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Effect of energy-gathered ultrasound on Alcalase

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ABSTRACT

This research was to explore the mechanism of ultrasonic impact on protease activity. The effects of energy-gathered ultrasound on the activity, kinetics, thermodynamics and molecular structure of Alcalase were investigated with the aid of the chemical reaction kinetics model, Arrhenius equation, Eyring transition state theory, fluorescence spectroscopy and circular dichroism (CD) spectroscopy. Results showed that ultrasound had effect on the activity of Alcalase. The highest Alcalase activity was achieved when the sample was treated with energy-gathered ultrasound at 80 W for 4 min, under which the enzyme activity was increased by 5.8% over the control. After the treatment, thermodynamics parameters Ea, ΔH, ΔS and ΔG were reduced by 70.0%, 75.8%, 34.0% and 1.3%, respectively. Besides, fluorescence and CD spectra revealed that the ultrasonic treatment had increased the number of tryptophan on Alcalase surface slightly, increased number of α-helix by 5.2%, and reduced the number of random coil by 13.6%.

1. Introduction

Ultrasound has attracted more and more attention in food science and technology, which can mainly be classified into two fields: high-frequency low-energy ultrasound in the MHz range, and low frequency high-energy ultrasound in the kHz range. The high-frequency ultrasound is usually used as an analytical technique for quality assurance, process control and non-destructive inspection, etc. [1–3]. Application of the low frequency high-energy ultrasound in food industry is relatively new, which has been used in the laboratory or food processing factory to improve food physicochemical properties in various areas such as nanoemulsion preparation [4], ultrasound-assisted extraction [5], enhancing oxidation [6], and improvement of foaming properties [7]. The ultrasound generated by periodic mechanical motions of a probe transfers ultrasonic energy into a fluid medium and triggers extremely high alterations in pressure leading to the formation of small rapidly growing bubbles [8], which expand during the negative pressure excursion, and implode violently during the positive excursion generating high temperatures, pressures and shear forces at the probe tip.

Recently, the interest of food technologists has turned to the use of ultrasound for altering enzyme activity. Effects of ultrasound on enzyme activity include activation and inactivation. There are already a lot of literatures focusing on inactivation of enzyme activity by ultrasound [9–15]. In comparison with the extensive researches devoted to the inactivation of enzymes, the activation of enzymes are just reported by a few technologists [16–17].

Proteases are important class of enzymes, which constitute more than 65% of the total industrial enzyme market. They can hydrolyze proteins to short peptides or free amino acids and catalyze peptide synthesis in organic solvents or in solvents with low water content [18]. Proteases have a large variety of applications in food industries. These include beer chill proofing, meat tenderization cheese manufacture, flavor development in fermentation, baking, and health products. Considering the commercial importance of proteases, it is necessary to find an effective method to improve the activity of protease.

Ovsianko et al. have indicated that ultrasound had a positive impact on the activity of protease [19]. However, the mechanism of ultrasound action on protease has not been reported. Therefore, in order to explore the enzyme activation mechanism, Alcalase was taken as the studied protease, and the effects of energy-gathered ultrasound on activity, kinetics, thermodynamics and molecular structure of Alcalase were investigated with the aid of the chemical reaction kinetics model, Arrhenius equation, Eyring transition state theory, fluorescence spectroscopy and circular dichroism (CD) spectroscopy.

2. Materials and methods

2.1. Materials

Alcalase was purchased from Novozymes Biotechnology Co. Ltd (Tianjing, China). The enzyme activity was 132,500 U/g. All other

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chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2. Test of effect of energy-gathered ultrasound on the Alcalase activity

2.2.1. The ultrasonic treatment of Alcalase solution

Sample solutions were sonicated in a ultrasound processor (Shangjia Biotechnology Co., wuxi, China; Model GA92-II DB) with a 2.0 cm flat tip probe at 20 kHz. The instrument can deliver a maximum power of 1800 W. Alcalase powder was dissolved in water with magnetic stirring for 5 min and the final concentration was 0.33 g/l.200 ml Alcalase solution was put into a 250 ml beaker, and the beaker was placed in a water bath at different initial temperatures. The surface of the Alcalase solution in the beaker was 2 cm lower than that of the water bath. The on-time of pulsed ultrasound was 2 s and off-time was 2 s. Each treatment was replicated three times.

The first group was used to investigate the effect of ultrasound at different power (0, 40, 80, 120, 160, 200, 300, 400 W) for 5 min at initial temperature of 35 °C. The second group was used to investigate the effect of ultrasound at different irradiation time (2, 4, 6, 8, 10, 30 and 60 min) at 80 W at initial temperature of 35 °C.

2.2.2. Assay of Alcalase activity

The activity of treated Alcalase was determined using a Folin-phenol method described by Zhou [20]. Casein was used as the substrate for hydrolysis by Alcalase. The absorbance was measured at A_{660} nm with UV–visible spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary 100), which was used to calculate the concentration of tyrosine released from casein. One unit of enzyme activity was defined as 1 μg tyrosine released from the substrate per minute at pH 8.0 and 50 °C. The activities of enzyme were expressed as means ± S D of three determinations.

