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Purification and Characterization of a Thermostable Alkaline Protease from Alkalophilic *Thermoactinomyces* sp. HS682

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Protease secreted into the culture medium by alkalophilic *Thermoactinomyces* sp. HS682 was purified to an electrophoretically homogeneous state through only two chromatograhies using Butyl-Toyopearl 650M and SP-Toyopearl 650S columns. The purified enzyme has an apparent relative molecular mass of 25,000 according to gel filtration on a Sephadex G-75 column and SDS-PAGE and an isoelectric point above 11.0.

Its proteolytic activity was inhibited by active-site inhibitors of serine protease, DFP and PMSF, and metal ions, Cu^{2+} and Hg^{2+}. The enzyme was stable toward some detergents, sodium perborate, sodium triphosphate, sodium-*n*-dodecylbenzenesulfonate, and sodium dodecyl sulfate, at a concentration of 0.1% and pH 11.5 and 37°C for 60 min. The optimum pH was pH 11.5—13.0 at 37°C and the optimum temperature was 70°C at pH 11.5. Calcium divalent cation raised the pH and heat stabilities of the enzyme. In the presence of 5 mM $CaCl_2$, it showed maximum proteolytic activity at 80°C and stability from pH 4—12.5 at 60°C and below 75°C at pH 11.5. The stabilization by Ca^{2+} was observed in secondary conformation deduced from the circular dichroic spectrum of the enzyme. The protease hydrolyzed the ester bond of benzoyl leucine ester well. The amino acid terminal sequence of the enzyme showed high homology with those of microbial serine protease, although alanine of the NH_2-terminal amino acid was deleted.

Proteases with high activity and stability in the regions of high alkali and temperature are interesting for bioengineering and biotechnological applications as well as protein chemistry. Alkaline serine proteases from alkalophiles are generally stable in the highly alkaline region,[1] but they are not very stable to heat. On the other hand, alkaline proteases from thermophiles are generally stable to heat but unstable in high pH. Extremely thermostable alkaline proteases with high pH stability from alkalophilic *Bacillus* sp. no. AH-101[2] and extremely thermophilic *Thermus aquaticus* YT-1[3] have been reported. Also, the protease from alkalophilic *Thermoactinomyces* sp. HS682 isolated in our laboratory[4] is extremely stable in both regions of high alkali and high temperature. The protease from strain HS682 is thermostable similarly to those of no. AH-101 and YT-1 strains but more stable in extremely high pH than those. Although Ca^{2+} enhances these stabilities of HS682 protease as well as those of no. AH-101 and YT-1, it stabilizes a secondary conformation of HS682 protease, which was deduced from a circular dichroic spectrum, in extremely high pH. This phenomenon has not been observed in proteases of no. AH-191 and YT-1. It is interesting to compare the properties of these proteases.

In this paper, we describe the purification and some properties of the protease from alkalophilic *Thermoactinomyces* sp. HS682.

Materials and Methods

Materials. Casamino acid (Vitamin assay) was obtained from Difco Laboratories Inc. Polypeptone was from Nihon Seiyaku Co., Ltd. Yeast extract and molecular weight marker for SDS-PAGE were purchased from Oriental Yeast Co., Ltd. Bovine serum albumin, cytochrome *c*, TPCK, and TLCK were obtained from Sigma Chemical Co. Trypsinogen, ovalbumin, *N,N'*-methylene-*bis*-acrylamide, and acrylamide were purchased from Seikagaku Kogyo Co., Ltd. Butyl-Toyopearl 650M and SP-Toyopearl 650S were produced by Tosoh Co., Ltd. Sephadex G-75 (superfine) was from Pharmacia LKB Biotechnology. DFP was from Fulka Chemie AG. TEMED, 4 N methanesulfonic acid, SDS, sodium perborate, sodium tripolyphosphate, sodium-*n*-dodecylbenzenesulfonate, 2-mercaptoethanol, milk casein (Hammersten), PMSF, PCMB, MIA, PTH-amino acid standards, and reagents for amino acid sequence analysis were obtained from Wako Pure Chemicals. Triethylamine of amino acid sequence analysis grade was from Pierce. Bz-Arg-OMe, Ac-Lys-OMe, Ac-Phe-OEt, Gly-OEt, and Leu-OEt were obtained from Peptide Institute Inc. Bz-Tyr-OEt and Met-OEt were from Aldorich Chemical Inc. Other amino acid esters were synthesizd by our laboratory by the methods of Hein and Nieman,[12] and Whitaker.[13] All other chemicals and reagents were of analytical grade and obtained from commercial sources.

