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FT-Raman spectroscopic analysis of enhanced activity of supercritical carbon dioxide treated bacterial alpha-amylase

Kaninika Paul, Sayantani Dutta, Paramita Bhattacharjee*

Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata 700032, India

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ABSTRACT

Our previous investigation on high pressure supercritical carbon dioxide treatment of a bacterial α-amylase had revealed enhanced activity of the same. ^{1}H NMR analysis of the activity enhanced enzyme led the authors to hypothesize that the enhancement was possibly owing to alterations in the active site of the enzyme. In the present study, the changes in the active site of the treated enzyme was analysed by Fourier-transform Raman (FT-Raman) spectroscopy. The spectra obtained revealed shifting of bands in the active site of α-amylase indicating a nudging effect of the bonds in this region consequent to high pressure treatment. Also, shifts in bands in the OH stretching vibration of water were observed in the enzyme spectra. These variations in the spectra confirmed changes in the active site as well as in the water associated with the same that perhaps had a concerted effect on the increased activity of α-amylase.

1. Introduction

Two milligrams of lyophilized α-amylase powder from *Bacillus licheniformis* was subjected to supercritical carbon dioxide (SC-CO_2) conditions of 300 bar, 60 °C for 2.25 h under continuous flow of 1 L/min of gaseous CO_2. Post SC-CO_2 treatment, the treated enzyme was immediately recovered in 20 mM sodium phosphate buffer with 6.7 mM NaCl of pH 6.9. It was found that the specific activity of the enzyme increased by 2.13 ± 0.03 times. It was observed that the treated enzyme retained the same increased activity (2.13 ± 0.03 times) up to three months. The enzyme was dissolved in DMSO-*d*6 and ^{1}H NMR spectra (relative to tetramethylsilane, TMS) was recorded at 300 MHz on a Bruker Avance DPX-300 instrument at the Indian Association for Cultivation of Science, Kolkata, West Bengal, India. ^{1}H NMR analysis revealed that the enhancement of specific activity of the enzyme was due to an alteration in the conformational arrangement and it was hypothesized that the alterations occurred at the active site of the enzyme [1].

It is known that there are six types of water associated with protein macromolecules and is the most important factor which affects functional properties of proteins. Therefore changes in water associated with active sites of enzymes would greatly affect enzyme catalytic activity. Fourier-transform Raman (FT-Raman) spectroscopy reportedly detects changes in OH-stretching in water and also secondary structures of proteins [2]. Thus, in the current study, FT-Raman spectroscopy was employed to study changes in the water associated with the activity enhanced enzyme. This would allow confirmation of enhanced specific activity of the bacterial α-amylase, post SC−CO_2 treatment.

2. Materials and methods

2.1. Materials

The materials for the present study were α-amylase from *B. licheniformis* (lyophilized powder, 500–1500 units/mg protein, 93–100% SDS-PAGE) of Sigma Aldrich and the same with 2.13 ± 0.03 times enhanced specific activity by SC−CO_2 treatment at 300 bar, 60 °C with flow rate of 1 L/min of gaseous CO_2, as reported in our previous investigation.

2.2. Sample preparation

Three sets of 2 mg sample of lyophilized α-amylase were subjected to SC−CO_2 treatment (mentioned above) and thus three independent sets of treated samples were obtained for FT-Raman spectroscopic analysis. Along with these three samples, the untreated enzyme sample was also analysed to decipher the differences with the treated samples. All treated samples exhibited 2.13 ± 0.03 times enhanced activity.

* Corresponding author.
E-mail address: pb@ftbe.jdvu.ac.in (P. Bhattacharjee).

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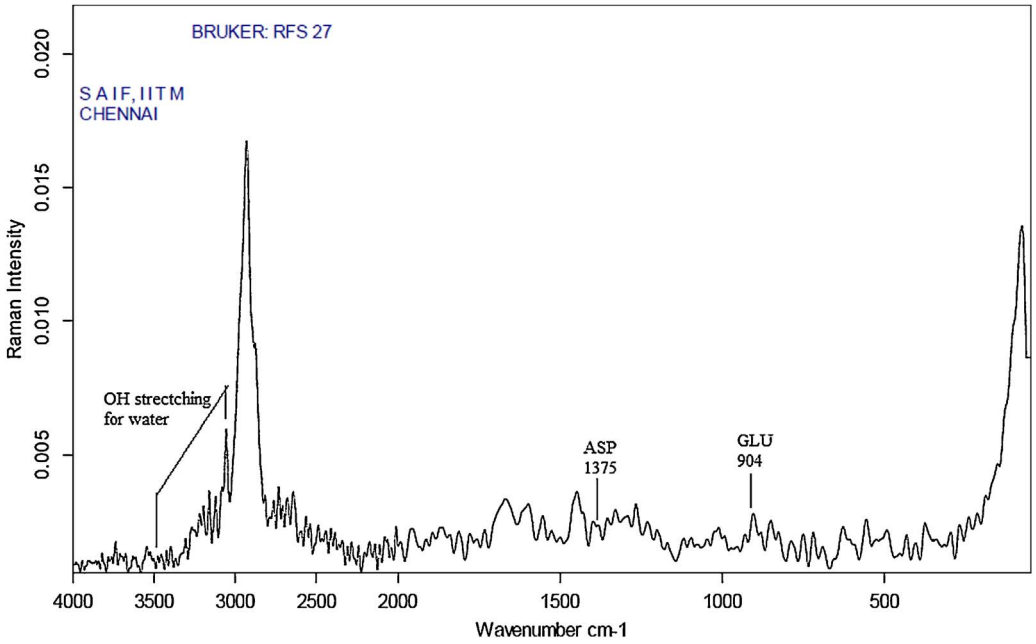

