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Interaction of L-glutamate oxidase with triazine dyes: selection of ligands for affinity chromatography

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Abstract

Glutamate oxidase (GOX, EC 1.4.3.11) from *Streptomyces* catalyses the oxidation of L-glutamate to α-ketoglutarate. Its kinetic constants for L-glutamate were measured equal to 2 mM for K_m and 85.8 s^{-1} for k_{cat}. BLAST search and amino acid sequence alignments revealed low homology to other L-amino acid oxidases (18–38%). Threading methodology, homology modeling and CASTp analysis resulted in certain conclusions concerning the structure of catalytic α-subunit and led to the prediction of a binding pocket that provides favorable conditions of accommodating negatively charged aromatic ligands, such as sulphonated triazine dyes. Eleven commercial textile dyes and four biomimetic dyes or minodyes, bearing a ketocarboxylated-structure as their terminal biomimetic moiety, immobilized on cross-linked agarose gel. The resulted mini-library of affinity adsorbents was screened for binding and eluting L-glutamate oxidase activity. All but Cibacron® Blue 3GA (CB3GA) affinity adsorbents were able to bind GOX at pH 5.6. One immobilized minodye–ligand, bearing as its terminal biomimetic moiety *p*-aminobenzyloxanylic acid (BM1), displayed the higher affinity for GOX. Kinetic inhibition studies showed that BM1 inhibits GOX in a non-competitive manner with a K_i of 10.5 μM, indicating that the dye–enzyme interaction does not involve the substrate-binding site. Adsorption equilibrium data, obtained from a batch system with BM1 adsorbent, corresponded well to the Freundlich isotherm with a rate constant *k* of 2.7 $mg^{1/2}$ $ml^{1/2}$/g and Freundlich isotherm exponent *n* of 1. The interaction of GOX with the BM1 adsorbent was further studied with regards to adsorption and elution conditions. The results obtained were exploited in the development of a facile purification protocol for GOX, which led to 335-fold purification in a single step with high enzyme recovery (95%). The present purification procedure is the most efficient reported so far for L-glutamate oxidase.

Keywords: L-Glutamate oxidase; Enzymes; Triazine dyes

1. Introduction

The biological significance of the majority of L-amino specific oxidases remains uncertain due to the lack of sufficient data [1]. *Streptomyces* L-glutamate oxidase (GOX) is an extracellular heterotrimeric enzyme consisting of α, β and γ subunits of molecular masses equal to 39, 19 and 16 kDa, respectively [1–3]. The enzyme attracts attention because of its biotechnological applications. It is used as a diagnostic tool for the determination of L-glutamic acid in physiological fluids, and as an analytical reagent for the evaluation of food quality [4,5]. Furthermore, an important application of GOX is its engagement in the clinical laboratory as an auxiliary enzyme to the determination of serum L-alanine aminotransferase (ALAT, earlier known as GPT) and L-aspartate aminotransferase (ASAT, earlier known as GOT) [6]. Despite its routine analytical application for many years, the enzyme remains expensive, thus creating a bottleneck for a cost-effective application. This is mainly due to its limited supply in a native form and unavailability in a recombinant form. To these problems, one must add the laborious and inefficient purification procedures available so far, involving four steps of which three being chromatographic [1,2,7], leading to modest enzyme recovery.

Abbreviations: BM, biomimetic-dye or mimodye/adsorbent; CB3GA, Cibacron® Blue 3GA; GOX, L-glutamate oxidase; Mes, 2-(morpholino)-ethanesulfonic acid; *p*-NHBenNHCOCOO-VBAR, BM1 dye or adsorbent; VBAR, Vilmafix blue A-R

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Reducing the cost of manufacturing biopharmaceutical and analytical proteins has become a critical challenge for the industry. Therefore, it appears that the development of a facile and efficient purification protocol for GOX would be of practical and economical importance.

Affinity chromatography is the most effective high-separation technique [8], able to offer safe solutions whenever high protein purity is needed. Most affinity ligands are of biological nature, offering good affinity and selectivity for the target proteins. However, these ligands suffer from low binding capacities, limited life cycles and low scale-up potential. On the other hand, synthetic affinity ligands are developed to show high capacity, durability and low cost, combined with good selectivity. Sulphonated chloro-triazyne dyes represent perhaps the most important group of synthetic affinity ligands, not only with laboratory, but more important, with industrial-scale potential. Although there have been concerns regarding the modest selectivity of dyes, and safety issues on their involvement in the production process of therapeutic proteins, these synthetic molecules continue to find wide application for over two decades, in purification processes of diagnostic, analytical and molecular biology enzymes and proteins [9–11].

One way to cope with the modest selectivity of textile dye–ligands is to use a specific eluent on the affinity column to selectively release the protein of interest [9]. Another strategy is to design new dye–ligands of superior affinity for the target protein, named 'biomimetic dyes' or 'mimodyes' [9]. These mimodyes aim to mimic the structure and binding mode of natural biological ligands of the target protein. Contemporary ligand design makes extensive use of biocomputing. For example, protein molecular modeling and ligand docking have been applied for the de novo design of chimaeric mimodyes for enzymes of analytical interest such as the ketocarboxyl-group recognizing L-malate dehydrogenase [12] and L-lactate dehydrogenase [13], the glutathione-group recognizing formaldehyde dehydrogenase [14], and the galactose-group recognizing L-galactose dehydrogenase [15,16].