Microbial growth and media. Thermoactinomyces sp. HS682 which was isolated as an alkalophile,[4] was incubated in an alkaline medium consisting of 1% glucose, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 1.5% Na_2CO_3, and 2% agar at 50°C overnight. The medium for protease production consisted of 10 g of maltose, 5 g of Casamino acid, 0.4 g of yeast extract, 5 g of K_2HPO_4, 2 g of NaCl, and 15 g of Na_2CO_3 in 1 liter tap water (pH 10.3). Forty ml of the culture broth in a 200-ml flask cultivated with shaking at 50°C for 24 hr was inoculated into the same medium in a 2-liter jar fermentor (MB-2, Iwashiya A.D.M.) and cultivated at 50°C for 35 hr by agitating at 500 rpm and aerating at 1 vvm. Then the cells were removed from the culture medium by centrifugation, and the superantant was used as a crude enzyme solution for the purification.

Protease activities. The proteolytic activity was measured by the

Abbreviations: Ac-, acetyl-; Bz-, *N*-benzoyl-; DBS, sodium-*n*-dodecylbenzenesulfonate; DTT, 1,4-dithiothreitol; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; MIA, monoiodoacetic acid; -OEt, ethyl ester; -OMe, methyl ester; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohidantoin; SDS, sodium dodecyl sulfate; SPB, sodium perborate; STPP, sodium triphosphate; TEMED, *N,N,N',N'*-tetramethylethylenediamide; TLCK, *N*-α-tosyl-L-lysyl chloromethyl ketone; TPCK, *N*-α-tosyl-L-phenylalanyl chloromethyl ketone.

method reported previously.[4]

The esterase activity was assayed as specific initial rates of hydrolysis of synthetic amino acid esters, which were dissolved at a final concentration of 0.25 mM in 0.1 M KCl containing 10 mM $CaCl_2$ and 10% CH_3CN. After incubating the substrate solution of 7.8 ml for 5 min at pH 8.0 and 30°C, the reaction was started by adding 0.2 ml of the enzyme solution of appropriate concentration and monitored by titrating with 0.001 or 0.002 M NaOH using a pH-stat apparatus (AT-117, Kyoto Electrics Manufacturing Co., Ltd.) at pH 8.0 and 30°C.

Purification procedures. All procedures were done at 4°C.

Step 1. Butyl-Toyopearl chromatography. To the culture supernatant, solid ammonium sulfate was slowly added with stirring to 15% saturation, adjusting the pH of the solution to 9.2. After the precipitate was removed by centrifugation, the supernatant was put on a column (4 × 12 cm) of Butyl-Toyopearl 650M that had been equilibrated with 20 mM borate buffer (pH 9.2) containing 15%-saturated ammonium sulfate. After the column was washed with 20 mM borate buffer (pH 9.2), the enzyme was eluted with a stepwise pH gradient using in turn 20 mM borax buffer of pH 10.0, 10.5, 11.0, and 11.5.

Step 2. SP-Toyopearl chromatography. The enzyme fraction obtained above was dialyzed against 15 mM sodium citrate buffer (pH 6.4) containing 5 mM $CaCl_2$ and 0.05 M NaCl and concentrated by ultrafiltration with a Diaflo membrane YM5 (Amicon, exclusion limit 5000). The enzyme solution was put on a column (1.5 × 30 cm) of SP-Toyopearl 650S equilibrated with the same buffer, and eluted with a linear gradient of 0.05—0.12 M NaCl in the same buffer. A major active fraction was collected, dialyzed against 10 mM NH_4OH solution, lyophilized, and stored at −20°C until use.

Protein measurement. Protein was measured by the Lowry's method modified by Miller[5] with bovine serum albumin as a standard.

Gel filtration. For molecular weight estimation, gel filtration on a column (2.1 × 101 cm) of Sephadex G-75 (superfine) was done by the method of Andrews,[6] eluting with 20 mM glycine–NaOH buffer (pH 10.5) containing 10 mM $CaCl_2$. Bovine serum albumin (67,000), ovalbumin (45,000), trysinogen (24,000), and cytochrome *c* (13,000) were used as the references.

