Control of Hydrolysis and Condensation Activities of Thermolysin by Ultrasound Irradiation

Takayoshi Kawasaki, Yu Hoshino, Yukari Ishizu, Yu Mizushiro, and Yoshio Okahata*
Department of Biomolecular Engineering and Frontier Collaborative Research Center, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501

(Received September 15, 2005; CL-051182)

Hydrolysis and condensation reactions of peptides catalyzed by thermolysin can be reversibly controlled by on/off ultrasound irradiation depending on its frequency region.

The flexibility and conformational mobility of proteins may be one of essential factors for enzyme catalyses,[1] however, little mechanical evidence has been demonstrated. For example, a time scale of conformational motions of a loop domain that covers the substrate and co-enzyme binding sites of dihydrofolate reductase (DHFR) is close to that of the turnover number of DHFR.[2,3] Kitahara et al. have succeeded in controlling the hinge motion of active site of DHFR with static pressure.[4] It is well known in polymer science that polymer solutions also absorb sound or ultrasound waves depending on their molecular weight and modes of mechanical relaxations of polymer molecules.[5] This fact strongly suggests that not only the static structure but also site-directed conformational motions of proteins can be controlled by periodic pressure fluctuation such as ultrasound irradiation. Thus, ultrasound waves from kHz to MHz can be a good candidate to perturb loops or domain structures of proteins and control enzyme activities in solution.[6]

In this communication, we report that enzymatic activities (hydrolysis and condensation) of thermolysin can be controlled reversibly by ultrasound irradiation with different wavelengths in solution. The reasons why we chose thermolysin, a thermostable zinc endopeptidase from *Bacillus thermoproteolytics Rokko*, is that its enzymatic activity can be changed by a single mutation of amino acids[7] or by adding organic solvents[8] due to the flexibility change of domains in proteins.

A schematic illustration of experimental setup and reaction schemes is shown in Figure 1. A PZT ($Pb(Zr_xTi_{1-x})O_3$) oscillator with resonant frequency at 500 kHz was used to generate the ultrasound, and the frequency (10–1000 kHz) and generation power ($1\,V_{p-p}$) of the square-waves were regulated with a function synthesizer. Oscillating characteristics of the oscillator was measured with a network analyzer and a laser Doppler vibrometer in water solution. We did not use the 200–500 kHz region to avoid non-linear responses of the oscillator. For the hydrolysis reaction, *N*-carbobenzyloxy-L-aspartic acid-L-phenylalanine methyl ester (Z-aspartheme; ZAPM) was used as a substrate. In addition, *N*-carbobenzyloxy-L-aspartic acid (Z-Asp) and L-phenylalanine methyl ester (Phe-OMe) were used as substrates for the condensation reaction. Reactions were followed by HPLC (solvent: water/CH_3CN = 3:1; flow rate: 1 mL min^{-1}; column: Xterra MSC_8, 4.6 × 150 mm; Waters HPLC 600 with dual UV detector). Initial reaction rates (less than 10% of conversion) were obtained from the slope without ultrasound irradiation (V_{off}) and under its irradiation (V_{on}) for 1–2 h (Figure 1). The reaction rate could revert to the original value after stopping the irradiation. The uncatalyzed hydrolysis and condensation rates were negligibly small (less than 1%). The temperature of a reaction cell (4 mL volume) did not change within ±0.01 °C during the ultrasound irradiation for 1 to 2 h. The reaction mixture was sufficiently stirred with a magnetic bar to avoid any effects of ultrasound irradiation on diffusion of reaction mixtures.

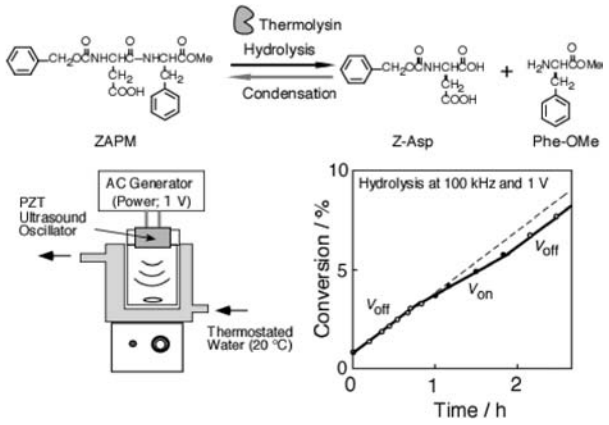

Figure 1. Schematic illustrations of ultrasound irradiation on thermolysin-catalyzed hydrolysis and condensation reactions, and typical time courses of hydrolysis of ZAPM catalyzed by thermolysin without ultrasound irradiation (V_{off}) and under its irradiation (V_{on}) at 100 kHz and 1 V for 1 h. [ZAPM] = 2.5 mM, [thermolysin] = 0.75 mM, 20 °C, 50 mM MES buffer (pH 6.2), 5 mM $MgCl_2$, 2 mM $ZnCl_2$.

