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Production and Characterization of Neutral and Alkaline Protease from Different *Bacillus subtilis* Strains

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An extracellular neutral and alkaline protease from two different *Bacillus subtilis* strains were studied. The optimal activity occured when the pH level was 7.0 and 10.5 at a temperature of 35 and 45 ºC for neutral protease and alkaline protease, respectively. When neutral protease was stable in the temperature range 35-60 ºC, alkaline protease was found stable between 35-55 ºC for 0.5 h. Divalent cations, especially Ca^{2+} increased enzymes activity and were inhibited by Mn^{2+},Ni^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+} and Hg^{2+} for neutral protease and by Zn^{2+}, Cd^{2+},Co^{2+}, Cu^{2+} and Hg^{2+} for alkaline protease. The neutral protease was also inhibited by ethylenediaminetetraacetic acid, 1,10 phenanthroline and dithiothreitol whereas alkaline protease was inhibited with phenylmethyl-sulfonyl fluoride. The obtained K_m values were $2.28 \times 10^{-3} \pm 3.65 \times 10^{-4}$, $2.28 \times 10^{-4} \pm 4.21 \times 10^{-5}$ and $1.70 \times 10^{-4} \pm 5.18 \times 10^{-5}$ M for azocasein, BSA and casein for neutral protease, respectively. The K_m values with azocasein, BSA, casein, N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA were $2.02 \times 10^{-3} \pm 7.3 \times 10^{-5}$, $2.13 \times 10^{-4} \pm 6.04 \times 10^{-5}$, $8.75 \times 10^{-4} \pm 1.36 \times 10^{-4}$, $1.71 \times 10^{-3} \pm 3.93 \times 10^{-4}$ and $2.64 \times 10^{-4} \pm 4.93 \times 10^{-5}$ for alkaline protease.

Key Words: *Bacillus subtilis*, Neutral protease, Alkaline protease, Properties.

INTRODUCTION

The proteolytic enzymes are the most important group of enzymes produced commercially[1]. Their major application is in the laundry detergents, leather preparation, protein recovery or solubilization, meat tenderization, biscuit and cracker industry[2,3]. Although many of the above applications have traditionally relied on proteases of animal and plant origin, microbial proteases have become more widely acceptable and available[4]. Many proteases have been purified and characterized from bacteria[3-5]. *Bacillus subtilis*

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neutral protease was a zinc containing enzyme. There is direct proportionality between the zinc content and specific activity of the enzyme during the purification procedure. Zinc is essential for catalytic activity and calcium is required to maintain the structural rigidity of the enzyme molecule. The molecule contains 1 g of zinc per mole of enzyme[3]. Alkaline proteases are generally stable in the high alkaline region. Especially, biosynthesis of alkaline protease is dependent on the sources of growth medium[6].

This work describes production, properties and some characterization of two extracellular proteases produced by two different *B. subtilis* strains.

EXPERIMENTAL

Microbial strain: *Bacillus subtilis*-1 and *Bacillus subtilis*-2 provided from Faculty of Science-Art, Dicle University, culture collection isolated from hot-spring water and soil were used for neutral and alkaline protease production, respectively.

All the chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA). Culture media components were obtained from DIFCO.

Neutral protease production: The medium for neutral protease, *Bacillus subtilis*-1 inoculum was prepared by transferring a loop-full of freshly prepared culture from nutrient agar plates into 250 mL flask, containing 50 mL of nutrient broth medium containing (g/L); KCl, 1; $MgSO_4.7H_2O$, 0.125; $MnCl_2$, 10^{-5}; $CaCl_2$, 10^{-3}; $FeSO_4$, 10^{-6}; $ZnCl_2$, 10^{-5}; nutrient broth, 8 (pH: 7.2) and it was incubated at 200 rpm at 37 ºC for 24 h. The bacterial strain with 5 % (v/v) was cultured in 250 mL of Spinzer growth medium containing (g/L); K_2HPO_4, 14; KH_2PO_4, 6; $(NH_4)_2SO_4$, 2; Na_3-citrate, 1; $MgSO_4.7H_2O$, 0.2 and 5 g of glucose was sterilized separately and then added to the medium[7]. The erlenmeyer flask was incubated at 37 ºC with agitation for 51 h.

