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Safeguarding the catalytic activity and stability of polyaniline chitosan silver nanocomposite bound beta-galactosidase against product inhibitors and structurally related compound

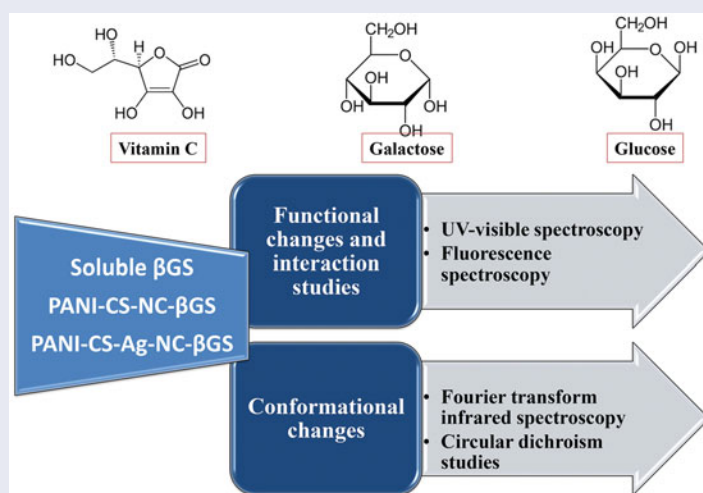
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ABSTRACT

In this study, an attempt has been made to evaluate the effect of products of β -galactosidase (β GS) catalyzed reaction i.e. glucose and galactose and their structurally related compound vitamin C (VC) on the catalytic activity of native and PANI-CS-NC and PANI-CS-Ag-NC adsorbed β GS. Results indicated a decline in catalytic activity of soluble enzyme in the presence of all investigated compounds. The order of inhibition was found to be VC < glucose < galactose. However, the immobilized preparations were found more resistant to inactivation caused by the added compounds. About 48% activity was retained by PANI-CS-Ag-NC- β GS in the presence of galactose (5%, w/v), while the native enzyme exhibited only 18% of its original activity. A significant decrease in absorbance and fluorescence intensity was evaluated in soluble enzyme incubated in the presence of all investigated compounds. Three-dimensional fluorescence graphs, CD and FT-IR spectroscopic studies illustrated noteworthy conformational changes in the secondary structure and microenvironment of the soluble enzyme in the presence of VC and tested sugars. These results suggest that both PANI-CS-NC and PANI-CS-Ag-NC bound β GS are more resistant to the exposure caused by the higher concentration of added glucose, galactose, and VC and, therefore, can be effectively utilized for the production of a hassle-free lactose nano-biosensor.

GRAPHICAL ABSTRACT



ARTICLE HISTORY

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KEYWORDS

β -galactosidase; immobilization; lactose intolerance; 3-D fluorescence; circular dichroism

Introduction

β -galactosidase, β GS (β -D-galactoside galactohydrolase, E.C. 3.2.1.23) is an important hydrolytic enzyme that presents numerous interesting applications in the food industry. Its major application is to hydrolyze lactose in milk or derived

products [1,2]. Lactose is the chief sugar (4–5%) present in milk and its hydrolysis makes milk and its products fit for consumption to lactose intolerant people [3]. β GS has been obtained from a variety of microorganisms and sources including fungi, bacteria, yeasts, plants, etc. The microbial

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sources represent the major group because the enzymes derived from other sources are of high cost and of low-production yield [4]. The hydrolyzed lactose-free products thus formed have enhanced sweetness, solubility and flavour [5,6].

However, free enzymes cannot be used directly in biotechnological industries due to the long processing time, difficult recovery and non-reusability [7]. Considerable research endeavours have been made to circumvent the aforementioned problems associated with the utilization of free enzymes. Immobilization of enzymes is a widely used technique that provides a number of advantages over the use of soluble enzymes. It allows continuous and automated processing, easier separation of reaction products, easy recovery and reutilization of the enzyme, making enzyme-based processes cost-effective and industrially viable [8–10]. Various methods for immobilization include physical adsorption, covalent attachment, entrapment etc.

Recently, with the rapid development of nanotechnology, NM has attracted enzymologists as a versatile enzyme immobilization support due to their large specific surface area, tailor-made size at the nanometer scale and similar size with enzyme molecules [11–13]. Nano-immobilization can provide a solution to the demerits possessed by the conventional supports used for immobilization like the poor yield of immobilization and steric hindrances caused by the substrate [14,15]. Until now, various nanostructures, such as nanoparticles [16,17], nanofiber [18], nanotube [19], nanosheet [20] and most recently, nanoflowers [21] have been explored as support for the immobilization of industrially important enzymes [22]. Among them, polymer-based NCs have attracted considerable attention in the last few years due to their exceptional stability against chemical, electrical and mechanical exposure [23,24].

Galactose is one of the products of β GS catalyzed reaction and also a well known competitive inhibitor (and rarely mixed inhibitor) for the majority of β -galactosidases. Several studies have been conducted showing the inhibition of β GS by the reaction products galactose and glucose [25,26]. For the first time, Park and Oh [27] have shown that both galactose and glucose exhibit an uncompetitive inhibition. Geven et al. [28] purified this enzyme from *thermoacidophilic Alicyclobacillus acidocaldarius* subsp. *Rittmannii* isolated from Antarctica. The enzyme was greatly inhibited by the reaction product galactose using substrates, *o*-nitrophenyl β -galactopyranoside (ONPG) and lactose. Lineweaver-Burk kinetics showed that galactose is a linear mixed-type inhibitor.

In this study, an effort has been made to systematically investigate the effect of substrate, products and VC on the activity of free and polyaniline chitosan nanocomposite (PANI-CS-NC) and polyaniline chitosan silver nanocomposite (PANI-CS-Ag-NC) bound β GS. This work mainly focuses on the effect of ascorbic acid, glucose, and galactose on the structural and functional integrity of soluble and immobilized β GS preparations. The interactions between the enzymes and the added compounds along with conformational changes in the protein were examined with the help of UV-visible, fluorescence, Fourier-transform infrared (FT-IR) and circular dichroism (CD) spectroscopic studies.

