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Designed chimaeric galactosyl–mimodye ligands for the purification of *Pseudomonas fluorescens* β-galactose dehydrogenase

C.F. Mazitsos [a], D.J. Rigden [b], Y.D. Clonis [a,*]

[a] *Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, 75 Iera Odos Street, GR-11855 Athens, Greece*
[b] *Embrapa Recursos Genéticos e Biotecnologia, Brasilia, Brazil*

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Abstract

Two chimaeric galactosyl–mimodye ligands were designed and applied to the purification of *Pseudomonas fluorescens* galactose dehydrogenase (GaDH). The chimaeric affinity ligands comprised a triazine ring on which were anchored: (i) an anthraquinone moiety that pseudomimics the adenine part of NAD^+, (ii) a galactosyl-mimetic moiety (D-galactosamine for ligand BM1 or shikimate for ligand BM2), bearing an aliphatic 'linker', that mimics the natural substrate galactose, and (iii) a long hydrophilic 'spacer'. The mimodye-ligands were immobilised to 1,1-carbonyldiimidazole-activated agarose chromatography support, via the spacer's terminal amino-group, to produce the respective mimodye adsorbents. Both immobilized mimodyes successfully bound *P. fluorescens* GaDH but failed to bind the enzyme from rabbit muscle. Adsorbent BM1 bound GaDH from green peas and Baker's yeast, but adsorbent BM2 failed to do so. The mimodye-ligand comprising D(+)-galactosamine (BM1), compared to BM2, exhibited higher purifying ability and enzyme recovery for *P. fluorescens* GaDH. The dissociation constants (K_D) of BM1 and BM2 for *P. fluorescens* GaDH were determined by analytical affinity chromatography to be 5.9 μM and 15.4 μM, respectively. The binding capacities of adsorbents BM1 and BM2 were 18 U/mg adsorbent and 6 U/mg adsorbent, respectively. Adsorbents BM1 and BM2 were integrated in two different protocols for the purification *P. fluorescens* GaDH. Both protocols comprised as a common first step DEAE anion-exchange chromatography, with a second step of affinity chromatography on BM1 or BM2, respectively. The purified GaDH obtained from the protocols using BM1 and BM2 showed specific activities equal to 1077 and 854 U/mg, respectively. The former is the highest reported so far and the enzyme appeared as a single band after sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

Keywords: *Pseudomonas fluorescens*; Biomimetic ligands; Molecular modeling; Affinity adsorbents; Galactose dehydrogenase; Enzymes; Dyes; Triazines

1. Introduction

The production of pure and efficacious proteins from native or genetically modified sources is an ongoing challenge. Affinity chromatography remains one of the most important techniques for the high purification of proteins, especially those of analytical and pharmaceutical interest. This technique helps to lower the number of steps in a purification process, hence offering the prospects of increased yields and higher returns. As changes in the regulatory climate have shifted the focus of regulation from production processes per se to the concept of the 'well-characterized biologic', inevitably more attention is paid towards affinity ligand selection and design [1]. Biological ligands such as peptides, proteins and oligonucleotides are readily obtained from combinatorial libraries employing biopanning and amplification techniques. However, these ligands often require purification in their own right, suffer from low binding capacity and are unstable under conventional sterilization schedules [1]. In contrast, the arsenal of synthetic chemical affinity ligands is more potent and promising due to the robustness and effectiveness of these ligands.

Modern trends to synthetic ligand design are based on the combination of knowledge of X-ray crystallographic and NMR structures with defined or combinatorial chemical synthesis [2]. In the absence of direct structural data of the targeted protein, molecular modelling can be engaged to produce a computationally derived protein model

* Corresponding author. Tel.: +30-210-5294311; fax: +30-210-5294307.
E-mail address: clonis@aua.gr (Y.D. Clonis).