In the present study, commercial textile dyes and anthraquinone mimodyes were immobilized on agarose and the resulted mini-library of affinity adsorbents was screened for binding and eluting L-glutamate oxidase activity. The work focused on the mode of interaction of the selected 'winner' dye with GOX, and its application to the purification of the target enzyme.

2. Experimental

2.1. Materials

GOX (9.9 units/mg solid), horseradish peroxidase, Reactive blue 2 (Cibacron® Blue 3GA), Reactive brown 10 (Procion® Brown MX-5BR), Reactive red 2 (Procion® Red MX-5B), Reactive yellow 86 (eq., Procion® Yellow MX-4G), and *o*-dianisidine were purchased from Sigma (USA).

2.2. Bioinformatics

The meta-server (http://www.infobiosud.cnrs.fr/bioserver) was used to screen structure databases aiming to find a compatible fold for GOX. Sequences homologous to GOX were sought in the Genbank and Unfinished Microbial Genome databases at the NCBI using BLAST [17] and PSI-BLAST [18]. The resulting sequence set was aligned with CLUSTALW [19]. Threading methodology was also carried out using several www servers, such as Jpred2, a secondary-structure prediction-server [20]; 3D-PSSM, a threader using 1D and 3D sequence profiles coupled with secondary-structure and solvation-potential information [21]; mGenThreader, a multiple-sequence profile-based threader [22]. A new threader, FUGUE [23], searching the HOMSTRAD profile database [24], was also employed. The best alignment was then directly submitted to TITO [25] to validate the chosen structure-sequence alignments. Template secondary structures were automatically assigned during TITO processing. Modelling of the α-subunit of GOX was carried out with MODELLER 6 [26] using the structures of L-amino acid oxidase from *Calloselasma rhodostoma* (PDB code 1f8r) [27], as a template. Given the low sequence similarity between target and templates, a rigorous iterative modeling scheme was employed in which four models were constructed and analyzed for each variant alignment. These models were analyzed with Verify3D [28], and with PROSA II [29] for packing and solvent exposure characteristics. Model regions corresponding to positive PROSA II profile peaks were treated as possibly resulting from misalignments. Alterations in alignments were tested for these regions. When no further improvements were possible, the model with the best PROSA II score was considered as the final model. The resulted model was submitted for Coulomb electrostatic potential analysis [30] and CASTp analysis, for the prediction of available binding pockets [31].

2.3. Synthesis of mimodye–ligands and their immobilization on beaded agarose gel

Four mimodyes were synthesized by nucleophilic substitution of the desired biomimetic carboxylated structure at the dichlorotriazine ring of the parent dye VBAR [32]. Immobilization of the dye–ligands and determination of immobilized-ligand concentration were executed as described in [24,33]. The immobilized dye concentrations employed here varied within a narrow range (2.2 ± 0.2 μmol dye/g moist wet gel) [33,34].

2.4. Assay of enzyme activity and protein

GOX assays were performed in accordance with a published method [35] at a Hitachi U-2000 double beam

UV–Vis spectrophotometer equipped with a thermostated cell-holder (10 mm path length). One unit of GOX is defined as the amount of enzyme that forms 1 μmol of hydrogen peroxide per min at 37 °C, using L-glutamate as substrate.

2.5. Screening procedure of dye–ligand adsorbents for GOX binding

All chromatography procedures were performed at 4 °C. GOX binding was assessed using analytical chromatography columns each packed with 0.4 ml dye-adsorbent equilibrated with 20 mM MES buffer, pH 5.6. Enzyme solution (0.5 ml, 0.015 U, pH 5.6) was loaded on each dye adsorbent. The adsorbent was then washed with equilibration buffer (2 ml) and elution of bound GOX was effected with 1.2 ml equilibration buffer containing KCl (1 M). Fractions were collected and assayed for GOX activity.

2.6. Effect of pH on the adsorption of GOX on BM1 mimodye adsorbent

All procedures were performed at 4 °C. GOX desorption was assessed using an analytical column packed with 0.4 ml dye-adsorbent equilibrated with buffers of different pH values (20 mM Mes, pH 5.6; 20 mM Mes, pH 6.0; 20 mM Mes, pH 6.4; 20 mM Mops, pH 6.8; 20 mM Mops, pH 7.2; 20 mM Mops, pH 7.6; 20 mM Tris pH 8.0). Enzyme solution (0.5 ml, 0.015 U), previously equilibrated in the same buffer as that used for the adsorbent, was loaded on the column, prior to washing with equilibration buffer (2.2 ml). Bound GOX was eluted with 1.3 ml equilibration buffer containing KCl (3 M). Collected fractions were assayed for GOX activity.