Materials and methods

Materials

Aspergillus oryzae β GS (8.4 U mg^{-1} solid) was obtained from Sigma-Aldrich Chemicals Co. St. Louis, Missouri, USA. ONPG, sodium carbonate, glucose, galactose and ascorbic acid were purchased from Sisco Research Laboratories (SRL), Mumbai, India. Chitosan, silver nitrate, hydrazinium hydrate, hexadecyltrimethyl ammonium bromide surfactant, ammonium persulphate and hydrochloric acid were obtained from Thermo Fisher Scientific, Waltham, Massachusetts, USA. All solvents and other reagents were of analytical grade and supplied by SRL.

Methodology

Synthesis of PANI-CS-NC and PANI-CS-Ag-NC

The synthesis of PANI-CS-NC and PANI-CS-Ag-NC has already been described earlier [24].

Immobilization of β -galactosidase on PANI-CS-NC and PANI-CS-Ag-NC

The immobilization of β GS on these NCs has been previously mentioned [24].

Effect of glucose, galactose and VC on the activities of soluble and immobilized β -galactosidase preparations

Soluble and immobilized β GS preparations (0.12 U) were independently incubated with various concentrations of sugars (1.0–5.0%, w/v) and ascorbic acid (0.01 mg mL^{-1} –0.1 mg mL^{-1}) in 0.1 M sodium acetate buffer, pH 4.5 for 1 h at 37 °C. The effect of these effectors on the activity of soluble and immobilized enzyme preparations was monitored by conducting routine β GS assay at 37 °C for 15 min. The activity of the enzyme without these added effectors was taken as control (100%) for the calculation of residual percent activity. The enzyme activity was estimated using UV-1800 spectrophotometer, Shimadzu, Japan at 405 nm.

Spectroscopic studies

UV-visible spectroscopy. The spectral changes in the free and immobilized β GS preparations were monitored using the instrument (Shimadzu, Kyoto, Japan, UV-visible spectrophotometer 1800). The spectra of soluble, PANI-CS and PANI-Ag-CS-NC bound enzyme were compared in the presence of different investigated compounds. β GS was mixed with varying concentrations of VC, glucose and galactose in order to examine their interaction with the free and immobilized enzyme. Concentrations of all effectors have been mentioned in the above section. Detailed parameters have been previously described [29].

Fluorescence measurements. Fluorescence measurements were recorded on a Shimadzu RF6000 spectrofluorophotometer, Japan. For fluorescence intensity measurements, 1 mg mL^{-1} β GS solution was mixed with varying concentrations of

VC, glucose and galactose. The concentrations of all modulators have been previously described [29].

Three-dimensional fluorescence spectroscopy analysis.

These studies were performed to visualize the structural changes taking place in β GS in the presence of all the effectors before and after immobilization [29].

The changes in the molecular structure of soluble and immobilized β GS were also analyzed with the help of FT-IR studies, using Perkin Elmer instrument, Waltham, Massachusetts, USA, Spectrum- II, wave number 4000–400. For FT-IR, defined amounts of treated and untreated soluble and immobilized enzymes (liquid samples) were incubated with added sugars and VC. The concentrations of enzymes and added compounds used have already been described in Section “Activity assay of β -galactosidase” [29].

Circular dichroism studies. The changes in the secondary structure of the native and immobilized enzyme in the presence of added VC, glucose and galactose were evaluated by CD spectroscopy (Jasco J-815 CD spectropolarimeter, Jasco, Tokyo, Japan). For soluble, PANI-CS and PANI-Ag-CS-NC immobilized β GSs, 0.012 U were taken for all comparative measurements. The α -helical and β -sheet contents in the respective protein have been calculated by the K2D3 software [29].

Activity assay of β -galactosidase

The activity of β GS was determined using *o*-nitrophenyl-D-galactopyranoside (ONPG) as a substrate and by measuring the release of *o*-nitrophenol from the substrate at 405 nm [26].

Protein estimation

Protein concentration was calculated according to the method described by Lowry et al. [30]. Bovine serum albumin was employed as standard.

Statistical analysis

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, $\leq 5\%$. The statistical analysis for comparison between free and both immobilized enzyme preparations was performed by One Way ANOVA test. The data expressed in various experimental studies were plotted using origin 6.1 and *p* values $\leq .05$ were considered as statistically significant.

Results and discussion

Effect of glucose, galactose and VC on the activities of soluble, PANI-CS-NC and PANI-CS-Ag-NC bound β -galactosidase preparations

The two sugars, glucose and galactose are the products of β GS catalyzed reaction. Therefore, it is essential to portray the effect of high concentrations of these sugars on the catalytic activity of native and immobilized β GS. UV-visible

spectrophotometry was performed to examine the effect of such compounds on the activity of β GS. Galactose competes with substrate for active sites and its competitive inhibitory role has already been described earlier [31]. Table S1 depicts the relative activity of soluble and immobilized β GS in the presence of equimolar concentrations (1–5%, w/v) of both sugars. The suppression of β GS activity was found to be dose dependent and in order of glucose < galactose. PANI-CS-NC- β GS and PANI-CS-Ag-NC- β GS retained 28 and 48% of their original activity in presence of 5% (w/v) galactose, while the free enzyme could maintain only 18% of its activity under similar experimental conditions.

The soluble enzyme exhibited 57% of its activity in the presence of 5% (w/v) of glucose. Immobilization had a positive effect on the activity of PANI-CS-NC- β GS and PANI-CS-Ag-NC- β GS. PANI-CS-NC-enzyme retained 69% activity, while PANI-CS-Ag-NC- β GS was found more resistant to inhibition mediated by added glucose and reserved 83% of activity under identical incubation conditions. The presence of silver ions in PANI-CS-Ag-NC- β GS somehow protects the active site of enzyme by preventing alterations in conformation. Free enzyme undergoes unfavourable conformational changes under denaturing conditions which is responsible for the decreased activity of the enzyme [32]. The immobilized enzyme showed marked resistance in inhibition caused by the tested compounds which might be due to conformational changes occurring outside the active site of the enzyme after binding to NC. Silver-containing NC bound β GS appeared more rigid to inhibition caused by higher concentrations of such compounds and retained a greater fraction of catalytic activity.

