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Production and Further Characterization of an Alkaline Elastase Produced by Alkalophilic *Bacillus* Strain Ya-B

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The characteristics of the obligate alkalophilic *Bacillus* sp. strain Ya-B, which produces alkaline elastase extracellularly, were examined. This strain grew at pH 7.0 only in the presence of 1% or more NaCl. Its fatty acid distribution pattern was similar to that of other *Bacillus* species in which iso-C_{15} and anteiso-C_{15} were the most abundant fatty acids. About 120 mg of enzyme was recovered from 1 liter of culture broth in a medium (pH 10.1) containing mainly glucose, soymeal, and glycerol. The antiserum against this enzyme did not recognize microbial proteinases, such as subtilisins, but reacted with proteinase C, which was purified from commercial pronase. Chemical modification studies revealed that certain histidine and tyrosine residues might be involved in the enzyme activity. This enzyme underwent a partial unfolding at pHs higher than 12.0, as indicated by the circular dichroism study.

Studies of alkalophilic microorganisms have led to the discovery of many new types of extracellular enzymes which are characterized by optimal pHs on the alkaline side (11). Horikoshi (9) reported that alkalophilic *Bacillus* sp. strain 221 produced an alkaline proteinase with an optimal pH of 11.5. Two other alkaline proteinases were isolated from the culture broth of alkalophilic *Bacillus* sp. strains AB42 and PB12 by Aunstrup et al. (3). These two proteinases had optimal pHs ranging from 9.0 to 12.0. Tobe et al. (24) reported that an alkaline proteinase of alkalophilic *Bacillus* sp. strain AJ3368 had an optimal pH of 11.5 and a molecular mass of 17,000 daltons. The most unique characteristic of these extracellular alkaline proteinases of alkalophilic *Bacillus* species was that their optimal pHs were much higher than those of the alkaline proteinases produced by common *Bacillus* species. Recently, we conducted a program screening for alkalophilic microorganisms which were able to hydrolyze elastin. The isolated alkalophilic *Bacillus* sp. strain Ya-B secreted a new type of alkaline proteinase, named alkaline elastase, which showed an unusual elastin hydrolysis ability (25–28). Unlike other alkaline proteinases, this enzyme showed a high specificity for alanine residues and rapid hydrolysis of elastase-specific substrates, such as succinyl-Ala_3-*p*-nitroanilide and succinyl-Ala-Pro-Ala-*p*-nitroanilide (27). We report the production and some properties of alkaline elastase and the characterization of the enzyme-producing strain, alkalophilic *Bacillus* sp. strain Ya-B.

MATERIALS AND METHODS

Materials. Elastin, elastin-orcein, subtilisin BPN′, and subtilisin Carlsberg were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tetranitromethane, rose bengal, chloramine T, phenylglyoxal, polypeptone, and methylene blue were purchased from Wako Pure Chemical Industries (Osaka, Japan). Standard fatty acid methyl esters were purchased from Alltech Associates, Inc., Applied Science Div. (State College, Pa.). Soymeal was purchased from a local market. Pronase was purchased from Kaken Chemical Co. (Tokyo, Japan). Alkaline proteinase C was purified from pronase by carboxymethyl cellulose column chromatography as described by Narahashi et al. (20). Porcine pancreatic elastase was purchased from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany). Elastase of *Pseudomonas aeruginosa* was kindly provided by K. Morihara of Toho Pharmaceutical Industry Co. (Kyoto, Japan), alkaline proteinase of alkalophilic *Bacillus* sp. strain 221 was provided by K. Horikoshi of the Institute of Physical and Chemical Research (Tokyo, Japan), and alkaline proteinase of *Streptomyces* sp. was provided by T. Yamamoto of Osaka City University (Osaka, Japan).

Isolation of elastolytic enzyme-producing alkalophilic bacteria. Soil specimens (1 g) collected around the Tokyo area were suspended in 10 ml of sterilized water. After appropriate dilution, the soil extracts were spread on the isolation medium agar plate and incubated at 37°C for 24 to 48 h. Strains which hydrolyzed elastin to make clear zones around the colonies on the elastin agar plate were selected. The isolation medium (medium A) contained glucose (10 g), elastin (5 g), yeast extract (5 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and Na_2CO_3 (10 g) in 1 liter of distilled water. This medium was solidified by the addition of 1.5% agar.

