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Biochemical Characterization of Elastase From *Pseudomonas aeruginosa* SES 938-1

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Abstract: We have investigated some biochemical properties of elastase from a new strain of *Pseudomonas aeruginosa* (SES 938-1). Activities were measured at 410 nm using N-succinyl-L-(ala)$_3$-p-nitroanilide as substrate. The elastase activity followed Michaelis-Menten kinetics over the substrate range of 0.067-0.540 mM with the apparent K_m value of 0.375 mM. Optimum activity was observed at 36 °C and pH 7.5. elastase activity was inhibited by metal chelating agents and high concentrations of Mn^{2+}, Zn^{2+} and Ni^{2+}. The results obtained suggest that elastase from P. aeruginosa SES 938-1 is a neutral metalloproteinase.

Pseudomonas aeruginosa SES-938-1'den İzole Edilen Elastazın Biyokimyasal Karakterizasyonu

Özet: Yeni Bir Pseudomonas aeruginosa suşundan (SES931-1-1) izole edilen elastazin bazı biyokimyasal özelliklerini araştırdık. Aktivite ölçümleri 420 nm de N-succinyl-L-(al)$_3$-p-nitroanilide substrat olarak kullanılarak yapılmıştır. elastaz aktivitesi 0.067-0.540 mM subtstrat konsantrasyonu sınırında Michealis-Menten kinetiğine uymakta olup görünür K_m değeri 0.375 mM olarak bulunmuştur. Optimum enzim aktivitesi 36 °C ve pH 7.5'da gözlenmiştir. Enzim aktivitesi metal tutucu ajanlar ve yüksek konsantrasyolarda Mn^{+2}, Zn^{+2} ve Ni^{+2} tarafından inhibe edilmektedir. Bu sonuçlar *P. aeruginosa* SES938-1 elastazının bir nötral metalloproteinaz olduğunu göstermektedir.

Introduction

Among the various factors produced by the opportunistic human pathogen *Pseudomonas aeruginosa* elastase has the primary importance (1-3). Purified elastase is capable of degrading several molecules of biological significance, including elastin (4), collogens (5), immunoglobulins (6), complement components (7), serum α_1-proteinase inhibitor (8), and α_2-macroglobin inhibitors (9), laminin and fibrin (10). In accordance with substrates it utilizes, elastase of *P. aeruginosa* is also able to inhibit several processes involved in defence mechanisms of hosts (11). That is why, characterization of the elastase will provide insights for our better understanding

of the elastolytic catalysis and molecular mechanism of *Pseudomonas* infections. Although isolated and purified first time, 30 years ago by Morihara (2), there are limited information regarding the kinetic analysis of *Pseudomonas* elastase. Previously, we have isolated the enzyme elastase from a new strain of *P. aeruginosa* (SES-938-1) and showed that this enzyme is strictly dependent on divalent cations (i.e., Zn^{2+} and Ca^{2+}) for catalytic activity and/or stability (12). In the present study, kinetic properties of the enzyme were determined by using a synthetic oligopeptide, N-succinyl-L-$(ala)_3$-p-nitroanilide, as substrate.

Materials and Methods

Bacteria

Twenty eight *P. aeruginosa* isolates from different clinical specimens submitted to Hacettepe University Hospital, Microbiology Laboratory, in 1992 have been tested for their elastase activities. Plate assay method adopted from Morihara *et al*., (2) and a colorimetric assay described by Ohman *et al*., (13) were concomitantly, used to screen the above strains for elastase producton. *P. aeruginosa* SES-938-1 was showen to have the highest elastolytic activity of the *P. aeruginosa* strains tested, and employed as elastase source throughout the study.

Preparation of Crude Elastase

Crude elastase was prepared from the culture supernatant of *P. aeruginosa* (SES-938-1) *via* salting out by ammonium sulfate, and then desalting and concentrating the sample using a centricon microconcentrator, as described before (12). The concentrated samples were kept at -20 °C.