Fig. 1. Spectra of untreated α-amylase over the range of 4000–500 cm^{-1}.

2.3. Assessment of enhanced specific activity of SC−CO_2 treated α-amylase by Fourier transform Raman spectroscopy (FT-Raman)

Four samples of α-amylase (control and three samples treated under SC−CO_2 conditions) were subjected to FT-Raman analysis. The spectra were collected using a Bruker RFS 27 spectrometer (Karlsruhe, Germany) module equipped with a Nd^{3+}:YAG laser emitting at a wavelength of 1064 nm. Laser power was approximately 1000 mW for solid samples. The spectra obtained were averages of 100 scans at 2 cm^{-1} resolution over the range of 4000–500 cm^{-1}, at 25 ± 1 °C.

3. Results and discussion

3.1. FT-Raman of untreated α-amylase and SC−CO_2 treated α-amylase

Figs. 1 and 2 and show the spectra of untreated α-amylase and SC−CO_2 treated α-amylase over the range of 4000–500 cm^{-1}, respectively. α-amylase predominantly possesses β-sheet secondary structure which is reportedly shown in Raman spectrum in the range 1660–1670 cm^{-1} [3]. FT-Raman spectrum of both untreated α-amylase (Fig. 1) and treated samples exhibited the amide I band at 1669 cm^{-1}, indicating unchanged β-sheet secondary structure of the enzyme. This revealed that SC-CO_2 treatment did not grossly disrupt enzyme structure. Although SC-CO_2 treatment did not alter the β-sheet structure in the enzyme, the enzyme activity had been enhanced 2.13 ± 0.03 times. This finding therefore leads us to opine that changes in the active

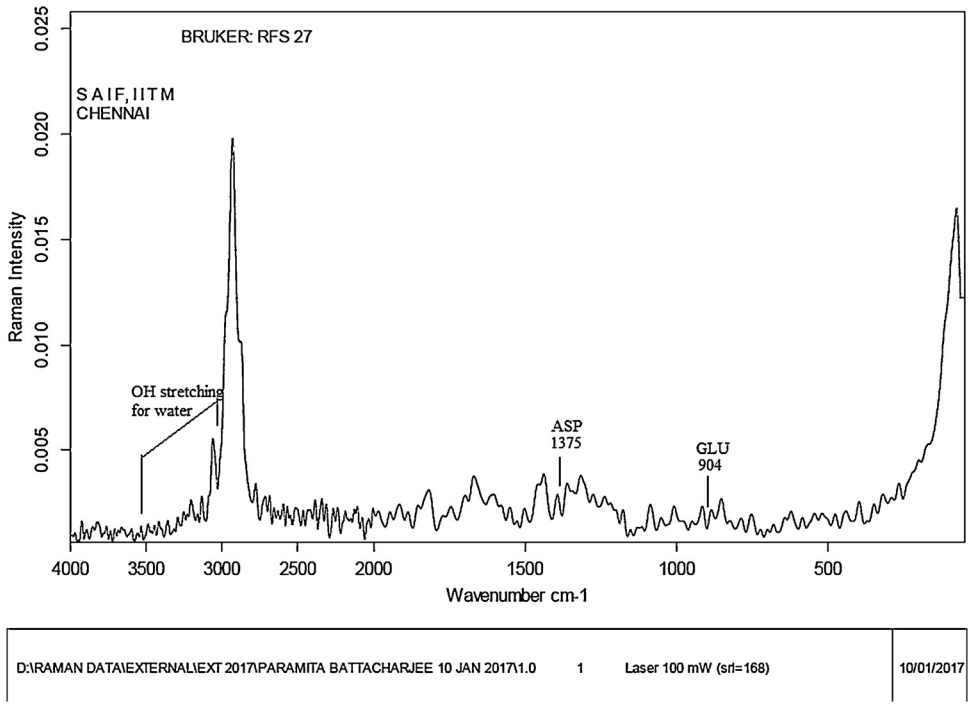

Fig. 2. Spectra of SC−CO_2 treated α-amylase over the range of 4000–500 cm^{-1}.