Alkaline protease production: *B. subtilis*-2 was cultured in 250 mL of erlenmeyer flask containing 50 mL culture medium containing (g/L); citric acid, 10; $NaNO_3$, 10; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.2; NaCl, 5; Na_2CO_3, 10 (pH: 9.0). The erlenmeyer flask was inoculated with 10 % (v/v) of 24 h old *B. subtilis*-2 culture and the flask was incubated at 200 rpm at 37 ºC for 54 h[2].

After incubation, the culture mediums were centrifuged at 15000x g for 15 min at 4 ºC. Enzyme activity was measured from supernatant for these two enzymes.

Protein assay: Proteolytic activity was determined at 0.02 M *tris*-HCl (pH: 7) and carbonate/bicarbonate (pH: 10.5) buffers for neutral and alkaline protease activity, respectively, with azocasein (1 %, w/v) as substrate[8]. Incubation was made at 37 ºC for 0.5 h. Protease activity, as U/mL, was

calculated as the increase in absorbance at 420 nm caused by 1.0 mL of enzyme per hour of incubation under assay condition.

Activity on casein and bovin serum albumin (BSA) were assayed according to the same protocol[9]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of tyrosine equivalent per minute.

The alkaline protease was also tested for its ability to hydrolyze synthetic peptide substrates conjugated to *p*-nitroaniline (pNA). N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA were used. Stock solutions of the substrates were prepared in DMSO. Subtrate (25 µL) was added to 465 µL of buffer (0.05 M glycine-NaOH, pH: 10.5) mixed and pre-incubated at 37 ºC for 5 min. The reaction was started by addition of 10 µL enzyme solution. After 10 min at 37 ºC, the reaction was stopped by addition of 500 µL of 10 % (w/v) citric acid and the absorbance was measured at 410 nm. One unit of *p*-nitroanilide-hydrolyzing activity was defined as the amount of enzyme that liberated 1 mmol of *p*-nitroaniline per minute at 37 ºC and pH: 10.55.

Determination of optimum pH profiles: The optimum pH of two proteases was determined with 1 % azocasein as substrate dissolved in different buffer systems (0.02 M acetate, pH: 5-6.5; 0.02 M *tris*-HCl, pH: 7-8; 0.02 M carbonate/bicarbonate, pH: 9-11.5).

Effect of temperature: Optimum temperature was determined by assay of enzyme activity by the standard method at different temperatures (25 to 75 ºC). The effect of temperature on enzyme stability was determined in 0.02 M *tris*-HCl (pH: 7) and 0.02 M carbonate/bicarbonate (pH: 10.5) buffers for neutral and alkaline protease, respectively. The enzyme solutions were kept for 0.5 h at different temperatures (20-75 ºC), cooled rapidly on ice and then reaction was started by adding the azocasein to the assay mixture after preincubation. The remaining activity was determined at 37 ºC as usual.

Effect of metal ions and inhibitors: The effect of metal ions (5 mM) on protease activity was examined. Proteases were preincubated for 0.5 h at 37 ºC with tested cations (Ba^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+}) in above buffers at 5 mM final concentration and the reaction was started by adding the azocasein solution to the assay mixture after preincubation. The remaining activity was assayed by using the standard method.

Enzyme solution and assay buffer containing various chemical reagents were mixed and preincubated at 37 ºC for 0.5 h. This was followed by addition of azocasein and then proteolytic activity was measured.

The following inhibitors were used: 2 mM phenylmethylsulfonyl fluoride, (PMSF) 10 mM ethylenendiaminetetraacetic acid (EDTA), 10 mM 1,10-phenanthroline and 10 mM dithiothreitol (DTT).