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from knowledge of the aminoacid sequence of the targeted protein and the X-ray crystallographic structure of a homologous template-protein. In any case, three distinct routes to ligand design can be realized: (i) de novo design based on template-ligands, that is, investigation of the structure of a natural protein–ligand complex and the use of this ligand as template on which to model a new biomimetic (BM) ligand [3–8], (ii) ligand design by constructing a molecule that shows complementarity to exposed residues in the target site of the protein [9], and (iii) ligand design by mimicking natural biological recognition [10]. The de novo design based on template-ligands has found by far the most application with peptidal-templates [3,5] and nucleotide coenzyme–substrate templates [4,6,7]. The latter form of templates has allowed the creation of the so-called 'bi-functional' or 'chimaeric' biomimetic dye-ligands ('mimodyes') [11,12]. These synthetic ligands are characterized by the presence of two recognition moieties anchored around a chloro-triazine ring: an anthraquinone-diaminobenzosulphonate pseudomimetic moiety that mimics a natural nucleotide coenzyme and a biomimetic moiety that acts as a substrate-mimetic analogue. The mimodyes were evolved from the archetypal structures of the commercial textile dyes Cibacron blue 3GA and Vilmafix blue A-R (VBAR) which, in common with most other conventional triazinyl dyes [12,13], offer high stability, low cost, ease of immobilisation and high binding capacity. Unfortunately these dyes display modest binding selectivity. In contrast, mimodyes have shown increased selectivity and purifying ability, being useful tools for the design of simple and effective enzyme purification protocols. This technological advance has successfully been exploited to the purification of (keto)carboxyl group-recognising enzymes, such as lactate [6] and malate [14] dehydrogenases, and glutathione-recognising enzymes, such as aldehyde dehydrogenaase and glutathione *S*-transferase [7]. In a recent study we reported the use of biocomputing for the construction of a galactose dehydrogenase (GaDH) model and the de novo design of bi-functional (chimaeric) galactosyl–mimodye ligands [15]. These new affinity ligands comprised a triazine ring around which are anchored (i) an anthraquinone-based pseudomimetic moiety that mimics the adenine part of the coenzyme NAD^+, (ii) a galactose-mimetic moiety, bearing an aliphatic 'linker' terminating with D-galactosamine or shikimate that mimic the natural substrate galactose, and (iii) an appropriate 'spacer' molecule via which the ligand can be immobilised to a chromatography support and used for the purification of galactose dehydrogenase.

The present work provides a detailed evaluation of the aforementioned galactosyl–mimodye adsorbents for the purification of galactose dehydrogenase from *Pseudomonas fluorescens*, the commercially exploited source, but also from other non-bacterial sources. For this purpose, along side the designed mimodye adsorbents, non-biomimetic and control adsorbents were evaluated, together with conventional ion-exchange chromatography media. A simple and effective purification protocol can be proposed for the preparation of GaDH of the highest specific activity reported so far. The study of GaDH is justified by the lack of a facile purification protocol able to yield high-purity enzyme with good recovery, and of the analytical and biotechnological importance of the enzyme. Galactose dehydrogenase catalyses the dehydrogenation of β-D-galactose in the presence of NAD^+, acting on the C1 position of the sugar substrate. The enzyme is used to the determination of β-D-galactose, α-D-galactose (together with mutarotase) [16], and arabinose [17] in biological samples. Together with β-galactosidase (lactase), the enzyme is useful in the assay of lactose in foods, pharmaceuticals or biological samples [16,18]. Galactose dehydrogenase may also be used in oral tests of galactose tolerance for monitoring liver function [19].

2. Experimental

2.1. Materials

CM-Sepharose CL-6B, DEAE-Sepharose CL-6B and β-galactose dehydrogenase (GaDH, EC 1.1.1.48, expressed in *E. coli* using *P. fluorescens* gene) were obtained from Sigma (St. Louis, MO, USA), whereas crystalline bovine serum albumin (fraction V) was from Boehringer Mannheim (Germany). Hexylamine and nutrient broth (for microbiology) were obtained from Merck (Germany). The agarose chromatography gel Sepharose CL-6B was purchased from Pharmacia. F324 *P. fluorescens* biovar V1 was kindly donated by Professor G.J. Nychas (Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, Greece). Baker's yeast, green peas and rabbit liver were purchased at the local market.

2.2. Synthesis of galactosyl–mimodye adsorbents

The chemical synthesis and purification of the chimaeric galactosyl–mimodye ligands, their immobilization to carbonyldiimidazole-activated agarose via the spacer's terminal amino-group and the determination of the concentration of the immobilized ligands were all performed as described by Mazitsos et al. [15].

2.3. Assay of enzyme activity and protein

Galactose dehydrogenase (D-galactose: NAD^+ 1-oxidoreductase; EC 1.1.1.48) assays were performed at 25 °C according to [20], using a double beam UV-Vis spectrophotometer equipped with a thermostated cell holder (10 mm pathlength). One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μmol of substrate to product per min. Protein concentration was determined by the method of Bradford [21] or by a modified Bradford method [22], using bovine serum albumin (fraction V) as standard.