Characterization of bacteria. The morphological properties and taxonomic characteristics of the bacteria were investigated by conventional methods (5, 7). Unless stated otherwise, the media used for identification were supplemented with 1% Na_2CO_3 to bring the pH to 10.1.

The G+C content was estimated from the melting point of chromosomal DNA as described by Marmur and Doty (17). DNA was prepared by the method of Saito and Miura (23). The DNA preparation was dissolved in dilute saline-citrate (15 mM NaCl plus 1.5 mM trisodium citrate). The melting temperature of the DNA was monitored by measuring the increase in the A_{260} during heating.

Fatty acid analysis. *Bacillus* sp. strain Ya-B was cultivated overnight in liquid medium B, which was the same as medium A, except that the elastin in medium A was replaced by polypeptone. Log-phase cells were harvested by centrifugation, washed free of medium with 0.15 M NaCl solution,

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and lyophilized to a constant weight. Lyophilized cells (20 mg) were used for fatty acid analysis by the method described by Ikemoto et al. (12) with 5% (wt/vol) HCl-methanol as the transesterification agent. The fatty acid methyl esters were analyzed on a Shimadzu model GC-6APF gas chromatograph equipped with a hydrogen flame ionization detector. A glass column (3 mm by 5 m) packed with 10% diethylene glycol succinate coated on 60/80-mesh, acid-washed Chromosorb W (Wako Pure Chemical Industries) was used. The injector and detector temperatures were 205°C, and the column temperature was 185°C. Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of standard fatty acid methyl esters. *Bacillus subtilis* ATCC 6057 was used as a reference strain. This strain was cultivated in nutrient broth, and the cellular fatty acids were extracted and analyzed as described above.

Enzyme assays. Elastolytic and caseinolytic activities with elastin-orcein and casein as substrates, respectively, were determined by previously described methods (28).

Enzyme production. Strain Ya-B was grown for 24 h at 37°C in liquid medium B. The culture was inoculated, to a final concentration of 2%, into 2.5 liters of a medium containing 2% glucose, 2% soymeal, 2% glycerol, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% Na_2CO_3 in a 5-liter mini-jar fermentor (Mizuwa Rikagaku Kogyo Co., Osaka, Japan). The initial cell density was about 6.8×10^8 CFU/ml. Fermentation was carried out at 37°C with 300-rpm agitation and 0.5 vol/vol per min aeration. After 20 h, the culture was chilled rapidly to 4°C, and the pH was adjusted to 7.0 with acetic acid. The cells were then removed by centrifugation.

Enzyme purification. The enzyme was purified by a previously described procedure (28), with some modifications. All operations were carried out at 4°C. Culture fluid (10 liters) was concentrated with the Pellicon Ultrafiltration System (Millipore Corp., Bedford, Mass.). The molecular weight cutoff value of the membrane was 10,000. Almost half of the enzyme activity remained in the filtrate, which was ultrafiltrated again to increase the recovery. The concentrate was fractionated by ammonium sulfate precipitation (between 35 and 70% saturation). The resulting precipitate was dissolved in a minimal volume of 50 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM $CaCl_2$ and dialyzed overnight against the same buffer. At a 70% saturation of ammonium sulfate, the enzyme floated on the surface of the solution and formed an easily collected cake after centrifugation. The dialyzed enzyme solution was concentrated by ultrafiltration and sequentially purified on a DEAE–Sephadex A-25 column, a carboxymethyl–Sephadex C-50 column, and a Toyopearl HW50S column as described previously (28). The purified enzyme was homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel electrofocusing.

Production of antisera. Antisera were raised against the purified alkaline elastase in rabbits by intramuscular injection of 1 mg of alkaline elastase in 0.5 ml of 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and mixed with an equal volume of complete Freund adjuvant. Two boosts at 10-day intervals with 1 mg of enzyme prepared in incomplete Freund adjuvant were given. Test bleeds were obtained at weekly intervals and analyzed for the presence of antibodies by Ouchterlony immunodiffusion against purified alkaline elastase.