The relative reactivity (V_{on}/V_{off}) of thermolysin at frequencies of 10–200 kHz and 500–1000 kHz (the active spectra of thermolysin under ultrasound irradiation) in both hydrolysis and condensation reactions are summarized in Figure 2. In the high-frequency region (500–1000 kHz), both hydrolysis and condensation reactions were accelerated with 1.0–1.2 times, especially at frequencies between 500 and 700 kHz and near 850 kHz. In contrast, only the condensation reaction was activated in the low frequency region with 1.0–1.1 times, especially at 100–150 kHz. Furthermore, the hydrolysis reaction was suppressed in the same region. This suppression effect suggests that the ultrasound effect does not always positively accelerate enzyme reactions.

Lower frequency ultrasound absorption of 100–150 kHz generally corresponds to the kinetic relaxation of a high molecular weight polymer (MW: 10^4–10^6).[5] Therefore, it seems reasonable to suppose that the large domain of an enzyme can be modulated by these frequency areas. Thermolysin is demonstrated from crystallographic studies to have two main large domains and the active site is located in a groove between these two domains.[7] Therefore, different ultrasound effects on the condensation and the hydrolysis in the low frequency region may be a result of mechanical distinction of the substance binding and product release caused by the enzyme motion in each reaction. It is

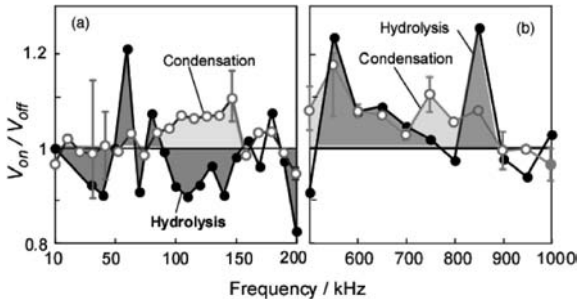

Figure 2. Ultrasound frequency dependences on the relative reaction rates (V_{on}/V_{off}) of hydrolysis (●) and condensation (○) catalyzed by thermolysin in the (a) low and (b) high frequency regions. Applied voltage: 1 V, For hydrolysis, [ZAPM] = 2.5 mM and [thermolysin] = 0.75 mM, For condensation, [Z-Asp] = [Phe-OMe] = 0.15 M and [thermolysin] = 2.9 mM, 20 °C, 50 mM MES buffer (pH 6.2), 5 mM $CaCl_2$.

worth indicating that the hydrolysis reaction was distinctively activated with a 60 kHz ultrasound perhaps due to another sub-mode motion or another domain motion at this frequency. In order to know the effect of ultrasound on substrate binding and/or product release of enzymes, we measured the binding of inhibitors to thermolysin.

Phosphoramidon, *N*-(α-L-rhamnopyranosyloxyphospho)-L-leucyl-L-tryptophan, is known to bind to zinc at the active site of thermolysin via phosphoramidate oxygen.[9,10] The phosphoramidate coordination at the inhibitor-enzyme complex is close to the geometry of the transition state of the ZAPM substrate to this enzyme. Given the structural similarity, we expected this inhibitor to act as a substrate mimic of the hydrolysis reaction. Since phosphoramidon has a triptophan moiety, the inhibitor binding can be monitored with the fluorescence spectroscopy.[11] The time course of fluorescence at 360 nm indicating the phosphoramidon binding to thermolysin could be mathematically fitted with single exponential functions (first-order binding kinetics). The relative binding rates (V_{on}/V_{off}) with and without ultrasound irradiation at different frequencies are shown in Figure 3. Although it is difficult to observe any distinguishable tendency of phosphoramidon binding to the enzyme with ultrasound wave irradiation in the high-frequency region, the substrate binding is somewhat suppressed at the low frequency region centered at 100 kHz. Nevertheless, the phosphoramidon binding rate is somewhat accelerated at 60 kHz. These results are consistent with the active spectrum of the hydrolysis reaction of ZAPM (Figure 2a). Since the phosphoramidon inhibitor is recognized by thermolysin as the ZAPM substrate of the hydrolysis reaction, both the suppression at 100–150 kHz and the acceleration at 60 kHz of the inhibitor binding are in good agreement with those suppression (100–150 kHz) and acceleration (60 kHz) of the ZAPM hydrolysis. Thus, the ultrasound irradiation at 100–150 kHz induces the release of the ZAPM hydrolysis substrate (the suppression of the hydrolysis) and the release of the condensation product (the acceleration of condensation), via the domain fluctuation or the static structure transition of thermolysin in the low frequency region due to the ultrasound.

