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Hyperactivation of serine proteases by the Hofmeister effect

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ABSTRACT

α-Chymotrypsin (CHT) is a serine protease that hydrolyzes peptide bonds at the carboxyl end of hydrophobic amino acids. It has been shown that the enzyme activity of CHT increases one order of magnitude in the presence of some types of amine compounds and inorganic ions. Here we show that hyperactivation occurs for several kinds of serine proteases in the presence of a kosmotrope. Among the eight model enzymes, the catalytic activities of four serine protease increased 1.3–30-fold by the addition of 1.5 M sodium sulfate. Enzyme kinetics and circular dichroism analyses revealed that the hyperactivation is caused by both increased k_{cat} and decreased K_M, which reflect stabilization of the active form of the enzyme and the affinity between the enzyme and hydrophobic substrate, respectively. These findings demonstrate the effectiveness of this simple method for enzyme activation without cost and contribute to our fundamental understanding of enzyme activity in solution.

Introduction

Enzymes catalyze reactions in aqueous solution under mild conditions with high specificity for their substrates. Various types of enzymes are used in many industries [1], such as those involving analytical reagents [2,3], biopharmaceutics [4], food processing [1,5], preservatives [6], and fermentation [7,8]. The improvement of activity and stability of enzymes typically used in pathological diagnosis [9,10] and biosensing [11] are valuable for these industries. Methods used to increase enzyme activity include protein engineering [12] and addition of activators in solution. Enzyme activators of various types have been developed, such as detergent micelles [13–15], polyelectrolytes [16–18], functional nanoparticles [19–22], kosmotropes [23], and ion liquids [24,25]. The mechanism of the increasing activities is different; detergent micelles and polyelectrolytes result mainly from the electrostatic interaction between protein and substrate, while kosmotropic ion and ion liquid result from the hydrophobicity of the substrate and coactivator.

α-chymotrypsin (CHT) hydrolyzes peptide bonds at the carboxyl end of hydrophobic amino acids. CHT has been studied for enzyme kinetics [26,27], protein engineering [28,29], protein folding [30,31], stability [32–34], as well as its various applications [35]. In earlier research, we found that the enzyme activity of CHT can be controlled via three types of approaches: The first approach type uses a polyelectrolyte in wrap-strip technology [36–38]. The positively charged polyelectrolyte binds non-covalently on the enzyme surface, which increases the enzyme activity of CHT by 18-fold toward the negatively charged small substrate [17]. Similarly, negatively charged polyelectrolytes increase CHT activity by 7-fold toward positively charged substrates. The second approach uses small amines and naturally-occurring polyamines [18]. The small amines increase the enzyme activity of CHT by increasing its hydrophobicity and multivalency, meaning that the artificial activators bind to enzymes similar to aggregation suppressors [39], which increase the affinity between substrates and enzymes. The third approach uses inorganic salts. Kosmotropes increase the enzyme activity of CHT toward hydrophobic substrates by 10-fold [23]. These studies showed that activators increased the affinity between CHT and substrates by electrostatic and/or hydrophobic interactions.

In the present study, we investigated the fundamental mechanism of hyperactivation for enzymes. We selected eight kinds of enzymes, including CHT, in the presence of inorganic salts. The results showed that serine proteases increased their enzyme activities in the presence of kosmotropes. Enzyme activation was found to be more favorable for hydrophobic substrates as reflected by decreased K_M and increased k_{cat} values.

Materials and methods

CHT from bovine pancreas, trypsin from bovine pancreas (TRP), subtilisin from Bacillus licheniformis (SUB), elastase from porcine pancreas (ELA), leucine aminopeptidase microsomal from porcine kidney (LAP), chicken egg white lysozyme (LYZ), papain from papaya latex (PAP), *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SPNA), l-lysine-

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p-nitroanilide dihydrobromide (Lys-pNA), l-leucine-*p*-nitroanilide dihydrobromide, Micrococcus luteus, and 3-(N-morpholino) propanesulfonic acid (MOPS) were obtained from Sigma Chemical Co. (St. Louis, MO). Lysyl endopeptidase from Achromobacter lyticus M497-1 (API), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium thiocyanate (NaSCN), hydrogen chloride (HCl), and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Ethanol (EtOH) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA), and Suc-Ala-Ala-Ala-pNA was obtained from Peptide Institute, Inc. (Osaka, Japan). All chemicals used were of high-quality analytical grade and were used as received.

Enzyme assay

Catalytic activities of model enzymes were measured as follows. The concentrations of enzymes were determined from the absorbance at 280 nm by using a spectrophotometer (ND-1000; Nanodrop Technologies, Inc., Wilmington, DE).

Enzyme assays for CHT and SUB

Thirty microliters of substrate solution containing 0–20 mM substrate in 90/10 (v/v) EtOH/DMSO was mixed with 260 μL of additive in 50 mM MOPS (pH 7.5) and 10 μL of 150 μM enzyme in 1.0 mM HCl (final concentrations 5.0 μM enzyme, 0–1.5 M salt, and 2.0 mM SPNA). The initial reaction velocities (v_0) were determined from the slope of the initial increase in the absorbance at 410 nm by using a Jasco spectrophotometer (V-550; Japan Spectroscopic Co., Ltd., Tokyo, Japan). The absorbance was converted to concentration by using the molar extinction coefficient for p-nitroaniline (pNA) of 8800 M^{-1} cm^{-1}. Normalized enzyme activity was defined as the ratio of v_0 in the presence of additive to v_0 in the absence of additive. The K_M and k_{cat} values were determined by the initial reaction velocity on theoretical Michaelis–Menten curves by nonlinear regression.

