

Spectroscopic Techniques Used for Enzyme Evaluation in Food Industry

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Abstract. Since applications of enzymes in the food industry are many and diverse, monitoring the activity and structure of these bio-catalysts has become a major concern. For this purpose, an *in vitro* study was conducted on beef liver catalase (BLC; EC. 1.11.1.6), as an enzyme model. The enzymatic activity was measured by following H₂O₂ dismutation to H₂O and O₂ under steady-state kinetics conditions. Whereas structural alterations were assessed by series of spectroscopic techniques such as electronic absorption, fluorescence, and circular dichroism at 25 °C in 0.1 M phosphate buffer solution at pH 7.0. Our results suggested that enzymes used in the food industry could be followed by various spectroscopic techniques to ensure the quality and safety of food products.

Keywords: spectroscopic techniques, enzyme, food industry

1. Introduction

The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes [1]. Applications of enzymes in many sectors of the food industry particularly in the dairy, fruit and wine, distilling, brewery, baking and starch industries are many and diverse. Enzymes also find application in the meat, fish, plant protein, and vegetable oil sectors [2]. In addition, the enzymes used in food processing vary from highly purified commercial formulations to relatively crude preparations in the form of leaves, plant exudates or chopped fruits. They may either be directly incorporated into food systems, or immobilized on inert supports to allow the enzyme to interact with food systems during processing [2]-[4].

According to diverse array of food industrial enzyme applications, the ability to determine the enzymatic activity is extremely important for ensuring the quality and safety of the food products [5]. An array of spectroscopic techniques, such as Ultraviolet-Visible (UV-Vis) absorption spectroscopy, Circular Dichroism (CD), and fluorescence spectroscopy can be utilized to study the correlation between structure and function of bio-molecules. UV-Vis can be used as a sensitive measure of subtle changes in enzyme structure and also to determine the enzymatic activity, kinetic parameters, concentration and purity of an enzyme solution. CD in the far UV region (180–260 nm) provides information regarding different forms of regular secondary structure found in proteins and finally fluorescence spectroscopy can also provide tertiary structural information. Since the conformational changes of enzymes due to any alterations such as physico-chemical conditions, modifications, and ligand bindings could impact the UV/Vis absorption, CD, and fluorescence spectra, use of various spectroscopic techniques provide invaluable information [6]-[8]. In this work, an *in vitro* study on beef liver catalase (BLC; EC. 1.11.1.6), as a vital enzyme model, actively involved in the decomposition of hydrogen peroxide to water and oxygen was investigated. Series of spectroscopic

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techniques such as UV-Vis electronic absorption, fluorescence, and CD were used to assess the enzymatic activity and also the BLC structure. The findings suggested that mentioned techniques could be used as potential, accurate, and sensitive methods for evaluation of enzymes used in the food industry.

2. Materials and Methods

2.1. Materials

Beef liver catalase as a freeze-dried powder was obtained from Sigma Chemical Company (St. Louis, MO). Hydrogen peroxide (30% solution) and all other chemicals used in this work were obtained from Merck (Darmstadt, Germany) and were of reagent grade.

2.2. Enzyme Assay

Catalase activity was measured spectrophotometrically, under steady-state kinetics conditions, by monitoring the H_2O_2 dismutation at 240 nm, using an extinction coefficient of $27 \text{ mM}^{-1}\text{cm}^{-1}$. H_2O_2 stock solutions (1 M) were prepared daily by appropriate dilution of 30% H_2O_2 in distilled water. Catalase solutions ($0.0125 \mu\text{M}$) were prepared by dissolving the enzyme in distilled water. Enzyme concentrations were determined spectrophotometrically, using an extinction coefficient of $100 \text{ mM}^{-1}\text{cm}^{-1}$ at 405 nm and a molecular weight of 250,000 [9]. All results were the average of at least three separate experiments.

2.3. Spectroscopic Studies

Electronic absorption spectra were recorded from 200 to 700 nm on a Cary 100 BioUV VIS spectrophotometer. Phosphate buffer (0.1 M, pH 7.0) and catalase ($6 \mu\text{M}$) were added to the sample cuvette, and phosphate buffer (0.1 M, pH 7.0) was added to the reference cuvette. All measurements were performed at 25°C . Intrinsic fluorescence was detected on a Cary Eclipse Fluorescence spectrophotometer equipped with temperature controller. An excitation wavelength of 297 nm, specific for tryptophan residue, and another one of 280 nm specific for tyrosine and tryptophan residues, were chosen. Emission spectra were recorded between 310 and 450 nm. The enzyme concentration was $1 \mu\text{M}$ and all measurements were done at 25°C in 0.1 M phosphate buffer, pH 7.0. Circular dichroism (CD) spectra (far UV region; 190-260 nm) were recorded with an Aviv Model 215 CD spectrometer. All measurements were done in 0.1 M phosphate buffer, pH 7.0, at 25°C , using a 1-mm light path cell for far-UV studies. The enzyme concentration was $5 \mu\text{M}$.

