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Chromatographic behaviour of glucose 1- and 2-oxidases from fungal strains on immobilized metal chelates

V Pacheco and A Karmali

Laboratório de Engenharia Bioquímica do Departamento de Engenharia Química do Instituto Superior de Engenharia de Lisboa, Rua Conselheiro Emídio Navarro, 1900 Lisboa, Portugal

Glucose 2-oxidase (EC 1.1.3.10) from *Coriolus versicolor* and *Phanerochaete chrysosporium* and glucose 1-oxidase (EC 1.1.3.4) from *Aspergillus niger* bound to a CU(II)-IDA column in the pH range of 6–8. However, glucose 1-oxidase from *Penicillium amagasakiense* bound only partially to a CU(II)-IDA column at pH 8.0. Metal chelates containing either Ni(II) or Zn(II) were useful in the adsorption of glucose 2-oxidase from *Phanerochaete chrysosporium*. The binding of glucose 2-oxidase from *P. chrysosporium* to Ni(II) and Zn(II)-IDA agarose columns increases as a function of pH of the buffer system. The adsorption of glucose oxidases on metal(II)-IDA chelates was due to the available histidine residues on enzyme molecules since the addition of imidazole in the buffer system abolished the binding of glucose oxidases to these columns. Both glucose oxidases from *C.versicolor*, *P. chrysosporium* and *A. niger* were purified in one step by immobilized metal affinity chromatography on metal(II)-IDA agarose columns with a recovery of enzyme activity in the range of 80–91%. Purified preparations of glucose oxidases from fungal strains were apparently homogeneous on native PAGE and SDS-PAGE. Immobilized metal affinity chromatography was used to separate glucose 1-oxidase from the 2-oxidase on metal(II)-IDA agarose columns which was confirmed by analysis of the reaction products by HPLC. The different chromatographic behaviour of glucose oxidases on metal(II)-IDA chelates is apparently due to the number and spatial distribution of available histidine residues on these enzyme molecules.

Keywords: glucose 2-oxidase; lignin degradation; one-step purification; glucose 1-oxidase; basidiomycete fungi; immobilized metal affinity chromatography

Introduction

Glucose 1-oxidase (EC 1.1.3.4) catalyses the oxidation of d-glucose at carbon 1 into d-glucono-1,5-lactone and hydrogen peroxide [10]. This enzyme has been isolated from a number of fungal strains including *Penicillium amagasakiense* and *Aspergillus niger* among others [19,30]. Glucose 1-oxidase has been purified from these fungal strains using several purification steps such as ammonium sulphate precipitation, ion-exchange and gel filtration chromatography [17,18]. This enzyme has been used in food preservation as well as in biosensors for the quantitative determination of glucose in body fluids, foodstuffs, beverages and fermentation broth [5,29].

On the other hand, glucose 2-oxidase (carbohydrate oxidase, pyranose oxidase, pyranose:oxygen-2-oxidoreductase, EC 1.1.3.10) catalyses the oxidation of d-glucose and some other carbohydrates in their pyranose forms at carbon 2 in the presence of molecular oxygen producing the corresponding carbonyl sugar and hydrogen peroxide [21,28,31]. The oxidation of d-glucose results in the formation of d-glucosone (2-keto-d-glucose; d-arabino-2-hexosulose) and hydrogen peroxide [25]. This enzyme is synthesized by a number of basidiomycete fungi such as *Coriolus versicolor*, *Phanerochaete chrysosporium*, *Peniophora gigantea* and *Oudemansiella mucida* [7,6,32]. Many research workers [1,7,13,21,24] have purified glucose 2-oxidase from several basidiomycete fungi to apparent homogeneity using precipitation with ammonium sulphate, ion-exchange, hydrophobic interaction and gel filtration chromatographic techniques. Glucose 2-oxidase may be used as an analytical reagent in the diagnosis of diabetes mellitus as well as a biocatalyst for preparative carbohydrate chemistry [33,34]. On the other hand, this enzyme also plays an important biological role since d-glucosone (2-keto-d-glucose) acts as a precursor of the antibiotic cortalcerone which is widely distributed among white-rot fungi [33].