Enzyme assays for TRP and API

Thirty microliters of substrate solution containing 0–20 mM substrate in 50 mM MOPS (pH 7.5) was mixed with 260 μL of additive in 50 mM MOPS (pH 7.5) and 10 μL of 150 μM enzyme in 1.0 mM HCl (final concentrations 5.0 μM enzyme, 0–1.5 M salt, and 2.0 mM Lys-pNA). The initial reaction velocities (v_0) were determined from the slope of the initial increase in the absorbance at 410 nm by using a Jasco spectrophotometer V-550 (Japan Spectroscopic Co., Ltd). Normalized enzyme activity was defined as the ratio of v_0 in the presence of additive to v_0 in the absence of additive. The K_M and k_{cat} values were determined by the initial reaction velocity on a theoretical Michaelis–Menten curve by nonlinear regression.

Enzyme assay for LYZ

One thousand nine hundred and ninety microliters of 0.3 mg/mL of M. luteus containing 50 mM MOPS buffer (pH 7.5) and 1.5 M salt was mixed with 10 μL of lysozyme solution. The decrease in the light-scattering intensity of the solution was monitored by measuring the absorbance at 600 nm on a Jasco model V-630 spectrophotometer (Japan Spectroscopic Co., Tokyo) at 25 °C. The residual activity was calculated from the slope of the initial decrease in absorbance.

Enzyme assay for PAP

One thousand two hundred and fifty microliters of 0.3 M salt in 50 mM MOPS buffer (pH 7.5) and 200 μL 15 mM L-BAPA in water were mixed with 50 μL of papain solution. Generation of p-nitroaniline was monitored by measuring the absorbance at 410 nm on a Jasco spectrophotometer V-630 at 25 °C. The residual activity was calculated from the slope of the initial increase of absorbance.

Enzyme assay for ELA

Ninety microliters of 4.66 mM Suc-Ala-Ala-Ala-pNA in 50 mM MOPS buffer (pH 7.5) was mixed with 10 μL of 3.0 μM elastase and 1.5 M salt solution. The initial reaction velocities (v_0) were determined from the slope of the initial increase in the absorbance at 410 nm by using a spectrophotometer V-550. Normalized enzyme activity was defined as the ratio of v_0 in the presence of additive to v_0 in the absence of additive.

Enzyme assay for LAP

Thirty microliters substrate solution containing 0–20 mM substrate in 90/10 (v/v) EtOH/DMSO was mixed with 260 μL additive in 50 mM MOPS (pH 7.5) and 10 μL of 3.0 μM LAP in 1.0 mM HCl (final concentrations 0.1 μM LAP, 0–1.5 M salt, and 2.0 mM Leu-pNa). The initial reaction velocities (v_0) were determined from the slope of the initial increase in the absorbance at 410 nm by using a Jasco spectrophotometer V-550 (Japan Spectroscopic Co., Ltd). Normalized enzyme activity was defined as the ratio of v_0 in the presence of additive to v_0 in the absence of additive.

Circular dichroism

Circular dichroism (CD) experiments were performed by placing samples in a 10-mm path length quartz cuvette and measured by using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Solutions containing 50 μM enzyme, 0–1.5 M salt, and 50 mM MOPS buffer (pH 7.5) were prepared, and the spectra were measured at 25 °C. The CD spectra of samples were corrected by subtracting the corresponding spectra of the buffers in the absence of enzyme. The thermal unfolding profiles were measured by CD intensity at 296 nm with increasing temperature of 1 °C/min^{-1}. The data obtained were fitted to a conventional two-state equation and determined denaturing temperature (T_m)

Results

Activation of serine protease by the Hofmeister effect

We have previously shown that the enzyme activity of CHT is increased by polyelectrolytes [17], small polyamines [18], and kosmotropes [23]. In the present study, we investigated the hyperactivation of various kinds of enzymes caused by kosmotropes. Eight kinds of enzymes were selected; (i) trypsin (TRP), *Achromobacter* protease I (API), CHT, subtilisin (SUB), and elastase (ELA) as CHT-like serine protease; (ii) leucine aminopeptidase (LAP) as metalloproteinase; (iii) papain (PAP) as cysteine protease; and (iv) lysozyme (LYZ) as a hydrolase for polysaccharide. Three kinds of salts were used; kosmotropic salt of Na_2SO_4, chaotropic salt of NaSCN, and NaCl as a control.

Fig. 1 shows the activity of eight kinds of enzymes in the presence of 1.5 M salts; Table 1 summarizes the activation rates and substrates used. The enzyme activity of CHT with Na_2SO_4 increased approximately 10.2-fold, whereas that with NaSCN decreased 0.1-fold (Fig. 1A), which is a similar result to that in a previous paper [23]. These enzyme activations by Na_2SO_4 and inhibition by NaSCN were observed for other enzymes including SUB, TRP, and API, which are classified into CHT-like serine protease. The enzyme activities of the enzymes with Na_2SO_4 increased approximately 1.3–30 fold, whereas that with NaSCN decreased 0.1–0.4 fold (Fig. 1B–D). In contrast to the above four enzymes, the similar activation/inhibition patterns were not observed for other enzymes (Fig. 1E–H). Note that Na_2SO_4 is classified as a kosmotrope that stabilizes the tertiary structures of proteins, whereas NaSCN is one of the chaotropes that destabilize protein structures in the Hofmeister series [40]. As expected, SCN ion decreased the activities of enzymes, excepting ELA, probably due to the destabilization of tertiary structures. By contrast, our data suggested that increment of enzyme activity by kosmotropic Na_2SO_4 was likely to occur