2.7. Effect of eluting conditions on the desorption of GOX from BM1 mimodye adsorbent

All procedures were performed at 4 °C. GOX binding was assessed using an analytical column packed with 0.4 ml dye adsorbent, equilibrated with buffer (20 mM Mes, pH 5.6). Enzyme solution (0.5 ml, 0.25 U) was loaded on the column, prior to washing with equilibration buffer (2 ml). Bound GOX was eluted with 1.2 ml equilibration buffer containing, in separate experiments, one of the following agents: 1 M KCl, 4 M KCl, 5 mM L-glutamate, 10 mM L-glutamate, 20% (v/v) glycerol, 40% (v/v) glycerol and distilled water. Collected fractions were assayed for GOX activity.

2.8. Adsorption equilibrium of GOX with BM1 mimodye adsorbent

In a total volume of 1 ml varying amounts of enzyme solution (0.60 U/ml), previously dialyzed against 20 mM Mes pH 5.6, were mixed with 20 mg of BM1 adsorbent. The suspensions were shaken for 75 min at 4 °C in order for the system to reach equilibrium. The mixture was then centrifuged (5000 rpm, 2 min) and the supernatant was assayed for GOX activity. For each experiment, a control was carried out to ensure that there was no loss of enzyme activity under these conditions. The data were analyzed according to the method of Livingston and Chase [36].

2.9. Purification of GOX on BM1 mimodye adsorbent

An artificial enzyme extract was produced by dissolving GOX (0.5 U) in a culture broth of *Streptomyces coelicolor*, since GOX is an extracellular enzyme. The resulting crude mixture (4 ml, 2.5 mg total protein/ml) was dialyzed against 1000-volume of 20 mM Mes/NaOH buffer (pH 5.6) and was applied on the BM1 affinity column (0.5 ml), which was previously equilibrated with 20 mM Mes/NaOH buffer (pH 5.6). Non-adsorbed proteins were washed with equilibration buffer (5 ml). Bound GOX was eluted with glycerol solution (3 ml, 40%).

2.10. Kinetic inhibition studies with free p-aminobenzyloxanilic-VBAR mimodye

Initial velocities for the GOX-catalyzed reaction with L-glutamate as a variable substrate were measured in a total assay volume of 1 ml at 37 °C containing: Mes/NaOH buffer, 100 mM, pH 5.6; in the absence of and in the presence of 5, 10 and 15 μM biomimetic dye. The kinetic and inhibition constants were deduced from Lineweaver–Burk plots.

2.11. HPLC analysis

The purity of the enzyme preparation was assessed by HPLC analysis using a computerized Gilson gradient bioHPLC system and a gel filtration column (Protein PAK 300SW, 300 mm × 7.8 mm (i.d.), Waters. The column was equilibrated with 100 mM potassium phosphate buffer, pH 6.5, containing 100 mM KCl. The sample was run isocratically at a flow rate of 0.03 ml/min. Eluting protein was monitored at 220 nm.

3. Results and discussion

3.1. Bioinformatics analysis

Prior to dye–ligand screening, a protein model of GOX was constructed in order to identify the presence of a possible binding pocket, able to accommodate large affinity ligands. Previous studies have suggested that in the case of oxidases, dye–ligands tend to bind to the protein regions that are probably near or overlap the FAD binding site [34]. Fig. 1 shows the amino acid sequence alignments resulted from the BLAST search of *Streptomyces platensis* GOX (accession number AAK15071). GOX showed 84% sequence identity with *Streptomyces* X-119 glutamate oxidase and 18–38% sequence identity with other bacterial L-amino acid