Electrophoresis. Electrophoresis was done by the method of Taber and Sherman[7] using 7.5% polyacrylamide gel in the buffer system of pH 6.6.

SDS–polyacrylamide gel electrophoresis was done by the method of Weber *et al.*[8] using 10% polyacrylamide gel. The enzyme solution inhibited with 40 mM PMSF was mixed with the sample buffer, which was preheated for 1 min at 100°C, and then incubated for SDS-treatment for 3 min at 100°C. For molecular weight measurement, oligomers of cytochrome *c* of molecular weight of 12,400—74,400 were used as the references.

Isoelectric gel electrophoresis was done with Ampholine carrier ampholite solution, which was mixed one volume of Ampholine for pH 3.5—9.5 with three volumes of that for pH 9—11, using 5% polyacrylamide gel in a glass tube of 5 × 110 mm at 4°C for 5 hr at 200 V. After electrophoresis, the gel was sliced into 5 mm pieces and incubated in 0.5 ml of deionized water for 2 hr at room temperature. Then the pH of the solutions was measured at 4°C and the protease activity of the solutions was assayed by reacting with 1.3% casein solution for 60 min at 50°C.

Amino acid composition. The enzyme preparations were hydrolyzed with 6 N HCl in evacuated sealed glass tubes at 115°C for 24, 48, and 72 hr. Amino acid analyses were done with an analyzer (JOEL model JTL-200). The contents of Ser, Thr, and Tyr were estimated by extrapolation of data at 24, 48, and 72 hr to zero time. For estimation of Trp content, hydrolysis was done with 4 N methane-sulfonic acid at 115°C for 24 hr.

NH_2-terminal amino acid sequence. The enzyme was sequenced by a modification[9] of Edman degradation.[10] PTH-amino acids were identified with a Tosoh HPLC system (CCP & 8010 series) on a TSKgel ODS-80Tm (4.6 × 250 mm) using an isocratic solvent system at a flow rate of 1 ml/min and 42°C and monitoring at 269 nm. The developing solvent was 10 mM formic acid buffer (pH 3.1)–acetonitrile (630 : 380) containing 10 mg DTT.[11]

Circular dichroism. Circular dichroic (CD) spectra were obtained with a JASCO J-20 CD spectrometer using a quartz cell of 2-mm light path with a jacket circulating water of various temperatures by the Haake bath.

Results

Purification of HS682 protease

The protease was purified from the culture supernatant of alkalophilic *Thermoactinomyces* sp. HS682 as described in Materials and Methods. Butyl-Toyopearl chromatography could effectively eliminate most impurities and raise the specific activity to 6.8 times by only one step (Fig. 1). The specific activity of the purified enzyme obtained by the next SP-Toyopearl chromatography was 19.68 units/mg, which was 10.6-fold higher than that of the supernatant, with 39% recovery. The purification of the protease is summarized in Table I. The purified enzyme migrated as a single protein band on SDS-PAGE (Fig. 2) as well as PAGE and isoelectrophoresis. The apparent molecular weight of the protease was estimated to be 25,000 by both gel filtration on Sephadex G-75 and SDS-PAGE. The isoelectric point of the protease was above pH 11.0 (data not shown).

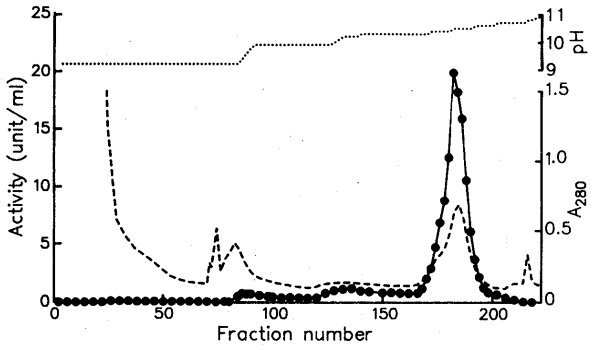

Fig. 1. Butyl-Toyopearl 650M Chromatography of Alkaline Protease of Alkalophilic *Thermoactinomyces* sp. HS682.