Immunodiffusion. Ouchterlony double-immunodiffusion studies were performed in 1.2% agarose gels prepared in 20 mM Tris hydrochloride buffer (pH 7.4) containing 0.9% NaCl. Protein samples and antisera were applied to each well, and diffusion was allowed to proceed for 48 h at room temperature. Gels were stained with Coomassie brilliant blue after immersion in 0.9% NaCl for 2 days.

Circular dichroism. A Jasco ORD/UV-5 instrument with a Sproul Scientific SS-10 CD modification was used for circular dichroism measurements. A 0.1-cm-path-length cell was used, and the temperature of the cell compartment was 25°C. A mean residue weight of 99 was used to calculate molar ellipticities (θ, in degrees · square centimeters per decimole).

Chemical modification. Alkaline elastase (20 μg) was incubated with each reagent in 0.5 ml of buffer under the following reaction conditions: tetranitromethane, 2 mM in 0.1 M Tris hydrochloride buffer (pH 8.0) at 25°C for 2 h; phenylglyoxal, 0.1 M in 0.2 M ethylmorpholine buffer (pH 8.0) at 25°C for 1 h in the dark; and chloramine T, 20 mM in 0.1 M Tris hydrochloride buffer (pH 8.0) at 25°C for 30 min. Photooxidation was carried out by irradiating 0.5-ml samples containing 20 μg of enzyme and 0.01% methylene blue or 0.001% rose bengal in 0.1 M phosphate buffer (pH 6.8) at 4°C with the dye added in the dark. Illumination was provided by a single 300-W spotlight at a distance of 5 to 8 cm. The sample was rapidly stirred during irradiation to ensure an adequate oxygen supply. After 30 min of irradiation, the modification reagent was removed by passing the reaction mixture through a Sephadex G-25 column (1 by 30 cm) after incubation. The residual caseinolytic activity and the amino acid composition were then determined. The amino acid composition was determined by the method of Liu and Chang (16); 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indol was used as the hydrolysis agent. Hydrolysis was carried out in a sealed, evacuated glass tube at 110°C for 24 h.

RESULTS

Characterization of strain Ya-B. Strain Ya-B was isolated from soil by the method described in Materials and Methods. This strain was gram positive, rod shaped (0.6 to 0.8 by 2.0 to 10.0 μm), obligately aerobic, and motile; cells had rounded ends and were peritrichous. Cells appeared singly or in pairs (occasionally in short chains or filaments) and formed elliptical endospores located terminally to subterminally. The sporangia were not definitely swollen or acid fast. Colonies on nutrient agar medium were circular, flat, entire or ciliate, glistening, butyraceous, and yellow.

Strain Ya-B grew well in the alkaline nutrient broth and on the alkaline nutrient agar. The pH range for growth in the nutrient broth was between 8.0 and 10.5 (optimal, 9.5), and the temperature range was between 14 and 40°C (optimal, 32°C). The strain was positive for the following tests: cytochrome oxidase test; catalase test; hydrolysis of starch, casein, and gelatin; growth in 10% NaCl; growth on Christensen citrate agar; acid production from triple sugar iron medium on slant cultures; and gas production from glucose. The strain was negative for the following tests: Voges-Proskauer test; indole test; LV test; Hugh-Leifson test; reduction of nitrate; reduction of methylene blue; deamination of phenylalanine and tyrosine; formation of dihydroacetone; growth on MacConkey agar; growth in 0.001% lysozyme; growth on Simmons citrate agar; H_2S formation in triple sugar iron medium; and acid production from triple sugar iron medium in stab cultures. This strain utilized and produced acid from glucose, arabinose, xylose, mannitol,

