Biocatalysis of Glucose 2-Oxidase from Coriolus Versicolor at High Pressures

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Glucose 2-oxidase (pyranose oxidase, pyranose:oxygen-2-oxidoreductase, EC 1.1.3.10) from *Coriolus versicolor* catalyses the oxidation of D-.glucose at carbon 2 in the presence of molecular oxygen producing D-glucosone (2-keto-glucose and D-arabino-2-hexosulose) and hydrogen peroxide. This enzyme was used to convert D- glucose into D-glucosone at high pressures with compressed air in a modified commercial batch reactor. Several parameters affecting biocatalysis at high pressures were investigated as follows: pressure, enzyme concentration, glucose concentration, supercritical fluid and the presence of catalase. Glucose 2-oxidase was purified by immobilized metal affinity chromatography on epoxy-activated Sepharose 6B-IDA-Cu(II) column at pH 6.0. The conversion of D-glucose into D-glucosone was dependent on the pressure since an increase in the pressure with compressed air resulted in higher rates of conversion. On the other hand, the presence of catalase increased the rate of reaction which strongly suggests that hydrogen peroxide inhibited the rate of reaction. The rate of conversion of D-glucose into D- glucosone by glucose 2-oxidase in the presence of either nitrogen or supercritical CO_2 at 110 bar was very low compared with the use of compressed air at the same pressure.

INTRODUCTION

Enzymes are of great commercial interest because they have a number of applications in industry and Medicine such as textile, leather, detergent, starch, diagnostics, therapy and bioremediation. However, enzymes are not widely used in fine chemical industry mainly due to their poor stability.
Glucose 2-oxidase (pyranose oxidase, pyranose:oxygen-2-oxidoreductase, EC 1.1.3.10) catalyses the oxidation of glucose at the carbon position 2 in the presence of oxygen producing D-glucosone and hydrogen peroxide (1). This enzyme is synthesized by white rot fungi such as *Coriolus versicolor* and *Phanerochaete chrysosporium* and plays an important role in lignin biodegradation (2). On the other hand, this enzyme is of great importance since the reaction product (D-glucosone) is an important precursor for biosynthesis of the antibiotic cortalcerone (3). The use of compressed air in this enzyme reaction would increase the rate of reaction since oxygen is one of the reaction substrates. However, high pressures also affect the enzyme structure altering its enzyme activity (4).
Enzyme reactions can be carried out under high pressure or in supercritical fluids which is a new and promising field of enzyme engineering (5). High pressure is responsible for direct conformational changes in enzymes which affect their biological activities. Quaternary structure is very sensitive to pressure it is generally maintained by hydrophobic interactions which are rather weak interactions. In fact, moderate pressures have been found to favour the dissociation of oligomeric proteins such as lactate dehydrogenase. On the other hand, the secondary and tertiary structures are more resistant to pressure than the quaternary structure

since higher pressures are required to observe structural changes (6). However, reversible unfolding of proteins can occur at higher pressure suggesting that the volume and compressibility changes during denaturation are not completely dominated by hydrophobic interactions. In fact, protein denaturation affecting the tertiary structure is a complex process which involves sometimes intermediates states/forms leading to multiple denatured forms. Moreover, pressure also influences the reaction rate constant which changes according to transition state theory and standard thermodynamics.

MATERIAL AND METHODS

Materials

Coriolus versicolor was obtained from INETI, Portugal. Corn-steep liquor was kindly donated by COPAM, Portugal. O-dianisidine, iminodiacetic acid, 1,4 butanediol diglycydil ether, catalase and peroxidase were purchased from Sigma Chemical Company. Recombinant glucose 2-oxidase from *E. coli* was obtained from Biozyme laboratories; U. K. Sepharose CL-4B was obtained from GE Helath Care, Sweden. DE-52 and Sugar Pak column was purchased from Whatman (UK) and Waters, USA, respectively.

Methods

Apparatus set up

The experimental set-up, as shown in Figure 1, consists basically of one batch reactor (Micro Reactor Parr Instruments CO, 4843) with 25 ml of capacity equipped with agitation, temperature and pressure reading devices. Two principal's valves inlet and outlet connections and a rupture disk set a 25 MPa were used to perform the tests.