There is some controversy concerning the roles of glucose 1- and 2-oxidases as possible donors of H_2O_2 in lignin biodegradation [6]. The presence of glucose 2-oxidase in white-rot fungi has been reported to play an important role in lignin degradation since it apparently supplies the lignolytic enzyme systems with hydrogen peroxide [6,32]. However, other workers suggest that glucose 1-oxidase is also an important source of hydrogen peroxide for reactions catalysed by lignin-depolymerizing peroxidases [16,17]. Furthermore, it is not clear whether these fungal strains produce simultaneously both glucose oxidases during lignin biodegradation [32]. The presence of these enzymes in lignin degradation could be detected by identification of their reaction products using either TLC or HPLC [24,32]. However, this study is further complicated because glucose 1-oxidase also acts on d-glucosone as the substrate whereas

Correspondence: Dr A Karmali, Laboratório de Engenharia Bioquímica do Departamento de Engenharia Química do Instituto Superior de Engenharia de Lisboa, Rua Conselheiro Emídio Navarro, 1900 Lisboa, Portugal
Abbreviations: IMAC, immobilized metal affinity chromatography; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography; Tris, 2-amino-2-(hydroxy-methyl)-1,3-propanediol.
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glucose 2-oxidase also catalyses the oxidation of d-glucono-1,5-lactone [8,14]. Therefore, it is of great importance to devise techniques to separate and identify both glucose oxidases from fungal sources in order to analyse their role in lignin biodegradation. Since glucose 2-oxidase is widely used in the production of d-glucosone for the synthesis of cortalcerone [33], it is of critical importance that glucose 2-oxidase is free from glucose 1-oxidase activity. The separation of glucose 1-oxidase from 2-oxidase is difficult because these enzymes exhibit similar physico-chemical properties such as carbohydrate contents, pI, M_r and biospecificity [1,15]. Therefore, it was of great interest to investigate the use of immobilized metal affinity chromatography (IMAC) for the isolation and separation of both glucose oxidases as well as to study the topology of glucose oxidase molecules [11,26]. In fact, the amino acid sequence of glucose 1- and 2-oxidases have revealed that these enzymes contain 20–76 histidine residues per enzyme molecule [10,23] which could be used to devise a specific method of separating glucose 1-oxidase from 2-oxidase from fungal strains.

The present work is concerned with the production of glucose 1- and 2-oxidases from several fungal strains as well as the purification of both enzymes in one step by IMAC involving different transition metal ions. On the other hand, the chromatographic behaviour of both glucose oxidases from fungal strains was analysed by IMAC at different pH values in order to separate the glucose 1- and glucose 2-oxidases.

Materials and methods

Materials

A strain of *Coriolus versicolor* was isolated from a *Quercus suber* stump. *Penicillium amagasakiense* ATCC 28686, *Phanerochaete chrysosporium* ATCC 34541, and *Aspergillus niger* ATCC 9029 were also used. d-Glucosone, horseradish peroxidase, *o*-dianisidine, catalase, chymotrypsin, ovalbumin, iminodiacetic acid, bovine serum albumin, alcohol dehydrogenase (yeast) and benzamidine were obtained from Sigma Chemical Company (St Louis, MO, USA). Sepharose 6B, IDA-agarose gel and a kit of protein markers were purchased from Pharmacia LKB International (Uppsala, Sweden). Membranes (P10) for ultrafiltration units were supplied by Amicon (Lexington, MA, USA). Corn steep liquor and paper mill wastes were generous gifts from Copam and Portucel Companies (Lisbon, Portugal), respectively. All other reagents used were of analytical grade.

Growth and maintenance of fungal strains

The strains of *Coriolus versicolor*, *Penicillium amagasakiense*, *Aspergillus niger* and *Phanerochaete chrysosporium* were grown and maintained on solid media. For enzyme production, they were grown in liquid media as described previously [4,22,24,31].

Preparation of chromatographic matrices

The activation of Sepharose 6B with 1,4-butanedioldiglycidyl ether was carried out as reported previously [12]. Epoxi-activated Sepharose 6B (20 g, wet weight) was reacted with iminodiacetic acid (14 mmol) using the procedure mentioned previously [12]. Alternatively, immobilized metal affinity chromatography was performed on commercial iminodiacetic acid agarose gel obtained from Sigma.

Extraction and purification of glucose 1- and 2-oxidases

Frozen mycelia (20 g, wet weight) were thawed in 2 vol of 50 mM phosphate buffer pH 6.5 containing 1 mM benzamidine, homogenized in a potter-Elvejheim apparatus for 3 min and sonicated at 160 W for 90 s at 4°C. The cell extract was centrifuged at 13 000 × *g* for 30 min, the supernatant phase was collected and kept at –20°C. The cell-free extract was diluted (1:5) with 20 mM phosphate buffer at the appropriate pH (6, 7 or 8) containing 1 M NaCl and 1 mM benzamidine and centrifuged at 13 000 × *g* for 10 min. The supernatant phase was applied at room temperature to a column (1 × 5 cm) containing 2.5 ml of sedimented iminodiacetic acid (IDA) agarose. Immobilized metal affinity chromatography (IMAC) was carried out by

