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Processing of protease under sub- and supercritical conditions for activity and stability enhancement

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ABSTRACT

Enzymatic reactions with supercritical carbon dioxide (SC-CO_2) have received increased attention during the last decade. The objective of this study was to alter the activity and stability of *Bacillus* sp. protease under SC-CO_2 conditions. The activity and stability of protease were evaluated according to the effects of operational variables, temperature (28–80 °C), pressure (60–300 bar), CO_2 flow (2–10 g/min) and process duration (60–180 min). Best conditions were identified as 300 bar, 54 °C, 6 g/min CO_2 flow and 120 min of process time efficient 54.4% (417.50 μmol/ml/min) as against to the untreated enzyme. Activity enhancement was observed with the raise of pressure while low flow rates decreased the activity by 5.1% with a flow rate of 2 g/min under supercritical conditions. Potential mechanisms for pressure stimulated activation and stabilization were investigated by NMR, SEM, FTIR, SDS-PAGE and XPS analyses. As a result, applications of SC-CO_2 medium for enzymatic processes are expected to become sustainable and important with economical synthetic and environmentally friendly protocols.

1. Introduction

Enzymes have the important advantages of high chemo-, stereo- and regio-selectivity in organic synthesis, generating the main class of synthetically relevant biological catalysts [1]. Today, non-aqueous medium for enzymatic applications is a well-accepted procedure which presents opportunities for the fundamental studies in regards to functions and structures of enzymes [2]. The use of supercritical fluids (SCF) seems to be a promising strategy to satisfy the requirements of green chemistry concept in industrial processes. The advantages of SCF include environmental, health, safety and chemical benefits [3]. The usage of supercritical carbon dioxide (SC-CO_2) is favorable with its non-toxic, non-flammable, available of low cost and environmental acceptable properties and is proper for biochemical and pharmaceutical applications, therefore has been utilized for enzymatic reactions medium [4]. Also, the mass transfer limitations and the low surface tension reduces by the high diffusivity of reactants and the relatively low viscosity of the mixture [5].

Supercritical fluids have the properties of liquid-like solubilizing power and gas-like low viscosities with high diffusivities and these features can be controlled by modification of the pressure and temperature. The small changes near the critical point in temperature or pressure can cause significant changes in density and density-dependent solvent properties such as the partition coefficient, the dielectric constant and the solubility parameter [6]. The type and source of enzyme, water content of the solution and the temperature and pressure of the reaction system can affect the activity and the stability of the enzymes treated with SC-CO_2 under high pressure. The three-dimensional structure of enzymes is very important and activity can be altered by small changes in the surrounding of the active site. The reduction in the enzyme activity can probably be affected by depressurization step; activity reduces with growing number of depressurizations, with the long-term enzyme application [7,8]. Apart from that progression from supercritical environment to ambient conditions is “enzyme friendly” [9].

Considering the industrial enzymes, the most important group is the proteases with a 60% share in the market and alkaline proteases accounted for 89% of the total protease sale. From biotechnological point of view, alkaline proteases derived from microorganisms are the most interesting and investigated in scientific areas of protein chemistry and engineering. The most significant proteases are bacterial enzymes having wide range of industrial applications compared to animal and fungal proteases. A significant share of the enzyme market is captured by production of extracellular proteases from many *Bacillus* species [10,11]. The hydrolysis of proteins into

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peptides and amino acids is catalyzed by a protease which is one of the most useful enzyme groups. This group of proteases also has industrial applied fields in such as foods, tannery, pharmaceutical, detergents, and leather industries [12,13].

In this research the intent was to optimize the process parameters which are effective on activity and stability of protease from *Bacillus* sp. treated under sub- and supercritical CO_2 conditions. For optimization of activity and stability with protease from the source of *Bacillus* sp., no publications were available based on our scientific databases research. Elemental analysis and spectroscopic techniques such as FTIR, high resolution ^{1}H NMR, SEM, SDS-PAGE and XPS have been used to investigate the possible mechanisms for pressure induced stabilization and activation for protease under supercritical CO_2 conditions.

2. Materials and methods

2.1. Materials

Bacterial protease from *Bacillus* sp. (P0029) was obtained in its free form from Sigma (St. Louis, MO, USA) and stored at +4 °C. Potassium phosphate, dibasic, trihydrate (P5504), casein (C7078), trichloroacetic acid (T0699), Folin & Ciocalteu's phenol reagent (F9252), sodium carbonate, anhydrous (S2127) and L-tyrosine, free base (T3754) were purchased from Sigma. CO_2 (99%) tube was taken from Habas, Izmir, Turkey. Ultrapure water used in the analysis was taken from ultrapure water system (Sartorius Arium 611, Sartorius-Stedim, Gottingen, Germany).