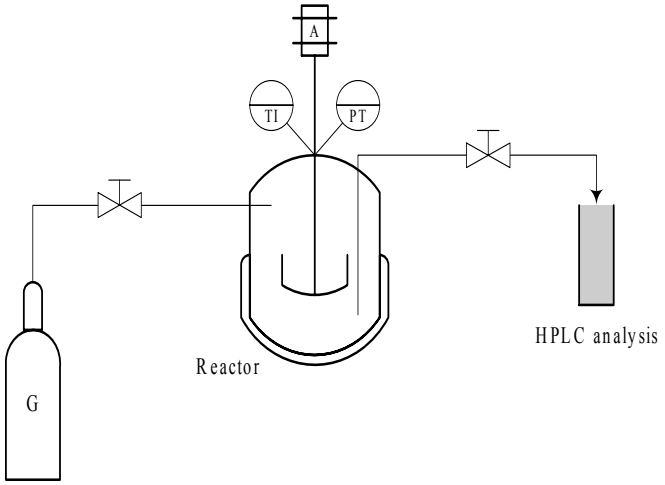

Figure 1: Schematic diagram of the experimental apparatus – Enzyme Reactor unit: G, Gas Cylinder; A, magnetic stirrer device; TI and PI, temperature and pressure indicator.

When nitrogen and compress air was used the gas cylinder was connected directly to the reactor (as was shown in Fig.1). If carbon dioxide was used the liquid CO_2 flowing from the cylinder was compressed to the desire pressure with a pump (Applied Separations, Spe-ed^{TM} SFE) into the reaction vessel. In each experiment samples were withdraw at various intervals to determine product concentration, using an HPLC (Jasco Instruments).

Production and purification of Glucose-2-oxidase from Coriolus versicolor: A fungal strain of *Coriolus versicolor* was cultured and maintained in malt extract agar. The production and purification of glucose 2-oxidase from *Coriolus versicolor* was carried out as described previously (1).

Immobilization of Glucose-2-oxidase*:* Either cell-free extract containing glucose 2-oxidase activity or purified enzyme was immobilized by non-covalent binding in anionic exchange resin DE-52. DE-52 cellulose (5g) was equilibrated in 50mM phosphate buffer pH 6.5. Subsequently, either cell-free extract (5ml, specific activity 0.21U/mg protein.ml) or purified enzyme (1ml, 8.2 U/mg protein.ml) and 4ml of 50 mM phosphate buffer pH 6.5. The mixture was agitated for 10 minutes and centrifuged for at 3000g for 2 min. The supernatant was removed and the resin was washed three times with 1 volume of the same buffer system . The immobilized enzyme preparation was stored in the same buffer system at 4ºC.

Enzyme and protein assay: Glucose 2-oxidase activity was determined by a linked assay method in the presence of peroxidase and o-dianisidine as described previously (1).Protein concentration was determined by coomassie blue dye binding method (1).

Electrophoretic analysis: Cell-free extract and purified preparation of enzymes were analysed for purity by SDS-PAGE and native PAGE as reported previously (1).

Biocatalysis at high pressure: Biocatalysis at high pressure was carried out by using either cell-free extract from *Coriolus versicolor* (0.21U/mg protein), or purified commercial enzyme (8.2 U/mg protein) and purified enzyme from *Coriolus versicolor* (8.9 U/mg protein). Immobilized forms of cell-free extract and purified enzyme were also used in biocatalysis at high pressure.

The reaction mixture contained appropriate concentration of D-glucose in 50mM phosphate buffer pH 6.5 (19.9ml) and 100µl of appropriate form of enzyme which was stirred at 110 rpm. The reaction was performed at several working pressures at room temperature by using either compressed air, compressed nitrogen or supercritical CO2 at 32ºC. In some experiments, catalase (20mg, 1000U/mg protein) was added to the reaction mixture. Aliquots (100µl) were removed from the batch reactor at suitable time intervals and the reaction was stopped by addition of HCl 0.6N (10µl). When immobilized forms of enzymes were used, aliquots were centrifuged at 5000g for 20 s and the supernatant was separated from the sediment and stored in an ice bath.

The reaction mixture was also carried out at NPT conditions in an end-over end mixer and its composition was the same as at high pressures. Aliquots were removed from the reaction mixture and processed the same way as before.

Calibration curve for D-glucose and D-glucosone: Several concentrations of D- glucose and D-glucosone were prepared in 50mM phosphate buffer pH 6.5. These samples were analysed in an HPLC system (Jasco) by using a Sugar Pak column at 75 bar , 80ºC, 0.1mM Ca(II) EDTA as the solvent and at a flow rate of 0.5ml/min.

HPLC analysis: Samples (25µl) were injected into the HPLC system and the chromatograms were obtained in 12 minute's time since the retention time of D-glucose and D-glucosone was 9.3 and 10.3 minutes, respectively. The peak height was determined as a function of either D-glucose or D-glucosone concentrations.

RESULTS AND DISCUSSION

The formation of D- glucosone and the consumption of D-glucose were followed by HPLC when the reaction was carried out at NPT in the presence of the cell-free extract.

