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ORIGINAL RESEARCH PAPER

A SPECTROSCOPIC STUDY ON THE HEAT INDUCED CHANGES OF GLUCOSE OXIDASE AT ACIDIC pH VALUES

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The heat induced conformational and structural changes of glucose oxidase from *Aspergillus niger* at pH 5.0 and 4.0 were investigated using fluorescence spectroscopy. Experimental studies were conducted in buffer solution in the 25°C-70°C temperature range at constant time. At both pH values, the phase diagram was linear, indicating the presence of two molecular species induced by thermal treatment. When thermally treating the glucose oxidase at pH 4.0, the denaturation process was faster than the one at pH 5.0, probably due to the release of cofactor FAD and molten globule formation. The quenching experiments using acrylamide and iodide revealed a more flexible conformation of glucose oxidase at higher temperatures, especially at pH 4.0.

Keywords*:* glucose oxidase, fluorescence, heat treatment, quenching

Introduction

Glucose oxidase (GOx) (EC 1.1.3.4) is an L-D-glucose: oxygen-1-oxidoreductase that catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone and H_2O_2 using molecular oxygen as an electron acceptor (Hosseinkhani *et al.*, 2004). GOx has been purified mainly from the genus *Aspergillus* and *Penicillium* (Sukhacheva *et al.*, 2004), out of which *Aspergillus niger* is the most commonly utilized for the production of GOx. GOx from *Aspergillus niger* is a flavoenzyme with high content (10–16% of the molecular weight) of mannose type carbohydrate (Hayashi and Nakamura, 1981). GOx is a glycoprotein, a homodimer enzyme with a high degree of localization of negative charges on the enzyme surface and the dimer interface. Each homodimer contains one FAD molecule (not covalently bound with the apoenzyme) as prosthetic group that is buried in a deep pocket formed in the protein. This pocket is represented by the β-D-glucose binding domain (Hecht *et al.*, 1993).

Due to its importance, applications of GOx cover many fields such as biotechnology, food and beverages, pharmaceutics, clinical chemistry. GOx has been used successfully to extend the shelf life of food and beverages due to its

capacity of removing the residual glucose and oxygen, and to improve the color and flavor of the food matrix (Bankar *et al.*, 2009). Pluschkell *et al.* (1996) reported the use of GOx for the production of gluconic acid, and as a food preservative, Petruccioli *et al.*, (1999) demonstrated that GOx can be used as an ingredient of toothpaste, while Kapat *et al.* (2001) mentioned the antagonistic effect against different food-born pathogens.

Moreover, applications of GOx in designing biosensors for medicine are well known, whereas the use of this enzyme for environmental monitoring as well as for the next generation biofuel cell systems represents one of the most recent applications of GOx (Guiseppi-Elie *et al.*, 2009).

Although, there are many studies in the literature focusing on GOx, the understanding of the unfolding behavior of the enzyme in various pH conditions has not been fully explored.

Therefore, the aim of our study was to provide additional insight into the function of GOx, so as to allow for the knowledge based design of new products. In this study, the heat influence on GOx structure at two acidic pH values was tested by means of fluorescence spectroscopy. The intrinsic fluorescence, quenching experiments and synchronous spectra runs were performed to provide a deep characterization of GOx behavior.

Material and methods

Materials

Glucose oxidase from *Aspergillus niger* was purchased from Fluka, and acrylamide from Sigma Aldrich. All reagents were of analytical grade. Protein solution of 5 mg/mL in 50mM acetate buffer of pH 4.0 and 5.0 were freshly prepared before each experiment.

Heat Treatment

Plastic tubes (1 cm diameter) filled with 0.5 mL of GOx solutions were incubated at temperature of 25, 50, 60 and 70 °C for 10 minutes using a thermostatic water bath (Digibath-2 BAD 4, RaypaTrade, Barcelona, Spain). After heating, the tubes were immediately cooled in ice water to avoid further thermal denaturation.

