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Enhanced thermal stability and pH behavior of glucose oxidase on electrostatic interaction with polyethylenimine

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ABSTRACT

Electrostatic interactions, mediated by ionic-exchange, between polyethylenimine (PEI) and glucose oxidase (GOx) were used to form GOx–PEI macro-complex, which were evaluated for pH and thermal stability of GOx. Under the experimental conditions, the complex had a dominant GOx presence on its surface and a hydrodynamic diameter of 205 ± 16 nm. Activity was evaluated from 40 to 75 °C, and at pH 2–12. GOx activity in complex was maintained up to 70 °C and it was lost at 75 °C. In contrast, free GOx showed a maximum activity at 50 °C, which was completely lost at 70 °C. This difference, observed by fluorescence analysis, was associated with the compact unfolded structure of GOx in the complex. This GOx stability was not observed under pH variations, and complex formation was only possible at pH ≥ 5 where enzymatic activity was diminished by the presence of PEI.

1. Introduction

Instability under processing conditions has become one of the major barriers to the use of enzymes in industrial biotechnology. The strategies to enhance the lifetime of enzymes may be through the isolation of novel enzymes functioning under extreme conditions; the modification of enzymes by molecular engineering; the modification of the solvent environment to decrease the denaturing effect of enzymes; and the immobilization on inert matrices [1]. From the last example, enzyme immobilization is widely used to improve stability of commercially and/or medically important enzymes [2]. Physical adsorption of enzymes onto a supporting material is the simplest method of enzyme immobilization [1,3–5] where stabilization has been attributed to a more rigid conformation of the immobilized enzyme that prevents enzyme denaturation and distortion of its active site. Thus, trapping enzymes in biologically inert matrices has led to prevent enzyme denaturation and increase their shelf life [6].

However, immobilization of enzymes may produce alterations in their observed activity, specificity, or selectivity; i.e. in many cases an impoverishment of the enzyme properties is observed, while in other cases such properties may be enhanced [7]. Enzyme entrapment is typically achieved using a polymer network [1,5] where enzyme immobilization is achieved by adsorption, covalent binding, cross linking, entrapment and chemical modification [1]. Many polymers have been tested to immobilize enzymes. Among these polymers, polyelectrolytes have been frequently selected to trap and immobilize several important enzymes [8,9]. In this regard, increasing interest has been observed in the use of polyelectrolytes as an excipient in the pharmaceutical industry and as a part of the formulation of biologically active agents such as proteins [10].

Linear polymers used in enzyme immobilization such as polyacrylic acid have been extensively studied both theoretically and experimentally [11,12]. Branched polyelectrolytes like polyethyleneimine (PEI) are more difficult to study, but have provided better results in enzyme stabilization than linear polymers [13,14]. The above has led to their extensive use mainly in pharmaceutical formulations as an excipient, personal care products as an antistatic agent, the food industry, and home and industrial detergents [12,15,16].

The main advantage of polyelectrolytes forming complexes with enzymes comes from their solubility in aqueous media. In contrast, the main challenge is to avoid undesired macromolecular interactions between polyelectrolytes and enzymes, favoring the retention of activity and stability of enzymes [17], as has been shown in

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reticulated polyelectrolyte microcapsules [18]. For these reasons, the study of branched PEI to immobilize an enzyme is very interesting.

In this context, the direct formation of an enzyme–polyelectrolyte complex should be dependent on experimental conditions—the stoichiometric relation polyelectrolyte/enzyme—and is indeed highly dependent on the pH in the medium [19], the dissociation of polyelectrolyte and enzyme counter ions [20], and the electrostatic conditions upon the polyelectrolyte complex formation [21].

Glucose oxidase (GOx) is an important enzyme widely used in industrial, medical, diagnostics, and analytics [22]. It is a dimeric enzyme with 80 kDa subunits that are highly glycosylated at the surface, mainly by β-D-mannose, α-D-mannose and N-acetyl-D-glucosamine, to contribute to the three-dimensional stability of the enzyme through intra- and inter-chain electrostatic interactions [23]. GOx contains 66 negatively charged amino acid residues contributing to a low isoelectric point (pI) of 4.2. The optimum pH for GOx activity is 5.5 [24]. Dimeric GOx contains two non-covalently bound flavin adenine dinucleotide (FAD) molecules that play a key role in the oxidation–reduction properties of the enzyme that are essential for the activity and structure of the enzyme [25]. The dissociation of FAD from GOx has been observed as a consequence of thermal and chemical denaturation of the enzyme [26]. Here, we report on the thermal stability and pH behavior of the GOx complexed with PEI. It also probes the resulting structure alterations of GOx due to complexation.