2.4. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli [23] on a 1.0 mm-thick vertical slab gel (10 cm × 8 cm) containing 10.0% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel, using a Hoefer SE 250 dual-slab cell unit. Protein samples, after dialysis against 25 mM Tris–HCl, pH 6.8, were mixed with an equal volume of treatment Tris–HCl buffer (125 mM, pH 6.8) containing SDS (4% (v/v)), glycerol (20% (v/v)), β-mercaptoethanol (10% (v/v)), and bromophenol blue (0.002% (w/v)). The samples were incubated at 75 °C for 5 min, applied to the wells, and run at a current of 20 mA per gel for 1 h. Protein bands were stained with Coomassie Blue R-250.

2.5. Cell cultures

P. fluorescens precultures were prepared by inoculating the bacterium into nutrient broth (Merck) (8.0 g in 1 l of deionized water, pH 7.0 ± 0.2, 25 °C). Stock cultures were maintained in agar-nutrient broth dishes, stored at 4 °C. Cultures (200 ml) were prepared in Erlenmeyer flasks, which were incubated on a rotary shaker (120 rpm) for 48 h at 25 °C. The cells were harvested by centrifugation at 4 °C (10 000 × *g* for 25 min), washed three times with potassium phosphate buffer (50 mM, pH 7.6) and recentrifuged. This procedure yielded approximately 4 g cell paste/l culture. The cell paste was freezed, lyophilized, and the dry, lyophilized mass was stored at −20 °C. Typically, this procedure yielded 1–1.5 g dry cell mass/l culture.

2.6. Preparation of cell extracts

P. fluorescens dry cells (1.5 mg) were suspended in 1 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 6.5, 7.0 or 7.5, and ultrasonically disintegrated (Vibra Cell, 400 W, Sonics & Materials) (amplitude: 40%, 2 s sonication—5 s pause, 8 cycles, 4 °C). Cell debris was removed by centrifugation (5000 × *g*, 20 min, 4 °C) and the supernatant was dialyzed overnight at 4 °C against 2 l of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 6.5, pH 7.0 or pH 7.5. The dialysate was clarified through a Millipore cellulose membrane filter (0.45 μm pore size). When necessary, the supernatant was enriched with commercial enzyme (*P. fluorescens* gene expressed in *E. coli*) in order to achieve an initial specific activity of about 1.1 units GaDH/mg.

Commercial baker's yeast cells (9 g paste) were suspended in 12 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0 or pH 7.5, or 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5, before ultrasonically disintegrated (amplitude 40%, 5 s sonication—5 s pause, 12 cycles, 4 °C). Cell debris was removed by centrifugation (14 000 × *g*, 50 min, 4 °C) and the supernatant was dialyzed overnight at 4 °C against 2 l of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0 or pH 7.5, or 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5. The dialysate was clarified through a Millipore cellulose membrane filter (0.45 μm pore size), affording, typically, an activity of 0.06 units GaDH/ml extract (0.08 units GaDH/g cell paste).

Green peas (13 g) were suspended in 20 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0 or pH 7.5, or 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5, before being pulped using pestle and mortar, and homogenized (Virtishear mechanical homogenizer, 10 000 rpm, 1 min, 4 °C). The homogenized suspension was filtered using cheesecloth and the filtrate was centrifuged (18 000 × *g*, 40 min, 4 °C). The supernatant was dialyzed overnight at 4 °C against 5 l of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0 or pH 7.5, or 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5. The dialysate was clarified through a Millipore cellulose membrane filter (0.45 μm pore size), affording, typically, an activity of 0.02 units GaDH/ml extract (0.03 units GaDH/g).

Rabbit liver (5 g) was suspended in 20 ml of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 6.5 or pH 7.0, and homogenized (Virtishear mechanical homogenizer, 10 000 rpm, 3 min, 4 °C). The homogenized suspension was centrifuged (750 × *g* for 15 min, 4 °C) and the supernatant was re-centrifuged (14 000 × *g*, 50 min, 4 °C). The supernatant was dialyzed overnight at 4 °C against 5 l of 10 mM potassium phosphate buffer, pH 6.5 or pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45 μm pore size), affording, typically, an activity of 0.03 units GaDH/ml extract (0.12 units GaDH/g).