Intrinsic fluorescence spectroscopy experiments

The fluorescence of GOx was evaluated at two pH values, 4.0 and 5.0. 30 µL of protein solution were suspended in 3 mL of appropriate buffer for measuring the tryptophan (Trp) and tyrosine (Tyr) fluorescence. In order to measure the flavin adenin dinucleotide (FAD) fluorescence, a volume of 0.2 mL was used. The steady state fluorescence spectra of GOx were obtained using LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA). Changes in the emmision spectra of the Trp, Tyr and FAD were recorded using excitation wavelength of 292 nm, 274 nm and 450 nm respectively (Pryzbyt *et al.*, 2011). All fluorescence scans were performed using a quartz cell of 10 mm path length, excitation and emmision slits being set at 10 nm each. The experiments were

repeated at least twice to ensure the reproducibility of the data. The *phase diagram* method was developed according to Stănciuc *et al.* (2012). Synchronous fluorescence spectra were recorded at excitation wavelength ranging from 250 to 500 nm with intervals 15 nm and 60 nm.

Quenching experiments

Fluorescence quenching experiments were performed with acrylamide (8M) and potassium iodide (5M). Freshly prepared stock solutions of quencher were added in aliquots (25-150 µL) to the cuvette containing buffer and protein solution. Assuming a single population of fluorophores (Eftnik and Ghirom, 1981), data were analyzed using the Stern-Volmer equation (Eq 1), where F_o and F are the fluorescence intensities in the absence and presence of the quencher, K_{SV} is the Stern-Volmer quenching constant, [Q] is the concentration of the quencher

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad \text{(Eq 1)}$$

The Stern-Volmer plots were analyzed by linear regression using the computer software MS Office.

Results and discussion

Phase diagram

Fluorescence emission spectra are very sensitive to the polarity of fluorophores surroundings whose intensities at two wavelengths can be plotted in the form of a *phase diagram* (Chen *et al.*, 2014). The *phase diagram* method is used for the detection of any intermediate states. The phase diagram was constructed to analyze the mechanism by which thermal treatment in acidic conditions induced conformational changes in GOx molecules, by monitoring the change of the intensity at 365 nm versus 320 nm. As can be seen in Figure 1, a linear dependence was obtained for both tested pH values indicating an *all-or-none* transition process. In the *all-or-none* transition process, also known as a two-state system, only the completed unfolded and the native state are populated on the unfolding and refolding pathways.

Based on the presented results, it can be stated that the heat treatment induced sequential conformational transitions upon the GOx solutions for both pH values. Regardless of the pH value, the Gox solutions heating in the temperature range of 25°C to 70°C resulted in a two state denaturation model (Figure 1), the linear correlation indicating the presence of two molecular species.

At pH 5.0, the unfolding of the native enzyme state predominates up to 50°C causing an exposure of the hydrophilic residues as resulted from the decrease in fluorescence intensity. The increase in fluorescence intensity at temperatures higher than 50°C is due to the folding of protein molecules. On the other hand, at pH 4.0, the fluorescence intensity decreased with temperature increase suggesting that the enzyme follows a specific unfolding pathway.

As suggested by Haq Khatun *et al.* (2003), the acid induced state of GOx at low pH, although retaining a significant amount of native-like secondary and tertiary

structures, also has sizeable amounts of exposed hydrophobic region that indicates the accumulation of a compact molten globule-like intermediate. Molten globules are partially structured protein folding intermediates that adopt a native-like overall backbone topology in the absence of extensive detectable tertiary interactions.

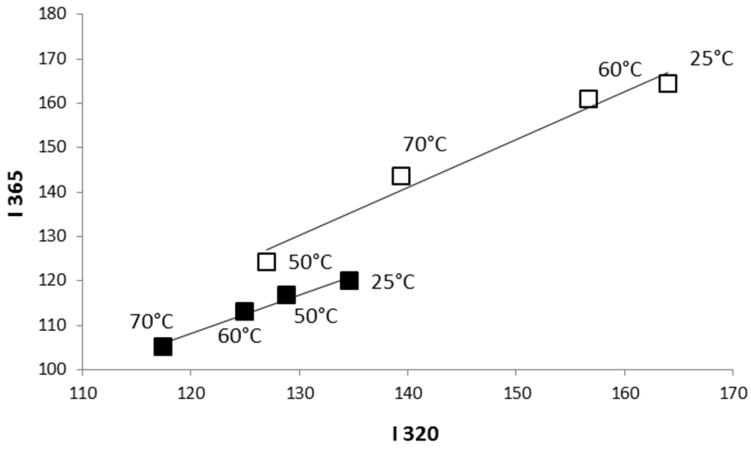

Figure 1. *Phase diagram* analysis of heat-induced conformational changes of Gox at pH 5.0 (□) and pH 4.0 (■) based on intrinsic fluorescence intensity values measured at 320 and 365 nm wavelengths. The temperature values are indicated in the vicinity of the corresponding symbol.