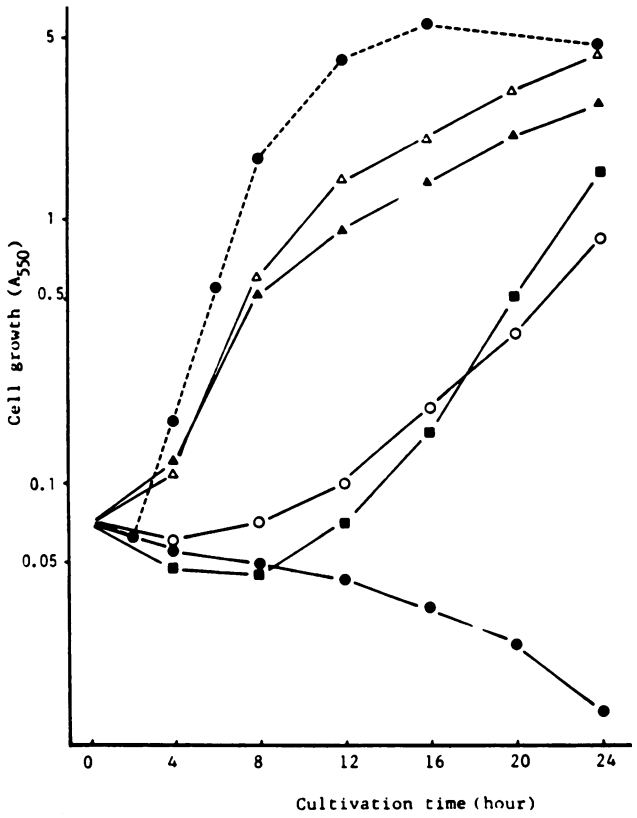

FIG. 1. Effect of NaCl on the growth of alkalophilic *Bacillus* sp. strain Ya-B. Strain Ya-B was grown in liquid medium B (pH 10.1) overnight at 37°C. The culture was inoculated, to a final concentration of 1%, into 500-ml shaking flasks each containing 100 ml of medium B without 1% Na_2CO_3 (pH 7.0) but with increasing concentrations of NaCl: 0% (●——●), 1% (○), 3% (▲), 5% (△), and 7% (■). ●-----●, Control, measuring cell growth in medium B (pH 10.1). Cell growth was determined by measuring the optical density at 550 nm.

lactose, sucrose, salicin, trehalose, galactose, α-methylglucoside, cellobiose, starch, inositol, dextrin, melibiose, and sorbose but not from glycerol, sorbitol, dulcitol, inulin, erythritol, or adonitol.

In general, the characteristics of strain Ya-B indicated that it was closely related to *Bacillus firmus*. The G+C content of the DNA of strain Ya-B was estimated to be 42 mol%; this value was very close to that of *B. firmus* ATCC 14575^T (41.4 mol%). However, strain Ya-B differs from *B. firmus* in several properties, such as the production of acid from melibiose and salicin, the production of gas from glucose, utilization of arabinose and xylose and, most of all, alkalophilicity.

A unique feature of the alkalophilic bacteria is that they require sodium ions for growth. Strain Ya-B did not grow at pH 7.0, but in the presence of as little as 1% NaCl it started to grow, although at a rate slower than that at pH 10.1 (Fig. 1). The optimal concentration of NaCl for growth at pH 7.0 was 5%. Strain Ya-B did not grow in the presence of 5% KCl at pH 7.0 (data not shown).

Fatty acid composition of strain Ya-B. The cellular fatty acid composition of strain Ya-B was analyzed by gas chromatography. This strain produced nine major fatty acids, six branched (iso-C_{14}, iso-C_{15}, iso-C_{16}, iso-C_{17}, anteiso-C_{15}, and anteiso-C_{17}) and three normal (n-C_{14}, n-C_{15}, and n-C_{16}). Among these fatty acids, iso-C_{15} and anteiso-C_{15} were the most abundant ones, making up over 60% of the total fatty acids. The fatty acid distribution pattern was similar to those of other *Bacillus* species reported by Kaneda (15) and to those of other alkalophilic *Bacillus* strains reported by Clejan et al. (6).

