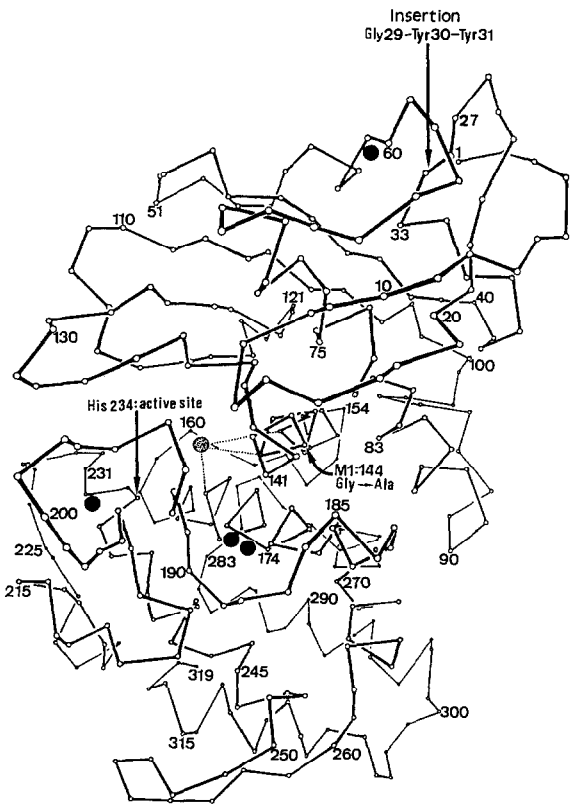

Fig.1. Three-dimensional structure of thermolysin [1] which is homologous (85%) to the NprT [8]. Open circles show α-carbon positions. The zinc atom is drawn as a stippled circle with its three protein ligands shown diagrammatically as dotted lines. Four calcium atoms are shown as filled circles. The amino acid number from the N-terminus is for NprT; originally, Gly-Tyr-Tyr at positions 29–31 were absent from thermolysin. Positions of the M1 mutation (Gly 144 → Ala) and the active site (His 234) are indicated by arrows.

purified from the culture supernatant as in [6]. After the second column chromatography using DEAE-Sephadex A50, enzyme fractions were collected and purified by high-performance liquid chromatography (HPLC) on TSK-gel G2000SW.

The casein hydrolytic activity of protease was assayed as reported [5], the specific activity being calculated as U/mg protein. Protein concentration was determined with BCA protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. When soluble reduced lysozyme and insulin B chain were used as substrates, peptide products were detected by the ninhydrin reaction according to [9]. The specific activity was defined as the increase in absorbance at a wavelength of 570 nm/min per mg enzyme protein (ΔA_{570}/min per mg protein). In the case of insulin A chain as substrate, the peptide products were purified and analyzed on reverse-phase HPLC with ODS-80TM followed by N-terminal amino acid sequencing using a protein sequencer (model 870A, Applied Biosystems, CA) [10]. The kinetic parameters for M1 and WT were determined using Z-Gly-Leu-NH_2 as substrate [11,12].

3. RESULTS AND DISCUSSION

The purity of neutral proteases (M1 and WT) was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in which both samples gave single bands (not shown). The specific activity of the wild-type enzyme for casein was 33 600 U/mg protein, that of mutant protein (M1) being 50 500 U/mg protein. The results indicate that the mutation (Gly 144 → Ala) led to enhancement of casein hydrolytic activity as well as thermostability.