Determination of Elastase Activity

Elastolytic activity was determined according to a modified continuous assay method of Bieth *et al*., (14), using N-succinyl-L-$(ala)_3$-p-nitroanilide as substrate. The reaction mixture (650 µ1) contained 100 mM Tris HC1, 10 mM $CaCl_2$, pH 7.5, 0.135 mM substrate and crude elastase (0.90-1.13 U/ml assay mixture). The reaction was carried out at 36 °C, and the absorbancy was measured continuously at 410 nm. Initial velocity was expressed in unites of Δ A 410 nm/min, unless otherwise stated. One unit (U) of enzyme was the amount that catalyzed the formation of 1 nmole of product per minute. control experiments were done by omiting the substrate or the enzyme from the reaction mixture. Protein was assayed spectrophotometrically at 235 and 280 nm according to Whitaker and Granum (15).

Lineweaver-Burk Plot

The dependence of initial reaction rate on concentration of the substrate (N-succiniyl-L-$(ala)_3$-p-nitroanilide (0.067-0.540 mM), the increase in the absorbancy at 410 nm, was recorded for 90 min. The linear portion of the progress cruve was used for the initial velicity determination. Plots of 1/V versus 1/S (Lineweaver-Burk Plot) permitted the fitting of definite straight lines and K_m was derived from such plots.

Effect of Temperature

To test the effects of various preincubation temperatures, enzyme samples in 100 mM Tris HC1, 10 mM CaCl2, pH 7.5 buffer were placed in microfuge tubes and incubated at 4°, 23°, 36° and 56 °C, separately. After 15 min, the reactions were started by adding the substrate and the elastase activities were measured at 36 °C, as described above. Optimum temperature for elastase activity was determined by carrying out the enzyme assay at 30°, 36°, 45° and 56 °C, separately,

Effect of pH

Effect of pH on enzyme activity was tested in equal amounts of 100 mM of various buffers as described before. The buffers used in these experiments were: MOPS pH 6.17; Tris HCl pH 6.17; Tris HCl pH 7.0, pH 7.5 and pH 8.0; and Glycin pH 9.05, each contained 10 mM $CaCl_2$.

Effect of EDTA

Elastase activity was measured in the presence of 15 mM EDTA, to test the divalent metal ion requirement of the enzyme. The enzyme assay was otherwise same as described before. Control experiment was done by ommiting the EDTA from the reaction mixture.

Effect of Metals

Enzyme activities were assayed at 10 mM concentration of $ZnSO_4$, $MnCl_2$, $MgCl_2$, $NiCl_2$ and $CaCl_2$, separately, in 100 mM Tris HCl buffer pH 7.5, as described before. Control missing metal ion was included in each set of experiment.

Results

Lineweaver Burk plot

With N-succinyl-L-$(ala)_3$-p-nitroanalide as the substrate, within the concentration range of 0.067-0.540 mM, elastase followed Michaelis-menten kinetics (Figure 1). At a constant enzyme concentration (1.31 U/ ml assay mixture), when the initial velocities were measured at these substrate concentrations K_m was 0.370 mM. LB plot revealed that N-succinyl-L-$(ala)_3$-nitroanilide inhibited elastase activity at high concentrations (<0.50 mM).

Effect of temperature on elastase

To find out the optimum temperature for *P. aeruginosa* SES-938-1 elastase, the activities were measured with N-succinyl-L-$(ala)_3$-p-nitroanilide as the substrate, at various temperatures. Figure 2 illustrates that the enzyme has performed the highest activity between 30°-36 °C. Activities were also measured after preincubations at different temperatures for 15 min. The enzyme assay was carried out at 36 °C. Higher reaction rates were measured attre preincubations at lower temperatures. The enzyme has retained the following activities: at 4 °C, 100%, at 23 °C, 100%; at 36 °C, 100%; at 45 °C, 53% and at 56 °C, 0%.

Effect of pH on elastase

When the assays were measured at different pH, the highest activities were obtained within

a pH range of 7.0-8.0. The optimum pH of elastase was found to be 7.5, as shown in Figure 3.