The supernatant of 15% ammonium sulfate saturation was put on the column (4 × 12 cm) equilibrated with 20 mM borate buffer (pH 9.2) containing 15%-saturated ammonium sulfate. The protease was eluted with a stepwise pH gradient using in turn 20 mM borax buffer of pH 10.0, 10.5, 11.0, and 11.5 at a flow rate of 300 ml/hr. Fractions of 15 ml were collected. Symbols: ●—●, protease activity; -----, absorbance at 280 nm; ·····, pH of eluent.

Table I. Purification of Protease from Alkalophilic *Thermoactinomyces* sp. HS682

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Culture supernatant	1430	4190	2256	1.86	100	1.0
$(NH_4)_2SO_4$ supernatant	1500	3675	1778	2.07	88	1.1
Butyl-Toyopearl	310	2477	175	14.17	59	7.6
SP-Toyopearl	37	1614	82	19.68	39	10.6

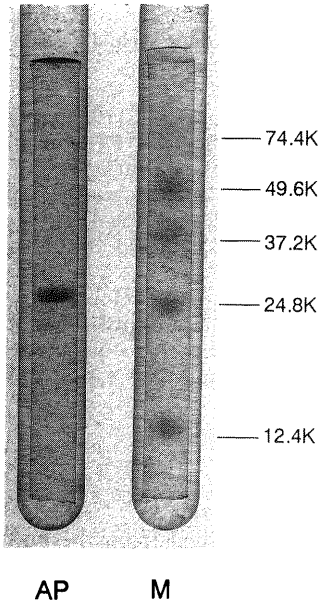

Fig. 2. SDS–Polyacrylamide Gel Electrophoresis of Alkaline Protease of Alkalophilic *Thermoactinomyces* sp. 682.

The purified enzyme was inhibited with 40 mM PMSF before incubation with SDS and 2-mercaptoethanol at 100°C. The electrophoresis was done on 10% polyacrylamide gel. AP, alkaline protease; M, M_r standards, oligomers of cytochrome *c* of relative molecular mass of 24,400—74,400.

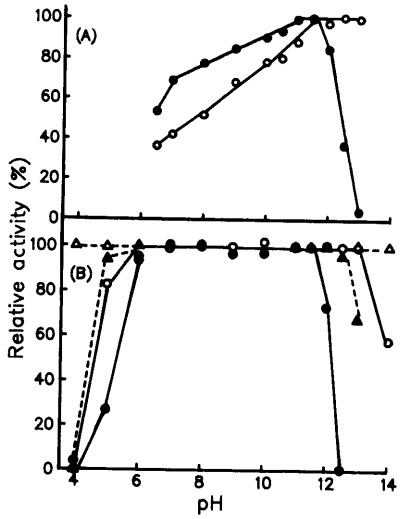

Fig. 3. Effects of pH on (A) the Proteolytic Activity and (B) the Stability of HS682 Protease.

(A) The proteolytic activities were measured at 37°C and 60°C in 1.0% casein solution of each pH. (B) The remaining activities were measured at 37°C and pH 11.5 after incubating the protease in the buffer solutions of each pH with or without 5 mM $CaCl_2$ at 37°C and 60°C for 10 min. Symbols: ○—○, without $CaCl_2$ at 37°C; ●—●, without $CaCl_2$ at 60°C; △—△, with 5 mM $CaCl_2$ at 37°C; ▲—▲, with 5 mM $CaCl_2$ at 60°C.

Table II. Effects of Inhibitors, Metal Ions, and Detergents on the Proteolytic Activity of HS682 Protease

The remaining activity was measured after incubating at pH 11.5 and 37°C for 10 min.

Reagents	Concentration (mM)	Remaining activity (%)	Reagents	Concentration (mM)	Remaining activity (%)
DFP	1	2.2	$MgCl_2$	10	87.2
PMSF	1	11.3	$CaCl_2$	10	97.0
TPCK	1	92.4	$MnCl_2$	10	87.2
TLCK	1	92.2	$FeCl_2$	10	100.0
PCMB	1	121.8	$FeCl_3$	10	96.8
MIA	10	115.4	$CoCl_2$	10	107.7
EDTA	10	92.7	$CuCl_2$	10	0.0
			$CuSO_4$	10	3.6
	(%)		$Cu(CH_3COO)_2$	10	0.8
SPB	0.1	88.6	$ZnCl_2$	10	83.0
STPP	0.1	93.4	$SnCl_2$	10	79.9
DBS	0.1	91.6	$BaCl_2$	10	99.2
SDS	0.1	92.8	$HgCl_2$	10	2.7
			$PbCl_2$	10	92.3

Effects of inhibitors, metal ions, and detergents on the proteolytic activity of HS 682 protease.