3. Results

BLC activity was assayed by following the rate of oxidation of H_2O_2 to H_2O and O_2 at 240 nm and under steady-state conditions. The pH activity profile of the enzyme exhibited a broad optimum pH from 6.5 to 8.0 (Figure 1) and below pH 5.0 and over pH 10.0 denaturation of enzyme is observed. The pH of 7.0 was chosen and all assays were performed at pH 7.0.

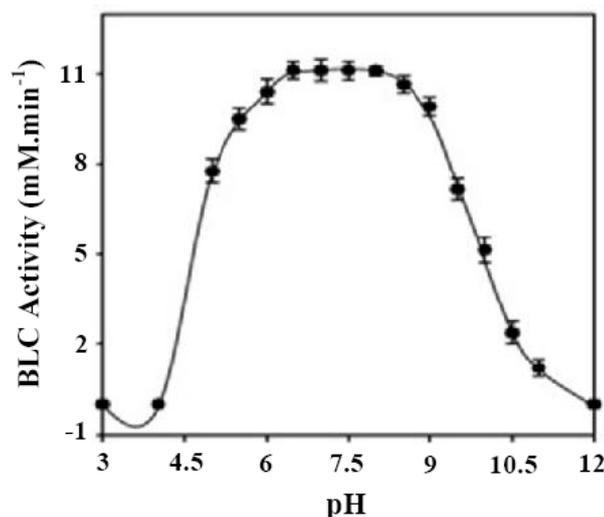


Fig. 1: pH profile of BLC in the presence of 23 mM H_2O_2 , $0.0125 \mu\text{M}$ BLC and 0.1 M T.S. buffer at 25°C .

Kinetics assays were performed and the values for the enzyme apparent V_{\max} , K_m , rate constant, specific activity, and catalytic efficiency were calculated based on the first-order dismutation of H_2O_2 (Table I).

Table I: kinetics parameters of BLC in the presence of 1-23 mM H_2O_2 , 0.0125 μM BLC, and 0.1 M phosphate buffer at pH 7.0, 25 °C.

Rate Constant (K; s ⁻¹)	Specific Activity (s ⁻¹ . μM^{-1})	K_m (mM)	V_{\max} (mM.min ⁻¹)	Catalytic Efficiency (K_{cat}/K_m) (μM^{-1} .min ⁻¹)
0.0032 ± 0.00003	0.253 ± 0.004	8 ± 0.19	7.69 ± 0.16	77 ± 3.23

The electronic absorption spectrum of BLC shown in Figure 2 exhibited essentially four maxima, respectively, at 275, 404, 503-535, and 623 nm, characteristic of the native enzyme and was similar to previously reported spectra [10], [11]. The highest peak, at 277 nm, was due to the aromatic amino acid side chains, the peak at 404 nm was attributed to the Soret absorption band (gamma band; heme b), and the shoulders at 503-535 nm (beta band) and the peak at 623 nm (alpha band) were due to the 3-banded high spin ferriheme.

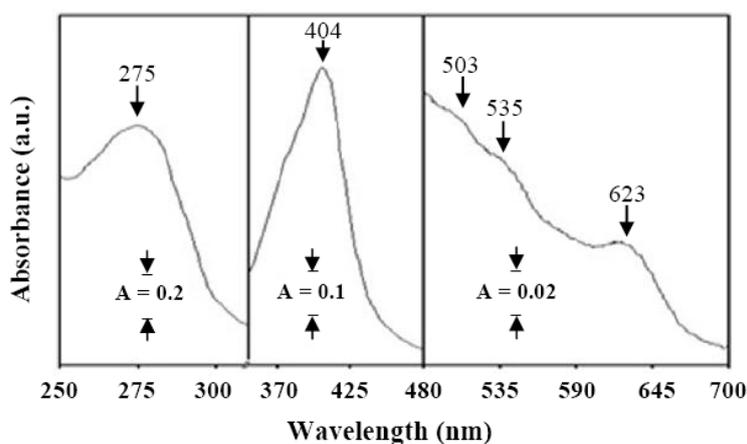


Fig. 2: Electronic absorption spectra of BLC. The spectrum was obtained with 6 μM BLC in 0.1 M phosphate buffer at pH 7.0, 25 °C.