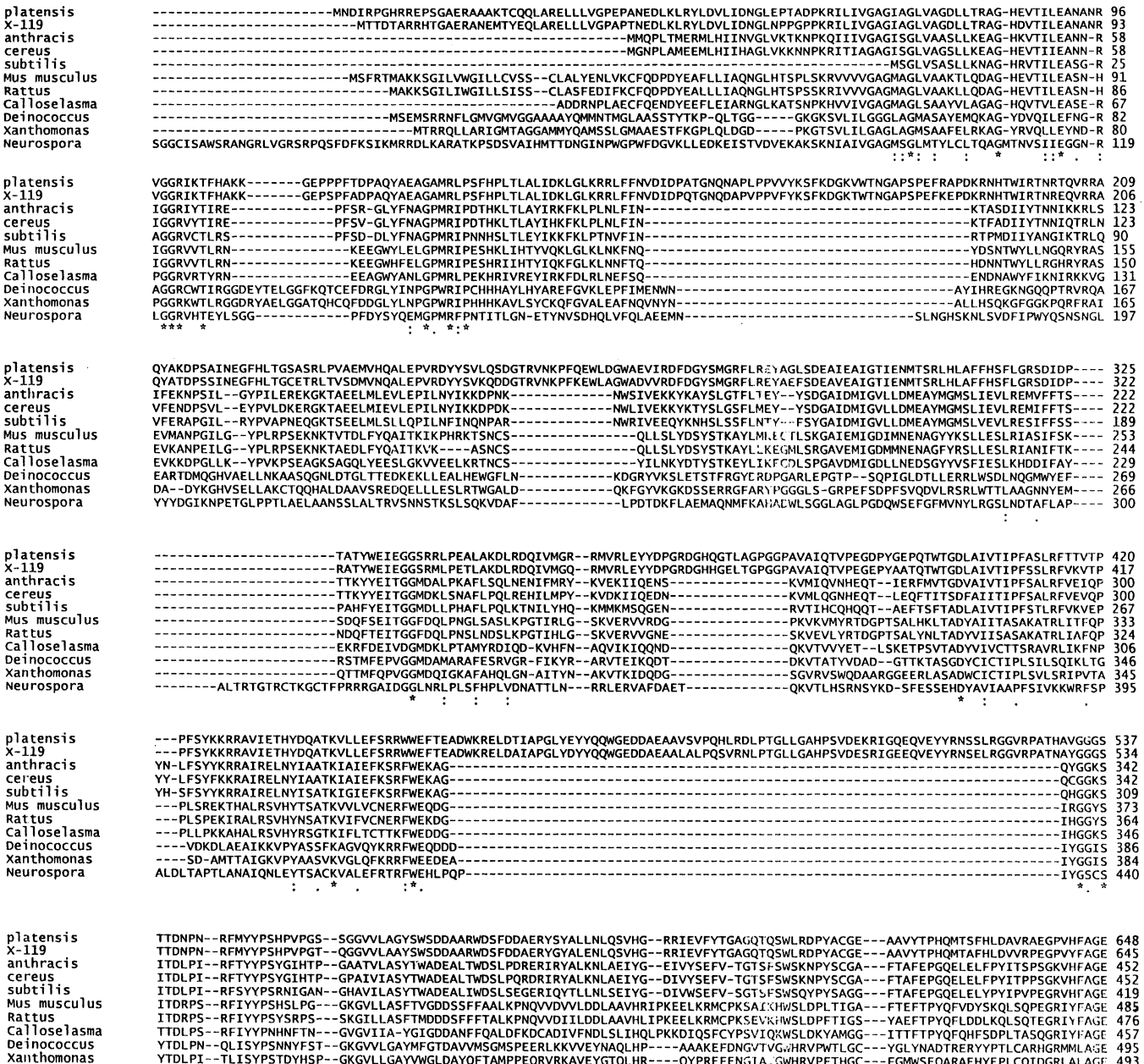


Fig. 1. Sequence alignment of GOX from *S. platensis* with 11 homologues oxidases. Genbank Ids and abbreviated species names are as following: *Streptomyces platensis*, AAK15071; *Streptomyces* sp. X-119-6 BAB93449; *Bacillus anthracis* str. A2012, NP_655788; *Bacillus cereus* ATCC 14579, NP_831695; *Bacillus subtilis*, NP_389783; *Mus musculus*, NP_598653; *Calloselasma rhodostoma*, Aj271725; *Rattus norvegicus*, XP_216521; *Xanthomonas axonopodis*, NP_643733 *Deinococcus radiodurans* R1, NP_285597; *Neurospora crassa*, A38314. Sequence alignments were achieved by CLUSTALW [19].

oxidases. Lower identity was observed with the L-amino acid oxidases homologues from *Neurospora crassa* (18%) and radioresistant bacterium *Deinococcus radiodurans* R1 (19%). The catalytic residues, such as Arg90 and Lys326 (numbering according to *C. rhodostoma* L-amino acid oxidase) are conserved. The consensus amino acid sequence -GXGXXG- important for the interaction with the ADP part of FAD is also conserved.

Since GOX is an extracellular enzyme, a precursor of GOX should possess a signal peptide sequence to lead its transport across the cytoplasmic membrane. A putative signal peptide can be found at the N-terminal of α-subunit (residues 1–18). Its sequence meets most of the criteria for a signal peptide characterized by Perlman and Halvorson [37].

Bioinformatic analysis was also conducted to identify the best template for constructing a structural model of GOX. Analysis was carried out by applying similarity and threading methods. The results of PDB BLAST (score $1e^{-103}$), 3D-PSSM (*E*-value $2.66e^{-04}$), SAM-T99 (score $1.926e^{-51}$), and FUGUE (*Z* score 17.27) analysis revealed that a confident model of mature α-subunit from *S. platensis* (amino acids 19–375) may be constructed based on the crystal structure of L-amino acid oxidase from *C. rhodostoma* (PDB accession code: 1f8r) [27]. The structural model of the mature α-subunit of GOX is illustrated in Fig. 2. Analysis of the GOX model with CASTp [31] revealed the presence of a large pocket with a solvent accessible surface area of 1243.816 (Richards' surface). This pocket is located at the protein's surface and comprises amino acid residues 152–160 and 265–267. Hydrophobicity [38] and Coulomb electrostatic potential analysis revealed that this pocket provides a mixed-type environment with high hydrophobicity (mean score 0.272) and partial positive electrostatic potential, thus providing favorable conditions of accommodating negatively charged hydrophobic ligands. This led us to evaluate a range of anionic aromatic textile and mimodye ligands for their ability to bind GOX.

