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Effects of high hydrostatic pressure or hydrophobic modification on thermal stability of xanthine oxidase

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ABSTRACT

The effect of high hydrostatic pressure (HHP) on the kinetics of thermal inactivation of xanthine oxidase (XOx) from bovine milk was studied. Inactivation of XOx followed pseudo-first-order kinetics at 0.1–300 MPa and 55.0–70.0 °C. High pressure up to at least 300 MPa stabilized XOx at all the studied temperatures. The highest stabilization effect of HHP on XOx was at 200–300 MPa at 55.0 and 58.6 °C, and at 250–300 MPa at 62.3–70.0 °C. The stability of XOx increased 9.5 times at 300 MPa and 70.0 °C compared to atmospheric pressure at the same temperature. The activation energy of inactivation of XOx decreased with pressure and was 1.9 times less at 300 MPa (97.0 ± 8.2 kJ mol^{-1}) than at 0.1 MPa (181.7 ± 12.1 kJ mol^{-1}). High pressure decreased the dependence of the rate constant of inactivation to temperature effects compared to atmospheric pressure. The stabilizing effect of HHP on XOx was highest at 70.0 °C where the activation volume of inactivation of XOx was 28.9 ± 2.9 cm^3 mol^{-1}. A second approach to try to increase XOx stability involved hydrophobic modification using aniline or benzoate. However, the thermal stability of XOx remained unaffected after 8–14 modifications of carboxyl side groups per XOx monomer with aniline, or 12–17 modifications of amino side groups per XOx monomer with benzoate.

1. Introduction

Bovine xanthine oxidase (XOx) is an oxidoreductase with one molecule of flavin adenine dinucleotide (FAD), one atom of molybdenum, and four atoms of iron per enzyme subunit [1]. The enzyme mainly catalyzes the oxidation of xanthine to uric acid by producing hydrogen peroxide in the presence of oxygen. The oxidation occurs using the molybdopterin center, the pair of iron-sulfur clusters, and bound FAD [1,2]. The ability of XOx to catalyze different substrates allows fabrication of biosensors to evaluate the freshness of fish by detection of hypoxanthine levels, which imparts a bitter spoiled taste to dead fish [3–5], and to detect caffeine (1,3,7-trimethylxanthine) for evaluation of the quality of commercial instant tea and coffee [6]. In addition, XOx has also been used as biosensor to detect theophylline (1,3-dimethylxanthine) content which is one of the most common medications for chronic asthma [7,8].

Enzyme stabilization is important for industrial and analytical applications to decrease biocatalyst consumption, or increase shelf life, and reuse. In the case of enzyme-based analytical devices, increasing enzyme stability increases operational life. Xanthine oxidase stabilization has been studied by various procedures including immobilization onto polymeric supports [9–11], application of organic media [12,13], and utilization of cosolute (trehalose) and osmolyte (betaine) additives [14]. Although substituting hydrophilic residues with hydrophobic reagents often destabilize proteins, interaction with existing surface hydrophobic groups can also stabilize enzymes [15]. High hydrostatic pressure (HHP) has stabilized several enzymes [16]. Recent reports include the effect of HHP on β-glucosidase [17,18], pectinases [19], polyphenoloxidase [20], inulin fructotransferase [21], and glucose oxidase [22]. However, neither the effect of hydrophobic modification nor the effect of high hydrostatic pressure on XOx stability at elevated temperatures have been explored. Therefore, the objective of this research was to elucidate the effects of HHP and hydrophobization on the stability of XOx at selected temperatures.

2. Materials and methods

2.1. Materials and equipment

Xanthine oxidase from bovine milk (EC 1.17.3.2, Product No. X4376), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide, sodium benzoate and aniline were purchased

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from Sigma-Aldrich (St. Louis, MO, USA). Xanthine was obtained from either MP Biomedical (Solon, OH, USA) or Sigma-Aldrich. Potassium phosphate and potassium hydroxide were purchased from Fisher Scientific (Pittsburg, PA, USA). The HHP system used in this research is described in a previous report [22]. Absorbance measurements were made in a multi-mode microplate reader (Model Synergy HTX) controlled by Gen5 data analysis software from BioTek Instruments (Winooski, VT, USA).

2.2. Methods

2.2.1. Thermal stability treatments at HHP

A volume of 100 μL of 0.15 units mL^{-1} (0.21 mg mL^{-1}) untreated XOx in 50 mM potassium phosphate buffer pH 7.5 was transferred into a 15 mm × 15 mm polyethylene plastic pouch. The pouch was then heat-sealed and placed in the high-pressure reactor held at 10 °C. The pressure was then raised to 50, 100, 150, 200, 250, or 300 MPa. Experiments at 0.1 MPa served as a control. The temperature was then raised to 55.0, 58.6, 62.3, 66.1, or 70.0 °C. Incubation temperatures were selected to achieve even increments of the reciprocal of the absolute temperature based on an Arrhenius approach. The incubation time began when 95% of the temperature set point was reached. Five incubation times were selected to obtain the kinetics of XOx inactivation under HHP for each incubation temperature. Upon completion of the incubation time, the samples were first cooled down to 15 °C and then the reactor was depressurized to ambient conditions (0.1 MPa). Fig. 1 illustrates heating/cooling and pressurizing/depressurizing sequences for a XOx sample treated at 200 MPa, 66.1 °C, for 0-min (open symbols) or 20-min (filled symbols) incubation.

