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# Alkaline-Resistance Model of Subtilisin ALP I, a Novel Alkaline Subtilisin

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**The alkaline-resistance mechanism of the alkaline-stable enzymes is not yet known. To clarify the mechanism of alkaline-resistance of alkaline subtilisin, structural changes of two typical subtilisins, subtilisin ALP I (ALP I) and subtilisin Sendai (Sendai), were studied by means of physicochemical methods. Subtilisin NAT (NAT), which exhibits no alkaline resistance, was examined as a control. ALP I gradually lost its activity, accompanied by protein degradation, but, on the contrary, Sendai was stable under alkaline conditions. CD spectral measurements at neutral and alkaline pH indicated no apparent differences between ALP I and Sendai. A significant difference was observed on measurement of fluorescence emission spectra of the tryptophan residues of ALP I that were exposed on the enzyme surface. The fluorescence intensity of ALP I was greatly reduced under alkaline conditions; moreover, the reduction was reversed when alkaline-treated ALP I was neutralized. The fluorescence spectrum of Sendai remained unchanged. The enzymatic and optical activities of NAT were lost at high pH, indicating a lack of functional and structural stability in an alkaline environment. Judging from these results, the alkaline resistance is closely related to the surface structure of the enzyme molecule.**

**Key words: alkaline-stability, autolysis, conformational restoration, serine protease, surface region.**

Subtilisins are serine proteases originating from strains of *Bacillus subtilis* or related bacteria (*1*, *2*). We previously isolated two alkaline proteases, subtilisin ALP I (ALP I) (*3*, *4*), and subtilisin Sendai (Sendai) (*5*), from alkalophilic *Bacillus* and a neutral protease, subtilisin NAT (NAT) (*6*), from *B. subtilis*.

It has been shown that alkaline subtilisins, namely, Savinase, 221 protease (*7*), and Sendai, are much more stable in an alkaline environment than neutral subtilisin BPN′ (*2*), subtilisin Carlsberg (*1*), and NAT. ALP I from alkalophilic *Bacillus* loses its activity completely at pH 12 (*4*), whereas Sendai is stable under the extreme alkaline condition of pH 12, similar to the other alkaline subtilisins (*5*).

From the results of studies on the stability of neutral subtilisin under extreme temperature, pH, and salinity conditions, inactivation of the enzyme was determined to be initiated by partial unfolding processes that render the enzyme susceptible to autolysis (*8–10*). Previous studies on the autolysis of broad-specificity proteases (*8*, *9*, *11*, *12*), together with observations concerning the conformational changes during protein unfolding (*13*, *14*), suggest that the local unfolding processes that lead to autolysis involve regions at the protein surface (*11*, *12*, *15*); in addition, differences in stability of the enzymes are determined mainly by differences in amino acid residues at the surface. The pH-dependent conformational changes of neutral subtilisin were shown to be irreversible with the use of inhibited subtilisin (*16–18*); however, it has never been determined how alkaline subtilisin is stabilized in a highly alkaline environment.

In the present study, we obtained initial data on the manner of inactivation of ALP I, a novel alkaline subtilisin secreted by alkalophilic *Bacillus* sp. NKS-21, in an extremely alkaline environment. Although the enzymatic profiles of ALP I resemble those of alkaline subtilisin, ALP I activity is lost at pH 11 (*3*). Its predicted amino acid sequence exhibits about 60% identity with those of other alkaline subtilisins and about 57% identity with those of neutral subtilisins (*4*); as a result, ALP I is classified differently to other subtilisins. In this study, we show that the inactivation mechanism for ALP I parallels the autolysis of the enzyme on SDS–PAGE. Moreover, to clarify the difference in alkaline stability between Sendai and ALP I, we studied the physicochemical properties of the two distinct subtilisins under alkaline conditions and found that the alkaline stability of the enzymes is due to the rigidity of limited surface regions of the enzyme molecules.

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Abbreviations: ALP I, subtilisin ALP I; BPBB, borate-phosphate broad-range buffer; CD, circular dichroism; DFP, diisopropyl fluorophosphate; DIP, diisopropylphosphoryl; NAT, subtilisin NAT; Sendai, subtilisin Sendai; Suc-Ala-Ala-Pro-Phe-MCA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-4-methylcoumaryl-7-amide.

## MATERIALS AND METHODS

*Materials*—Diisopropyl fluorophosphate (DFP) was from Fluka Chemie AG (Buchs, Switzerland). Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-4-methylcoumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA) was from Peptide Institute (Osaka). Subtilisin Sendai secreted from *Bacillus* sp. G-825-6 was grown in alkaline bonito extract broth (2% bonito extract, 2% polypeptone, 2% soybean powder, 1% $Na_2CO_3$, 0.2% milk casein). Subtilisin NAT secreted from *B. subtilis* NC2-1 ts25 was grown in bonito extract broth (2% bonito extract, 2% polypeptone, 0.7% NaCl, 0.2% milk casein).

*Purification of Subtilisin ALP I*—Subtilisin ALP I was purified according to the previously described method (*3*).