Production of alkaline elastase. Table 1 summarizes a preliminary assessment of the effect of medium nutrients on the production of alkaline elastase. Medium no. 1 was the screening medium. Soymeal proved to be a very good nitrogen source for enzyme production (media no. 4, 6, and 7). Soybean flakes were extracted with hot ethanol, and the yellowish powder obtained was referred to as the hot ethanol extract of soybean. The addition of a 0.2% concentration of this extract to the screening medium increased enzyme productivity about eight times (medium no. 5). The best media were no. 6 and no. 7, which contained glucose, soymeal, yeast extract, and the hot ethanol extract of soybean (no. 6) or glycerol (no. 7). A time course of the production of alkaline elastase in medium no. 7 is shown in Fig. 2. Alkaline elastase was secreted at the late logarithmic phase, followed by the pH change in the broth from 10.1 to 8.5. Under these cultivation conditions, about 120 mg of alkaline elastase could be obtained from 1 liter of culture broth by the purification procedure described in Materials and Methods.

Immunodiffusion. The anti-alkaline elastase antiserum did not recognize subtilisin BPN', subtilisin Carlsberg, porcine pancreatic elastase, *P. aeruginosa* elastase, alkaline proteinase of alkalophilic *Bacillus* sp. strain 221 (9), or alkaline proteinase of *Streptomyces* sp. (19), but it recognized pronase, which is a mixture of several proteinases produced by *Streptomyces griseus*. Pronase was further purified by carboxymethyl cellulose column chromatography as described by Narahashi et al. (20). The anti-alkaline elastase antiserum reacted with alkaline proteinase C, which was one of the serine proteinase components in pronase (Fig. 3). The crossing of the immunoprecipitin lines showed that these two enzymes were not identical; however, they might share some common antigenic determinants, because the antibody raised against alkaline elastase did react with alkaline proteinase C.

pH dependency of the molar ellipticity of alkaline elastase. Circular dichroism was used to analyze the effect of pH on the conformation of alkaline elastase. It revealed that the spectra of this protein change remarkably at pHs above 12.0. The pH dependency of the molar ellipticity at 222 nm, which reflects the helix conformation, is shown in Fig. 4. This result might reflect a partial unfolding of the enzyme structure at pHs higher than 12.0.

TABLE 1. Effect of medium composition on the production of alkaline elastase[a]

Addition	Concn (%) of addition in medium no.:						
	1	2	3	4	5	6	7
Glucose	1	1		2	1	2	2
Elastin		1					
Soymeal			2	2		2	2
Glycerol							2
Polypeptone	0.5		0.5		0.5		
Hot-ethanol-extracted soybean					0.2	0.2	
Elastolytic activity (U/ml)	6	4	4	45	50	83	86

[a] The basal medium contained yeast extract (0.5%), KH_2PO_4 (0.1%), $MgSO_4 7H_2O$ (0.02%), and Na_2CO_3 (1%).

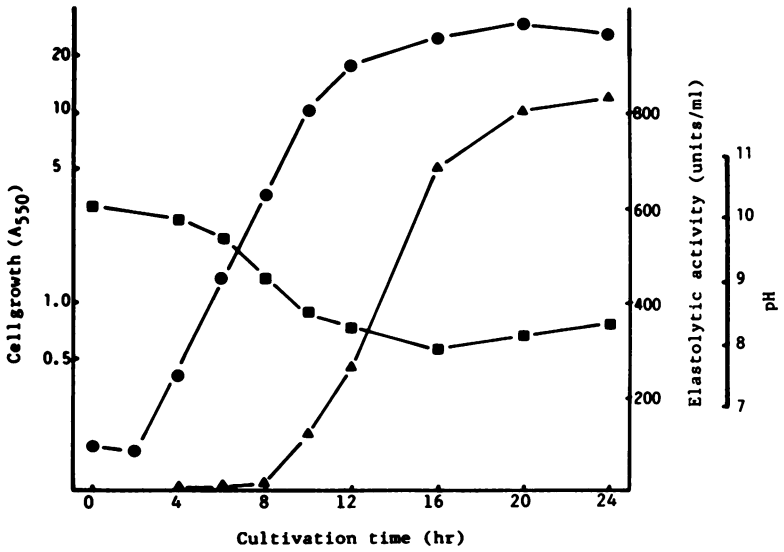

FIG. 2. Growth and alkaline elastase production in cultures of alkalophilic *Bacillus* sp. strain Ya-B. Culturing was carried out as described in Materials and Methods. Cell growth (●), pH of the broth (■), and alkaline elastase activity, determined with elastin-orcein as the substrate (▲), were determined.