Table 1 Chromatographic behaviour of glucose 1- and 2-oxidases from fungal strains on immobilized metal affinity chromatography on metal(II)-IDA agarose columns

Metal(II)-IDA agarose columns	*Coriolus versicolor*	*Phanerochaete chrysosporium*	*Aspergillus niger*	*Penicillium amagasakiense*
1. Cu(II)-IDA agarose				
1.1. pH 6.0	+	+	+	–
1.2. pH 7.0	+	+	+	–
1.3. pH 8.0	+	+	+	–,+
2. Ni(II)-IDA agarose				
2.1. pH 6.0	–	–,+	–	–
2.2. pH 7.0	–	+	–	–
2.3. pH 8.0	–,+	+	–	–
3. Zn(II)-IDA agarose				
3.1. pH 6.0	–	–,+	–	–
3.2. pH 7.0	–	+	–	–
3.3. pH 8.0	–	+	–	–

+ and –: Denotes that the enzyme binds and does not bind to the column, respectively; –,+ denotes that the enzyme binds partially or weakly to the column.

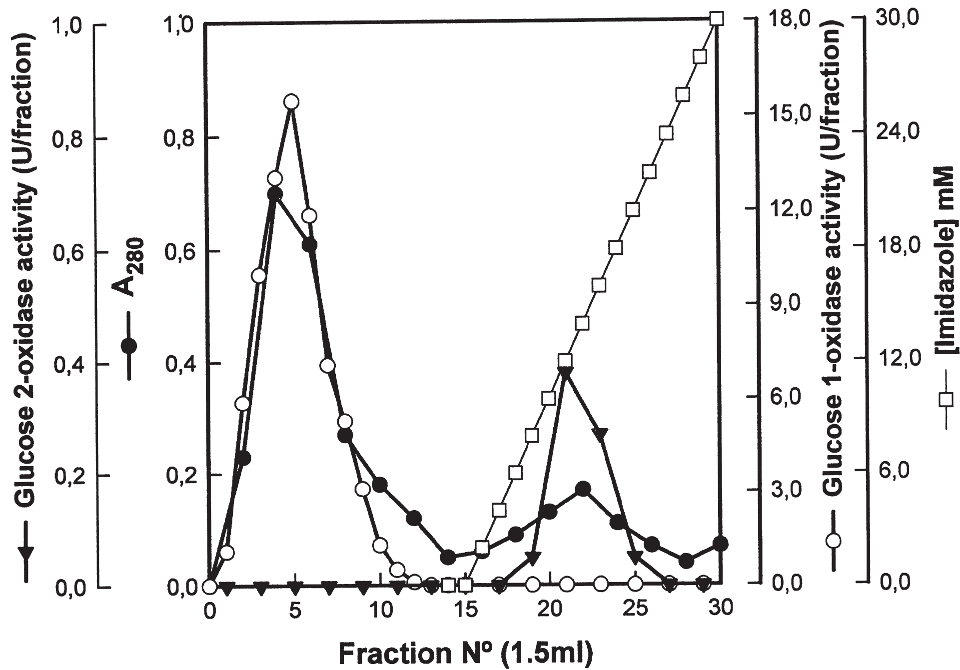

Figure 1 Separation of glucose 2-oxidase from *Coriolus versicolor* and glucose 1-oxidase from *Penicillium amagasakiense* by immobilized metal affinity chromatography. An artificial mixture was prepared containing cell-free extract from *C. versicolor* and the culture supernatant from *P. amagasakiense* which was applied to a column packed with Cu(II)-IDA-agarose at pH 6.0. Glucose oxidase activity was eluted from the column with a linear gradient of imidazole (0–30 mM) in the same buffer system as described in Materials and Methods. Column fractions (1.5 ml) were analysed for protein and glucose oxidase activity. The enzyme activity observed at the washing and elution steps was identified as 1- or 2-oxidase by analysis of their reaction products by HPLC.