*Purification of Subtilisin Sendai (5)*—All purification procedures were performed at 4°C. A crude enzyme solution was obtained by centrifugation of the culture broth of alkalophilic *Bacillus* sp. G-825-6 at 12,000 ×*g* for 20 min. The enzyme solution was dialyzed against 10 mM Atkins–Pantin buffer (pH 8.0) containing 0.5 mM $CaCl_2$. The dialyzed enzyme solution was loaded on a CM-Cellulofine C-500 column (4 × 25 cm) equilibrated with the same buffer; the enzyme-active fraction was eluted with a 0–1 M NaCl linear gradient, and fractions were pooled and dialyzed against the Atkins–Pantin buffer. The enzyme solution was then placed on a CM-Sephadex C-50 column (3 × 18 cm) equilibrated with the buffer. The column was washed with Atkins–Pantin buffer, and the enzyme-active fraction was eluted with a 0–0.5 M NaCl linear gradient. The active fractions were then pooled, dialyzed against $H_2O$, and lyophilized.

*Purification of Subtilisin NAT (6)*—All purification procedures were performed at 4°C. A crude enzyme solution was obtained by centrifugation of the culture broth of *B. subtilis* NC2-1 ts25 at 12,000 ×*g* for 20 min. The enzyme solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.0) containing 2 mM $CaCl_2$; the dialyzed enzyme solution was then loaded on a CM-Cellulofine C-500 column (4 × 25 cm) equilibrated with the same buffer. The column was washed with the same buffer, and the enzyme-active fraction was eluted with a 0–1 M NaCl linear gradient. The active fractions were pooled and then dialyzed against 10 mM Atkins-Pantin buffer (pH 8.0) containing 0.5 mM $CaCl_2$. The enzyme solution was then placed on a CM-Sephadex C-50 column (3 × 18 cm) equilibrated with the Atkins–Pantin buffer. The column was washed with the same buffer, and the enzyme-active fractions were eluted with a 0–0.5 M NaCl linear gradient. The active fractions were pooled, dialyzed against 10 mM phosphate buffer (pH 6.8), and then placed on a GIGAPITE column (3 × 10 cm) equilibrated with the phosphate buffer. The column was washed with the phosphate buffer, and the enzyme-active fractions were eluted, pooled, dialyzed against $H_2O$, and then lyophilized.

*Preparation of DIP-ALP I, DIP-Sendai, and DIP-NAT*—All preparation procedures were performed at 4°C. Purified subtilisin ALP I and Sendai were dissolved in 10 mM Tris-HCl buffer (pH 7.0) containing 2 mM $CaCl_2$ (0.2 mg/ml). The purified subtilisin NAT solution was diluted with 10 mM phosphate buffer (0.4 mg/ml). A specific inhibitor for serine proteases, diisopropyl fluorophosphate (DFP), was added (final concentration, 10 mM) to each enzyme solution, and the mixtures were left on ice for 2 h. The mixtures were dialyzed against either 10 mM Tris-HCl buffer (pH 9.0 for ALP I and pH 7.0 for Sendai) or 5 mM phosphate buffer, pH 7.0 (DIP-NAT). The dialyzed DIP-NAT solution was used as the purified DIP-NAT. The dialyzed diisopropylphosphoryl–ALP I (DIP-ALP I) reaction mixture was applied to a DEAE-Cellulofine A-500 column (3 × 9 cm) equilibrated with 10 mM Tris-HCl buffer (pH 9.0), and then eluted with a 0–0.5 M NaCl linear gradient. The dialyzed DIP-Sendai reaction mixture was applied to a CM-Cellulofine C-500 column (3 × 9 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0), and then eluted with a 0–0.5 M NaCl linear gradient. The eluted DIP–enzyme fractions from the CM or DEAE ion exchange chromatography column were each collected, dialyzed against $H_2O$, and lyophilized.

*Enzyme Assay*—A fluorometric assay with the substrate Suc-Ala-Ala-Pro-Phe-MCA was carried out as described previously (*19*).

*Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)*—SDS–PAGE was performed by the method of Laemmli (*20*) on a 15.0% polyacrylamide slab gel.

*pH Stability*—Subtilisins (0.1 mg/ml) were incubated in a 100 mM borate–phosphate broad-range buffer (pH 7–12; BPBB) for 10 min at 30°C. The remaining activity was measured as described using 5 μl of the treated mixture. Equal amounts of 100% (w/v) trichloroacetic acid (TCA) were added to a portion of the incubated mixture; the TCA mixture was left on ice for 2 h and then centrifuged at 18,500 ×*g* for 20 min. Precipitates were separated by SDS–PAGE. The proteins were stained with Coomassie Brilliant blue R-250; the intensities of the protein signals were then scanned with a Model GT-9000 Seiko Epson scanner and analyzed with NIH-Image 1.62 soft (National Institutes of Health, Bethesda, MD).

*Circular Dichroism Measurements*—Far- and near-UV circular dichroism (CD) spectra were measured with a Jasco J-700 spectropolarimeter at room temperature in 1- and 10-mm path length cells, respectively (*21*).