The remaining proteolytic activity was assayed after incubating with various inhibitors, metal ions, and detergents for 10 min at pH 11.5 ad 37°C (Table II). The protease was strongly inhibited by active-site inhibitors of serine protease, DFP and PMSF, but not by TPCK and TLCK, specific inhibitors for chymotrypsin and trypsin, respectively. The protease was not inactivated by the ibhibitors for thiol and metal proteases. The enzyme was inhibited only by Cu^{2+} and Hg^{2+} among metal ions tested in this study. Laundry detergents such as sodium perborate, sodium tripolyphosphate, sodium-*n*-dodecylbenzenesulfonate, and SDS did not affect the proteolytic activity of protease at 37°C for 10 min. Furthermore, the enzyme retained almost all of its activity even after incubation with sodium perborate and sodium tripolyphosphate at 0.1% at 50°C for 60 min (data not shown).

Effects of pH on the proteolytic activity and stability of HS682 protease

The optimum pH for the proteolytic activity of the protease was pH 11.5—13.0 at 37°C and pH 11.0—11.5 at 60°C in the absence of $CaCl_2$ (Fig. 3A). In view of the effects of protease inhibitors and the optimum pH, the protease should be classified as an alkaline serine protease.

The stability of HS682 protease was examined by incubating it in solutions of various pHs in the absence and presence of $CaCl_2$ for 10 min at 37°C and 60°C (Fig. 3B). In the absence of $CaCl_2$, the protease was stable in the pH range of 6.0—13.0 at 37°C, and in the pH range of 6.0—11.5 at 60°C. Even at pH 14.0 in 0.1 N NaOH solution at 37°C, the protease remained above 60% of the activity. By adding 5 mM $CaCl_2$, the stability of the enzyme was enhanced to the pH range of 4.0—14.0 at 37°C, and to the pH range of 5.0—12.5 at 60°C.

Effects of temperature on the proteolytic activity and stability of HS682 protease

The effects of temperature on the proteolytic activity of HS682 were examined in the absence and presence of $CaCl_2$ at pH 11.5 for 10 min (Fig. 4A). The maximum activity was obtained at 80°C in the presence of 5 mM $CaCl_2$. The enzyme activity at 80°C was 17 times higher than that at 37°C. In the absence of $CaCl_2$, the activity was maximum at 70°C and inactivated completely at 80°C. Below 70°C, the proteolytic activity of the enzyme without $CaCl_2$ was the same as that with 5 mM $CaCl_2$.

The effects of temperature on the stability of HS682 protease were examined in the absence and presence of $CaCl_2$ at pH 11.5 for 10 min (Fig. 4B). The protease was

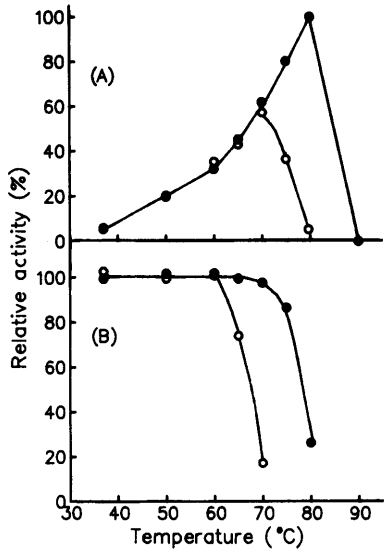

Fig. 4. Effects of Temperature on (A) the Proteolytic Activity and (B) the Stability of HS682 Protease.

(A) The proteolytic activities were measured at each temperature for 20 min at pH 11.5. (B) The remaining activities were measured at 37°C and pH 11.5 after incubating the protease in the buffer solutions of pH 11.5 with or without 5 mM $CaCl_2$ at each temperature for 10 min. Symbols: ○—○, without $CaCl_2$; ●—●, with 5 mM $CaCl_2$.

stable below 60°C and showed the remaining activity of only 20% at 70°C without $CaCl_2$. By addition of 5 mM $CaCl_2$, the heat stability was enhanced up to 70°C and the remaining activity was 85% at 75°C and 25% at 80°C.