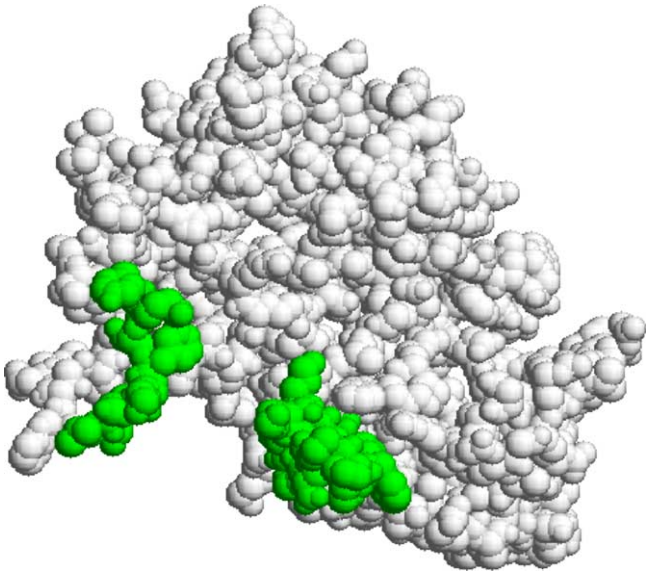
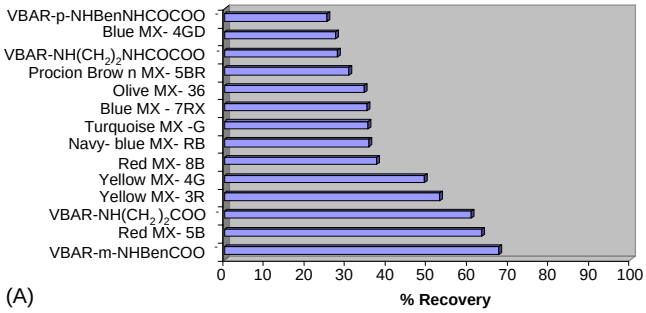


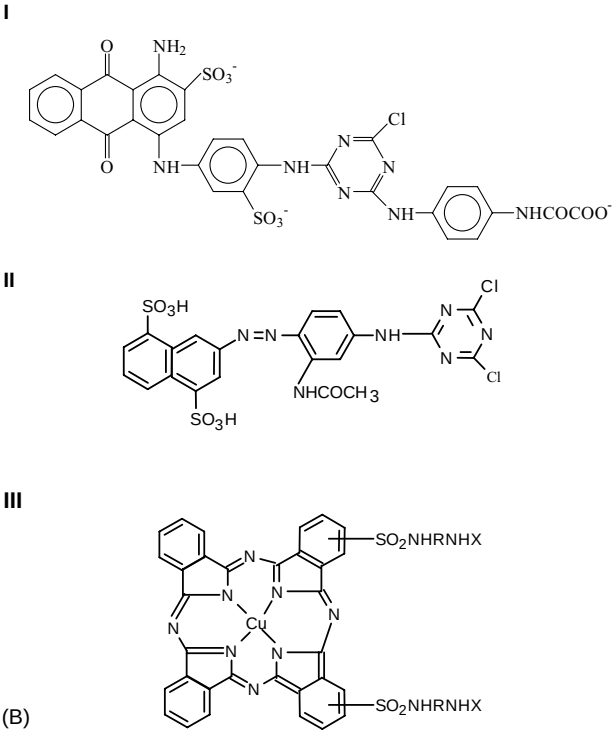
Fig. 2. Spacefill representation of the modeled α-subunit of GOX from *S. platensis*. Key residues comprising the binding pocket are colored dark grey. The model was constructed by MODELLER 6 using as template the structures of L-amino acid oxidase from *Calloselasma rhodostoma* (PDB code 1f8r).

3.2. Dye-adsorbent screening for GOX binding

In the present study, 15 triazine dyes (textile and mimodyes) were immobilized on agarose and evaluated for their ability to bind GOX (Fig. 3A). Four dye structures were regarded as biomimetic, since they bear a terminal (keto)carboxyl-moiety linked on the triazine ring, thus mimicking the natural substrate of GOX, glutamic acid. Enzyme solution (0.5 ml, 0.015 U, pH 5.6) was loaded on each of



(A)



(B)

Fig. 3. (A) Evaluation of dye adsorbents for binding GOX. Procedures were performed as described in the text (4 °C). Recovery was calculated on the basis of bound GOX units (100%). (B) Structures of three representative triazine dyes, anthraquinone mimodye BM1 (I) and two textile dyes containing azo (II, Procion Yellow) and phthalocyanine (III, Turquoise MX-G) chromophores.

the dye adsorbents. The column was washed with buffer before enzyme elution with KCl (1 M). This experimental approach is typical for assessing the binding strength of dye–ligands for a target enzyme [39]. The results are summarized in Fig. 3. All but Cibacron® Blue 3GA adsorbents were able to bind GOX at pH 5.6. The lower is the GOX activity recovered from each column, the tighter is the enzyme binding and, thus, the higher is the relative affinity. The mimodye adsorbent BM1, bearing an α-ketoacid as biomimetic moiety, bound tighter the enzyme (Fig. 3B), indicative of higher relative affinity. It appears that the presence of a terminal aminobenzyl-sulphonate group on CB3GA, instead of a *p*-aminobenzyloxanylic acid present on BM1, dramatically affects the binding ability of the ligand for the target enzyme GOX.