Effect of chemical modification reagents. The results of chemical modification studies are summarized in Table 2. In the presence of 0.001% rose bengal or 0.01% methylene blue, photooxidation inhibited the activity of the enzyme completely. The amino acid composition revealed that three of the eight histidine residues in the enzyme were oxidized. Tetranitromethane, the mild tyrosine-specific modification agent, modified one or two of the four tyrosine residues in the enzyme and inhibited 65% of the enzyme activity. Chloramine T also modified two tyrosine residues at a concentration of 20 mM and caused the enzyme to lose 90% activity. Arginine residues might not be involved in the activity of the enzyme, because the enzyme activity was not significantly inhibited after the modification of six of eight arginine residues by phenylglyoxal.

DISCUSSION

Alkalophilic microorganisms are widely distributed in nature. The most abundant alkalophilic bacteria isolated so far belong to the genus *Bacillus* (10). Alkalophilic *Bacillus* sp. strain Ya-B differs from *Bacillus alcalophilus* (5) in characteristics such as growth in 5% NaCl, methylene blue reduction, acid formation from glycerin and sorbitol, etc. It differs from *B. alcalophilus* subsp. *halodurans* (4) in nitrate and methylene blue reduction. It differs from the alkalophilic *Bacillus* species isolated by Horikoshi and Akiba (10) mainly in nitrate reduction and sporangium swelling. Although most of the characteristics are quite similar between alkalophilic *Bacillus* sp. strain Ya-B and *B. firmus*, the alkalophilicity is considered to be the major difference between these two strains. The difference between alkalophilic and neutrophilic bacteria must be in the cell surface. The cell wall composition of alkalophilic *Bacillus* strains was investigated by Aono

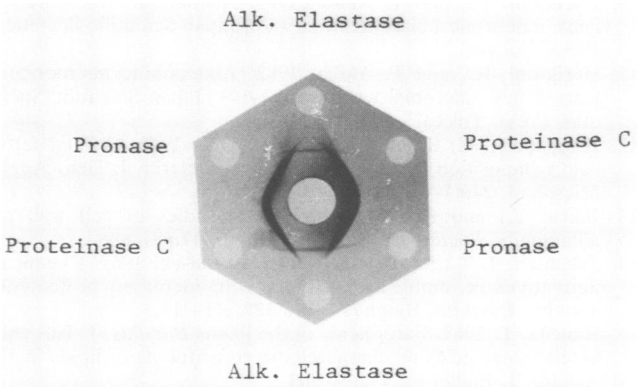

FIG. 3. Double diffusion of alkaline (Alk.) elastase, pronase, and proteinase C against rabbit antibodies to alkaline elastase. Outer wells contained approximately 2 μg of alkaline elastase or 5 μg each of pronase or proteinase C. For details, see Materials and Methods.

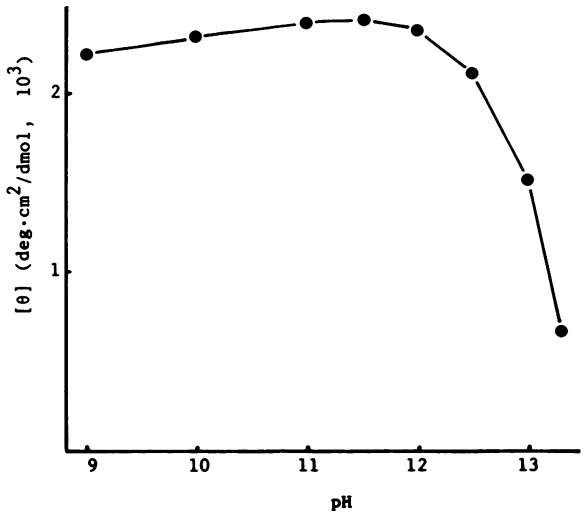

FIG. 4. Effect of pH on the molar ellipticity at 222 nm of alkaline elastase. Alkaline elastase was diluted into the following buffer systems to a final concentration of 1 mg/ml just before scanning: Tris hydrochloride (pH 9.0), Na_2CO_3-$NaHCO_3$ (pH 10.0 to 11.5), Na_2HPO_4-NaOH (pH 11.5 to 13.0), and 0.2 N NaOH (pH 13.0). The molar ellipticities at 222 nm were determined. deg, Degrees.