Table S2 demonstrates inhibition constants (K_i) for soluble and immobilized β GS incubated in the presence of higher concentrations of glucose and galactose. K_i values for β GS bound to NC containing Ag in the presence of glucose and galactose were found to be on the higher side which infers that this enzyme preparation is significantly more resistant to the added compounds. VC is one of the compounds found in milk and it is structurally related to glucose and galactose. Therefore, we have evaluated the effect of VC on the activity of free and immobilized β GS (Table S3). The soluble enzyme lost about 28% of its initial activity in the presence of 0.1 mg mL^{-1} of VC whereas it had a little effect on the activity of PANI-CS-NC- β GS and did not influence the activity of PANI-CS-Ag-NC- β GS.

Spectroscopic studies

UV-visible spectroscopy

To further develop this study, UV-visible spectra were recorded in order to determine the interaction of these products of the enzyme-catalyzed reaction and VC with native and immobilized β GS. Figure 1 depicts UV-visible spectra for soluble and PANI-CS-NC- β GS and PANI-CS-Ag-NC- β GS in presence of glucose, galactose and VC. β GS exhibited λ_{max} at 280 nm after exposure to a maximum concentration of the investigated compounds. Native and immobilized enzyme displayed a hypochromic effect. A peak shift was observed only in case of PANI-CS-Ag-NC- β GS in the presence of VC. The order of hypochromicity was similar to that observed for the inhibitory

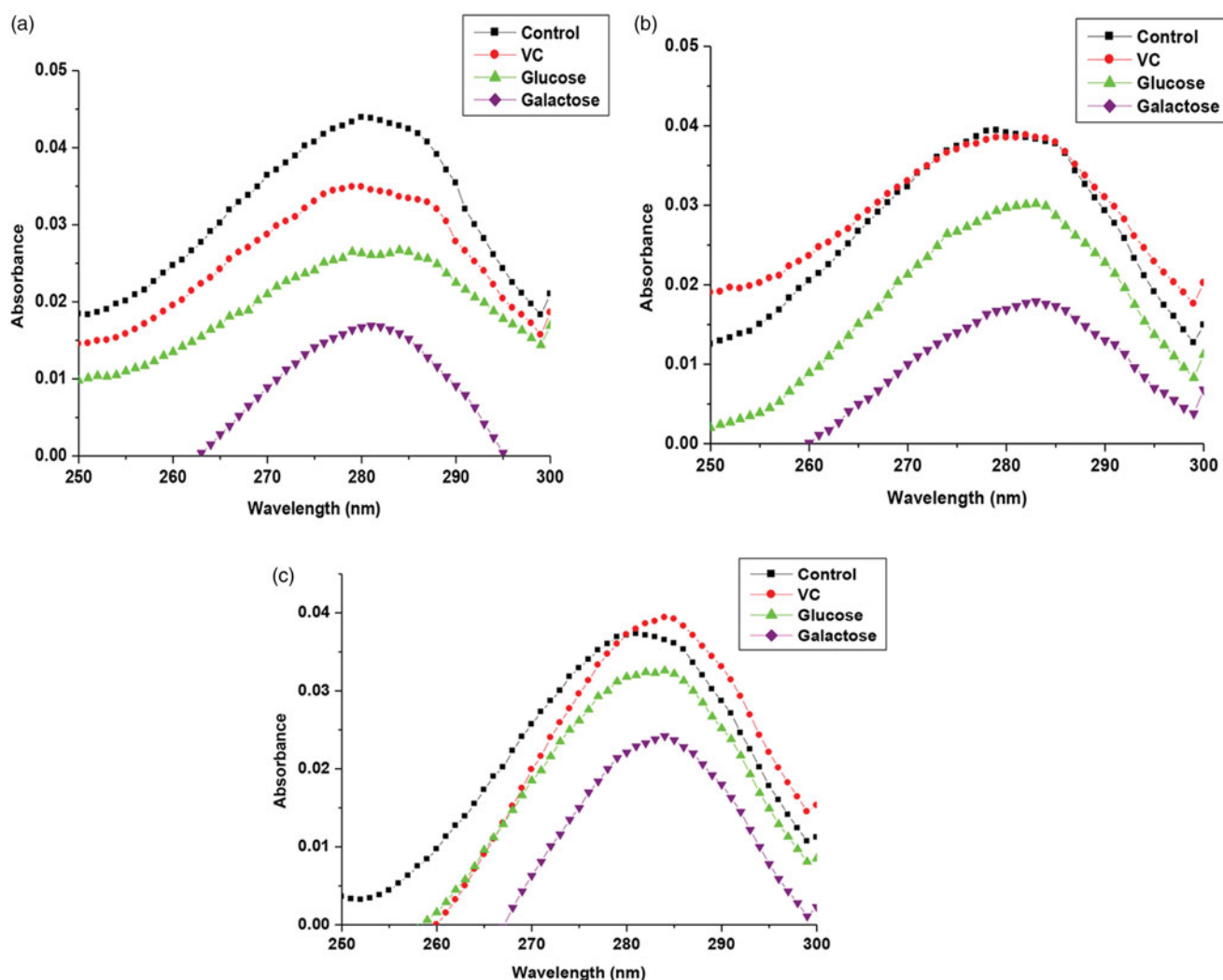


Figure 1. UV-vis spectra of soluble and immobilized β GS in the presence of investigated compounds. The spectra show (a) free (b) PANI-CS-NC- β GS and (c) PANI-CS-Ag-NC- β GS (0.06 U). The concentration of VC was 0.1 mg mL^{-1} and each sugar was 5% (w/v).

effect, i.e. $\text{VC} < \text{glucose} < \text{galactose}$. The most prominent changes in absorbance spectra were recorded for free enzyme in the presence of added compounds with respect to control. The recorded spectra for PANI-CS-Ag-NC- β GS showed least hypochromic effect. It indicates stronger binding of such compounds with the soluble enzyme as compared to immobilized β GS. This corresponds to tabulated data of activity retention, where the free enzyme was found to be most affected by the addition of compounds. Since humans are increasingly exposed to various food colorants like carmoisine, the binding of carmoisine and its inhibitory effect on amyloid fibrillation in lysozyme have been recently investigated. The results exhibited a decrease in the absorbance of lysozyme on addition of carmoisine. However, there was no shift in the position of maximum wavelength, λ_{max} [33].