Sattari *et al.* (2013) observed that GOx presented a dimeric form up to a temperature of 55°C. Above this temperature, authors reported an increased exposure of hydrophobic surface to the solvent followed by the formation of aggregates.

Intrinsic fluorescence

Fluorescence spectroscopy is a spectral technique mainly sensitive to changes in the tertiary structure and the conformational stability of protein in solution (Tang *et al.*, 2009). GOx contains one tightly bound FAD cofactor per monomer and 10 Trp residues per subunit. In particular, 4 of Trp residues are located in the FAD domain (Hecht *et al.*, 1993). The spectral parameters of Trp, Tyr and FAD fluorescence are dependent on the dynamic and electronic properties of its environment; hence cromophore fluorescence was used to obtain information on the structural changes and the dynamical microenvironment of GOx. In Figure 2 and Figure 3 are presented the fluorescence intensity of Trp (a), Tyr (b) and FAD (c) at pH 5.0 and 4.0 for different temperatures after 10 minutes of heating. When excited at 292 nm, the Trp residues, the un-treated samples had a λ_{max} at 344 nm (pH 5.0), and 347 nm (pH 4.0), respectively. In general, shifts in λ_{max} are due to the changes that appear in the tertiary structure of the protein. Regardless of temperature, the fluorescence intensity of Trp and Tyr residues was always higher at pH 5.0. Thus, at 25°C, the fluorescence intensity of Trp residues was by about 25% higher compared with that at pH 4.0. In its native state (pH 6.0), GOx has a λ_{max} of 351 nm (data unpublished),

that is typical for buried Trp side chains in nonpolar environment. Therefore it can be stated that in acidic conditions, the Trp residues microenvironment becomes nonpolar. As described by Haouz *et al.* (1998), the increase in the Trp and Tyr fluorescence with increasing pH in GOx molecule is associated with the conformational change of the GOx molecule that depends on the Föster energy transfer from Trp and Tyr residues to flavin moiety.

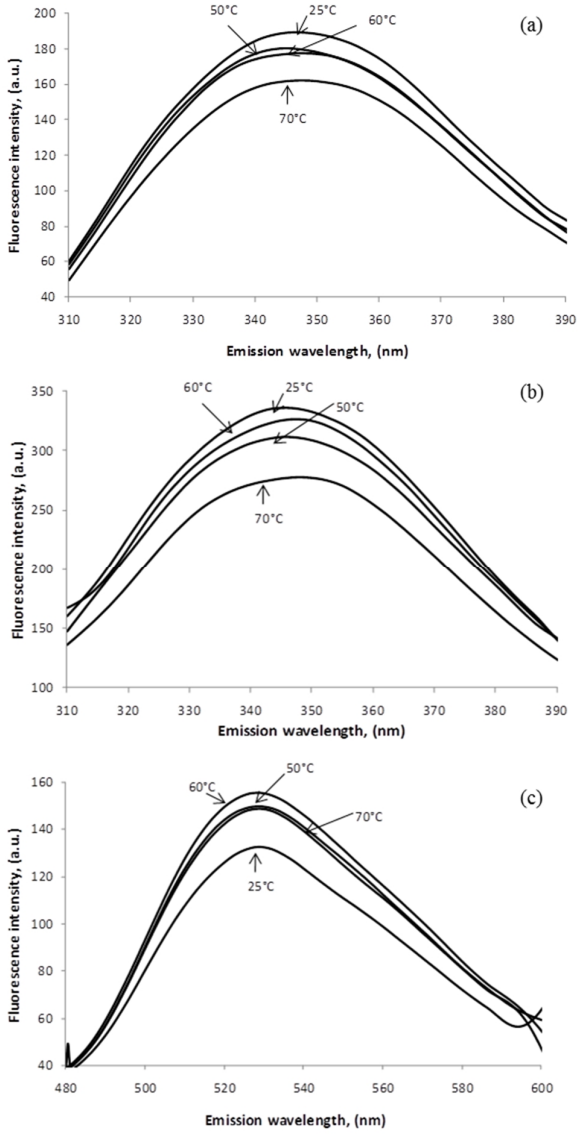

Figure 2. Structural changes of Gox monitored by emission spectrum at pH 5.0. The excitation wavelength was 292nm (a) 274 nm (b) and 450 nm (c)