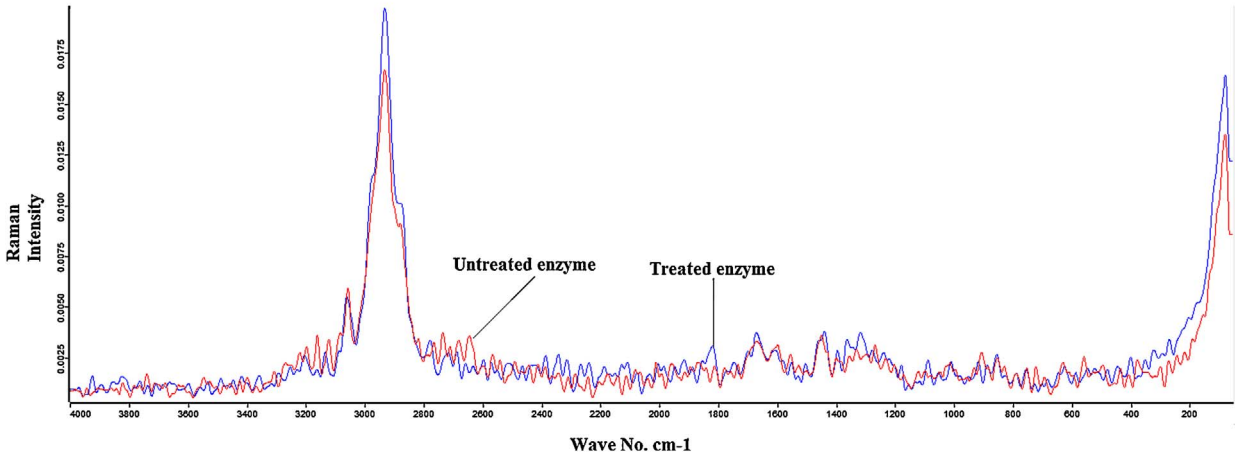

Fig. 3. The overlapped spectra of the treated and the untreated enzymes over the range of 4000–500 cm^{-1}.

site of the enzyme possibly enhanced the enzyme activity.

The active site of α-amylase chiefly comprises of glutamate and aspartate [4]. The bands at 904 and 1375 cm^{-1} indicate presence of glutamate and aspartate, respectively [5]. The spectra of the treated and the untreated enzymes were overlapped to record changes at 904 cm^{-1} and 1375 cm^{-1} (Fig. 3). The overlapped spectra revealed shifting of bands in this zone indicating a nudging effect of the bonds in this region consequent to high pressure treatment and also increased intensity of all seven bands in the spectra of the treated enzyme in the said region. These changes had possibly led to increased catalytic activity of α-amylase.

Further, shifts in bands in the OH stretching vibration of water (3000–3500 cm^{-1}), observed in the overlapped images of the enzyme spectra confirmed changes in water associated with the SC-CO_2 treated enzyme. Among the six types of water associated with proteins, mainly structural water and hydrodynamic hydration water play important roles in functional properties of proteins [6]. Hydrophobic hydration water is the bound water among these six types of associated water and even if affected adversely by SC-CO_2 treatment, would contribute less compared to the remaining five fractions of associated water. The variations in the spectra confirmed changes in the active site of the SC-CO_2 treated enzyme as well as in the water associated with the same, that perhaps had a concerted effect on the increased (2.13 ± 0.03 time enhanced) activity of α-amylase.

Further changes in group frequencies were observed for the high pressure treated enzyme such as increased intensities of bands in the regions 1775–1810 and 2100–2260 cm^{-1}, depicting C═O stretching (1775–1810 cm^{-1}) and presence of triple bonds, mainly C—C and C—N (2100–2260 cm^{-1}) [7]. Additionally, two new peaks were observed in the range 1300–1350 cm^{-1} which represent N—C—O stretching and bending [7]. Also, the aliphatic groups in the region 2700–3000 cm^{-1} showed increased C—H stretching vibrations in the treated enzyme vis-à-vis those in the untreated one. All these increased intensities in the specified regions of α-amylase possibly cumulatively increased catalytic activity of SC—CO_2 treated α-amylase.

4. Conclusion

This study had validated the hypothesis of our previous investigations on enhancement of catalytic activity of supercritical carbon dioxide treated α-amylase. The FT-Raman spectra of the said enzyme vis-à-vis the untreated enzyme, revealed shifting in bands related to amino acids, aspartate and glutamate at the active site and also in OH stretching vibration of water, possibly in structural and hydrodynamic hydration water associated with the active site of the SC-CO_2 treated enzyme. FT-Raman spectroscopy therefore confirmed enhancement of activity of the bacterial α-amylase by supercritical carbon dioxide processing. The SC—CO_2 treated α-amylase with enhanced catalytic activity can be used *per se* in food, starch, textile, paper and detergent manufacturing industries and in ethanol production. This treated enzyme can also be subjected to immobilization for various industrial applications.

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