Fluorescence emission spectroscopy

Fluorescence spectroscopy is one of the most excellent techniques to investigate the binding of bioactive molecules to proteins as it is more sensitive compared to other optical methods [34,35]. Three amino acid residues; viz, tryptophan

(Trp), tyrosine (Tyr) and phenylalanine (Phe) are primarily responsible for the intrinsic fluorescence of β GS. In fact, the intrinsic fluorescence of β GS is mainly contributed by tryptophan, because Phe has a very low quantum yield and the fluorescence of Tyr is almost totally quenched if it is ionized, or near an amino group, a carboxyl group, or a Trp. This viewpoint has been supported by the experimental observations made by other researchers [36,37].

Fluorescence measurement studies were carried out to understand the interactions between hydrolyzed products and native and immobilized β GS. For this purpose, the intrinsic fluorescence spectra of β GS in the presence of added compounds were recorded. When β GS was excited at 380 nm, it exhibited a strong emission peak at 436 nm. Upon the addition of VC, glucose and galactose to β GS solution, decreased fluorescence intensity was observed with no significant shift in its emission maxima which was observed at 436 nm (Figure 2). However, in immobilized β GS preparations, the quenching was apparently reduced inferring less interaction taking place between β GS bound to PANI-CS-NC and the added compounds. Again, Ag containing NC bound β GS demonstrated the least decrease in fluorescent intensity in the presence of

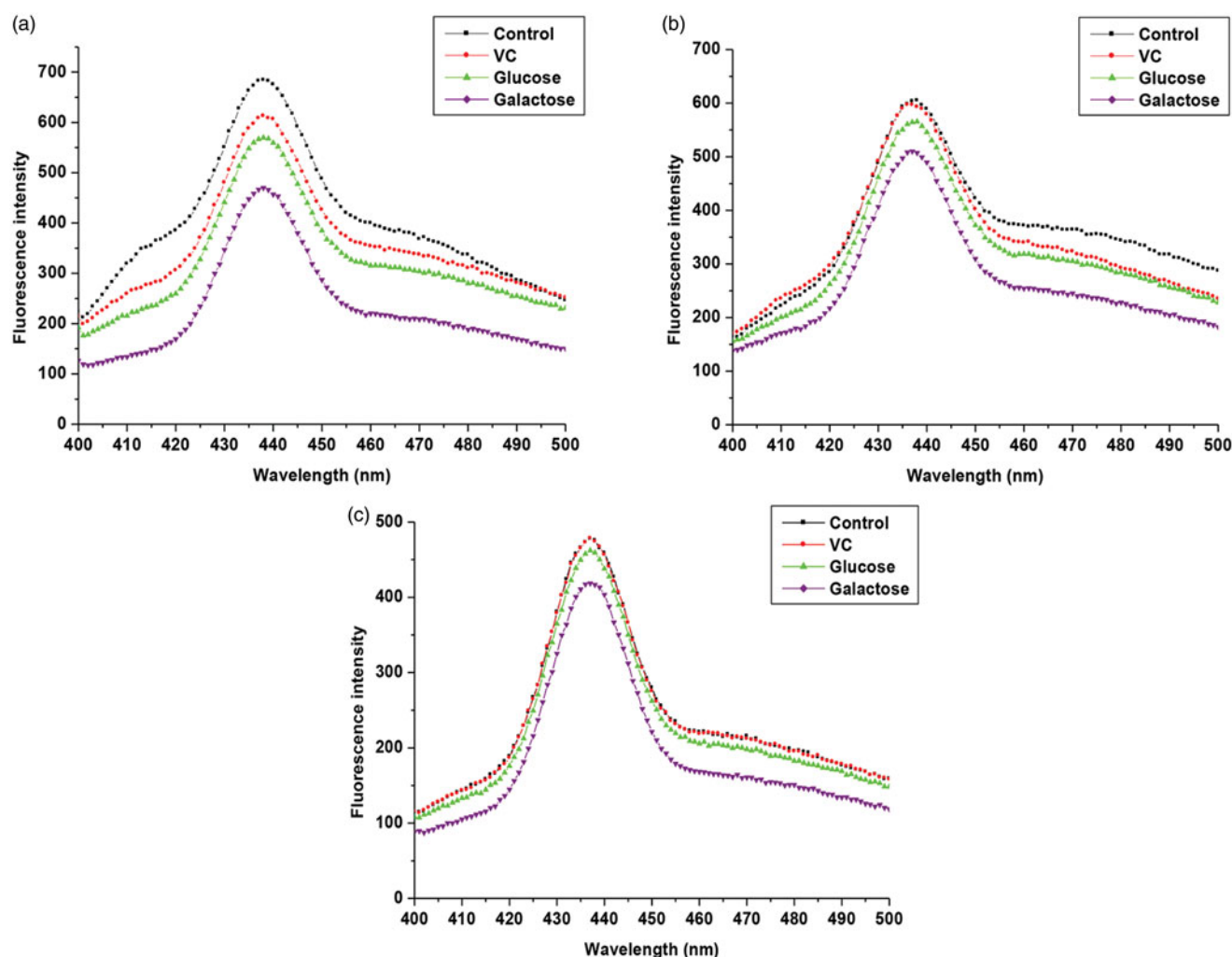


Figure 2. Fluorescence emission spectra of free and NC immobilized β GS in the presence of added compounds. The spectra illustrate (a) free (b) PANI-CS-NC- β GS and (c) PANI-CS-Ag-NC- β GS. The concentration of VC was 0.1 mg mL^{-1} and each sugar was 5% (w/v).

all investigated compounds. Tryptophan fluorescence is known to be more sensitive to the polarity of its local environment [38]. Quenching in fluorescence has been previously reported by various researchers to validate the interactions between protein and added compounds [33,39].