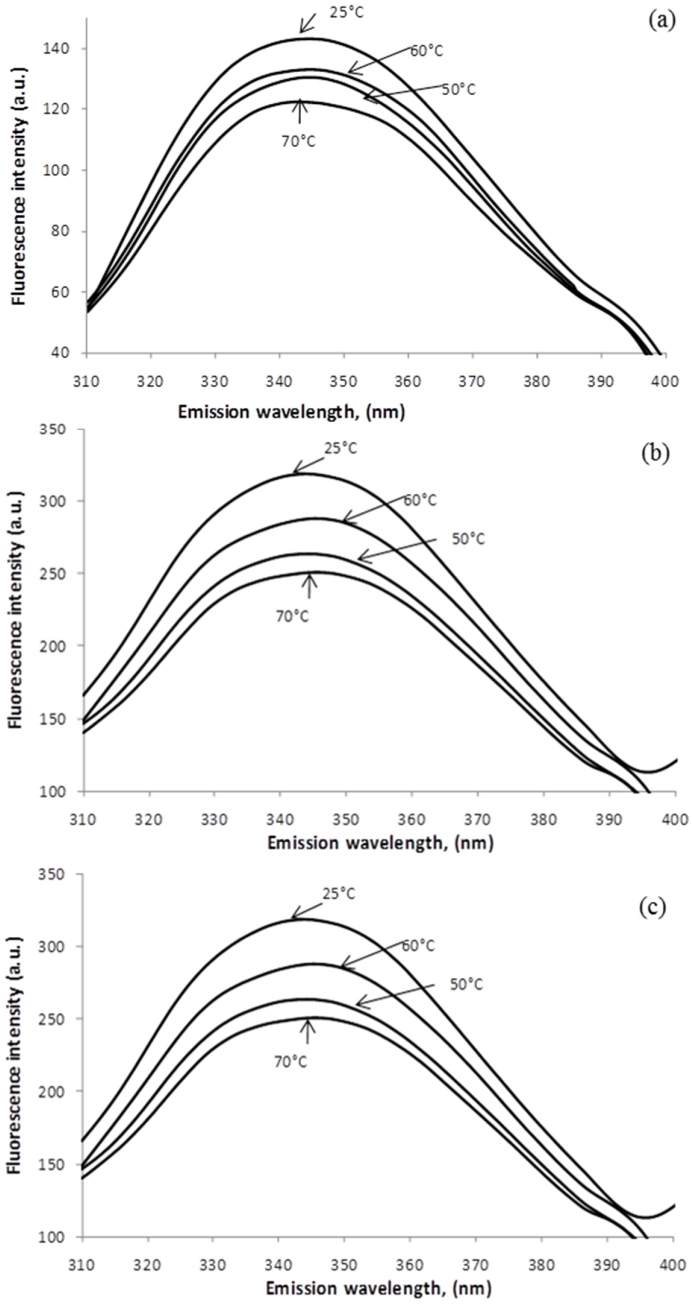

Figure 3. Structural changes of Gox monitored by emission spectrum at pH 4.0 The excitation wavelength was 292 nm (a) 274 nm (b) and 450 nm (c).

When studying the heat influence (increasing temperature up to 50°C) upon the Trp residues of GOx molecule, a blue shift of 2 nm in λ_{max} was noticed at pH 5.0 (Figure 2a). This was followed by a red shift of 2 nm at 70°C, indicating that Trp residues are relocating from a hydrophobic to a more hydrophilic environment at higher temperatures. At pH 4.0, the fluorescence intensity (Figure 3a) decreased with increasing temperature, indicating that at higher temperature the Trp residues are more exposed to the solvent. The λ_{max} values remained constant for the whole tested temperature range. According to Gasymov *et al.* (2000), the aminoacids with high mobility are more exposed to the polar environment, while those that are buried are surrounded by an apolar environment.