2.3. Test of effect of energy-gathered ultrasound on the Alcalase kinetics and thermodynamics

2.3.1. Ultrasonic treatment test

For the determination of rate constants, 200 ml Alcalase solution (0.33 g/l) was treated with ultrasound probe (20 kHz) at 80 W under different initial temperature (20, 30, 40, 50 °C) for 4 min. After ultrasonic treatment, 0.2 ml of enzyme solution was added into the 1 ml of 2% casein protein solution. The pH of the enzymolysis experiment was adjusted to 8.0 with 0.05 M NaOH, and the temperature was controlled at 50 °C in water bath. For the determination of thermodynamics parameters, Alcalase was pretreated with ultrasound probe (20 kHz) at 80 W under 35 °C for 4 min, and enzymolysis experiments were conducted under pH 8.0 at temperature of 20, 30, 40 and 50 °C, respectively.

2.3.2. Measurement of kinetics parameter rate constants

The chemical kinetic models of Alcalase can be described as first-order kinetics [10].

$$\ln\left(\frac{C}{C_0}\right) = -k_{in}t \tag{1}$$

where C is the concentration of casein at time $t = t$ (μg/ml), C_o is the initial concentration of casein, t is time, k_{in} is the effective (total) rate constant involving the rate constants of ultrasonic k_{us} and thermal k_1 of Alcalase (Eq. 2). As it is difficult to measure the decrement of casein, the reaction rate can be reflected by the increased amount of tyrosine released by casein. The observed value of k_{in} can be determined by Eq. (3):

$$k_{in} = k_1 + k_{us} \tag{2}$$

$$\ln(V_\infty - V_t) = -k_{in}t + \ln V_\infty \tag{3}$$

where V_t is the concentration of tyrosine at time $t = t$ (μg/ml), V_∞ is the ultimate concentration of tyrosine (μg/ml), which is obtained from the enzymolysis experiment conducted under pH 8.0 at 50 °C for 10 h.

2.3.3. Determination of thermodynamics parameters Ea, ΔG, ΔH and ΔS

The dependence of the constant rate k_{in} on temperature can be described by Arrhenius equation:

$$k_{in} = Ae^{\frac{-E_a}{RT}} \tag{4}$$

where A is pre-exponential or collision factor and Ea the activation energy (J/mol). R is the universal gas constant (8.314 J/mol K).

In order to understand the effect of temperature on Alcalase activity and also the macroscopic changes observed in this study, the Eyring transition state theory was used:

$$k_{in} = \frac{k_B T}{h} exp\left(-\frac{\Delta G}{RT}\right) = \frac{k_B T}{h} exp\left(-\frac{\Delta H}{RT} + \frac{\Delta S}{R}\right) \tag{5}$$

where T is the absolute temperature in K, k_B is Boltzman constant (1.38×10^{-23} J/K), h is Planck constant (6.6256×10^{-34} J/s), R is the universal thermodynamic constant, ΔG, ΔH and ΔS are the parameters of changes in free energy, enthalpy, and entropy for the process of ultrasonic activation of enzyme.

2.4. Test of effect of energy-gathered ultrasound on the structure of Alcalase

2.4.1. The ultrasonic treatment of Alcalase solution

Two-hundred milliliter Alcalase solution (0.33 g/l) was treated with ultrasound probe (20 kHz) at 80 W for 4 min. The initial temperature was controlled at 35 °C.

2.4.2. Measurement of intrinsic fluorescence

Intrinsic fluorescence spectra of untreated (control) and ultrasound-treated samples in water were measured at room temperature (25 ± 1 °C) with fluorescence spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary Eclipse) at 280 nm (excitation wavelength, slit = 5 nm), 300–500 nm (emission wavelength, slit = 5 nm) and 10 nm/s of scanning speed. Water used to dissolve Alcalase was used as blank solution for the sample.

2.4.3. Circular dichroism analysis of Alcalase

Circular dichroism (CD) spectra were recorded with a spectropolarimeter (French Biologic Company, Grenoble, French; Model MOS-450), using a quartz cuvette of 1 mm optical path length at room temperature (25 ± 1 °C). CD spectra were scanned in the far UV range (250 to 190 nm) with three replicates at 100 nm/min and with 0.1 nm as bandwidth. The CD data were expressed in terms of mean residue ellipticity, [θ], in deg cm^2 dmol^{-1}. The α-helix content of Alcalase was calculated from the [θ] value at 208 nm using the equation described by Greenfield and Merelo [21,22].

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed to compare the effects of ultrasound under the significance level of $p < 0.05$. All graphs and calculations were performed with OriginPro 8.0 and Microsoft Office Excel 2007, respectively.