Three-dimensional fluorescence analysis

Three-dimensional fluorescence spectroscopy is an informative tool to study conformational changes in biomolecules, especially proteins. The decrease in peak intensities or change in the appearance of peaks in the 3D graph indicates the presence of effectors causing conformational changes in the protein [40]. The 3D spectra and corresponding contour diagrams for free and immobilized β GS in presence of VC, glucose and galactose have been shown in Figures 3–5. As illustrated in figures, 3D spectra of β GS exhibited three typical peaks, Rayleigh scattering peak (peak A) and two fluorescence peaks (peak B and C). Out of these two peaks, peak B indicates spectral characteristics of Trp and Tyr residues of protein whereas, peak C reveals polypeptide backbone structure of β GS and the intensity of this peak is correlated with β GS secondary structure [41].

The consequences of added compounds namely VC, glucose and galactose on conformational properties of β GS were studied by recording 3D fluorescence spectra for free and NC bound β GS in presence of specified compounds. The fluorescence intensities of both peaks A and B in free β GS declined upon addition of all three effectors. The order of inhibition was found to be $\text{VC} < \text{glucose} < \text{galactose}$ (Figure 3–5). These spectral changes revealed that interaction between added compounds and β GS resulted in micro-environmental and conformational changes in the soluble protein. On the contrary, PANI-CS-NC- β GS and PANI-CS-Ag-NC- β GS showed a less inhibitory effect in the presence of tested compounds. Alterations were mostly observed in peaks A and B. Changes in peak A are suggestive of an increase in the diameter of β GS which translates into an enhanced Rayleigh scattering effect, thus signifying complex formation, while alteration in peak B is attributed to alterations in the micro-environment of Tyr and Trp residues [42]. PANI-CS-Ag-NC- β GS was found to be least affected in the presence of all examined compounds exhibiting minor conformational changes. The alternations in peak C related to the conformational changes in peptide backbone associated with helix-coil were mostly observed in galactose mediated inhibition.

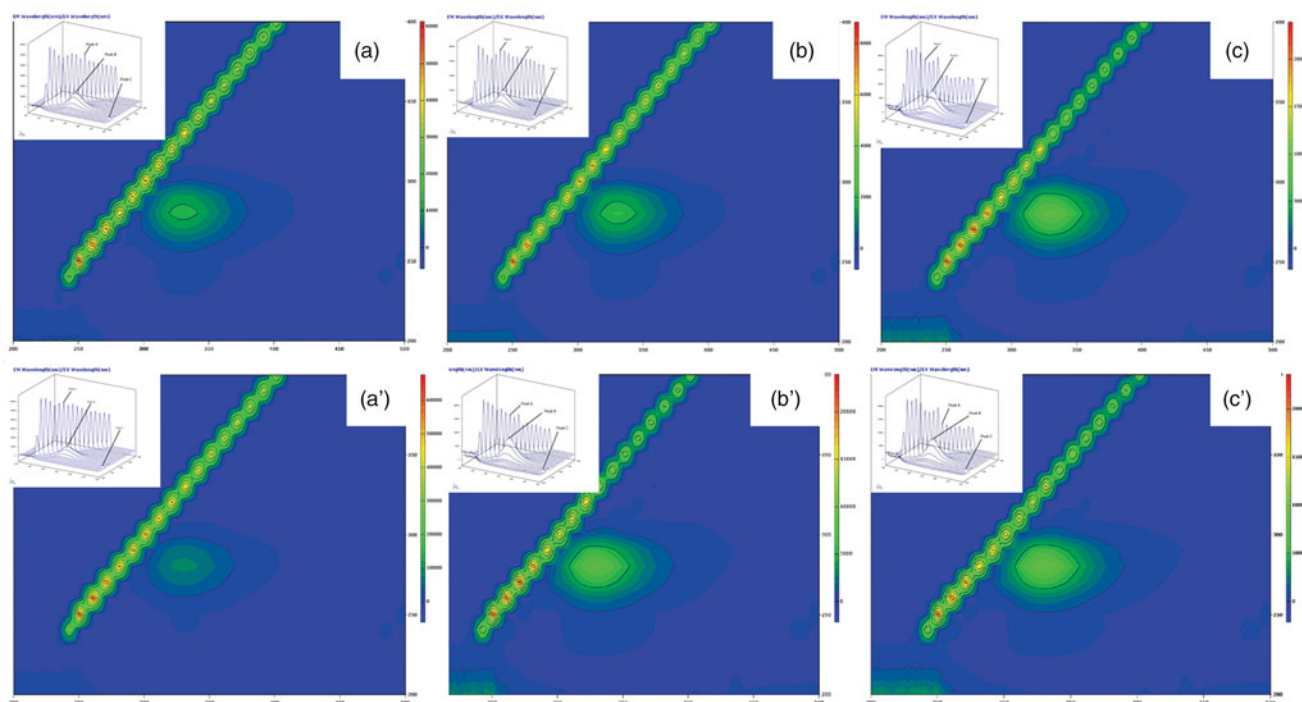


Figure 3. 3D fluorescence spectra and corresponding contour map of soluble and immobilized β GS in presence of VC. The spectra illustrate (a), (b) and (c) for controls of soluble, PANI-CS-NC- β GS, and PANI-CS-Ag-NC- β GS and (a'), (b') and (c') for native, PANI-CS-NC- β GS, and PANI-CS-Ag-NC- β GS treated with VC.