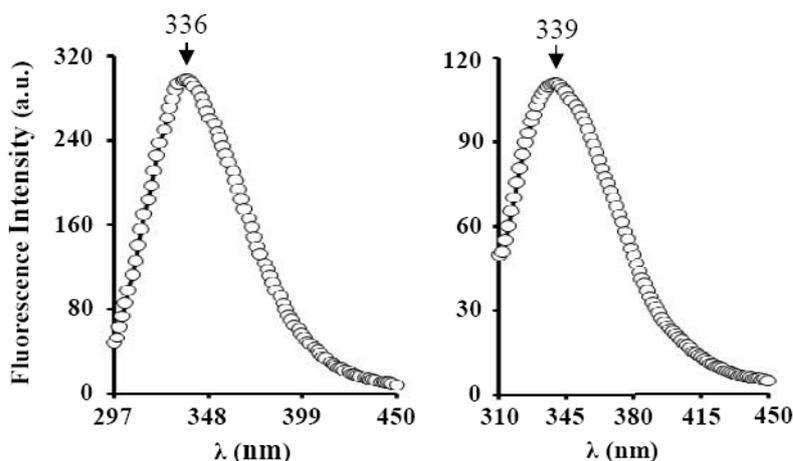


Fig. 3: Fluorescence emission spectra upon excitation at 280 nm (A) and 297 nm (B) with 1 μM BLC in 0.1 M phosphate buffer at pH 7.0, 25 °C.

The amino acid sequence of each subunit in Beef liver catalase includes 20 tyrosine and 5 tryptophan residues [9]; upon excitation at 280 nm, the enzyme exhibited a single fluorescence emission spectrum (due to tyrosine and tryptophan residues) with a maximum at 336 nm (Figure 3A), and upon excitation at 297 nm, a fluorescence emission spectrum (essentially due to tryptophan residues) with maximum at 339 nm was

obtained (Figure 3B). Changes in the protein conformation that would affect the tryptophan and tyrosine environment would alter the protein's fluorescence.

Far-UV CD spectrum of BLC taken immediately is shown in Figure 4. Native BLC present in the ultraviolet region at 208 and 222 nm two negative bands, characteristic for the α -helical structure [12]. Secondary structure fractions were calculated using the CD spectra deconvolution program CDNN version 2.1. whereas the α -helical content, β -sheet, β -turn, and the random coil fraction were 26%, 19.5%, 18.5%, and 36%, respectively.

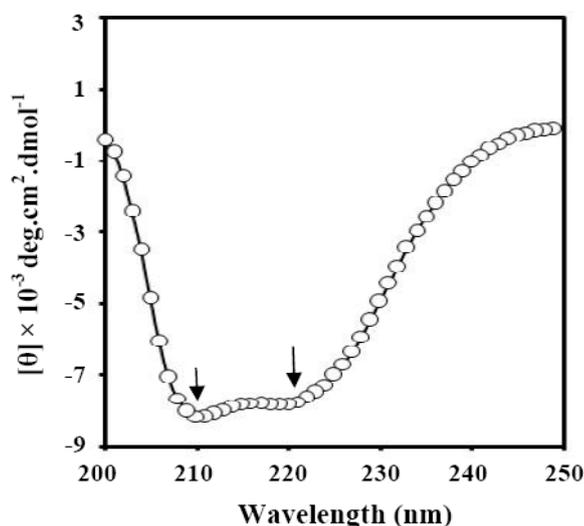


Fig. 4: Far-Ultraviolet CD spectrum of 5 μ M BLC in 0.1 M phosphate buffer solution at pH 7.0, 25 $^{\circ}$ C.

4. Conclusion

Enzymes play an important role in the food industry in both traditional and novel products. Having a better insight into correlation between the native structure and function of the enzymes has become a major concern since it was recognized that the unfolded or hydrolyzed enzyme molecules can exert very different functionality to food products compared to folded enzymes [13].

Steady-state kinetics studies coupled with the use of various spectroscopic techniques, as here, can be utilized as a universal tool for the study of the structure and function of enzymes; as structural changes can have a major impact on their activity, stability and toxicity, and consequently can compromise the efficacy and shelf life of products.

5. Acknowledgements

A considerable amount of research has been conducted in the field of spectroscopic techniques to study enzyme folding and stability. Unfortunately, space limitations preclude our referring to all of them. This work was supported in part by the Standard Research Institute (SRI), Karaj, Iran and in part by the Institute of Biochemistry & Biophysics (IBB) of Tehran University, Tehran, Iran.

6. References

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