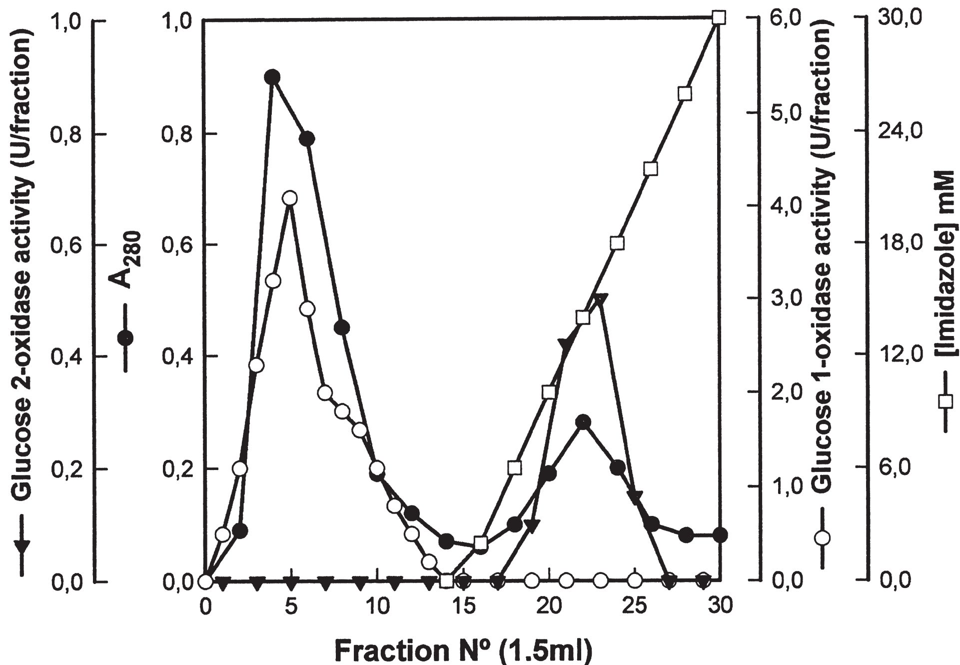

Figure 2 Separation of glucose 2-oxidase from *Phanerochaete chrysosporium* and glucose 1-oxidase from *Aspergillus niger* by immobilized metal affinity chromatography. A mixture was prepared containing cell-free extracts from *P. chrysosporium* and *A. niger* which was applied to a column packed with Ni(II)-IDA-agarose at pH 7.0. Glucose oxidase activity was eluted from the column with a linear gradient of imidazole (0–30 mM) in the same buffer system as described in Materials and Methods. Column fractions (1.5 ml) were analysed for protein and glcuose oxidase activity. The enzyme activity observed at the washing and elution steps was identified as 1- or 2-oxidase by analysis of their reaction products by HPLC.

charging the column with the appropriate 50-mM metal solution ($CuSO_4$, $NiCl_2$ or $ZnCl_2$), washed with distilled water and equilibrated with 20 mM phosphate buffer at the appropriate pH containing 1 M NaCl as described previously [27]. The cell-free extract was applied to the column at a flow rate of 10 ml h^{-1} and the column was washed with the same buffer system until A_{280} was less than 0.05. The enzyme was eluted from the column with a linear gradient of imidazole (0–30 mM) in the same buffer system and column fractions were analysed for protein and glucose oxidase activity. The fractions containing glucose oxidase activity were pooled, concentrated and dialysed against 50 mM phosphate buffer pH 6.5 by pressure dialysis using a P-10 membrane at 4°C. Since glucose 1-oxidase from *P. amagasakiense* is an extracellular enzyme, the culture supernatant from the fermentation broth was used as the source of enzyme which was diluted (1:5) with 20 mM phosphate buffer at the appropriate pH containing 1 mM benzamidine and 1 M NaCl and purified according to the procedure described above.

Chromatographic behaviour of glucose oxidases

In the present work, the binding of glucose oxidases to metal (II)-IDA agarose columns was considered to be partial or weak when the total activity applied to the column was recovered in 10 column volumes of buffer at the washing step exhibiting a broad activity peak. On the other hand, the enzyme did not bind to the column when the total activity applied to the column was recovered in five column volumes of buffer at the washing step, exhibiting a sharp activity peak.

Separation of glucose 1-oxidase from 2-oxidase

An artificial mixture was prepared which contained the cell-free extract of *C. versicolor* (1 ml) and the culture supernatant of *P. amagasakiense* (1 ml). Alternatively, the cell-free extract of *P. chrysosporium* (1 ml) was mixed with the cell-free extract of *A. niger* (1 ml). The enzyme activity of glucose oxidase in each separate cell-free extract or culture supernatant as well as in the artificial mixtures were determined and the mixture was applied to a column packed with the appropriate metal chelate agarose. The column was washed with 20 mM phosphate buffer at the appropriate pH containing 1 M NaCl and 1 mM benzamidine and the enzyme was eluted from the column with a linear gradient of imidazole (0–30 mM) in the same buffer system. Column fractions were analysed for protein and glucose oxidase activity according to the procedure described above. Glucose oxidase activity was detected at the washing step as well as at the elution step and was identified as 1-oxidase or 2-oxidase by HPLC [24]. Moreover, the recovery of enzyme activity at the washing and elution steps was determined in order to compare with the amount of glucose 1- and 2-oxidase activities applied to the column.