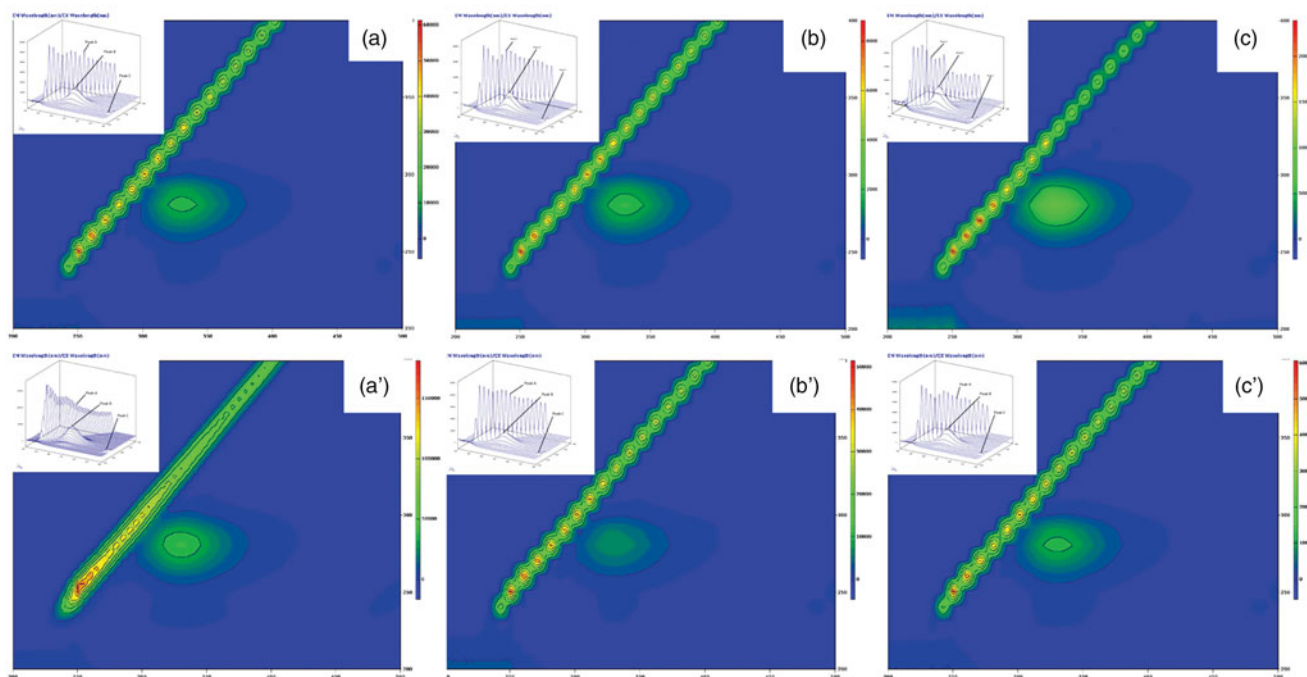


Figure 4. 3D fluorescence spectra and corresponding contour map of soluble and immobilized β GS in presence of glucose. The spectra show (a), (b) and (c) for controls of soluble, PANI-CS-NC- β GS and PANI-CS-Ag-NC- β GS and (a'), (b') and (c') for native, PANI-CS-NC- β GS, and PANI-CS-Ag-NC- β GS.

Fourier-transform infrared spectroscopy

FT-IR spectral results provide valuable information about the content of protein secondary structure. The IR spectrum of protein exhibits two characteristic absorption bands, viz., amide I and amide II. These are prominent vibrational bands of protein backbone. The amide I band appears in a region of $1700\text{--}1600\text{ cm}^{-1}$ due to C=O stretching vibrations of

peptide linkages, while the amide II band is attributable to C–N stretch coupled with N–H bending mode appearing at $1600\text{--}1500\text{ cm}^{-1}$. Amide I band is known to be more sensitive to protein secondary structural changes than the amide II band [43]. The conformational changes in soluble and immobilized β GS upon binding to VC and different sugars were evaluated by recording FT-IR spectra of free and NC

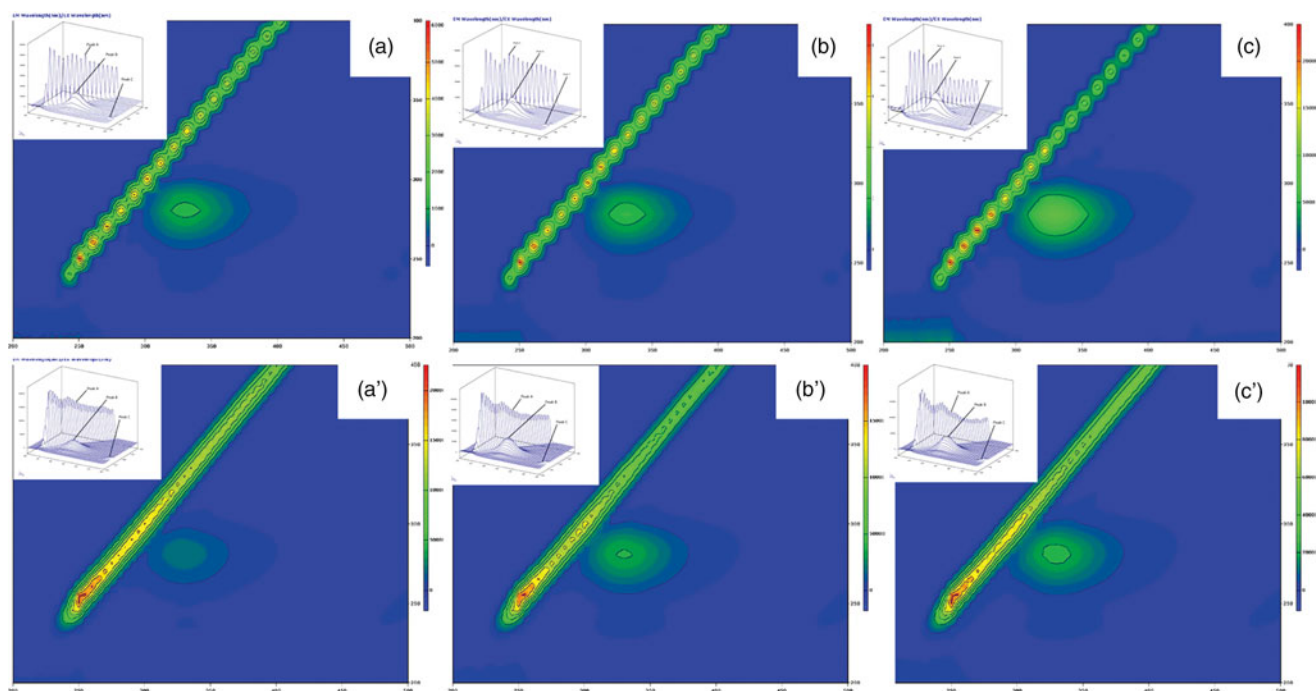


Figure 5. 3D fluorescence spectra and corresponding contour map for soluble and immobilized β GS in the presence of galactose. The spectra demonstrate (a), (b) and (c) as controls of soluble, PANI-CS-NC- β GS and PANI-CS/Ag-NC- β GS and (a'), (b') and (c') for native, PANI-CS-NC- β GS, and PANI-CS/Ag-NC- β GS.