Substrate specifity: Neutral protease activity towards azocasein, casein and BSA and alkaline protease activity towards azocasein, casein, BSA, N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA were examined. The kinetic parameters were calculated from the non-linear regression by using Fig P programme. The K_m, k_{cat} and hydrolytic coefficients (k_{cat}/K_m) of each subtrate were calculated.

RESULTS AND DISCUSSION

B. subtilis is recognized as a producer of extracellular enzymes and has the potential to grow in liquid medium. In present study, a neutral and alkaline proteases were recovered from the culture supernatant. The results showed that maximum enzyme production was 32.68 U/mL and 53.6 U/mL after 51 and 56 h for neutral and alkaline protease, respectively.

The protein nature of enzymes means that pH will affect the ionization state of the amino acids which dictate the primary and secondary structure of the enzyme and hence, controls its overall activity. A change in pH will have a progressive effect on the structure of the protein and the enzyme activity[10]. Several workers found optimum pH of neutral and alkaline protease pH range at pH 7, 8 and between pH 11-13 at 37 ºC, respectively[1,4,11]. The optimum pH of the neutral protease was found at pH: 7. Alkaline protease produced high activity in the pH range 9.5-11.0 and optimum pH was found to be 10.5 toward azocasein (1 %, w/v) (Fig. 1).

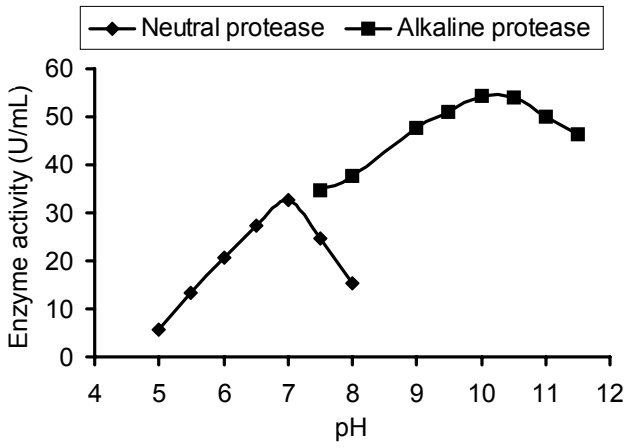

Fig. 1. Effect of pH on *Bacillus subtilis*-1 neutral protease and *Bacillus subtilis*-2 alkaline protease activity

The stability of proteins is intrinsic and resides in their primary structure. Thermostabilization of proteins is achieved through optimization of intramolecular interactions, packing densities, internalization of hydrophobic

residues and surface exposure of hydrophilic residues[5]. The effect of temperature on proteases activity is shown in Fig. 2. The proteolytic activity of neutral protease was thermostable in the temper-ature range 35-60 and 35-55 ºC for alkaline protease. Irreversible inactivation of both took place at 75 ºC. The optimum temperatures for neutral and alkaline protease were 35 and 45 ºC, respectively, when the enzyme were assayed with azocasein as the substrate. The looseness of proteolytic activity after preincubation at 60 ºC might be caused by a conformational process.

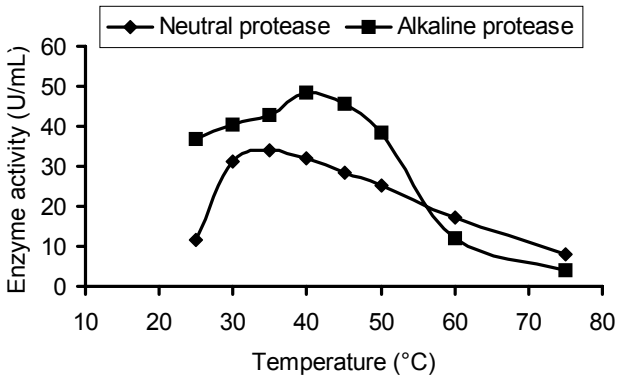

Fig. 2. Effect of temperature on *Bacillus subtilis*-1 neutral protease and *Bacillus subtilis*-2 alkaline protease activity

The effect of divalent cations on proteolytic activity was examined in order to find out which metal ions were inhibitors and stimulators of the catalytic process.