Enzyme assay

Both glucose 1- and 2-oxidase activities were determined in the presence of **d**-glucose using a linked assay system with peroxidase at 436 nm due to the oxidation of *o*-dianisidine as described previously [2]. One enzyme unit is defined as the amount of enzyme required to hydrolyse 1 μmole glucose per min at 25°C.

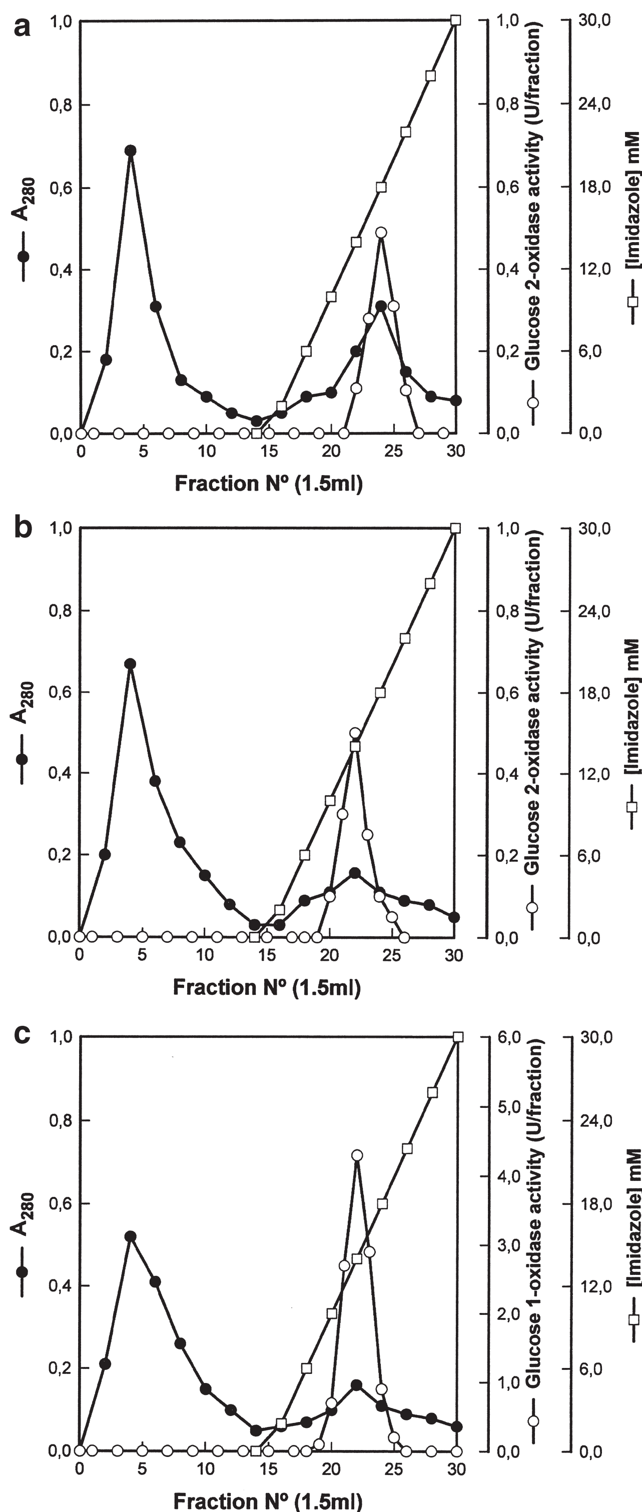

Figure 3 Immobilized metal affinity chromatography of cell-free extract from a fungal strain. The cell-free extract was applied to a column packed with Cu(II)-IDA-agarose at pH 6.0 and glucose oxidase activity was eluted from the column with a linear gradient of imidazole (0–30 mM) in the same buffer system as described in Materials and Methods. Column fractions (1.5 ml) were analysed for protein and glucose oxidase activity. (a) *P. chrysosporium;* (b) *C. versicolor*; (c) *A. niger*.

The oxidation products of the reactions catalysed by glucose 1- and 2-oxidases were identified by HPLC as described previously [24]. Protein was determined by the Coomassie blue dye binding method [3].

Electrophoretic analysis

Native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE of protein samples were carried out as described previously [9,20] and stained with silver nitrate. The specific detection of both glucose 1- and 2-oxidase activities in native gels was performed as reported previously [2].