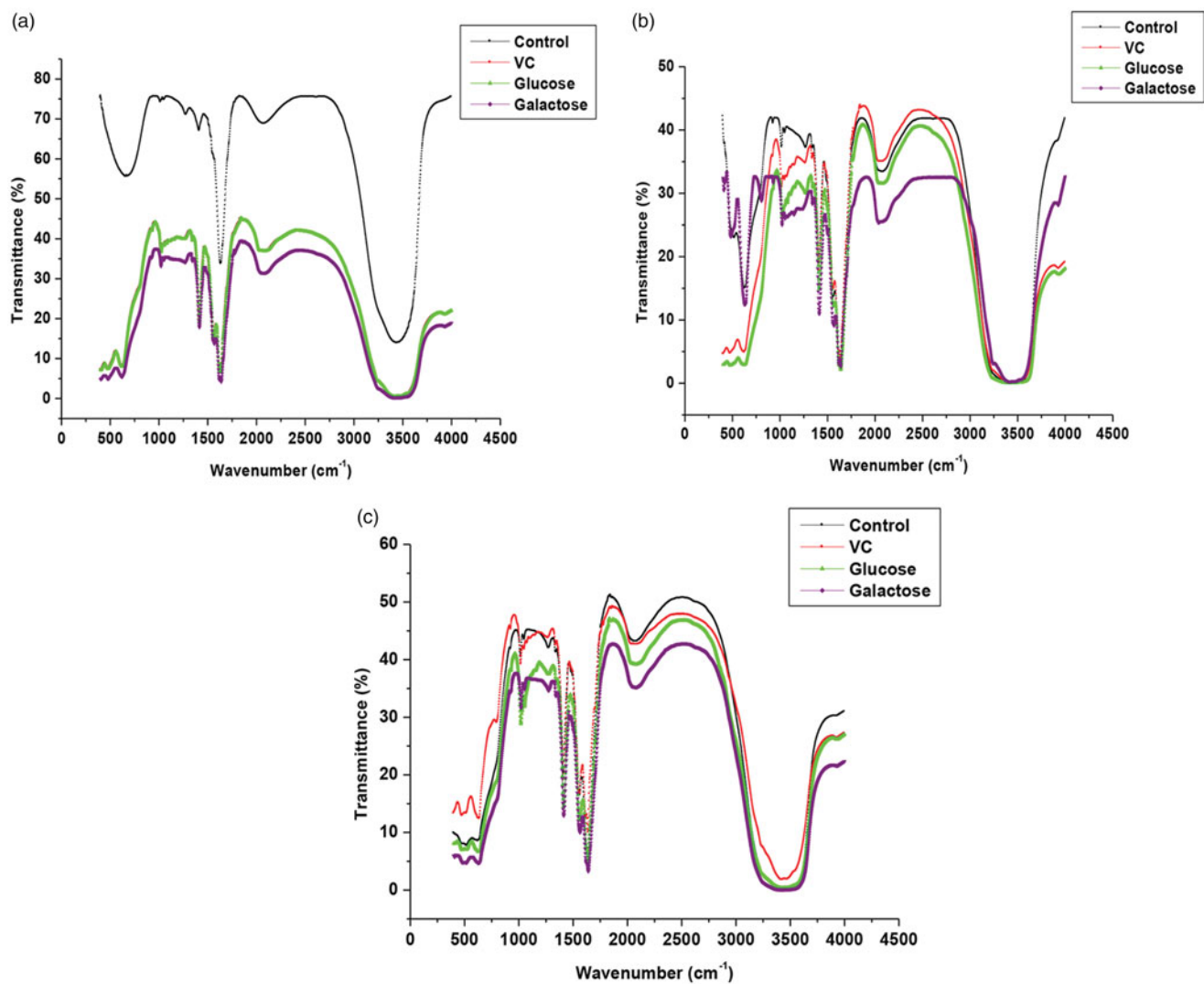


Figure 6. FT-IR spectra of soluble and NC immobilized β GS in the presence of VC and sugars. The spectra depict (a) free (b) PANI-CS-NC- β GS and (c) PANI-CS-Ag-NC- β GS.