*Tryptophan Fluorescence Measurements*—Tryptophan fluorescence was measured with a Model F-3000 Hitachi fluorescence spectrometer, with excitation at 283 nm and emission at 300–400 nm (*22*).

## RESULTS

*Effect of pH on the Stability of Subtilisins*—The activity of ALP I gradually decreased as the incubation pH increased to pH 11.0; this was accompanied by protein degradation, and both the protein band of the enzyme and the activity disappeared completely at pH 12.0 (Fig. 1). In the same manner, NAT activity decreased and degradation occurred as the pH increased to 11.0; however, both the relative activity and relative protein quantity were about 30% at pH 12.0 (Fig. 1). On the other hand, Sendai was quite stable at pH 12.0 and did not show a loss of activity or protein (Fig. 1). To avoid autolytic degradation, the catalytic sites of the enzymes were modified with DFP. Neither DIP-ALP I nor DIP-NAT was degraded at pH 12.0 in a manner similar to that above (data not shown).

*Secondary Structure Analysis of DIP-Subtilisins: Far-UV CD Spectra*—Far-UV CD spectra of DIP-ALP I, DIP-Sen-

dai, and DIP-NAT (Fig. 2, A, B, and C, respectively) were recorded from 200 to 250 nm at pH 7.0 and 12.0 after incubation at 30°C for 10 min; the spectrum of DIP-NAT was still observable at pH 12.0 after incubation for 20, 30, and 60 min. There were no apparent changes in the far-UV CD spectra of DIP-ALP I and DIP-Sendai obtained at pH 7.0 and pH 12.0 (Fig. 2, A and B). The far-UV CD spectra of DIP-NAT, in contrast, were sensitive to alkaline conditions from pH 7 to 12 (Fig. 2C). Spectra showing changes over a period of 10 min exhibited optical inactivation at all wavelengths at pH 12.0.

*Tertiary Structure Analysis of DIP-Subtilisins: Near-UV CD Spectra*—Near-UV CD spectra of DIP-ALP I, DIP-Sendai, and DIP-NAT were recorded from 250 to 300 nm at pH 7.0 and 12.0 after incubation at 30°C for 10 min. The residual molar ellipticities ([$\theta$]; deg cm$^2$ dmol$^{-1}$) of DIP-ALP I and DIP-Sendai at 280 nm were unchanged at pH 7.0 and 12.0 (DIP-ALP I, $-0.4 \times 10^4$ and $-0.7 \times 10^4$; DIP-Sendai, $-0.3 \times 10^4$ and $-0.5 \times 10^4$). In contrast, the near-UV CD spectra of DIP-NAT were different at pH 7.0 and 12.0: the residual molar ellipticities at pH 7.0 and 12.0 were $-1.7 \times 10^4$ and $0.2 \times 10^4$, respectively.

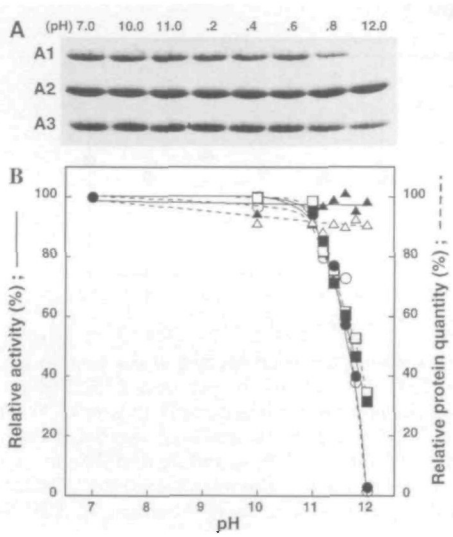


Fig. 1. **Effect of pH on the stability of subtilisins: ALP I, Sendai, and NAT.** Gels were run under reducing conditions and stained with Coomassie Brilliant Blue R-250. Subtilisins were incubated at pH 7.0, 10.0, 11.0, 11.2, 11.4, 11.6, 11.8, and 12.0 at 30°C for 10 min, and subsequently subjected to SDS-PAGE (A): A1, subtilisin ALP I; A2, subtilisin Sendai; A3, subtilisin NAT. The pH dependence of the relative activity and the relative protein quantity are indicated in B. The values shown are relative, compared with those at pH 7.0. Circles, triangles, and squares represent data for ALP I, Sendai, and NAT, respectively. The solid lines and black-filled symbols indicate the relative activity at pH 10.0 with Suc-Ala-Ala-Pro-Phe-MCA, at 30°C. The dashed lines and open symbols indicate the relative protein quantity, determined with NIH-Image 1.62 soft.

*Fluorescence Characteristics of DIP-Subtilisins*—To examine the structural changes on the molecular surface that occurred when the enzyme was in an alkaline environment, fluorescence emission spectra of the tryptophan residues of the DIP-subtilisins were recorded with excitation at 283 nm. The fluorescence intensities of DIP-Sendai at 342 nm were almost invariant between pH 7.0 and 12.0. In contrast, the fluorescence intensities of DIP-ALP I and DIP-NAT decreased as the pH increased to pH 12.0.