In conclusion, we could control the thermolysin activity (both hydrolysis and condensation) by ultrasound irradiation depending on its frequency region. Thus, at the low frequency region of 100–150 kHz, the condensation was accelerated but

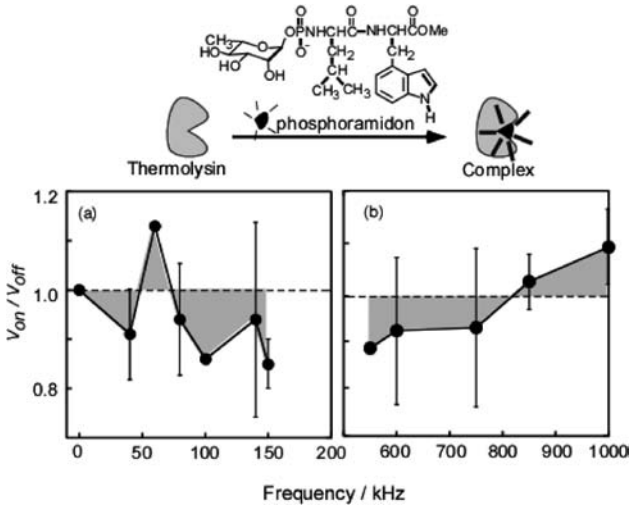

Figure 3. Ultrasound frequency dependences on the relative reaction rates (V_{on}/V_{off}) of binding of phosphoroamidon to thermolysin in the (a) low and (b) high frequency regions. Data were collected at least three times at the same frequency as shown in error bars. At several frequencies, the obtained data were converged and the error bars were hidden by the plot marks. Applied voltage: 1 V, [phosphoroamidon] = 1.3 μM, [thermolysin] = 0.55 μM, 20 °C, 50 mM Tris buffer (pH 7.5), 10 mM $CaCl_2$.

the hydrolysis was decelerated probably due to the inaccessibility and/or the release of a ZAMP substrate by motions of large domains. In the high-frequency region (500–1000 KHz), both the hydrolysis and condensation reactions were accelerated probably due to the perturbation of small segments such as active site residues. We believe that these sound/ultrasound effects on enzyme activities will launch novel techniques for domain specific control of enzyme functions.

References

1 A. Fersht, "Structure and Mechanism in Protein Science – A Guide to Enzyme Catalysis and Protein Folding," W. H. Freeman and Company, New York (1999).
2 T. Kamiyama and K. Gekko, *Biochim. Biophys. Acta*, **1478**, 257 (2000).
3 C. J. Falzone, P. E. Wright, and S. J. Benkovic, *Biochemistry*, **33**, 439 (1994).
4 R. Kitahara, S. Sareth, H. Yamada, E. Ohmae, K. Gekko, and K. Akasaka, *Biochemistry*, **39**, 12789 (2000).
5 G. R. Strobl, "The Physics of Polymers," Springer-Verlag Berlin, Heidelberg (1997), Chap. 5.
6 T. V. Chalikian, M. Totrov, R. Abagyan, and K. J. Breslauer, *J. Mol. Biol.*, **260**, 588 (1996).
7 S. Kidokoro, Y. Miki, K. Endo, A. Wada, H. Nagao, T. Miyake, A. Aoyama, T. Yoneya, K. Kai, and S. Ooe, *FEBS Lett.*, **367**, 73 (1995).
8 K. Watanabe and S. Ueji, *Biotechnol. Lett.*, **22**, 599 (2000).
9 A. Beaumont, M. J. O'Donohue, N. Paredes, N. Rousselet, M. Assicot, C. Bohuon, M.-C. Fournié-Zaluski, and B. Roques, *J. Biol. Chem.*, **270**, 16803 (1995).
10 D. E. Tronrud, A. F. Monzingo, and B. W. Matthews, *Eur. J. Biochem.*, **157**, 261 (1986).
11 K. Kitagishi and K. Hiromi, *J. Biochem.*, **95**, 529 (1984).

Published on the web (Advance View) October 27, 2005; DOI 10.1246/cl.2005.1602