2.2. Protease activity assay

The substrate casein was dissolved in 0.05 M Tris/HCl buffer pH 9.0 at 0.6% (w/v) by gentle heating. The enzyme solution (0.5 ml) was incubated with the substrate solution (2.5 ml) for 20 min at 37 °C. The reaction was ended by adding 0.44 M trichloroacetic acid (2.5 ml). After centrifugation (8 min at 5000 rpm) of the samples, 2.5 ml Na_2CO_3 (0.5 M) solution and 0.5 ml Folin–Ciocalteau reagent were added into 0.5 ml supernatant. The absorbance was measured at 660 nm after the reaction mixture had stood for 30 min at room temperature. One unit of enzyme releases 1 μmol of tyrosine from casein per 20 min at 37 °C. The activity of protease enzyme was calculated from the following equation:

$$(\text{U/ml})\,\text{Enzyme} = \frac{A/\text{slope} \times V_t}{V_e \times 20} \times D_f \quad (1)$$

where A: the concentration obtained from the tyrosine calibration curve at the measured absorbance value (μmol), V_t: volume of total reaction (ml), V_e: the enzyme volume (ml), 20: duration of reaction (min), D_f: dilution factor.

2.3. Supercritical fluid treatment

Supercritical CO_2 treatments for the activity enhancement were performed at SFE 100 System and we followed the method described in our previous study [14]. The independent variables were pressure (60–300 bar), temperature (28–80 °C), CO_2 flow (2–10 g/min) and process duration (60–180 min).

2.4. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were recorded on Varian AS400 at operating frequency of 400 MHz. The proton chemical shifts were related to tetramethlysilane (TMS) as an internal standard and samples were recorded in D_2O solution. The spectrometer frequency, acquisition time and spectral width were as follows: 400 MHz, 1.998 s, 6000 Hz, respectively.

2.5. Scanning electron microscopy (SEM)

A Quanta-250 FEG environmental scanning electron microscope system (FEI Company, USA) was used to investigate morphological changes of samples treated and untreated with SC-CO_2 with the required magnification. Before the observation, Emitech K550X sputter coater was used to mount the samples on metal grids with an aluminum tape and coated with gold under vacuum for 1 min.

2.6. Fourier transform infrared spectrometry (FTIR)

Perkin Elmer Spectrum BX-FTIR Spectrophotometer was used to record the FTIR spectra by using ATR (Attenuated Total Reflectance) system for enzymes in powder form (4000–650 cm^{-1}).

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

For determination of homogeneity and molecular weight, the protease enzyme preparation and known molecular weight markers were exposed to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The method of Bollag and Edelstein [15] was referred by using 10% polyacrylamide gel. The gel was stained with Coomassie Blue R-250 dye in methanol-acetic acid-water solution (4:1:5, by volume) for 1 h and destained in the same solution without dye after electrophoresis.

2.8. X-ray photoelectron spectroscopy (XPS)

XPS was performed for elemental characterization of the enzyme surface using the Thermo-Scientific, Al-*K*α electron spectrophotometer. The device was calibrated according to gold $4f_{7/2}$. About 10^{-8} mbar under vacuum was studied and 20 scanning were applied to a single point. Pass energy and energy step size were determined as 150 eV and 1 eV.

2.9. Statistics

Results were expressed as means ± standard deviations. The data were statistically analyzed using the Student's *t*-test. Statistically significant difference was defined with a probability value of $p \leq 0.05$ and the power of the significance was showed with $p \leq 0.01$.

3. Results and discussion

3.1. Effect of sub- and supercritical CO_2 treatment on the catalytic properties of protease

In order to determine the optimum conditions which enhanced the activity and stability of protease from *Bacillus* sp., the effects of pressure (60–300 bar), temperature (28–80 °C), CO_2 flow (2–10 g/min) and process time (60–180 min) were interpreted. The activity of untreated enzyme was measured as 270.40 μmol/ml/min. This value was used to compare the activities of treated enzymes.

The effect of pressure (Fig. 1A) was investigated by the SC-CO_2 treatment at 54 °C, with a flow rate of 6 g/min and a process time of 120 min at 60, 180 and 300 bar. The highest activity of the treatments was obtained at the pressure of 300 bar (417.51 μmol/ml/min) with an increase of about 54.4% when compared with the untreated enzyme. For this case, it can be said that the increase in the pressure, increases the solvating power which may lead to structural changes that can cause an enzyme activity enhancement. Various hypotheses and considerations emphasize that the enzyme structure gets more compact and hard with the