Because wavelength changes in fluorescence emission are excellent for monitoring the polarity of the tryptophan environment and hence are sensitive to protein conformation, the time courses of the maximum fluorescence wavelengths of DIP-subtilisins were recorded (Fig. 3, B1, B2, and B3). The fluorescence emission maximum of DIP-ALP I shifted remarkably, from 344 to 358 nm, over a period of only 10 min (Fig. 3B1). The maximum wavelengths of DIP-Sendai (Fig. 3B2) and DIP-NAT (Fig. 3B3), however, remained constant, from 348 to 350 nm over a period of 120 min and from 350 to 351 nm over a 10-min period, respectively.

*Conformational Restoration of Alkaline-Denatured DIP-ALP I and DIP-NAT at pH 7.0*—DIP-ALP I and DIP-NAT solutions were incubated at pH 12.0 (alkaline-denatured DIP-subtilisin) and then neutralized by dialysis against pH 7.0 buffer (alkaline-neutralized DIP-subtilisin). CD spectra and fluorescence emission spectra of native, alkaline-denatured, and alkaline-neutralized DIP-subtilisins, and measurement of the degradation times of alkaline-neutralized DIP-subtilisins confirmed that the native conformation was restored.

The CD and fluorescence spectra of alkaline-neutralized DIP-ALP I were the same as those of native DIP-ALP I (Figs. 4A and 5A). Neither native DIP-NAT nor alkaline-denatured DIP-NAT was similar to alkaline-neutralized DIP-NAT in either CD or fluorescence spectra (Figs. 4B and 5B). The alkaline-neutralized DIP-ALP I was not degraded with active ALP I over a period of 10 min (Fig.

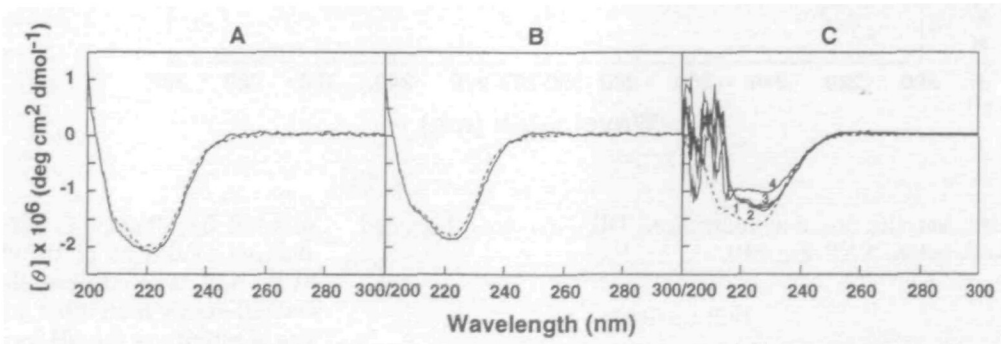


Fig. 2. **CD spectra of DIP-subtilisins: DIP-ALP I, DIP-Sendai, and DIP-NAT at pH 7.0 and 12.0.** The protein concentrations were kept at 7.5 mM for all experiments. The cell path length was 1 mm. The DIP-subtilisins were incubated at pH 7.0 and 12.0 at 30°C for 10 min (DIP-NAT was incubated for 60 min) and subsequently subjected to CD measurements. Panel A, DIP-ALP I; panel B, DIP-Sendai; panel C, DIP-NAT at both pHs, respectively. Dotted and solid lines represent the data at pH 7.0 and 12.0. In panel C, curves 1–4 represent the incubation times, 10, 20, 30, and 60 min, for DIP-NAT.