Effects of pH and temperature on the circular dichroic spectrum of HS682 protease.

The CD spectrum of the native enzyme dissolved in 20 mM borax buffer (pH 11.0) at a concentration of 0.05 mg/ml had a maximum at 222 nm and a prominent shoulder around 208—210 nm. The molar ellipticity, which reflects the α-helix structure of the enzyme, changed in the pH and temperature regions where the protease was inactivated in the adsence of $CaCl_2$. The changes of the molar ellipticity in these regions were diminished by the presence of 5 mM $CaCl_2$ (data not shown).

Hydrolysis of ester substrates

Table III shows the esterase activity of HS682 protease toward some ester substrates. The enzyme preferentially hydrolyzed the ester bond of Bz-Leu-OEt, and showed fairly high activity toward aromatic and hydrophobic amino acid ester, Phe, Tyr, Met, and Ala. However, the enzyme showed less activity toward ester substrates composed of an amino acid with a side chain at β-carbon, Thr, Val, and Ile, and basic amino acid, but not the ester bond of an amino acid with a free α-amino group, Leu-OEt and Met-OEt.

Amino acid composition and NH_2-terminal amino acid sequence of HS682 protease

The amino acid composition of HS682 protease is shown in Table IV, compared with those of alkaline proteases from mesophilic, thermophilic, and alkalophilic microorganisms. The amino acid composition of HS682 protease was similar to those of these alkaline proteases, except for aqualysin I contained Cys. The protease has low Lys and high Arg compared with subtilisin BPN'[16] and alkaline proteases of mesophilic and alkalophilic *Bacillus* sps. no. 221[14] and YaB[15] as well as that of aqualysin I from thermophilic *Thermus aquaticus* YT-1.[3]

Twenty-one residues of the NH_2-terminal sequence of HS682 protease were identified and compared with those of subtilisin-type proteases (Fig. 5). The NH_2-terminal amino acid of the protease was not Ala, which was that of many subtilisin-type proteases, but Gln like alkaline elastase YaB from alkalophilic *Bacillus* sp. YaB.[15]

Table III. Initial Rate of Hydrolysis of Amino Acid Esters by HS682 Protease

The initial rate was measured by the pH-stat method as described in Materials and Methods.

Substrate (0.25 mM)	Initial rate (μmol/min/mg-enzyme)
Bz-Phe-OEt	3.4
Bz-Tyr-OEt	2.1
Bz-Leu-OMe	12.0
Bz-Met-OMe	3.1
Bz-Ala-OMe	1.8
Bz-Thr-OMe	0.32
Bz-Val-OMe	0.19
Bz-Ile-OMe	0
Bz-Arg-OEt	0.19
Ac-Lys-OMe	0.35
Ac-Phe-OEt	1.1
Ac-Tyr-OEt	1.1
Leu-OEt	0
Met-OEt	0
Gly-OEt	0

Table IV. Comparison of Amino Acid Composition of HS682 Protease with Several Subtilisin-type Proteases

The amino acid composition of the protease was calculated on the basis of a molecular weight of 25,000.

Amino acid	Number of residues				
	HS682 protease	*Bacillus* sp. no. 221[14]	*Bacillus* sp. YaB[15]	Subtilisin BPN'[16]	Aqualisin I[3]
Lys	3	6	4	11	2
His	7	8	8	6	5
Arg	11	8	7	2	16
Trp	2	5	2	3	4
Asx	29	29	28	28	28
Thr	14	18	17	13	24
Ser	25	23	26	37	28
Glx	17	16	14	15	9
Pro	9	16	8	14	12
Gly	31	39	37	33	38
Ala	30	45	33	37	41
Val	20	27	21	30	27
Met	3	4	3	5	2
Ile	15	9	10	13	10
Leu	15	22	11	15	19
Tyr	10	7	4	10	12
Phe	4	2	6	3	3
Cys/2	ND[a]	0	0	0	4
Total	245	284	239	275	284

[a] Not determined.