2.7. Affinity chromatography evaluation of immobilised mimodyes (biomimetic (BMs) ligands) BM1 and BM2 with GaDH from baker's yeast, green peas and rabbit liver extracts

All procedures were performed at 4 °C. Baker's yeast and green peas extracts were dialyzed overnight against 2 and 5 l, respectively, of 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5. Rabbit liver extract was dialyzed overnight against 5 l of 10 mM potassium phosphate buffer, pH 6.5 or pH 7.0. A sample of baker's yeast dialysate (4–6 ml, 0.16–0.41 units GaDH, 7.9–9.65 mg protein), green peas dialysate (6–8 ml, 0.10–0.16 units GaDH, 63.0–114.8 mg protein) and rabbit liver dialysate (8 ml, 0.08–0.1 units GaDH, 35.76–39.5 mg protein) was applied to each analytical column (packed with 0.5 ml dye adsorbent), which was previously equilibrated with the respective buffer. Non-adsorbed protein was washed off with equilibration buffer (6–20 ml for baker's yeast, 20–45 ml for green peas, 25 ml for rabbit liver). Bound GaDH was eluted by 0.5 mM NAD^+/5 mM Na_2SO_3 (BM1) [15] or 0.8 mM NAD^+/8 mM Na_2SO_3 (BM2) [15] in the equilibration buffer (1–3 ml for baker's yeast, 1–3 ml for green peas, 2 ml for rabbit liver). Collected fractions (2 ml or 5 ml for baker's yeast, 5 ml for

green peas, 5 ml for rabbit liver) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity were pooled and the specific activity was determined.

2.8. Determination of dissociation constants (K_D) and GaDH-binding capacities of immobilized mimodyes BM1 and BM2

Determination of the K_D values of BM affinity adsorbents was performed essentially according to the method of Kasai and Ishii [24] and Kasai et al. [25]. The column contained 100 mg wet weight (0.16 ml wet volume gel) of affinity adsorbent equilibrated with 10 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0 for BM1, pH 7.5 for BM2). A dilute solution of GaDH from *P. fluorescens* (0.12 units GaDH/ml, M_r for GaDH 64 kDa) made in the above buffers, was continuously applied at a flow rate of 1.7 cm/min until the effluents had reached a maximum and steady GaDH activity. The fractions collected (typically 1 ml each) were assayed for GaDH activity. The mathematical calculations are described in Results and Discussion.

Adsorbent capacity was calculated from the plot of eluting GaDH activity versus effluent volume, and it is defined as the GaDH units present in extract volume equal to effluent volume corresponding to 10% of the maximum eluting activity from 0.16 ml of adsorbent.

2.9. DEAE anion-exchange chromatography of GaDH from Pseudomonas fluorescens extract

All procedures were performed at 4 °C. Analytical columns, each packed with 0.5 ml of DEAE-Sepharose CL-6B anion-exchanger, were equilibrated with 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 5.5, 6.0 and 6.5, and 20 mM potassium phosphate buffer containing 1 mM EDTA, pH 5.5. Dialyzed *P. fluorescens* enriched extract (1 ml, 0.11–0.35 units GaDH, 0.09–0.30 mg protein) was applied to each analytical column. Non-adsorbed protein was washed off with equilibration buffer (2–7 ml) followed by 2–4 ml of equilibration buffer containing 100 mM (pH 5.5), 120 mM (pH 6.0) or 140 mM (pH 6.5) KCl. Bound GaDH was eluted by equilibration buffer containing 140 mM (5 ml, pH 5.5), 160 mM (5 ml, pH 6.0), 180 mM (4 ml, pH 6.5) or 120 mM (7 ml, 20 mM buffer, pH 5.5) KCl. Collected fractions (1 ml) were assayed for GaDH activity and protein. The fractions with GaDH activity were pooled and the specific activity was determined.

2.10. Purification of GaDH from Pseudomonas fluorescens extract on immobilized mimodye-ligands

All procedures were performed at 4 °C.