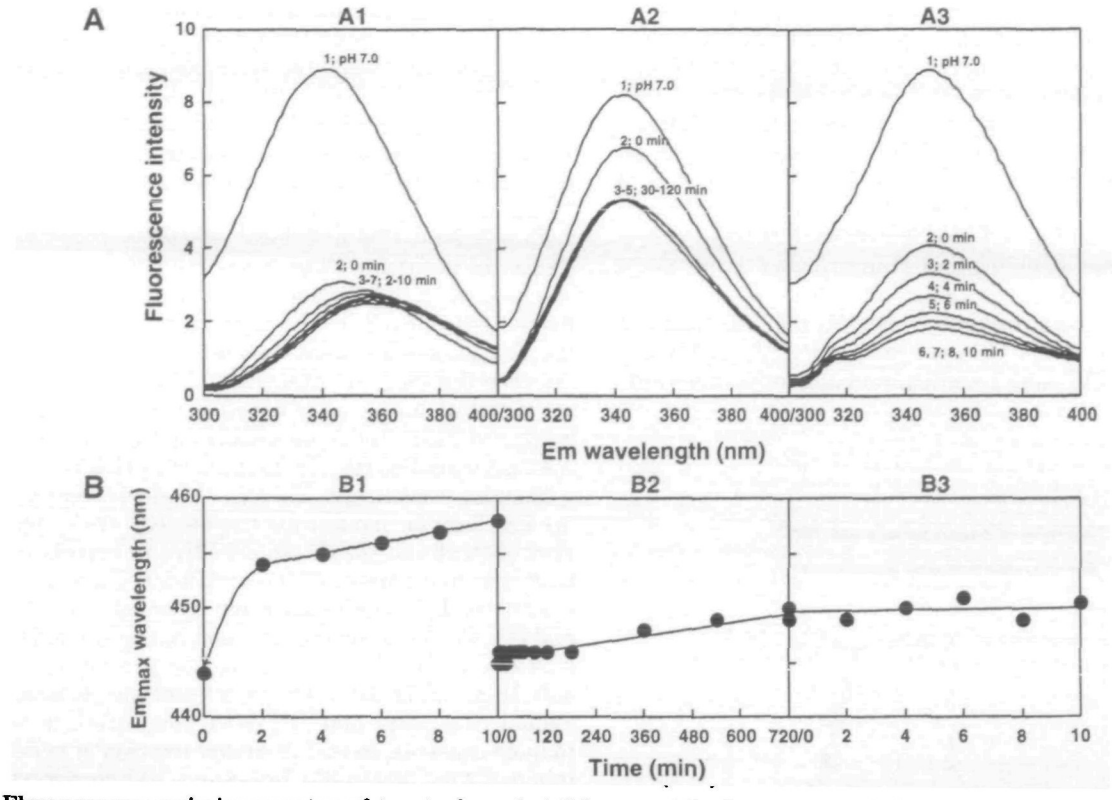


Fig. 3. **Fluorescence emission spectra of tryptophans in DIP-subtilisin: DIP-ALP I, DIP-Sendai, and DIP-NAT.** All experiments were performed with a protein concentration of 0.05 mg/ml. The excitation wavelength was 283 nm. The fluorescence emission wavelength was between 300 and 400 nm; the cell path length was 10 mm. DIP-subtilisin was incubated at pH 12.0 at 30°C and subsequently subjected to fluorescence measurements: A1, DIP-ALP I; A2, DIP-Sendai; A3, DIP-NAT. Curve 1 represents the fluorescence spectra of proteins incubated at pH 7.0 for 10 min. Curves 2–7 in A1 represent the fluorescence emission spectra of the protein incubated at pH 12.0 for 0, 2, 4, 6, 8, and 10 min, curves 2–5 in A2 show the emission spectra of the protein incubated at pH 12.0 for 0, 30, 60, and 120 min, and curves 2–7 in A3 are the emission spectra of the protein incubated at pH 12.0 for 0, 2, 4, 6, 8, and 10 min. The incubation time dependence of the variation in the fluorescence emission maximum is indicated in B: B1, DIP-ALP I; B2, DIP-Sendai; B3, DIP-NAT at pH 12.0.

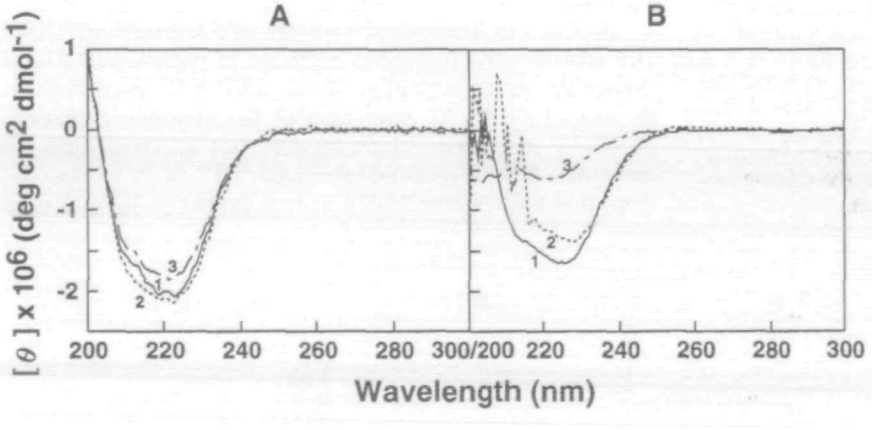


Fig. 4. **Differences in the CD spectra of native, alkaline-denatured, and alkaline-neutralized DIP-subtilisins.** The DIP-ALP I and DIP-NAT concentrations were kept at 7.5 mM for all experiments. The cell path length was 1 mm. Curves 1, 2, and 3 represent the spectra of native (pH 7.0), alkaline-denatured (pH 12.0), and alkaline-neutralized DIP-ALP I (A) and DIP-NAT (B).

6A), but the alkaline-neutralized DIP-NAT was degraded with active NAT (Fig. 6B).

## DISCUSSION

In the present study, we obtained initial data on the manner of inactivation of ALP I, a novel alkaline subtilisin secreted by alkalophilic *Bacillus* sp. NKS-21, under the extreme conditions of an alkaline environment. Although ALP I was isolated from alkalophilic *Bacillus*, it is classified differently from other subtilisins, due to both a gradual loss of activity as the pH increases to 11.0 and its predicted amino acid sequence (~60% identity with other alkaline subtilisins; ~57% identity with neutral subtilisins) (*4*).