To investigate the difference in specific activity in greater detail, soluble reduced lysozyme (129 amino acid residues, 14.3 kDa) and insulin B chain (30 amino acid residues, 3496 Da) were used as substrates and the enzyme activities evaluated (fig.2). In contrast to casein, the specific activities of M1 for lysozyme and insulin B chain were 0.29 and 0.33 (ΔA_{570}/min per mg protein), respectively, and 17.2 and 13.2% lower vs the corresponding values for WT (0.35 and 0.38 ΔA_{570}/min per mg protein) (fig.2A,B). The difference in specific ac-

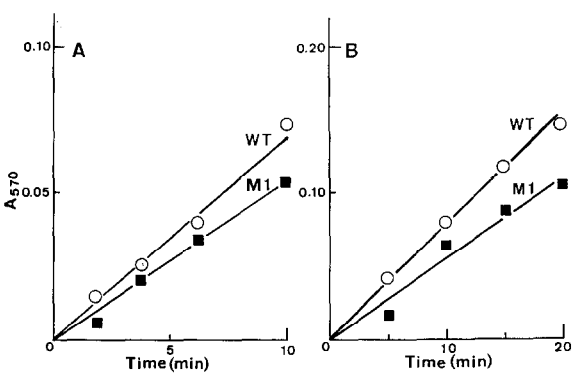

Fig.2. Hydrolysis of soluble reduced lysozyme (A) and insulin B chain (B) with neutral proteases. Each substrate was dissolved in 360 μl of 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.5, to a concentration of 2.5 mg/ml. 40 μl enzyme sample (20 μg protease) was mixed with substrate (total 400 μl) and the mixture incubated at 37°C. Aliquots (20 μl) were taken and 180 μl acetic acid was added to stop enzyme reaction. For detection of digestion products, 200 μl ninhydrin solution [2% (w/v) ninhydrin and 0.15% (w/v) hydrindantin dissolved in 25% (v/v) 4 M sodium acetate buffer (pH 5.5) and 75% (v/v) 2-methoxyethanol] was added, followed by incubation at 100°C for 15 min. Samples were diluted 6 times with 50% ethanol. The absorbance at 570 nm was measured and the specific activity (ΔA_{570}/min per mg protein) calculated after evaluating the protein concentration.

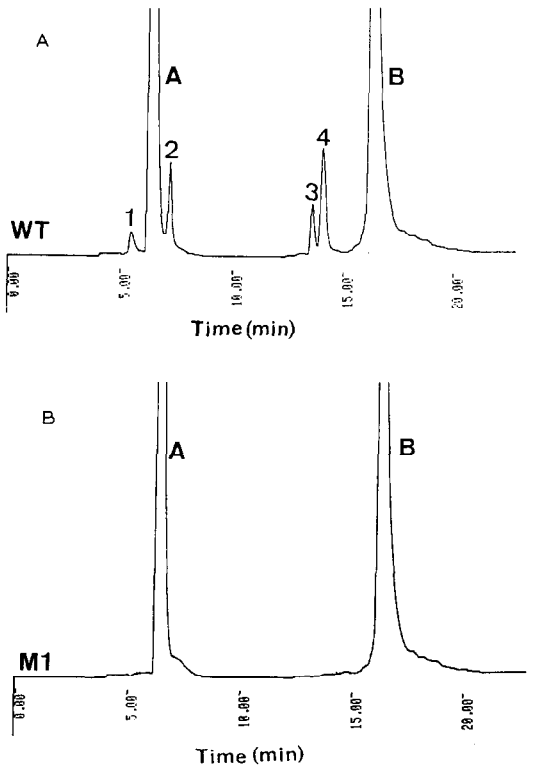

Fig.3. Hydrolysis of insulin A chain with WT (A) and M1 (B). Enzyme reaction was initiated as in fig.2 and the reaction continued for 20 min. Acetic acid (3.6 ml) was added to stop the reaction and the digestion products were analyzed on reverse-phase HPLC with ODS 80 TM (Tosoh, Tokyo). Products were monitored at 210 nm with a gradient of 20–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The two large peaks (A,B) correspond to acetic acid and insulin A chain, respectively.

tivities between WT and M1 was much more evident when insulin A chain (21 amino acid residues, 2530 Da) was used as substrate.