Step 1 (Ion-exchange chromatography on DEAE-Sepharose CL-6B). Dialyzed extract (1 ml, 0.72 units GaDH, 0.63 mg protein) was applied to a column of DEAE-Sepharose CL-6B anion-exchanger (0.5 ml) previously equilibrated with 20 mM potassium phosphate buffer containing 1 mM EDTA, pH 5.5. Non-adsorbed protein was washed off with 7 ml equilibration buffer, followed by 4 ml of equilibration buffer containing 100 mM KCl. Elution of GaDH activity was carried out with 8 ml of equilibration buffer containing 120 mM KCl. Collected fractions (1 ml) were assayed for GaDH activity and protein [22]. The fractions with GaDH activity were pooled (8 ml) and the specific activity was determined.

Step 2a (Affinity chromatography on immobilised mimodye BM1). GaDH activity eluted from the ion-exchanger was dialysed overnight against 2 l of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.0 [15]. The dialysate (8 ml, 0.7 units GaDH, 11.7 μg protein) was applied to the mimodye-agarose BM1 (0.5 ml) which was previously equilibrated with the same buffer. Non-adsorbed protein was washed off with 6 ml equilibration buffer. Bound GaDH was eluted in equilibration buffer containing 0.5 mM NAD^+/5 mM Na_2SO_3 (5 ml) [15]. Collected fractions (1 ml) were assayed for GaDH activity and protein (A_{280}, except for fractions with NAD^+/Na_2SO_3, where the protein was determined by a modified Bradford method [22]). The fractions with GaDH activity were pooled (5 ml) and the specific activity was determined.

Step 2b (Affinity chromatography on immobilised mimodye BM2). GaDH activity eluted from the ion-exchanger was dialysed overnight against 2 l of 10 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.5 [15]. The dialysate (8 ml, 0.7 units, 12 μg protein) was applied to the mimodye-agarose BM2 (0.5 ml) which was previously equilibrated with the same buffer. Non-adsorbed protein was washed off with 4 ml equilibration buffer. Bound GaDH was eluted in equilibration buffer containing 0.8 mM NAD^+/8 mM Na_2SO_3 (5 ml) [15]. Collected fractions (1 ml) were assayed for GaDH activity and protein (A_{280}, except for fractions with NAD^+/Na_2SO_3, where the protein was determined by a modified Bradford's method [22]). The fractions with GaDH activity were pooled (5 ml) and the specific activity was determined.

2.11. Ion-exchange chromatography of GaDH from baker's yeast and green peas extracts

All procedures were performed at 4 °C. Analytical columns were used, each packed with 0.5 ml of CM-Sepharose CL-6B cation-exchanger or DEAE-Sepharose CL-6B anion-exchanger.

2.11.1. GaDH from baker's yeast on CM-Sepharose CL-6B

The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.5 or pH 7.0. Dialyzed baker's yeast extract (5 ml, 0.13–0.14 units GaDH, 1.04–1.22 mg protein) was applied to the column. Non-adsorbed protein was

washed off with equilibration buffer (4 ml). Most of GaDH was not adsorbed. Bound GaDH was eluted by equilibration buffer containing 1 M KCl (4 ml). Collected fractions (2 ml) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity were pooled and the specific activity was determined.

2.11.2. GaDH from baker's yeast on DEAE-Sepharose CL-6B

The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.5 or pH 7.0. Dialyzed baker's yeast extract (5 ml, 0.13–0.39 units GaDH, 1.04–2.23 mg protein) was applied to the column. Non-adsorbed protein was washed off with equilibration buffer (6 ml). Bound GaDH was eluted by equilibration buffer containing 60 mM (pH 6.5) or 80 mM (pH 7.0) KCl (4 ml). Collected fractions (2 ml) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity were pooled and the specific activity was determined.

2.11.3. GaDH from green peas on CM-Sepharose CL-6B

The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5, or 10 mM Tris–HCl, pH 8.0. Dialyzed green peas extract (2–5 ml, 0.27–0.44 units GaDH, 8.2–8.5 mg protein) was applied to the column. Non-adsorbed protein was washed off with equilibration buffer (6–8 ml). Most of GaDH was not adsorbed. Bound GaDH was eluted by equilibration buffer containing 1 M KCl (2 ml). Collected fractions (2 ml) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity (from washings) were pooled and the specific activity was determined.

2.11.4. GaDH from green peas on DEAE-Sepharose CL-6B

The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.5, pH 7.0 or pH 7.5, or 10 mM Tris–HCl, pH 8.0. Dialyzed green peas extract (5–20 ml, 0.12–0.48 units GaDH, 19.45–66.7 mg protein) was applied to the column. Non-adsorbed protein was washed off with equilibration buffer (6–35 ml). Most of GaDH was not adsorbed. Bound GaDH was eluted by equilibration buffer containing 1 M KCl (2–4 ml). Collected fractions (2–5 ml) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity (from washings) were pooled and the specific activity was determined.