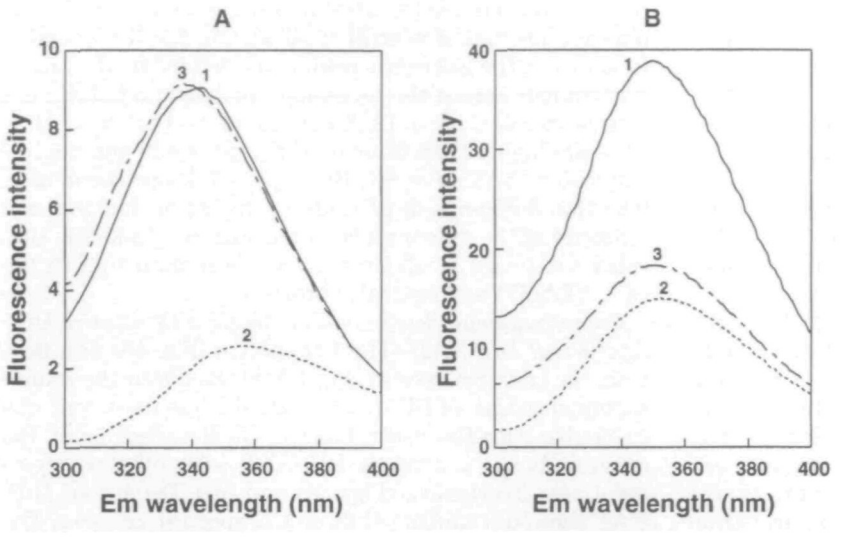


Fig. 5. **Differences in the fluorescence emission spectra of tryptophan in native, alkaline-denatured, and alkaline-neutralized DIP-subtilisins.** All experiments were performed with a protein concentration of 0.05 mg/ml. The excitation wavelength was 283 nm, and the emission wavelength was 300–400 nm. The cell path length was 10 mm. Curves 1, 2, and 3 represent the spectra of native (pH 7.0), alkaline-denatured (pH 12.0), and alkaline-neutralized DIP-ALP I (A) and DIP-NAT (B).

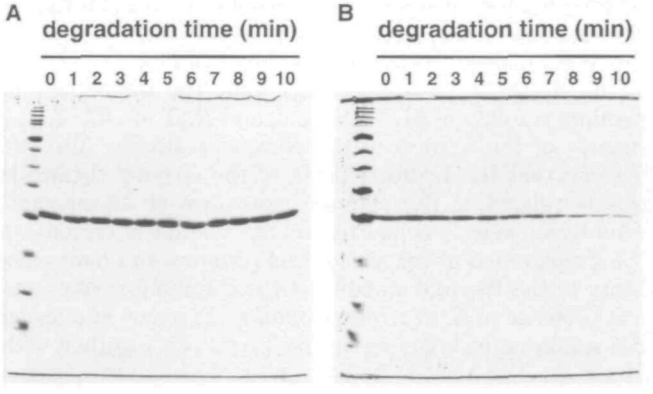


Fig. 6. **Degradation of alkaline-neutralized DIP-subtilisins with active subtilisin.** Gels were run under reducing conditions and stained with Coomassie Brilliant Blue R-250. Both alkaline-neutralized DIP-subtilisins, DIP-ALP I and DIP-NAT, were incubated at pH 7.0 and 30°C several times with active subtilisin ALP I and active subtilisin NAT, respectively, and subsequently analyzed by SDS–PAGE: A, alkaline-neutralized DIP-ALP I; B, alkaline-neutralized DIP-NAT. The enzyme/substrate ratio (alkaline-neutralized DIP-subtilisin) was 1/1,000.

*Effect of pH on the Stability of Subtilisins*—ALP I and NAT showed gradual decreases in activity as the pH increased to 11.0, this being accompanied by protein degradation (Fig. 1). Both the protein band and the activity of ALP I completely disappeared at pH 12.0, but both the relative activity and relative protein quantity of NAT were about 30%. This is also supported by the fact that the specific activity of ALP I for the protein substrate casein ($250 \times 10^{-3}$ katal/kg) (*3*) is higher than that of NAT ($41 \times 10^{-3}$ katal/kg) (*6*) at pH 10.0.

On the other hand, neither active-site-modified ALP I nor active-site-modified NAT (DIP-ALP I and DIP-NAT) was degraded in an alkaline environment (see "RESULTS"). Comparison of the degradation behavior of native and DIP-subtilisins indicated that inactivation of ALP I and NAT occurs through autolytic cleavage in an alkaline environment.

*Conformational Stability of Subtilisins: Higher Order Structure*—Thus far, the conformational change of inhibited neutral subtilisins (Dip-subtilisin Carlsberg and Dip-subtilisin BPN′) has been examined as a function of pH over the range of 4–12 (*16*). However, the alkaline stability of alkaline subtilisin has never been determined. We investigated the conformational change of diisopropylphosphoryl alkaline subtilisins, DIP-ALP I, DIP-Sendai, and DIP-NAT, using spectroscopic methods. In the far-UV region, CD spectral characteristics are determined primarily by the polypeptide backbone conformation, especially by its secondary structure (*21*). The far-UV CD spectra of DIP-ALP I and DIP-Sendai showed no apparent changes between pH 7.0 and 12.0 (Fig. 2, A and B), suggesting that the secondary structures of both subtilisins are not affected by an alkaline environment. The far-UV CD spectrum of DIP-NAT, in contrast, was sensitive to alkaline pH (from pH 7.0 to 12.0; Fig. 2C), suggesting that the alkaline pH induced changes in the secondary structure of DIP-NAT.