Each protein separation process must be individually optimised and no general rules can safely predict the factors and parameters for a specific separation [33,39,40]. However, some general factors that influence the performance of an affinity chromatography step need to be carefully considered. Therefore, mimodye adsorbent BM1 was further studied in terms of binding and elution conditions.

3.3. Effect of pH on the adsorption of GOX on BM1 mimodye adsorbent

Table 1 summarizes the effect of pH during the adsorption stage of GOX chromatography. At neutral to slightly alkaline pH (pH 7.6), BM1 displayed reduced capacity for GOX, whereas a dramatic fall was observed under more alkaline conditions (pH 8.0). On the contrary, in acidic pH values, the adsorbent exhibited good capacity and recovery, the higher values being obtained at pH 5.6. Values of pH lower than 5.6 were not considered, since the enzyme is unstable. This behavior is in agreement with previous findings that acidic conditions enhance protein binding to dye–ligand adsorbents [41].

3.4. Effect of elution conditions on the desorption of GOX from BM1 mimodye adsorbent

A suitable 'elution buffer' must effectively desorb the protein of interest in its native state, while leaving the column intact. Elution methods can be selective or non-selective [33,38]. Selective elution can be achieved with the inclusion of an agent that competes either with the target protein for the ligand, or with the ligand for the target protein. Non-selective elution usually involves buffers containing components that weaken protein-dye binding. Table 2 summarizes the effect of specific and common non-specific elution agents (L-glutamate, KCl, and glycerol) on GOX desorption from the BM1 mimodye adsorbent. From all agents tested, glycerol (40% (v/v)) led to the higher recovery (70.8%). By increasing the volume of the elution buffer (3 ml) an almost quantitative recovery (99.1%) was observed. L-Glutamate was unable to elute GOX from BM1, which strengthens the view that the interaction between BM1 and GOX does not take place at the substrate-binding region (see below).

Table 1
Effect of pH of the equilibration buffer on the adsorption and recovery (with 3 m KCl) of GOX for the BM1 adsorbent

pH	Adsorbed GOX (%)	Recovery[a] (%)
5.6	100	25.5
6.0	100	43.1
6.4	100	44.2
6.8	100	48.5
7.2	67.5	85.8
7.6	40.4	57.9
8.0	–	–

Procedures were performed as described in the text (4 °C).
[a] Calculated on the basis of bound GOX units (100%).

Table 2
Effect of the elution medium on the chromatography of GOX with the BM1 adsorbent at pH 5.6.

Elution medium	Recovery[a] (%)
KCl (1 M)	25.1
KCl (3 M)	25.5
KCl (4 M)	40.7
dd-H_2O	0
L-Glutamate (5 mM)	0
L-Glutamate (10 mM)	0
Glycerol (20%)	57.4
Glycerol (40%)	70.8

Procedures were performed as described in the text (4 °C).
[a] Calculated on the basis of bound GOX units (100%).

3.5. Kinetic studies

Kinetic analysis of GOX at pH 5.6 using L-Glu as a variable substrate obeyed Michaelis–Menten kinetics, with a K_m of 2 mM and a k_{cat} of 85.8 s^{-1}. This K_m value is in agreement with that reported for *Streptomyces endus* enzyme [1] but differs significantly to that reported for the *Streptomyces* sp. 80–5 enzyme (0.21 mM) [2].

Kinetic inhibition studies provided more evidence regarding the mode of interaction between the biomimetic dye and the binding site of GOX (Fig. 4). The dye showed a non-competitive type of inhibition with respect to L-Glu with K_i of 10.5 μM, indicating that the dye–enzyme interaction does not involve the substrate-binding region. This is in accordance with the observation that L-Glu was unable to elute the enzyme from the BM1 adsorbent and the proposed dye-binding pocket predicted by the CASTp analysis.

3.6. Equilibrium adsorption studies of GOX with BM1 mimodye adsorbent

To successfully design an affinity step, a thorough understanding of the fundamental mechanisms underlying such separations is an important requirement [42]. The adsorption isotherms and interaction mechanisms involved in dye–ligand chromatography are of great importance since

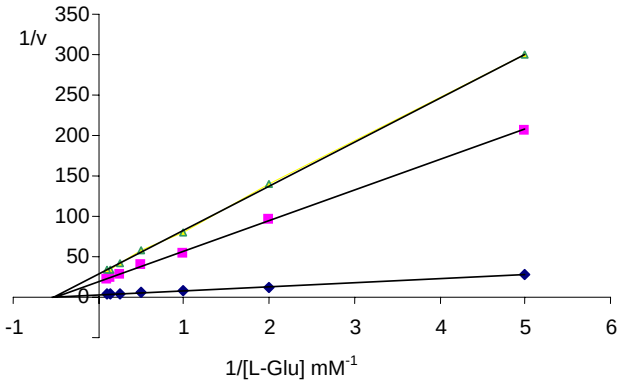


Fig. 4. Lineweaver–Burk plots for the determination of kinetic and inhibition constants. Inhibition of GOX by the BM1 mimodye at different L-Glu concentrations (37 °C). The enzyme was assayed in the presence of 5 μM (◆), 10 μM (■) and 15 μM (△), BM1.