2.12. Purification of GaDH from green peas extract on immobilized mimodye BM1

All procedures were performed at 4 °C.

Step 1 (Ion-exchange chromatography on DEAE-Sepharose CL-6B). Dialyzed extract (20 ml, 0.28 units GaDH, 66.7 mg protein) was applied to a column of DEAE-Sepharose CL-6B anion-exchanger (0.5 ml) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0. Non-adsorbed protein was washed with 10 ml equilibration buffer, followed by 4 ml of equilibration buffer containing 1 M KCl. Most of the GaDH activity did not bind to this column ('negative' chromatography) and appeared in the washings. Collected fractions (2 ml) were assayed for GaDH activity and protein [21]. The fractions with GaDH activity were pooled (20 ml) and the specific activity was determined.

Step 2 (Affinity chromatography on immobilised mimodye BM1). GaDH activity from the ion-exchanger (20 ml, 0.27 units, 24.6 mg protein) was directly applied to a column of immobilised mimodye BM1 (0.5 ml) which was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0 [15]. Non-adsorbed protein was washed off with 35 ml equilibration buffer. Bound GaDH was eluted in equilibration buffer containing 0.5 mM NAD^+/5 mM Na_2SO_3 (2 ml) [15]. Collected fractions (5 ml) were assayed for GaDH activity and protein (A_{280}, except for fractions with NAD^+/Na_2SO_3, where the protein was determined by Bradford's method [21]). The fractions with GaDH activity were pooled (2 ml) and the specific activity was determined.

3. Results and discussion

In a recent study we reported on the de novo design, synthesis and preliminary affinity chromatographic evaluation of two novel chimaeric galactosyl–mimodye ligands (Fig. 1), along side non-mimodye/control ligands, for the enzyme galactose dehydrogenase [15]. These galactosyl–mimodyes exhibit successful affinity binding with the targeted enzyme GaDH (Fig. 2). With the good objective quality of the GaDH model established [15], ligand-docking experiments were carried out by first docking into the GaDH model the three ring systems of the CB3GA-derived portion of the putative ligands (numbered 1–3: anthraquinone, diaminobenzosulphonic acid and triazine, respectively), followed sequentially by the galactose or the shikimic acid portion (biomimetic moiety), the 'linker' molecule between ring 3 and the galactose or the shikimic acid, and finally the chain ('spacer' molecule) by which the ligand attaches to the chromatograpic matrix [15]. In the present study we focus on purely chromatographic experimental aspects, and on the application of these mimodyes to the high-purification of GaDH from *P. fluorescens* and green peas, using simple and facile protocols.

3.1. Preliminary affinity chromatographic study of mimodye adsorbents with GaDH from different sources

Galactosyl–mimodye adsorbents, prior to be integrated in a purification protocol, were studied with respect to their ability to purify, at different pH, GaDH from *P. fluorescens*, green peas, baker's yeast and rabbit liver extracts (Table 1). For this purpose, an effective biospecific elution system had to be established for bringing GaDH off the affinity adsorbents. Different concentrations of NAD^+/Na_2SO_3 mixtures