The activity of the processed sample was measured immediately after it was removed from the HHP reactor at ambient conditions at 29 °C. The enzyme activity was determined by monitoring the formation of uric acid at 295 nm for 3 min following the Bergmeyer et al. [23] method but adapted to the microplate reader by reducing 10-fold the volume of the reaction mixture. The residual enzyme activity was calculated as a percent relative to the initial activity. The initial activity was determined as the activity of a sample at the same pressure-temperature condition but with 0 min incubation time, as shown in Fig. 1. This accounted for the possible changes in the activity during the heating/cooling and pressurizing/depressurizing transient periods.

Treatments were duplicated and performed in a randomized block design. Pressure and incubation time were randomly chosen while treatments were blocked by incubation temperature.

2.2.2. Kinetics of thermal inactivation at HHP

The thermal inactivation kinetics of bovine milk XOx were evaluated utilizing a first-order kinetics model. The rate constant of inactivation of XOx (k_{inact}) was calculated by linear regression analysis of the relationship between the natural logarithm of residual activity and incubation time. Experimental error in the calculation of k_{inact} was determined utilizing the standard error of the linear regression. To analyze whether there were significant differences in k_{inact} among pressure levels at each temperature, a general linear model (GLM) was applied, evaluating "pressure" and "incubation time" as random factors as well as the "pressure" × "incubation time" interaction with "incubation time" selected as covariate. Linear regression analysis and GLM were done using SAS statistical software (Cary, NC, USA). The R^2 values were used as indicators of the quality of the regression fit.

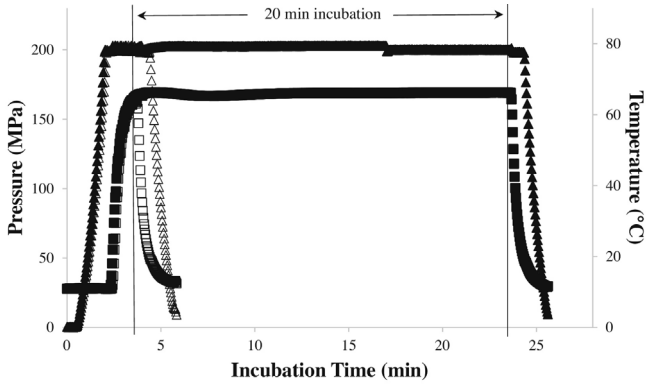

Fig. 1. High pressure reactor temperature (■) and pressure (▲) profiles for a 20 min incubation time at 66.1 °C and 200 MPa; and temperature (□) and pressure (△) profiles for a 0 min incubation time at 66.1 °C and 200 MPa.

2.2.3. Effect of temperature on k_{inact}

The Arrhenius equation was used to quantify the effect of temperature on k_{inact} [24,25]. Therefore, the activation energy of inactivation (E_a) in the Arrhenius equation was calculated by linear regression analysis of the natural logarithm of k_{inact} of XOx and the reciprocal of the absolute temperature. The error in the calculation of the E_a was reported as the standard error of the linear regression of the Arrhenius plot. The comparison of E_a among HHP levels was performed using GLM using "pressure" and "1/temperature" as random factors and evaluating "pressure" × "1/temperature" interaction with "1/temperature" selected as covariate.

2.2.4. Effect of pressure on k_{inact}

The Eyring equation was used to assess the effect of pressure on the k_{inact} [24,26]. This allowed calculation of the activation volume of inactivation ($\Delta V^{\neq}$) by linear regression analysis of a plot of the natural logarithm of the rate constant of inactivation of XOx and pressure. The experimental error in the calculation of $\Delta V^{\neq}$ was determined utilizing the standard error of the linear regression. The comparison of $\Delta V^{\neq}$ among temperature levels was done using GLM, using "temperature" and "pressure" as random factors and evaluating the "temperature" × "pressure" interaction with "pressure" selected as covariate.

2.2.5. Modification of XOx for stability

Xanthine oxidase was chemically modified as described previously for glucose oxidase [22] with a few minor modifications. Briefly, 100 mM sodium benzoate, or aniline, was added to a 1-mL solution of 5 mg mL^{-1} XOx in 50 mM K_2HPO_4, pH 7.5. The solution was incubated on ice for 5 min before addition of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and N-hydroxysulfosuccinimide to concentrations of 10 mg mL^{-1} to initiate the covalent coupling of benzoate or aniline to XOx. The reaction proceeded on ice for 5, 30 or 60 min. Duplicate reactions were performed for each time point. The reaction was stopped by immediately loading the solution onto a 10-DG Econopac column (BioRad) pre-equilibrated in the same 50 mM K_2HPO_4 buffer. This column removes excess reagents and products from the protein. Samples were stored at 4 °C until further use. Modification reactions performed for ≥2 h tended to completely abolish the activity of the enzyme.

2.2.6. Characterization of modified enzyme

Quantifying the level of modification of XOx was performed as previously reported [22]. Briefly, a trinitrobenzene sulfonic acid (TNBS) assay was used to monitor labeling of the lysines and the N-terminal amine of XOx by benzoate. Additionally, the number of modifications by benzoate and aniline were estimated using a Bruker MicroFlex MALDI-TOF mass spectrometer. The difference in mass between the modified and unmodified enzyme was divided by the mass of benzoate or aniline, minus the mass of water (18 Da).

Effects of modification on the thermal stability of XOx were measured on a MicroCal VP differential scanning calorimeter (DSC). Scans were performed on 0.5–1.5 μM XOx in 50 mM Na_2HPO_4, pH 8.0 from 25 to 95 °C at a scan rate of 1.5 °C min^{-1}. Origin v7.0 software supplied by MicroCal controlled the instrument and was also used to fit the data. All values are the average of two independent modification

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