The near-UV CD spectral region, particularly at 280 nm, reflects the tertiary structure of proteins, especially the change in the arrangement of aromatic amino acid residues, namely, phenylalanine, tryptophan, and tyrosine, in the molecule (*23–25*). The tertiary structure, visualized as the negative residual molar ellipticity at 280 nm, was retained in DIP-ALP I and DIP-Sendai between pH 7.0 and 12.0, suggesting that the higher order structures of the two subtilisins from alkalophilic *Bacillus* were retained in the alkaline environment. In contrast, the negative residual molar ellipticity at 280 nm of DIP-NAT shifted to positive with a change in pH from 7.0 to 12.0. In conclusion, in an alkaline environment, the tertiary structures of the two alkaline subtilisins remain intact, whereas the tertiary structure of neutral subtilisin decays.

*Conformational Stability of Subtilisins: Molecular Surface Structure*—The fluorescence emission spectra of tryptophan residues in a protein reflect the ionization of external tyrosine residues (*16, 18, 26*), and the accessibility of tryptophan residues to the solvent (*22*). In our study, the fluorescence intensities of DIP-ALP I and DIP-NAT decreased rapidly at pH 12.0. Because the decrease in tryptophan fluorescence emission is due to energy transfer from tyrosyl to tryptophyl residues (*16, 18, 26*), this decrease in

fluorescence intensity indicates that in both DIP-subtilisins, the ionization of exposed tyrosine is complete at a highly alkaline pH before conformational changes occur. This hypothesis is supported by the finding that tyrosyl residues of subtilisin BPN′, classified as a neutral subtilisin, are located at or near the surface of the enzyme in an X-ray model (*27–29*).

The fluorescence emission maximum of alkaline-denatured DIP-ALP I shifted from 344 to 358 nm (Fig. 3B1), implying that the previously buried tryptophyl residues were now exposed to the solvent. The maximum wavelengths of DIP-Sendai (Fig. 3B2) and DIP-NAT (Fig. 3B3) were red-shifted more slowly than that of DIP-ALP I, indicating that the state of tryptophyl residues on or near the surface had not changed (*30–33*). Furthermore, the constancy of the fluorescence intensity of DIP-Sendai (Fig. 3A2) leads to the conclusion that the tyrosyl residues are not ionized. Thus, the surface regions containing buried tyrosyl residues, located on or near the surface, are structurally stable. On the other hand, it could be seen that with the lapse of time, the maximum fluorescence intensity of DIP-NAT decreased, but the emission spectrum at 310 nm appeared with the elapse of time (Fig. 3A3) due to ionization of the exposed tyrosyl residues (*16*, *18*, *26*); thus, the surface regions containing buried tyrosyl residues move as these residues are exposed to the solvent.

Assuming that the structure of subtilisin consists of a surface layer and a core region, the fluorescence emission spectral results suggest that the molecular surface structure of DIP-Sendai is more rigid than those of DIP-ALP I and DIP-NAT due to the formation of a hydrophobic surface layer containing exposed tryptophan and buried tyrosine residues. This is also supported by the fact that the surface region of Savinase (*34*, *35*) and PB92 protease (PB92) (*36*), classified as alkaline subtilisins, is more hydrophobic than that of neutral subtilisins in the X-ray model. In addition, the isoelectric points (p*I*), defined by the amino acid composition at the molecular surface, of alkaline subtilisins (Sendai, p*I* > 12; Savinase, p*I* = 11.2; 221, p*I* = 11.0) are higher than those of ALP I (p*I* = 8.2) and neutral subtilisins (NAT, p*I* = 8.7; BPN′, p*I* = 7.8; Carlsberg, p*I* = 9.8); this difference in p*I* leads to the conclusion that the tendency of the amino acids on the surface of alkaline subtilisins to ionize at alkaline pH is lower than that in the cases of ALP I and neutral subtilisins.

*Conformational Restoration of Alkaline-Denatured DIP-ALP I and DIP-NAT*—The CD spectra (Fig. 4A) and fluorescence emission spectra (Fig. 5A) showed that the conformational change of DIP-ALP I with alkaline treatment was reversible with the neutralization. On the other hand, the conformational change of DIP-NAT with alkaline treatment was irreversible (Figs. 4B and 5B). Denatured DIP-NAT showed a random shift on CD spectral analyses. The conformation of neutral subtilisins such as NAT is irreversible at high pH (*16*), but alkaline-neutralized DIP-ALP I reverts to a native and stable conformation in the presence of active ALP I (Fig. 6A). This implies that the structure of the ALP I molecule possesses alkaline stability.