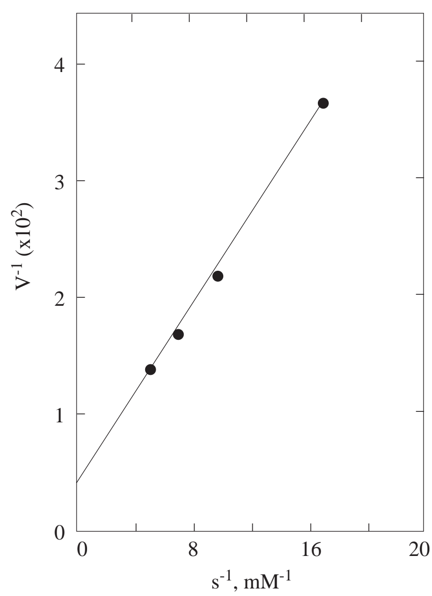

Figure 1. Lineweaver-Burk Plot of *P.aeruginosa* SES-938-1 Elastase for N-succinyl-L(ala)$_3$-p-nitroanilide. V is in unites of Δ A410 nm/min.

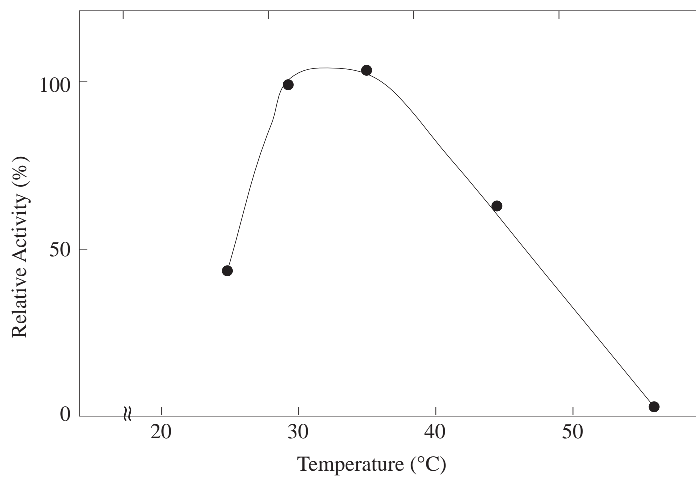

Figure 2. Temperature Optimum of Elastase from *P.aeruginosa* SES-938-1.

Effect of EDTA on elastase

Figure 4 shows that 15 mM EDTA has markedly (> 95%) inactivated elastase under the experimental conditions. This results shows that divalent ion(s) is essential for elastolytic activity.