they may predict the dynamic behavior of scaling-up and controlling the system [33]. Equilibrium adsorption studies were employed to characterize the interaction of the enzyme with the mimodye adsorbent BM1. This approach provides a relationship between the concentration of the enzyme in the solution and the amount of enzyme adsorbed to the solid phase, when the two phases are at equilibrium [36]. The model usually employed for affinity dye–ligand systems is the second-order reversible interaction, where the enzyme is assumed to interact with the ligand by a monovalent interaction (Eq. (1)), which has a characteristic binding energy:

$$\mathrm{E} + \mathrm{D} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ED} \quad (1)$$

where E is the enzyme in solution, D the dye adsorption site and ED is the enzyme–dye complex. The parameters k_1 and k_2 are the forward and reverse rate constant, respectively, for the adsorption process. From Eq. (1), it has been shown that at equilibrium a familiar Freundlich isotherm model, described by Eq. (2), can be obtained [36,43].

$$q^* = k(c^*)^n \quad (2)$$

where q^* is the adsorbate concentration at equilibrium (mg/g adsorbent), c^* is the equilibrium liquid phase concentration (mg/ml), k the velocity constant of the Freundlich isotherm ($\mathrm{mg}^{(n-1)}\,\mathrm{ml}^n/\mathrm{g}$), and n is the Freundlich isotherm exponent (dimensionless), which is correlated with the type of interactions (attraction or repulsion) between the adsorbed chemical kinds [36]. The Freundlich isotherm assumes that enthalpy during the adsorption of molecules is varying exponentially according to the available adsorption sites.

For the analysis of the present affinity system, a known mass of adsorbent was placed into a buffer containing a known concentration of solute. The change in solute concentration (after allowing 75 min for equilibrium to be attained) can be equated to the amount of solute adsorbed by the solid, and by using various starting solute concentrations, equilibrium data was obtained. When values of c^* versus q^* have

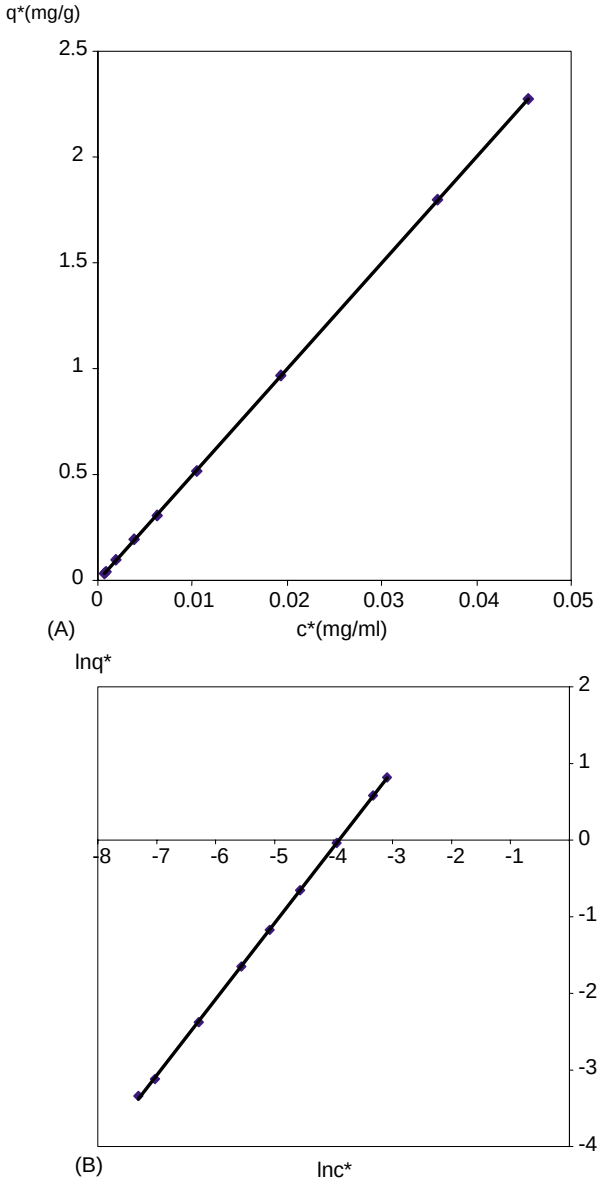


Fig. 5. (A) Equilibrium adsorption of GOX on the BM1 mimodye adsorbent in a batch system (pH 5.6, 4 °C). (B) A plot describing the equilibrium in liquid phase GOX concentration over the equilibrium in solid phase GOX concentration vs. the equilibrium in liquid phase GOX concentration, for the Freundlich isotherm.

been obtained, it is necessary to determine which theoretical isotherm fits the data best. For the Freundlich model, a plot of $\ln q^*$ versus $\ln c^*$ should yield a straight line. The batch adsorption of GOX on the biomimetic adsorbent BM1 is shown in Fig. 5. These lines correspond to the Freundlich isotherm that best fits the experimental data. The respective correlation coefficient R^2 was 0.999, indicating that the model, fits the data very well. From Fig. 5B a rate constant of $k = 2.7\,\mathrm{mg}^{1/2}\,\mathrm{ml}^{1/2}/\mathrm{g}$ and the Freundlich exponent of $n = 1$, were estimated. The Freundlich isotherm reveals that the heat of adsorption is varying according to the covered surface either due to the presence of energy-heterogeneity

at the positions of adsorption, or due to the development of side interactions between the adsorbed chemical molecules. This energy-heterogeneity of the adsorbent, is related to the fact that there are positions which have different abilities of adsorbing molecules [42].