The fluorescence intensity of Tyr residues (Figure 2b and Figure 3b) is always higher when compared with that of Trp residues. Thus, Trp residues fluorescence in untreated samples is 55.06±0.12% (pH 4.0) and 43.55±1.34% (pH 5.0) lower compared with Tyr fluorescence. For both pH values analyzed, Tyr fluorescence decreased with increasing temperature, suggesting the alteration of GOx conformation. Heating at 70°C caused the decrease of the Tyr fluorescence intensity by 20.14 ±1.24% (pH 4.0) and 17.25±1.87% (pH 5.0).

The FAD structure is composed by flavoprotein and adenine dinucleotide; in aqueous solution it exists in two conformations: a non-fluorescent stacked conformation, in which the isoalloxazine and adenine aromatic rings are in close proximity, and a fluorescent open conformation, in which the two aromatic rings are separated from each other (Islam *et al.*, 2013). In solution, the FAD molecule is predominantly present in the stacked conformation, while the open conformation gives rise to the fluorescent component of FAD (Feenstra, 2002). The FAD binding domain is covered by a β-sheet lid involved in the interaction between GOx monomers. During heating, due to the loss of most interaction at the dimer interface, this lid opens, causing FAD loss and dimer dissociation, followed by spontaneous aggregation of the resulting monomers (Caves *et al.*, 2011).

In the untreated samples (Figure 2c and Figure 3c), FAD fluorescence at pH 4.0 is about 40% higher than at pH 5.0. This behavior has been ascribed to the partial unfolding of the protein that is associated with the release of the FAD molecules with consequences upon fluorescence intensity that increases. At pH 5.0 the fluorescence intensity increased up to 60°C by about 17% and then at 70°C slowly decreased, suggesting that, after dissociation of FAD from the enzyme, several structural changes appear in the GOx molecule. On the other hand, heating at pH 4.0 resulted in a significant enhancement of flavin fluorescence intensity up to 50°C, followed by a small blue shift of 2 nm in the wavelength of peak emission from 529 nm at 25°C to 527 nm for all the other temperatures tested. More precisely, the FAD fluorescence intensity increased at 50°C by 40%. For both pH tested, increasing the temperature from 50°C to 70°C did not cause significant changes in FAD fluorescence and λ_{max} emission. It seems that, in the native conformation of the enzyme, seven Trp residues and FAD are in proximity; therefore the quenching of fluorescence is correlated with Föster energy transfered from Trp residues to the flavin group (Gouda *et al.*, 2003). In a study conducted by

Ma *et al.* (2009), FAD fluorescence was found to be dependent on the changes of the environment of GOx that alter the conformational motion and dynamics, the strength of the binding inside the GOx molecule. Thus, any changes in pH or temperature have implications upon flavin moiety that dissociates due to the disruption of non covalent interactions, resulting in a higher quantum yield.

At neutral pH and at higher temperatures, GOx from *Aspergillus niger* denaturates, leading to FAD release associated with the molten globule state of the enzyme in which the enzyme loses the tertiary structure and retains 60-70% of the secondary structure (Zoldak *et al.*, 2004).

Taking into account these observations, one can conclude that, in addition to the heated induced changes in fluorescence intensity, the influence exerted by FAD should also be considered.

Synchronous spectra

Synchronous fluorescence spectroscopy represents a common method in which the fluorescence emission varies, giving information about the microenvironment change around amino acid residues. This method proved to be useful for investigating the environment of Tyr and Trp residues, by measuring the possible shift in maximum emission wavelength (Cheng *et al.*, 2013). If $\Delta\lambda$=15 nm then synchronous spectra gives information about Tyr residues, whereas stabilized at 60 nm may give some characteristics of Trp residues and FAD molecule (Przybyt *et al.*, 2011) within the protein matrix.

In Figure 4 is depicted the synchronous spectra for GOx with $\Delta\lambda$ equal to 60 nm, at different pH values. A sharp maximum at 280 nm can be seen due to Trp and two very weak maxima at 370 nm and 460 nm corresponding to flavin moiety (Figure 4 inset). The peak at 460 nm increased with decreasing pH due to the dissociation of FAD from GOx molecule.