The effect of 5 mM metal ions on protease activity is shown in Table-1. The neutral protease activity did not change much by Ba^{2+}, Mg^{2+} and Zn^{2+}. The activity in the presence of Ca^{2+} was 5 % higher than that observed in the control solution. Ca^{2+} ions are known as stabilizers of many enzymes, protecting them against disadvantageous conformational changes. The Ca^{2+} binding sites examined for bacterial proteases contain a number of coordinating aspartate and glutamate[4]. Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+} and Hg^{2+} reduced neutral protease activity to 56.6, 21.5, 31.8, 54.6, 35.7, 60 and 35.5 %, while Zn^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+} inhibited alkaline protease activity to 31, 14, 41, 50.7 and 29.5 %, respectively. Other ions showed little inhibitory effect. Similar effects have also been observed by some researchers[2,4,11,12].

Metal ions which have stimulator effect capable of electron rearrangement can occur at higher energy level and can assume a deformed tetrahedral symmetry. Enzyme complexes with other ions with lower catalytic activity, *i.e.*, the enzyme complexes with Fe^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} Co^{2+}, Cd^{2+} and Hg^{2+}

TABLE-1
EFFECT OF METAL IONS AND VARIOUS CHEMICALS ON *Bacillus subtilis*-1 NEUTRAL PROTEASE AND *Bacillus subtilis*-2 ALKALINE PROTEASE ACTIVITY

Chemicals	Concentration (mM)	Neutral protease	Alkaline protease
		Residual protease activity (% of control)	
Ba^{2+}	5	91.06	89.8
Ca^{2+}	5	105.00	88.8
Mg^{2+}	5	95.80	95.0
Mn^{2+}	5	56.60	90.0
Fe^{2+}	5	21.50	79.0
Zn^{2+}	5	96.07	31.0
Co^{2+}	5	31.80	50.7
Cu^{2+}	5	60.00	29.5
Ni^{2+}	5	54.60	90.0
Cd^{2+}	5	35.70	41.0
Hg^{2+}	5	35.50	14.0
EDTA	10	9.00	85.0
DTT	10	7.00	86.0
1,10-Phenanthroline	10	6.80	83.0
PMSF	2	90.00	10.0

for neutral protease and Zn^{2+}, Hg^{2+}, Cd^{2+},Co^{2+} and Cu^{2+} for alkaline protease, can probably occur in a state corresponding to lower energy and can assume a proper tetrahedral symmetry, which fails to facilitate proper spatial orientation of the substrate. The inhibition effects of Hg^{2+} for alkaline protease (14 %) and Fe^{2+} for neutral protease (21.50 %) can also occur with the process of irreversible noncompetetive inhibition of enzyme.

The effect of different inhibitors on two proteases activity is also shown in Table-1 of the inhibitors, EDTA, PMSF, 1,10-phenanthroline and DTT were tested. EDTA, 1,10-phenanthroline and DTT are usually preferred as a diasnostic indicator of metalloprotease because of their higher stability constant for zinc than for calcium[5].

10 mM EDTA, 1,10-phenanthroline and DTT were the most potent inhibitor for neutral protease and these reagents reduced the activity from 100 % to 9.0, 6.8 and 7.0, respectively. Thiols such as DTT, known as metal chelators, are particularly capable of complexing zinc. An interaction with the active center-located zinc and the conformational change connected with this may be the cause of the inhibition observed[13].

2 mM PMSF inhibited alkaline protease activity. This reagent strongly inhibits the Ser residue in the active site causing complete loss of enzyme activity[5].