Results

The fungal strains used in the present work produced different glucose oxidases since *P. chrysosporium* and *C. versicolor* synthesized glucose 2-oxidase whereas glucose 1-oxidase was produced by *P. amagasakiense* and *A. niger* (data not shown) which is in agreement with published data [4,6,15] Moreover, glucose 1-oxidase from *P. amagasakiense* was the only glucose oxidase which was obtained extracellularly in the culture supernatant [4].

The chromatographic behaviour of both glucose oxidases from several fungal strains is summarized in Table 1. Glucose 2-oxidases from *C. versicolor* and *P. chrysosporium* each bound to a Cu(II)-IDA agarose column in the presence of salt at pH 6.0–8.0 (Table 1). However, glucose 1-oxidases from *A. niger* and *P. amagasakiense* exhibited different chromatographic behaviour on Cu(II)-IDA agarose columns since the enzyme from *A. niger* bound to the column at pH 6.0–8.0 whereas glucose 1-oxidase from *P. amagasakiense* bound only partially to this column at pH 8.0 (Table 1). A different chromatographic behaviour of glucose 2-oxidases from *C. versicolor* and *P. chrysosporium* was observed with Ni(II)-IDA agarose columns since the enzyme from *P. chrysosporium* bound partially to this column at pH 6.0 and completely at pH 7.0 and 8.0. Glucose 2-oxidase from *C. versicolor* bound partially to this column at pH 8.0 but it did not bind at pH 6.0–7.0 (Table 1). The separation of glucose 2-oxidase from 1-oxidase was accomplished by preparing an artificial mixture of cell-free extract from *C. versicolor* and the culture supernatant from *P. amagasakiense* which was applied to a column packed with Cu(II)-IDA agarose gel at pH 6.0. Glucose 1-oxidase did not bind to the column and was eluted at the washing step whereas glucose 2-oxidase bound to the column (Figure 1) which is in agreement with the data presented in Table 1. However, the separation of glucose 1-oxidase from *A. niger* from glucose 2-oxidase from *P. chrysosporium* could be accomplished on a column packed with Ni(II)-IDA agarose at pH 7.0. The enzyme from *A. niger* did not bind to the column and was eluted at the washing step, whereas glucose 2-oxidase from *P. chrysosporium* bound to the column (Figure 2) which is in agreement with the results presented in Table 1. On the other hand, the separation of glucose 2-oxidases from *C. versicolor* and *P. chrysosporium* was carried out by preparing an artificial mixture of cell-free extracts from these two fungal strains which was applied to a column packed with Ni(II)-IDA agarose gel at pH 7.0. The enzyme from *C. versicolor* did not bind to the column and was eluted at the washing step whereas glucose 2-oxidase from *P. chrysosporium* bound to the column (figure not shown) which is in agreement with the data presented in Table 1. Glucose 1- and 2-oxidases from several fungal strains were purified in one-step by IMAC on Cu(II)-IDA agarose columns (Figure 3) with a recovery of enzyme activity in the range of 80–91% and a purification factor of 23–44 (Table 2). The purified enzyme preparations from these fungal strains were apparently homogeneous on SDS-PAGE and native PAGE running with an M_r of 70 000 and 180 000 daltons, respectively (Figure 4a and b). However, the purified preparation of glucose 2-oxidase from *P. chrysosporium* exhibited an M_r of 260 000 daltons (Figure 4b). Furthermore, the native gel was also stained for glucose oxidase activity and the activity band was coincident with the protein band (Figure 4c). However, the purified preparations of glucose oxidases from *P. chrysosporium* and *A. niger* revealed a major protein band corresponding to glucose oxidase as well as a few minor protein bands due to protein contaminants (Figure 4a and b).

Discussion

Immobilized metal affinity chromatography has been widely used in the purification of a large number of serum proteins and enzymes with a high degree of purity and yield [11,27]. These proteins bind to metal ion (II)-IDA-agarose columns through the coordination between the electron

Table 2 One-step purification of glucose oxidases from several fungal strains by immobilized metal affinity chromatography on Cu(II)-IDA agarose columns at pH 6.0

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (U mg^{-1} protein)	Recovery (%)	Purification factor
1. Cell-free extract from *C. versicolor*	3.81	1.20	0.31	100	1
2. Column eluate at pH 6.0	0.08	1.09	13.62	90.8	43.9
1. Cell-free extract from *P. chrysosporium*	3.33	1.12	0.33	100	1
2. Column eluate at pH 6.0	0.12	0.92	7.66	82.1	23.2
1. Cell-free extract from *A. niger*	3.29	11.84	3.59	100	1
2. Column eluate at pH 6.0	0.08	9.59	119.87	80.9	33.3