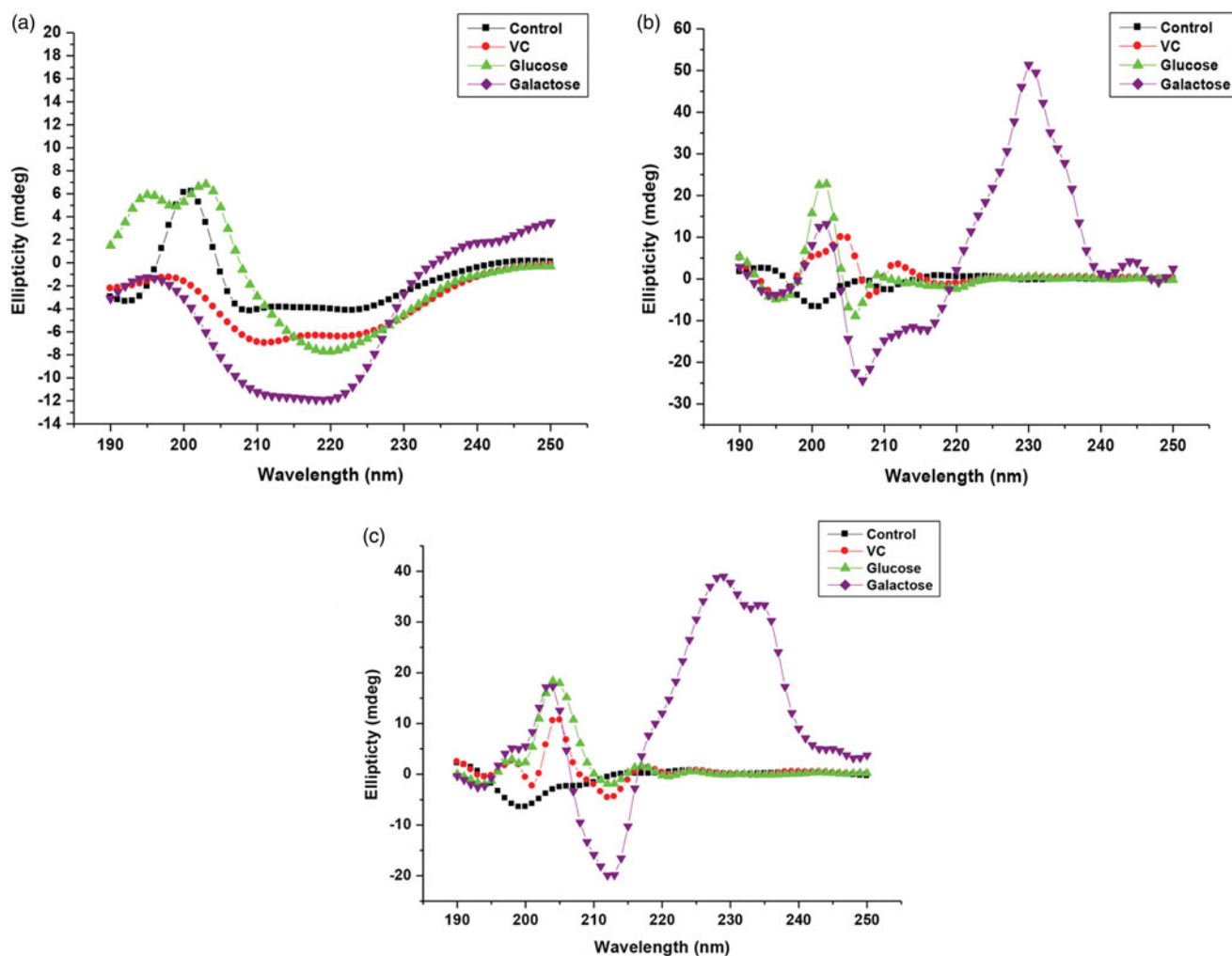


Figure 7. CD spectra of native and immobilized β GS in the presence of added compounds. The spectra illustrate (a) free (b) PANI/CS-NC- β GS and (c) PANI-CS-Ag-NC- β GS.

bound β GS incubated in the presence of all studied compounds (Figure 6). The FT-IR spectrum of free enzyme exhibited the only amide I band at 1629 cm^{-1} . After additions of investigated compounds, a shift in the position of amide I bands was visualized. The amide I band was shifted from 1629 cm^{-1} to 1647 cm^{-1} and an additional amide II band appeared at 1557 cm^{-1} . A sudden decrease in transmittance intensity with respect to untreated β GS was also witnessed as it is evident from the FT-IR spectra of the native enzyme. The band shift, additional band and decreased intensity were indicative of changes in protein secondary structure upon its interaction with VC and added sugars.

However, immobilized β GS preparations in the presence of VC, glucose, and galactose exhibited spectra identical to control. Both amide bands I and II were observed at same wave number which infer less conformational changes in immobilized β GS preparations in presence of all tested compounds. Galactose, a competitive inhibitor of β GS showed a maximum change in IR spectra of PANI-CS-NC- β GS. These results are in agreement to our previous findings which described that these compounds at higher concentrations were successful in suppressing the activity of soluble β GS,

while the PANI-CS-NC- β GS showed remarkably higher resistance to inhibition mediated by such compounds. Pawar et al. [39] investigated the binding of proton pump inhibitors to human serum albumin by means of spectroscopic and molecular modelling approaches. They observed shifts in protein amide bands due to conformational changes in the protein upon its interaction with omeprazole and esomeprazole.

Circular dichroism analysis

Figure 7 demonstrates CD spectroscopic measurements of soluble, PANI-CS-NC and PANI-CS-Ag-NC adsorbed β GS in the presence of investigated compounds. Among all the investigated compounds the highest changes in ellipticity were observed in the presence of galactose in all enzyme preparations with respect to control. Significant conformational changes were noticed in a soluble enzyme in the presence of galactose which led to a loss in its enzymatic activity. Table S4 summarizes α -helical and β -sheet content in all preparations in the presence of VC and sugars. α -Helical content was abruptly increased to 4 fold in a free enzyme in the presence of added galactose. On the other hand, insignificant changes

were observed in the secondary structure of the immobilized β GS in presence of both sugars and VC with respect to control. These results corroborate well with those obtained through UV-visible, fluorescence-based spectroscopic experiments and FT-IR studies. Other researchers have previously employed CD technique to substantiate secondary structural changes taking place upon immobilization in proteins. He et al. [44] evaluated conformational changes in the secondary structure of trypsin and chymotrypsin in the presence of Bowman-Birk inhibitor by CD measurements.

This study is an extension to our previously published work [24,29] where we have demonstrated the immobilization strategy and stability enhancement of the immobilized enzyme over the soluble counterpart. Furthermore, in one of the studies, we obtained a remarkable increase in the catalytic activity of NC bound enzymes in the presence of essential ions found in milk [29].

Conclusion

To summarize, this study demonstrates the effect of products of lactose-hydrolyzed reaction, glucose and galactose and their structurally related compound, VC on the functional and structural properties of β GS in free and bound form in a comprehensive manner. Herein, a systematic effort has been done to measure changes in the structure of free and immobilized β GS in the presence of VC glucose and galactose. The soluble β GS showed a major loss in its catalytic activity in the presence of investigated compounds. PANI-CS-Ag-NC- β GS was found to be the most resistant against the effect of VC and products of β GS catalyzed reaction. Major secondary structural changes were noted in native enzyme incubated in the presence of galactose. This is an indication of unfavourable changes in the secondary structure of the protein which caused destabilization that leads to a loss in enzyme activity. Both immobilized NBC retained significantly higher catalytic activity and exhibited minor conformational changes in the presence of VC, glucose and galactose. Thereby, the immobilized β GS preparations can be effectively employed for the production of hindrance-free nano-biosensor for lactose detection in milk and its products and to produce lactose-free dairy products to be fed upon by lactose intolerant people.

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QH designed the experiments and contributed in finalizing the manuscript; MK performed the experiments and analyzed the experimental data. Authors are extremely grateful to the instrumentation facility of Department of Chemistry, AMU Aligarh for conducting FT-IR and CD experiments.

Disclosure statement

The authors declare that they have no competing interests.

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