The maximum emission wavelength of GOx cromophores is related to its microenvironment adding some insights regarding the conformational changes of GOx protein. Figure 4 shows that fluorescence intensity of Trp residues declined with increasing temperature for both pH values tested. The same behavior was noticed for Tyr residues (data not shown). The peak of Tyr at pH 4.0 showed a red shift of 2 nm at 60°C indicating that the polarity around Tyr has changed and hydrophobicity decreased. The peak of Trp residues remained unchanged and fluorescence intensity decreased at 70°C by about 19% compared with the untreated sample. On the other hand, at pH 4.0, the peak of Tyr and Trp showed the same fluorescence intensity in the temperature range of 25-60°C, while at 70°C fluorescence intensity decreased by about 15% for both Trp and Tyr residues. These results indicate that at pH 5.0 the microenvironment of Tyr and Trp is less affected. Therefore, it can be concluded that GOx is more stable at pH 5.0 unlike the enzyme tested at pH 4.0. It seems that the greater stability of the enzyme is due to the lack of repulsion between monomers and protein close contact to zero net charge (Caves *et al.*, 2011).

All the above mentioned results are in agreement with those reported for intrinsic fluorescence.

Quenching experiments

Quenching experiments were performed for monitoring the temperature induced conformational changes of GOx in acidic conditions with the aim to get more information about the polarity of the Trp and FAD microenvironment and their accessibility to the solvent. For this purpose, acrylamide was used to identify both hydrophobic and hydrophilic regions of Trp residues within the protein, while KI, the fluorescence emitted by Trp and FAD, located at or near the surface of the molecules.

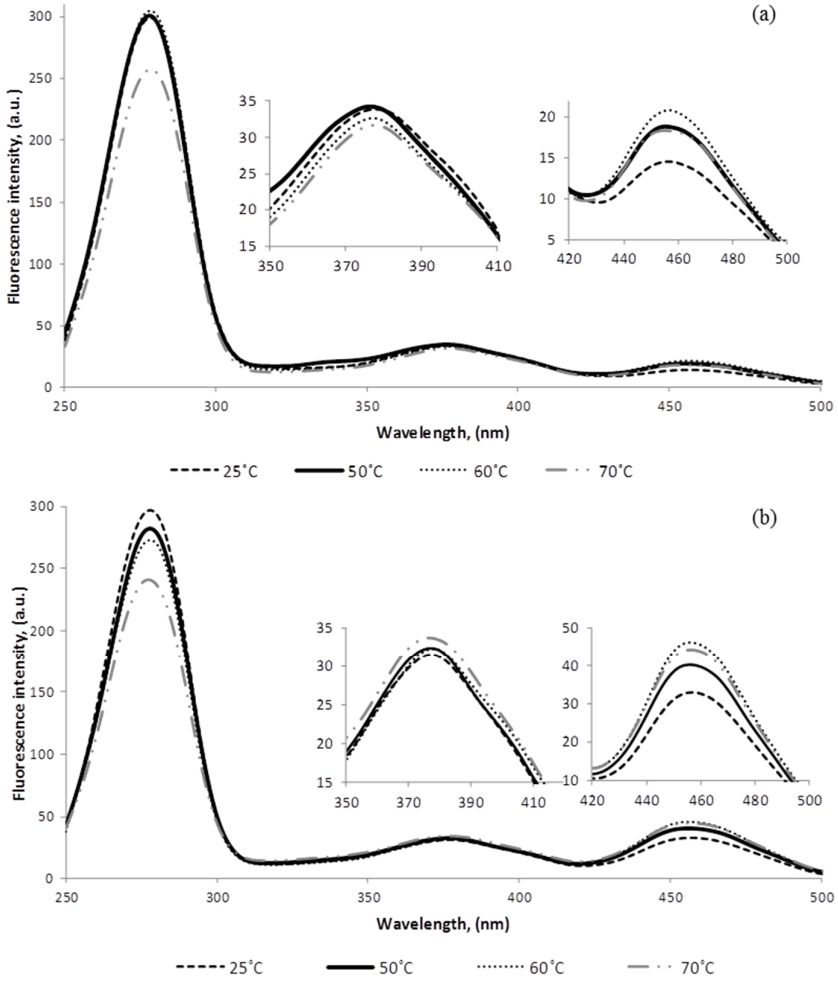

Figure 4. Synchronous spectra scans of glucose oxidase with Δλ=60 nm at pH 5.0 (a) and pH 4.0 (b)

In Table 1 are summarized the Stern-Volmer constants of GOx heat treated solutions at two pH values. When quenching with acrylamide, at pH 4.0 (25°C) and pH 5.0 (70°C), the highest quenching constants were calculated, 7.48±0.12 10^{-3} mol^{-1} L and 6.24±0.08 10^{-3} mol^{-1} L, respectively, indicating that at these temperatures, the Trp residues are maximally exposed to solvent.