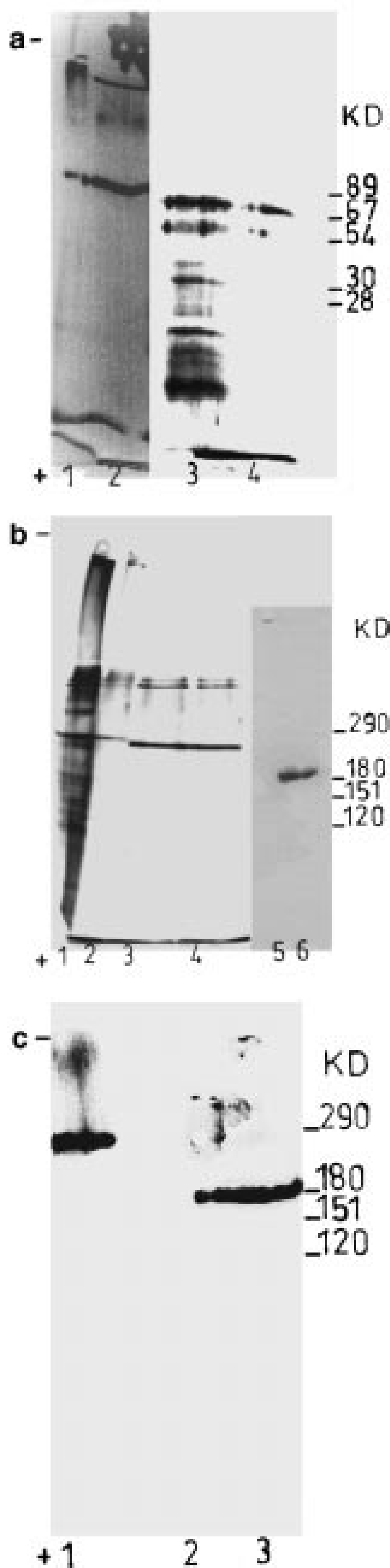

Figure 4 PAGE of purified glucose oxidases. (a) SDS-PAGE of glucose oxidases purified on Cu(II)-IDA column using a 10% separating gel. Lanes: 1, purified enzyme (5 μg) from *P. chrysosporium*; 2, purified enzyme (7 μg) from *A. niger*; 3, cell-free extract (50 μg) from *C. versicolor*; and 4, purified enzyme (5 μg) from *C. versicolor*. (b) Native PAGE of purified glucose oxidases from Cu(II)-IDA column using a 7.5% separating gel. Lanes: 1, cell-free extract (45 μg) from *P. chrysosporium*; 2, purified enzyme (3 μg) from *P. chrysosporium*; 3 and 4, purified enzyme (6 μg) from *P. chrysosporium*; 5, purified enzyme (4 μg) from *C. versicolor*; and 6, purified enzyme (4 μg) from *A. niger*. (c) The native gel was stained for activity. Lanes: 1, cell-free extract (30 μg) from *P. chrysosporium*; 2, purified enzyme (10 μg) from *A. niger* and 3, purified enzyme (10 μg) from *C. versicolor*. The molecular mass markers are indicated on the margins.

donor residues on a protein surface (histidine, tryptophan and cysteine) and chelated transition metal ions (IDA-metal ion). However to our knowledge, this technique has not been used for the isolation and separation of both glucose oxidases from microbial sources. The adsorption of both glucose oxidases on these chelated agarose columns (Table 1) may be due to the coordination between available histidine residues on glucose oxidase molecules and chelated transition metal ions/IDA-metal ion since the presence of 10 μmM imidazole in the equilibration buffer prevented the binding of these enzymes to metal (II)-IDA agarose columns (data not shown). The different chromatographic behaviour of both glucose oxidases from fungal strains on metal (II)-IDA agarose columns may be due to the number and spatial distribution of available histidine residues on the protein surface. In fact, a comparative study of the amino acid composition of glucose 2-oxidase from *P. chrysosporium* and *C. versicolor* revealed that they contain 76 and 42 histidine residues per enzyme molecule, respectively [1,23]. These data are in agreement with the different chromatographic behaviour of glucose 2-oxidases on metal(II)-IDA chelates since the enzyme from *P.chrysosporium* bound to Cu(II)-IDA, Ni(II)-IDA and Zn(II)-IDA agarose columns whereas the enzyme from *C. versicolor* bound only to Cu(II)-IDA columns (Table 1). Furthermore, the enzyme from *P. chrysosporium* was eluted from Cu(II)-IDA agarose columns at 18 mM of imidazole (Figure 3a) whereas glucose 2-oxidase from *C. versicolor* was eluted at 14 mM imidazole (Figure 3), which is in agreement with the different content of histidine residues in these enzymes. On the other hand, glucose 1-oxidase from *A. niger* bound to Cu(II)-IDA agarose columns in the pH range 6–8 whereas the enzyme from *P. amagasakiense* bound only partially to this column at pH 8.0. This chromatographic behaviour may be explained since the amino acid composition of glucose 1-oxidases from *A. niger* and *P. amagasakiense* has revealed that they contain 38 and 20 histidine residues per enzyme molecule, respectively [15].