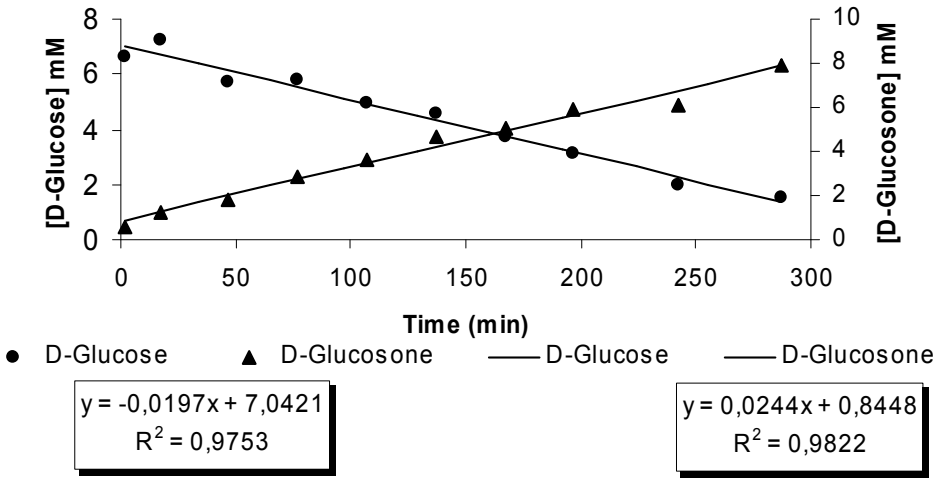

Fig.1- Progress curve showing the consumption of D-Glucose and formation of D-Glucosone at NPT by using cell-free extract (0.1ml, specific activity, 0.21U/mg protein) from *Coriolus Versicolor* without catalase.

Oxidation of D-glucose in the presence of glucose 2-oxidase was also carried out at several working pressures at room temperature as shown in Fig.2. This data suggest that by increasing the pressure there is an increase in the conversion degree compared with NPT conditions.

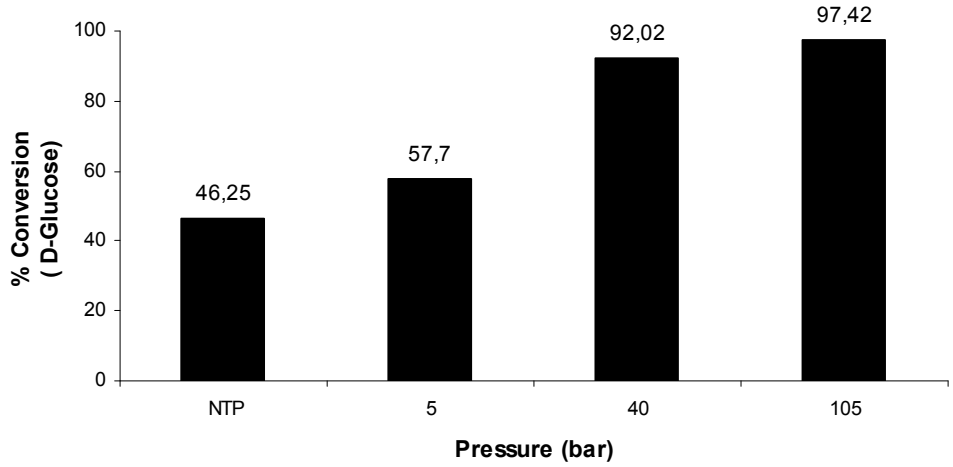

Fig.2 - Conversion of 10mM D-Glucose to D-Glucosone determined for a period of 90 minutes at room temperature by using 100 µl of commercial enzyme (10mg/ml, 8.2U/mg protein) without catalase.

Furthermore, the conversion of D-glucose into D-glucosone was investigated by using different enzyme preparations either in immobilized or free forms. The data presented in Fig.3 reveal that cell-free extract exhibits a higher degree of conversion compared with the purified enzyme. On the other hand, immobilized enzyme preparations were able to convert D-glucose into D-glucosone.

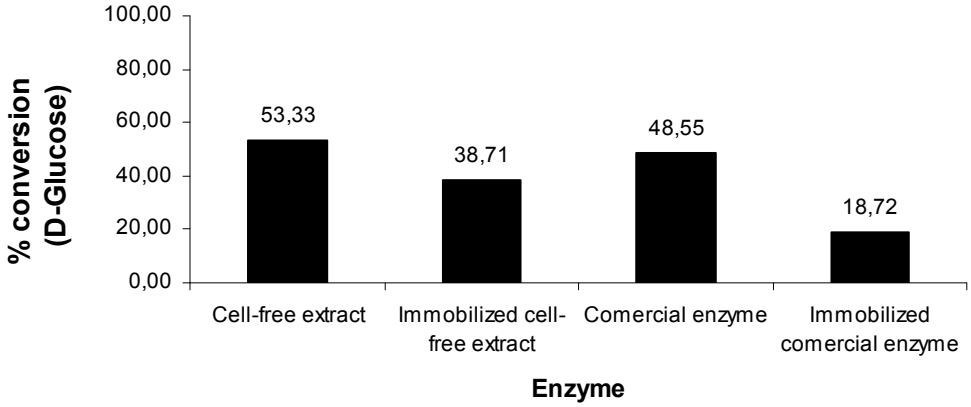

Fig. 3- Conversion of 10mM D-Glucose to D-Glucosone determined for a period of 90 minutes with different enzyme preparations at 100 bar. Immobilized enzyme (1,89U/mg protein) and immobilized cell-free extract (0,49U/mg protein).