TABLE 2. Effect of various reagents on the activity and amino acid composition of alkaline elastase

Reagent (concn)	Residual activity (%)[a]	Modified amino acid[b]
Methylene blue (0.01%)	0	Histidine (3)
Rose bengal (0.001%)	0	Histidine (3)
Chloramine T (20 mM)	10	Tyrosine (2)
Tetranitromethane (2 mM)	35	Tyrosine (1 to 2)
Phenylglyoxal (100 mM)	100	Arginine (6)

[a] Residual activity was assayed with casein as the substrate.
[b] Values in parentheses indicate the number of modified amino acids. There are eight histidine and four tyrosine residues in one molecule of alkaline elastase (23).

and Horikoshi (1) and by Ikura and Horikoshi (13). Both found that the amounts of neutral sugar and uronic acid in the cell wall were different from those in common *Bacillus* species, whereas the peptidoglycan composition was the same (2). Clejan et al. (6) reported that the membranes of the obligated *Bacillus* species contained a high concentration of branched-chain and unsaturated fatty acids comparable to those from the membranes of facultatively alkalophilic *Bacillus* species. However, the fatty acid composition of strain Ya-B, an obligate alkalophile, is quite similar to those of the facultatively alkalophilic strains OF1 and OF4 (6, 8). They all contain very few unsaturated fatty acids, such as n-$C_{16:1}$.

Alkaline elastase produced extracellularly by strain Ya-B possessed two main characteristics of the elastin-hydrolyzing enzymes: high elastin-binding ability and substrate specificity for small aliphatic amino acids (25, 27). The amino-terminal sequence of this enzyme showed a high level of homology with those of subtilisins (25). However, these enzymes differed from each other in several important characteristics, such as substrate specificity, reaction pH, elastin-binding ability, etc. The immunological cross-reactivity of this enzyme was also different from those of subtilisins, as shown in the immunodiffusion study. Interestingly, the antiserum against this enzyme reacted with proteinase C, one of the alkaline proteinases purified from pronase (19). The double-immunodiffusion pattern indicated that these two enzymes share some common antigenic determinants (Fig. 3).

From the chemical modification results, it could be assumed that some histidine and tyrosine residues of the enzyme are important for enzyme activity. Since this enzyme is a serine proteinase, it is quite natural that histidine is involved in catalytic activity. Ohtsuki et al. showed that *N*-bromosuccinimide inactivated subtilisin BPN′, presumably by oxidation of single tyrosine and methionine residues (21). Another report pointed out that 5 of 10 tyrosine residues in subtilisin BPN′ were fully nitrated and that 2 were partially nitrated by tetranitromethane without affecting the enzyme activity (22). In the case of subtilisin Carlsberg, the enzyme activity even increased six- to sevenfold after 9 of the 13 tyrosine residues were modified by tetranitromethane (14). Alkalophilic *Bacillus* sp. strain 221 alkaline protease is quite similar to our enzyme in some properties, such as reaction pH, elastin hydrolysis ability, pI, etc. (9). When seven of the nine tyrosine residues of this alkaline protease were modified by tetranitromethane, the enzyme activity was not affected at all (18). The response of alkaline elastase to tyrosine modification was totally different from those of the enzymes mentioned above. The enzyme activity was abolished when one or two tyrosine residues were modified by tetranitromethane or chloramine T. These observations strongly suggest that a specific tyrosine residue may play an important role in enzyme catalysis. Further investigations are required to determine whether this specific tyrosine residue is involved directly in enzyme catalysis or indirectly in substrate binding, enzyme stability, etc.

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