Table 1. Stern-Volmer quenching constants (10^{-3} mol^{-1} L) of Gox at different temperatures using KI and acrylamide

Temperature (˚C)	KI				Acrylamide	
	Ksv for Trp		*Ksv* for FAD		*Ksv* for Trp	
	pH 5.0	pH 4.0	pH 5.0	pH 4.0	pH 5.0	pH 4.0
25	3.15±0.45	5.31±0.15	15.27±1.12	15.47±1.65	5.54±0.03	7.48±0.12
50	3.73±0.13	4.87±0.12	18.87±1.49	22.89±0.22	5.73±0.08	6.40±0.20
60	3.86±0.05	5.12±0.17	20.06±1.22	24.14±0.36	5.90±0.07	6.71±0.48
70	4.52±0.17	5.94±0.28	20.12±0.42	24.95±0.73	6.24±0.08	6.40±0.12

As resulted from Table 1, heat treatment caused unfolding of the GOx molecules in the whole temperature range tested at pH 5.0. On the other hand, heating at pH 4.0 resulted in the folding of the protein, as indicated by the quenching constants that decreased with increasing temperature.

As expected, when quenching with both quenchers of Trp residues, the K_{SV} are always higher for acrylamide regardless of the pH and heat treatment applied. Quenching with both acrylamide and KI resulted in higher values of K_{SV} at pH 4.0 compared with pH 5.0, indicating that Trp residues are more exposed to the solvent under lower pH values. The increase of the quenching constants with increasing temperature indicates that the conformational transition of GOx decreases the distance between the Trp residues and the quenchers.

On the other hand, the quenching experiments of GOx flavin moiety fluorescence with KI are always higher compared with those for Trp residues (both acrylamide and KI). The calculated K_{SV} constants revealed that FAD is more exposed to the quencher with decreasing pH. Therefore, the increase of the quenching constants of FAD at higher temperatures indicates that GOx molecule is in a state with disrupted tertiary structure due to the dissociation of FAD that is almost free in solution. The quenching with KI of the FAD molecule followed the pattern observed for Trp residues indicating that FAD in the denaturated state is more accessible to the solvent than the untreated Gox samples. Zoldak *et al.* (2004) studied the thermal denaturation of glucose oxidase in its native state and calculated a quenching constant for FAD of 25.40±0.3 mol^{-1} L in the thermally denatured form of the enzyme. The same authors suggested that thermally denaturated Gox is a compact structure, a form of molten globule like apoenzyme. Thus, for both pH values, the highest K_{SV} constants of FAD were calculated at 70˚C

suggesting that the conformational transition of GOx decreases the distance between the FAD and the quencher. The K_{SV} constants of the Gox molecule are always lower at pH 5.0 compared with pH 4.0 indicating the lower accessibility of the quencher to FAD in the Gox molecule.

Conclusions

In the present paper, the spectroscopy approach was used to investigate the temperature induced changes in the glucose oxidase structure. The fluorescence measurements were performed in acidic environment, at pH 4.0 and 5.0 respectively, after 10 minutes of enzyme incubation at 25°C, 50°C, 60°C, 70°C. Regardless of the pH, the phase diagram was linear, indicating the presence of two molecular species induced by heating. Decreasing the pH of glucose oxidase solutions from 5.0 to 4.0 resulted in a red shift of 3 nm of Trp residues, indicating changes in the tertiary structure of the protein. Based on fluorescence intensity results, heating up to 50°C caused at pH 5.0 a blue shift of 2 nm in λ_{max}, followed by a red shift of 2 nm at 70°C, suggesting conformational rearrangements within the protein matrix at higher temperatures. To get additional information about the temperature dependence of the quenching process, acrylamide and KI have been used. Quenching studies with acrylamide indicated that Trp residues are maximally exposed to solvent at 25°C (pH 4.0) and 70°C (pH 5.0). Quenching studies with KI demonstrated that the higher accessibility of FAD to the solvent is correlated with the dissociation from the enzyme during the process of denaturation.

Acknowledgements

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