Furthermore, the chromatographic behaviour of both glucose oxidases on metal (II)-IDA agarose columns is in agreement with the number of histidine residues per enzyme molecule as follows: glucose 2-oxidase from *P. chrysosporium* contains 76 histidine residues and binds to Cu(II)-, Ni(II)-, and Zn(II)-IDA agarose columns; glucose 2-oxidase from *C. versicolor* contains 42 histidine residues and binds to Cu(II)- at pH 6.0–8.0 and partially to Ni(II)-IDA agarose columns at pH 8.0; glucose 1-oxidase from *A. niger* contains 38 histidine residues and binds only to

Cu(II)-IDA agarose columns at pH 6.0–8.0; glucose 1-oxidase from *P. amagasakiense* contains 20 histidine residues and binds only partially to CU(II)-IDA agarose columns at pH 8.0 (Table 1).

On the other hand, the different chromatographic behaviour of glucose oxidases on chelated agarose columns is a function of pH (Table 1) [26,27]. In fact, the binding of glucose 2-oxidase from *P. chrysosporium* to Ni(II)- and ZN(II)-IDA agarose columns increases as a function of pH since the elution of the enzyme from the column at pH 8.0 requires a higher concentration of imidazole than at pH 7.0 (data not shown).

The different chromatographic behaviour of both glucose oxidases on metal(II)-IDA agarose columns could be used to separate and identify glucose 1- and 2-oxidases from basidiomycetes under natural conditions of wood decay. Glucose 1-oxidase from *P. amagasakiense* did not bind to Cu(II)-IDA agarose columns at pH 6.0 whereas glucose 2-oxidase from *C. versicolor* bound to the columns and was eluted with imidazole (Figure 1) which is in agreement with the data presented in Table 1. Alternatively, glucose 2-oxidase from *P. chrysosporium* was separated from glucose 1-oxidase from *A. niger* on a Ni(II)-IDA agarose column at pH 7.0 (Figure 2) which is in agreement with the data presented in Table 1. Furthermore, both glucose oxidases from fungal strains were purified in one step on Cu(II)-IDA agarose columns (Figure 3) with a recovery of enzyme activity of about 90% (Table 2) and the purified preparations were apparently homogeneous on SDS and native PAGE (Figure 4). The specific activity of the purified enzyme from *C. versicolor* (Table 2) is in agreement with published data [21,24]. However, the specific activities exhibited by enzymes from *A. niger* and *P. chrysosporium* (Table 2) are lower than those reported in the literature [10,31]. The reason for this discrepancy is not known but the enzyme assay methods used by these workers were different from the one presented in this work.

Several workers have purified either glucose 1- or 2-oxidases from fungal strains using isolation schemes which involve several chromatographic steps [15,17,31,32]. However, these workers have not exploited the difference in the number of available histidine residues in these glucose oxidases in order to separate them by a simple chromatographic technique. Moreover, the isolation scheme presented here involves the use of a cheap chromatographic matrix which can be reused more than 50 times without any loss of capacity or resolution, and glucose 1- and 2-oxidases can be purified in one step to apparent homogeneity (Figure 4).

There is some controversy concerning the roles of glucose 1- and 2-oxidases in lignin biodegradation since these enzymes provide hydrogen peroxide for ligninolytic peroxidases [6,16,32]. This problem could be analysed by detecting both glucose oxidase activities under conditions of wood decay in natural environments as well as by separating one enzyme from another in order to evaluate their contribution in the oxidative depolymerisation of lignocellulose. The method described in the present work could be used to separate glucose 1-oxidase from 2-oxidase and study their roles in lignin degradation. On the other hand, an important application of glucose 2-oxidase free from glucose 1-oxidase activity is related to the production of **d**-glucosone for the synthesis of cortalcerone [33]. The method described in the present work could be used to remove glucose 1-oxidase from purified preparations of glucose 2-oxidase which could be used in the production of **d**-glucosone.

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