The hydrolysis of tested substrates and kinetics of hydrolysis are shown in Tables 2 and 3. Among the substrates tested, neutral and alkaline proteases hydrolyzed azocasein, casein and BSA. The substrate specificity of the alkaline protease was also investigated in detail with a number of synthetic substrates (N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA). The highest specific activity was observed with casein for neutral protease.

TABLE-2
SUBSTRATE SPECIFICITY OF *Bacillus subtilis*-1 NEUTRAL PROTEASE AND KINETIC PARAMETERS

Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Azocasein	$2.28 \times 10^{-3} \pm 3.65 \times 10^{-4}$	0.490 ± 0.033	214.90
BSA	$2.28 \times 10^{-4} \pm 4.21 \times 10^{-5}$	0.266 ± 0.009	1166.66
Casein	$1.70 \times 10^{-4} \pm 5.18 \times 10^{-5}$	0.205 ± 0.010	1205.88

TABLE-3
SUBSTRATE SPECIFICITY OF *Bacillus subtilis*-2 ALKALINE PROTEASE AND KINETIC PARAMETERS

Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Azocasein	$2.02 \times 10^{-3} \pm 7.30 \times 10^{-5}$	1.2000 ± 0.0180	594.05
BSA	$2.13 \times 10^{-4} \pm 6.04 \times 10^{-5}$	0.2290 ± 0.0100	1075.11
Casein	$8.75 \times 10^{-4} \pm 1.36 \times 10^{-4}$	0.5110 ± 0.0240	584.00
N-suc-Ala-Ala-Ala-pNA	$1.71 \times 10^{-3} \pm 3.93 \times 10^{-4}$	0.0998 ± 0.0088	58.36
N-cbz-Ala-Ala-Leu-pNA	$2.64 \times 10^{-4} \pm 4.93 \times 10^{-5}$	0.7220 ± 0.0270	2734.84

N-cbz-Ala-Ala-Leu-pNA, a common substrate for subtilisins, is hydrolyzed with high specifity by extracellular serin proteases from different microorganisms[5]. In present study, N-cbz-Ala-Ala-Leu-pNA was also found to be the substrate for alkaline protease. Michaelis-Menten kinetics were observed with azocasein, casein, BSA, N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA. The Km values obtained were $2.28 \times 10^{-3} \pm 3.65 \times 10^{-4}$, $2.28 \times 10^{-4} \pm 4.21 \times 10^{-5}$ and $1.70 \times 10^{-4} \pm 5.18 \times 10^{-5}$ (M) for azocasein, BSA and casein for neutral protese, respectively. The corresponding k_{cat} and k_{cat}/K_m values were found to be 0.490 ± 0.033, 0.266 ± 0.009 and 0.205 ± 0.010 (s^{-1}), 214.9, 1166.66 and 1205.88 ($M^{-1}1$ s^{-1}), respectively.

The K_m values with azocasein, BSA, casein, N-suc-Ala-Ala-Ala-pNA and N-cbz-Ala-Ala-Leu-pNA were $2.02 \times 10^{-3} \pm 7.3 \times 10^{-5}$, $2.13 \times 10^{-4} \pm 6.04 \times 10^{-5}$, $8.75 \times 10^{-4} \pm 1.36 \times 10^{-4}$, $1.71 \times 10^{-3} \pm 3.93 \times 10^{-4}$, $2.64 \times 10^{-4} \pm 4.93 \times 10^{-5}$ (M) for alkaline protease. The corresponding k_{cat} and k_{cat}/K_m values were also found to be 1.2 ± 0.018, 0.229 ± 0.01, 0.511 ± 0.024,

0.0998 ± 0.0088 and 0.722 ± 0.027 (s^{-1}), 594.05, 1075.11, 584, 58.36 and 2734.84 (M^{-1} s^{-1}), respectively.

An inhibitors reacts with the enzyme within the first seconds of the process and therefore one should think that the enzyme is made inactive within its 0.5 h preincubation with the inhibitor, rather than the latter reacting with substrate.

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