The presence of catalase in the reaction mixture was also investigated on the degree of conversion as shown in Fig.4. This data reveal that the presence of catalase increases significantly the degree of conversion suggesting that hydrogen peroxide acts as a powerful inhibitor for this enzyme which is in agreement with published reports (7)

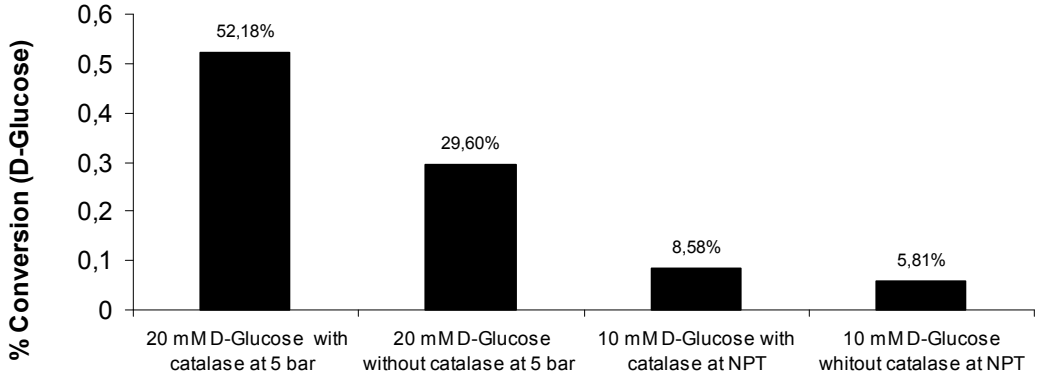

Fig. 4- Conversion degree of D-Glucose to D-Glucosone for the reaction with and without catalase determined at NTP and 5 bar at 25ºC for a period of 60 minutes of reaction.

This reaction was also studied by using either supercritical CO_2 at 35ºC or compressed nitrogen at 110 bar. The results obtained are shown in Fig. 5 which suggest that the degree of conversion is very low when either supercritical CO_2 or N_2 were used compared with the compressed air. This data can be explained by the fact that the oxidation of D-glucose catalysed by glucose 2-oxidase requires the presence of O_2 which is absent in either supercritical CO_2 or N_2.

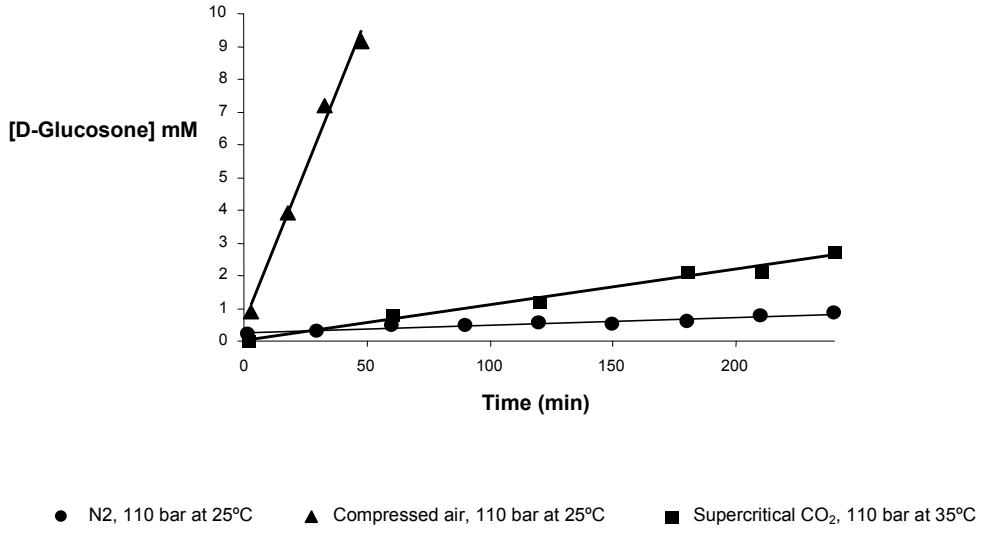

Fig. 5- Formation of D-Glucosone by using different gases at the same working conditions: Pressure: 110 bar; [Glucose] = 20 mM; 100µL commercial enzyme (10mg/ml, 8.2U/mg protein) without catalase.

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