A Freundlich isotherm implies that no theoretical limit exists for a maximum capacity of the adsorbent for GOX. However, this proves to be unrealistic in practice because the adsorbents used have physical limits regarding the amount of protein that can attach itself to their inner pore surface. It is possible that at higher concentrations of GOX than those used in this study, the solid phase loading would have reached a constant value. It is also possible that an affinity binding effect or non-specific interaction might occur between the ligand and GOX. The observed Freundlich isotherm may be the best representation of the superposition of these two effects.

Previous work, based on equilibrium adsorption studies on dye-adsorbents, has shown that, in most cases studied so far, the Langmuir isotherm model was observed [33,36]. However, the Freundlich isotherm seems to be the model of the adsorption of lysozyme on immobilised Procion MX-R on porous silica matrix [36].

3.7. Purification of GOX on BM1 mimodye adsorbent

A number of purification methods have been reported for GOX, unfortunately all laborious and of moderate industrial potential. Specifically, the enzyme was purified from *S. platensis* NTU 3304 by ammonium sulfate fractionation and three-column chromatography (DEAE-Fractogel, phenyl-toyopearl, Sephadex G-150) which led to a specific activity of 65.2 units/mg and an overall yield of 53.3% [1]. Another procedure for the *S. endus* enzyme involved four steps, three of them being chromatographic, leading to a specific activity of 6.0 U/mg and an overall yield of 20.8% [7]. Finally, GOX from *Streptomyces* sp. 80–5 was purified following a three-step chromatographic procedure, leading to a specific activity of 0.24 U/mg and an overall yield of 17.8% [2].

In the present study, crude culture broth from *S. coelicolor* was chromatographed on BM1 mimodye adsorbent (0.5 ml), employing the specified binding and elution conditions. The proposed procedure resulted in enzyme specific activity of 67 units/mg, 335-fold purification and 95% recovery, in a single chromatography step. When the purified GOX stored in 40% (v/v) glycerol solution, was stable for at least a month (4 °C) and could be used in analytical applications.

The data reported in Table 1 suggest that binding of GOX on BM1 adsorbent is pH dependent. This raises the possibility that varying the pH of the irrigating buffer may elute GOX from the BM1 adsorbent. Unfortunately, employment of either pH or KCl elution techniques has resulted in a 20 and 40% reduction of final specific enzyme activity, respectively, when compared to the specific enzyme activity obtained after glycerol elution. As far as the final purification protocol is concerned, GOX elution was uneffected by changes in pH or salt concentration. Importantly, elution with glycerol leads to a more purified GOX enzyme, however, this increase in enzyme purity is balanced against a reduction in GOX recovery.

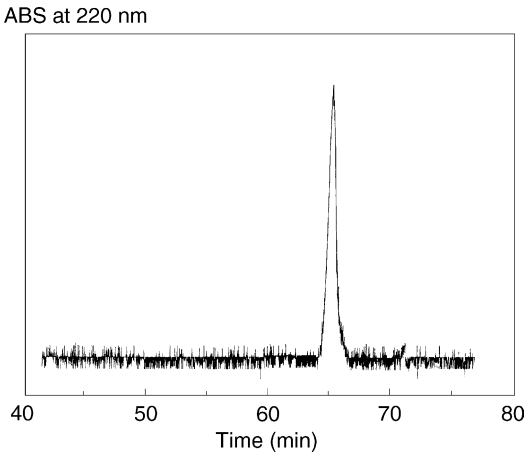


Fig. 6. High performance gel filtration chromatography of the enzyme eluted from the BM1 adsorbent. Protein sample was applied to a Protein PAK 300SW, 7.8 mm × 300 mm (i.d.), column (Waters) which was equilibrated with 100 mM potassium phosphate buffer, pH 6.5, containing 100 mM KCl. The protein was eluted in the same buffer mixture at a flow rate 0.03 ml/min. Eluting protein was monitored at 220 nm.

The purity of the enzyme preparation was assessed by HPLC analysis using a computerized Gilson gradient bio-HPLC system and a gel filtration column. The results revealed a single protein peak as shown in Fig. 6.

4. Conclusions

The present work investigated the interaction of L-glutamate oxidase with dye–ligands, employing four complementary techniques: molecular modeling, analytical affinity chromatography, kinetic inhibition and adsorption equilibrium. The results obtained provided the basis for developing a facile and efficient purification method for GOX. The method holds potential for application at preparative scale, since it employs low-cost materials and affords, in a single chromatography step, GOX at high specific activity and recovery.

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