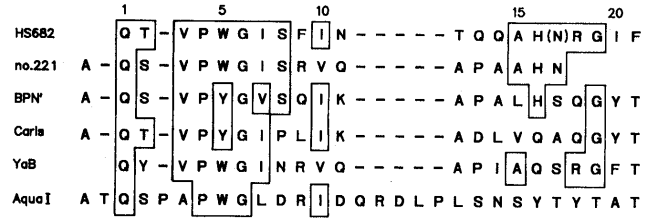

Fig. 5. NH_2-Terminal Amino Acid Sequence of HS682 Protease.

The residues of HS682 protease are numbered above the sequences. Amino acids of HS682 protease, identical to those of the other proteases, are boxed. Gaps are inserted in the sequences according to that of aqualysin I. BPN', subtilisin BPN'[16]; Carls, subtilisin Carlsberg[17]; no. 221, alkalophilic *Bacillus* sp. no. 221[18]; YaB, alkalophilic *Bacillus* Ya-B[15]; AquaI, Aqualysin I.[3]

Discussion

The protease from alkalophilic *Thermoactinomyces* sp. HS682 was easily purified from culture supernatant by only two chromatographies, on Butyl-Toyopearl 650M and SP-Toyopearl 650S columns. The hydrophobic chromatography on Butyl-Toyopearl 650M was very effective for the purification of HS682 protease, though ammonium sulfate had to be previously added to the culture at a concentration of 15% saturation. This chromatography could remove most of the impurities from the supernatant of 15% ammonium sulfate saturation and raise the specific activity 6.8 times with a recovery of 67%.

The specific activity of the purified enzyme toward casein is about 20 units/mg-protein, which is equivalent to 5,700 units/mg-protein under the assay method of Takami *et al.*[2] This value is lower than that of *Bacillus* sp. no. 221[14] (18,000 units/mg-protein) but higher than those of subtilisin BPN'[16] (2,300 units/mg-protein) and *Bacillus* sp. AH101[2] (2,500 units/mg-protein).

The isoelectric point of HS682 protease is above 11.0, which is higher than those of other microbial alkaline proteases.[2,3,14-17] Because the amount of basic amino acids residues in the protein was similar to those of other microbial alkaline proteases (Table IV), it is estimated from its high basicity that most of Asx and Glx may be asparagine and glutamine, respectively. The thermostable enzymes have generally high Arg and Low Lys among basic amino acid residues.[3] HS682 protease showed the same content of basic amino acid to that of the thermostable enzymes.

Many microbial alkaline proteases show high activity toward aromatic amino acid esters, followed by hydrophobic amino acid esters.[19-21] HS682 protease showed high activity toward Bz-Leu-OMe, which was 4 times those toward aromatic and other hydrophobic amino acid esters. Also, the enzyme showed less activity toward ester substrates composed of amino acids with a side chain at β-carbon, Thr, Val, and Ile, and basic amino acids, but not toward the ester bond of amino acids with a free α-amino group, Leu-OEt and Met-OEt.

The optimum pH of the protein toward casein is similar to those of alkalophiles[2,14] but higher than those of neutralophiles.[3,15-17] Though the pH stability of the protease is the range of pH 6—13 at 37°C in the absence of Ca^{2+}, the stability is risen up to pH 14 in 0.1 N NaOH by the presence of Ca^{2+}. Even at 60°C, the protease is stable in the range of 5—12.5. Thus pH stabilization by Ca^{2+} has not appeared in other microbial alkaline proteases so far reported.

The thermostability of most microbial alkaline serine proteases[2,3,14-17] are enhanced by the presence of Ca^{2+}. The protease of HS682 is stabilized by Ca^{2+} as well. The optimum temperature of the protease toward casein rises up to 80°C in the presence of 5 mM $CaCl_2$ due to the enhancement of thermostability up to 75°C by Ca^{2+}. These values of optimum temperature and thermostability are one of the highest among microbial alkaline serine proteases as well as those of AH101 protease[2] and aqualysin I.[3] The behaviors of the molar ellipticity at 222 nm of the protease toward pH and temperature are associated with those of the proteolytic activity. This might suggest that the high stabilities of HS682 protease toward pH and temperatue are due to the stabilization of secondary conformation by Ca^{2+}. Thus, the HS682 protease has characteristic properties of pH and heat-stabilities interesting to investigate at the molecular level.

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