The foregoing results indicate that the differences in alkaline stability of ALP I, Sendai, and NAT are due to the rigidity of the surface of the enzyme molecule. Also, we assume that the hydrophobicity of the enzyme surface is closely related to the alkaline resistance of the enzyme. This hypothesis is supported by the results of studies on the contribution of surface-located residues and hydrophobicity to the thermal stability of the thermolysin-like neutral protease of *B. stearothermophilus*. Previous studies on the autolysis of broad-specificity proteases, together with

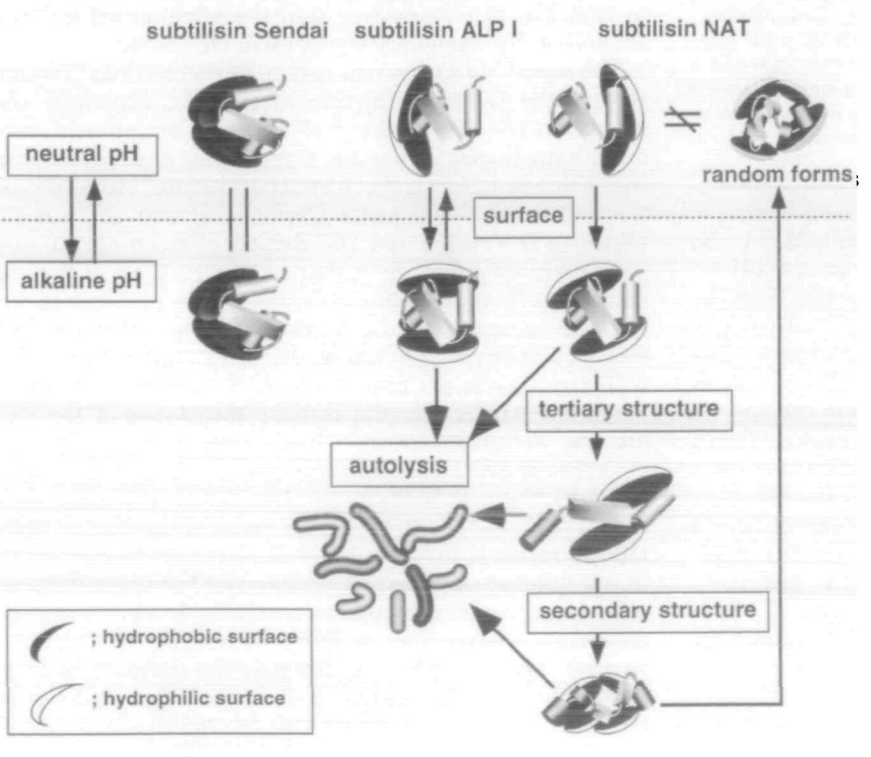


Fig. 7. **Model of the alkaline-stability mechanism of subtilisins.** Black and white shell forms depict the more hydrophobic and hydrophilic surface regions, respectively. Pillars represent the formation of the secondary structure. Subtilisin Sendai exhibits molecular structural stability in the pH region of 7.0–12.0, as shown by two vertical bars in this model. This stability is due to the presence of the more hydrophobic surface region. Subtilisin ALP I has an alkaline-stable molecular core, similar to subtilisin Sendai, and a flexible surface region. Its surface region shows a reversible reaction between neutral pH and alkaline pH, as shown by paired arrows in this model. The direct cause of inactivation is exposure of the fragile region in the presence of active subtilisin ALP I due to partial unfolding, which is limited to the molecular surface. Subtilisin NAT does not exhibit enzymatic stability or structural stability in an alkaline environment. Its conformational change on alkaline treatment is an irreversible reaction, as shown by the one-way downstream arrow in this model. In an alkaline environment, the unfolding of subtilisin NAT is due to the disintegration at successive surface regions, involving both the tertiary and secondary structures, which occurs parallel to autolysis. In addition, the unfolded subtilisin NAT does not regain the native subtilisin NAT conformation.

observations concerning the structural changes during protein unfolding, suggest that the local unfolding processes that lead to autolysis involve protein surface layer regions (*9*, *10*, *37*, *38*). The thermal stability of the *Bacillus* neutral protease was increased considerably when Arg, Lys, or bulky hydrophobic amino acids were introduced into the protein surface layer. The tightly packed hydrophobic layer separates the hydrophilic protein surface and the solvent from the rest of the protein (*39*).

In conclusion, we describe a model for the alkaline-stability mechanism of serine proteases of alkalophilic *Bacillus* (Fig. 7). The more hydrophobic surface layer of Sendai acts as a separation layer between the protein and the solvent, and forms a tight interaction between the surface and the hydrophobic core region. ALP I and NAT possess a relatively low degree of hydrophobic surface structure, and alkaline treatment results in ionization of the molecular surface that provokes partial unfolding, followed by the exposure of a fragile region in the presence of the active enzyme that leads to autolysis. Because of the unique and essential properties of ALP I, which in these studies exhibited molecular structural stability but not functional stability in an alkaline environment, the alkaline stability of this novel alkaline subtilisin has been the focus of intense research interest.

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