Products of the hydrolysis of insulin A chain with WT and M1 were analyzed on HPLC as shown in fig.3A,B, respectively. Four peaks were detectable after digestion with WT (fig.3A). However, no apparent digestion products were observed for the reaction mixture of M1 (fig.3B). Each peak fraction for a small peptide derived from the insulin A chain with WT was collected and the N-terminal amino acid sequences were determined. The sequences are aligned and compared with that of insulin A chain in fig.4. Each peak corresponds to a particular part of the insulin A chain, the data indicating that WT cleaved the substrate at a minimum of 3 sites where leucine or isoleucine exists as a P1 residue. Our results are consistent with the proposal that the presence of a hydrophobic amino acid residue in the substrate is essential as the P1 residue for cleavage with thermolysin, a thermostable neutral protease from *B. thermoproteolyticus* [12].

The inability of M1 to digest insulin A chain may be supposed to result from two possible causes: (i) a decrease in the affinity with substrate; (ii) a diminished catalytic rate. To determine whether insulin A chain interacts with the enzyme's active site, the enzyme sample firstly was mixed with insulin A chain (2.5 mg/ml) and kept at 37°C for 20 min. Secondly, 400 μl casein solution (2%) was added to the mixture and the hydrolytic activity measured. The enzyme activities with and without insulin A chain were compared. In the case of M1, enzyme activity was inhibited by 32% in the presence of insulin A chain, however no such inhibition was observed for WT. It was inferred that insulin A chain was bound to the active site of M1 but the enzyme could not cleave the substrate, and therefore the insulin A chain was not dissociated and inhibited casein hydrolytic activity. Thus, it is most likely that the amino acid substitution (Gly 144 → Ala) decreases the rate of catalysis rather than the substrate binding affinity.

To prove directly that a decrease in catalytic velocity was indeed the case, K_m (mM) and k_{cat} (min^{-1}) values were measured at 37°C using Z-Gly-Leu-NH_2 as substrate. The K_m values are almost identical for WT (26 mM) and M1 (25 mM), however, the k_{cat} value for M1 (240 min^{-1}) is about one-third of that for WT (830 min^{-1}). The result indicates that the mutation M1 (Gly 144 → Ala) had no effect on the affinity of substrate binding but did cause a significant decrease in catalytic velocity.

Previously, we reported that the amino acid substitution (Gly 144 → Ala), located in the α-helix joining two large domains, increased internal hydrophobicity and stabilized the α-helix structure [8]. Around the position of the mutation, three amino acid residues are found (His 145, Glu 146 and His 149) which have been reported to be important for enzyme function [13]. Of these, the two histidine residues are ligands for Zn^{2+} and Glu 146 plays an important role as a nucleophile in the promotion of hydrolysis. Furthermore, the three

Insulin A Chain	1 Gly ▼ Ile Val Glu Gln Cys Cys Ala Ser 10 Val Cys Ser ▼ Leu Tyr Gln ▼ Leu Glu Asn Tyr Cys 21 Asn
Peak 1	2 Ile Val Glu Gln
Peak 2	16 Leu Glu Asn Tyr
Peak 3	13 Leu Tyr Gln Leu
Peak 4	1 Gly Ile Val Glu

Fig.4. Amino-terminal amino acid sequences of digestion products. The four peaks corresponding to the products in fig.3A were collected and the results of four cycles of N-terminal amino acid sequencing are aligned together with the sequence of the insulin A chain. Peak nos 1–4 correspond to those in fig.3.

residues are conserved among four neutral proteases [8]. The α-helix is located at the bottom of the binding cleft and includes important residues as regards hydrolysis. Therefore, the substitution (Gly 144 → Ala: addition of a methyl group) might exert steric and hydrophobic effects on the enzyme-substrate interaction, thus resulting in low catalytic activity. The same kind of decrease in catalytic activity was reported for mutant subtilisin from *B. amyloliquefaciens* [14].

It is interesting to note that the relative enzyme activity of WT and M1 depends on the species of substrate. This property might be attributed to variations in amino acid sequence and the lack of a tertiary structure in small proteins or peptides. In order to clarify the reasons for enhanced thermostability and altered specific activity of the M1 enzyme from the standpoint of tertiary structure, a crystallographic study is currently in progress.

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