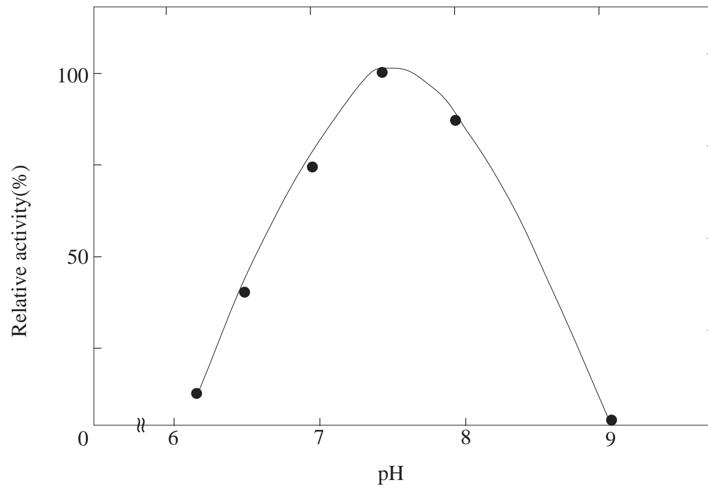

Figure 3. pH Optimum of Elastase from P. aeruginosa SES 938-1

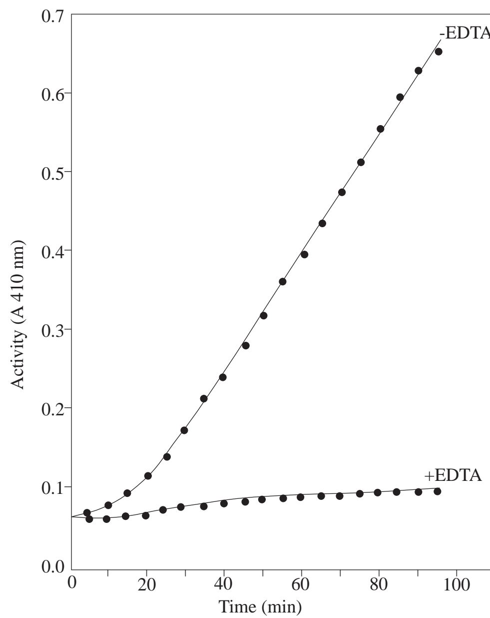

Figure 4. Effect of EDTA on Elastase Activity of P. aeruginosa SES-938-1. The enzyme activities were measued in 100 mM Tris HCl (pH 7.5) buffer, in the absence or in the presence of 15 mM EDTA, as described in the Materials and Methods

Effect of metal ions on elastase

Effects of various metal ions on P. aeruginosa SES-938-1 elastase were studied, by measuring the activities in the absence and in the presence of various metal salts. Table 1 shows that 10 mM $CaCl_2$ did not have a pronounced effect on elastase activity, which increased the enzyme activity only by 12.5 %. On the other hand, Ni^{2+}, Zn^{2+} and Mn^{2+}, at the same concentration, almost completely inhibited the elastase. The enzyme activity has decreased by 25 % when 10 mM $MgCl_2$ is added into the reaction mixture.

Table 1. Effects of metal ions, at 10 mM concentration, on P.aeruginosa SES 938-1 elastase

Metal ions (mM)	Remaining Activity (%)
Control	100
Mn2+	0
Ni2+	0
Zn2+	0
Mg2+	75
Ca2+	112.5

Control does not contain any metal ion.

Discussion

Our results show that *P. aeruginosa* SES-938-1 elastase is a typical neutral-metallo-proteinase. The optimum temperature for this enzyme is 30°-36 °C and optimum pH is 7.5. Metal complexing agent EDTA is an inhibitor for the elastase, indicating strong dependence on divalent metal ion(s) for the activity. This finding is in agreement with the previous reports which demonstrated that *Pseudomonas* elastase was inactivated by various chelating agents, such as EDTA and EGTA and this inactivation could be reversed by addition of Zn^{2+} or Co^{2+} (13, 16). Inactivation of elastase by EDTA may be due to removal of Zn^{2+} and/or Ca^{2+}, each one is tightly bound to enzyme (16). Zn^{2+} binding ligands involving His and Glu residues, are located at the active center (16-18). Although zinc is accounted for the reactivity, calcium is rather suspected to be associated with the stabilization of the enzyme molecule (17). Morihara and Tsuzuki (19) have found that Ca^{2+} can not be removed from elastase by dialysis and could dissociate at high temperatures, such as 55 °C, which means some denaturation of the enzyme is required. In our experiments, presence or absence of Ca^{2+} did not make a significant difference in the elastase activity. This could be due to inefficient removal of Ca^{2+} from the enzyme preparation or the enzyme molecule itself by centricon ultrafiltration. On the othar hand, Mn^{2+}, Ni^{2+} and Zn^{2+} at 10 mM concentration, almost completely inhibited the elastase. At lower concentrations (10^{-3} M) however, Mg^{2+} and Mn^{2+} are suggested as stimulators of the *Pseudomonas* elastase *(2,4)*.

It was reported that elastase, like neutral-metallo-proteinase shows its specificity against

aromatic or bulky aminoacid residues at the splitting point (16). Also, there are reports for independence of the microbial elastase activities of their side-chain specificities in proteolysis (20). Our results indicated that, *P. aeruginosa* elastase can easily hydrolyze the synthetic peptide N-succinate-L-$(ala)_3$-p-nitroanilide, in which alanine is at the carboxyl side of the splitting point. In this respect, *P.aeruginosa* SES-938-1 elastase is similar to elastases from pancreas and neutrophile (20).

Acknowledgement

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