2. Materials and methods

2.1. Reagents

Glucose oxidase (GOx) type VII from *Aspergillus niger* was from Sigma Chemical Co. (Saint Luis, MO, USA). Branched polyethylenimine (PEI) Mw of 60,000 was from Acros Organics (Geel, Belgium). Both, GOx and PEI were used without further purification. Other reagents such as glucose, horseradish peroxidase, 4-aminoantipyrine, Tris, MES, NaCl, sodium phosphate and citric acid were from Sigma Chemical Co.; phenol from Fermont (Monterrey, Mexico); acetic acid and hydrochloric acid from J.T. Baker (Phillisburg, NJ, USA); and sodium hydroxide from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada). All solutions were prepared in distilled water (18.3 MΩ cm). Stock solutions for GOx and PEI were prepared by dissolving each at 1 mg/mL in 25 mM MES buffer, 25 mM Tris, pH 7.2, 0.13 M NaCl. Stock solutions were filtered using a 0.2 μm cutoff poly (tetrafluoroethylene) membrane from Nalgene®.

2.2. Buffers used

Buffers pH 2 and pH 3 were prepared using citric acid 2 M. Buffer pH 5 consisted of 0.2 M acetic acid. Buffers pH 7 and pH 9, corresponded to a 0.1 M Tris solution. Buffers pH 11 and pH 12 were prepared using a 0.05 M Na_2HPO_4 solution. For each buffer, the pH of the initial mixture was adjusted using either NaOH or HCl, except for pH 5.0 in which sodium acetate was used. Ionic strength had a maximum value of 0.13 M at pH 12. The ionic strength was therefore adjusted to 0.13 M for all other buffer solutions using NaCl.

2.3. GOx–PEI complex preparation

GOx working solutions were prepared in buffer MES–Tris, NaCl, degassed and filtered. PEI was added dropwise to GOx solution. The order of the additions affects the type of complex formed [27]. PEI added on a GOx solution favors the formation of a complex with the GOx on its surface because of the local excess of GOx when the PEI is added. The immediate formation of GOx–PEI complexes was evidenced by the formation of a cloudy suspension; this complex was incubated for 3 h at room temperature (around 24 °C). After this time, two phases were observed and the cloudy phase at the bottom corresponded to the GOx–PEI complex, which was separated by centrifugation for 15 min at 9000 rpm. The clear upper phase was removed by decantation, and the complexes in the pellet were re-suspended in the MES–Tris–NaCl buffer at pH 7.2. The solution was homogenized by sonication, and a highly turbid solution was obtained with the suspended GOx–PEI complex.

2.4. GOx–PEI complex characterization

After preparation, the complex was characterized by turbidimetric measurements, which were performed at a wavelength of 500 nm at 25 °C using a SmartSpec™ spectrophotometer from Bio-Rad Laboratories (Berkeley, CA, USA). The tendency of the enzyme to form the complex was determined by titration with different PEI concentrations; spectrophotometric readings were obtained 2 min after PEI addition. An absorbance (0.5) was taken as a cutoff to consider GOx–PEI complex formation; thus, lower readings were not considered as effective complex formation. The average size and Zeta (ζ) potential of GOx, PEI and GOx–PEI complex was determined by dynamic light scattering (DLS) using a ZetasizerNano ZN ZEN 3660 Malvern instruments Ltd. (Malvern, UK) by using a green laser (532 nm wavelength) and a detection at 173°. The samples were filtered, degassed and homogenized before taking the measurements in a rectangular 1 cm path length cell. Surface electrical charges were determined by measuring the electrophoretic mobility of the particles in an electric field using the same device and a curved capillary cell with electrodes.

2.5. GOx activity assay

GOx activity was determined using a coupled assay as described by Trinder [28]. The assay consists of glucose oxidation via GOx with generation of H_2O_2, which then interacts with a chromogen (4-aminoantipyrine) in a reaction catalyzed by horseradish peroxidase. The reaction results in the formation of a colored quinone (quinoneimide dye), and its absorbance was determined at a wavelength of 510 nm using a 1 cm path length cell in UV-Vis spectrophotometer SmartSpec™ Plus from BioRad Laboratories (Berkeley, CA, USA). GOx reaction was monitored during the first 7 min after the enzyme addition and the initial rate was calculated from the straight line formed.

2.6. Thermal and pH tests of GOx–PEI complexes

GOx–PEI complex in a MES–Tris–NaCl buffer at pH 7.2 was incubated for 15 min at temperatures ranging from 40 to 75 °C. Then an aliquot (9 μg enzyme in 20 μL) was taken and tested for residual GOx activity using the colorimetric assay described in Section 2.4 (at 37 °C and containing 9.5 μg glucose/mL).

GOx–PEI complex was incubated for 15 min at room temperature in the buffers described above (pH from 2 to 12). Then an aliquot (9 μg enzyme in 20 μL) was taken and tested for residual GOx activity using the colorimetric assay described in Section 2.4 (at 37 °C and containing 9.5 μg glucose/mL). In both cases, the assays were triplicated, and free GOx was taken as reference.

2.7. Fluorescence spectroscopy

Intrinsic GOx and FAD fluorescence were evaluated for free GOx and GOx–PEI complex (both at 0.22 mg enzyme/mL) upon excitation at a wavelength of 295, corresponding to the excitation for

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