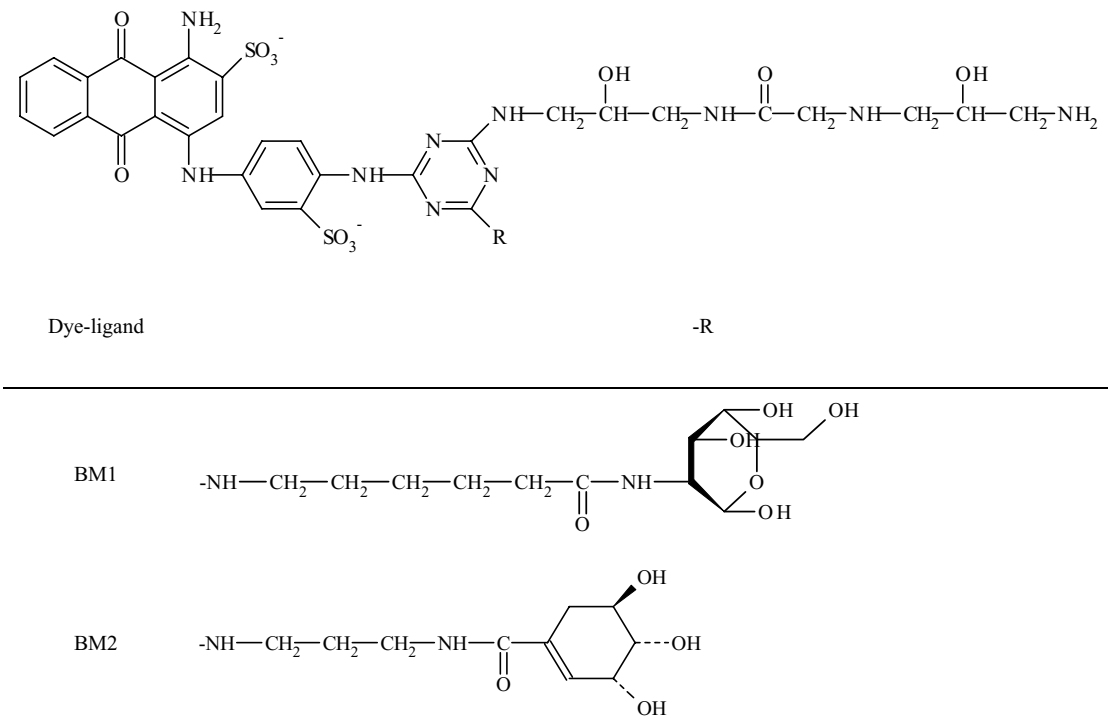

Fig. 1. Structure of chimaeric galactosyl-biomimetic dyes: galactosamine-mimodye (BM1) and shikimate-mimodye (BM2).

were tested as a biospecific eluent; 0.5 mM NAD^+/5 mM Na_2SO_3 proved the most effective with BM1 and 0.8 mM NAD^+/8 mM Na_2SO_3 with BM2. Immobilised BM1 and BM2, at pH 7.0 and 7.5, respectively, bind successfully GaDH from *P. fluorescens* [15], showing good purifying ability (41.9- and 37.5-fold, respectively) and high recovery (98–100%). Furthermore, of the two affinity adsorbents (Table 1), only BM1 seems to present a certain degree of affinity for GaDH from green peas and baker's yeast extracts, leading to 17.4- and 9.1-fold purification, respectively, at pH 7.0. GaDH from rabbit liver extract binds negligibly to BM1, whereas BM2 does not bind GaDH from sources other than *P. fluorescens*. At pH values higher or lower than 7.0, the purifying ability of the affinity adsorbents falls considerably. It appears, therefore, that the galactosyl–mimodyes are suitable affinity chromatography tools for the purification of GaDH, principally from *P. fluorescens*, but also, to a lesser degree, from green peas. These findings suggest that the structural differences among GaDHs from the studied sources are probably significant enough to seriously affect the binding of the mimodyes to the targeted enzyme sites. Since the primary structures of GaDHs from species other than *P. fluorescens* are not known, a more comprehensive explanation for these findings could not be attempted. It is clear, however, that only *P. fluorescens* GaDH shows effective binding to the mimodye-ligands, not surprisingly, since they were purpose designed for this particular enzyme.

Table 1
Affinity chromatography evaluation of galactosyl–mimodye adsorbents for binding GaDH activity from *Pseudomonas fluorescens*, green peas, rabbit liver and baker's yeast

pH	BM1		BM2	
	Purification (fold)	Recovery (%)	Purification (fold)	Recovery (%)
Pseudomonas fluorescens				
6.5	27.2	66	13.7	68
7.0	41.9	100	32.8	76
7.5	27.5	35	37.5	98
Green peas				
6.5	11.9	12.5	–	0
7.0	17.4	30.8	–	0
7.5	–	0	–	0
Baker's yeast				
6.5	6.0	42.8	–	0
7.0	9.1	17.5	–	0
7.5	2.5	3.3	–	0
Rabbit liver				
6.5	–	1.3	–	0
7.0	–	0.6	–	0

3.2. Dissociation constants (K_D) and GaDH-binding capacities of immobilized galactosyl–mimodyes

Since immobilized BM1 and BM2 ligands are suitable affinity chromatography tools for *P. fluorescens* GaDH,

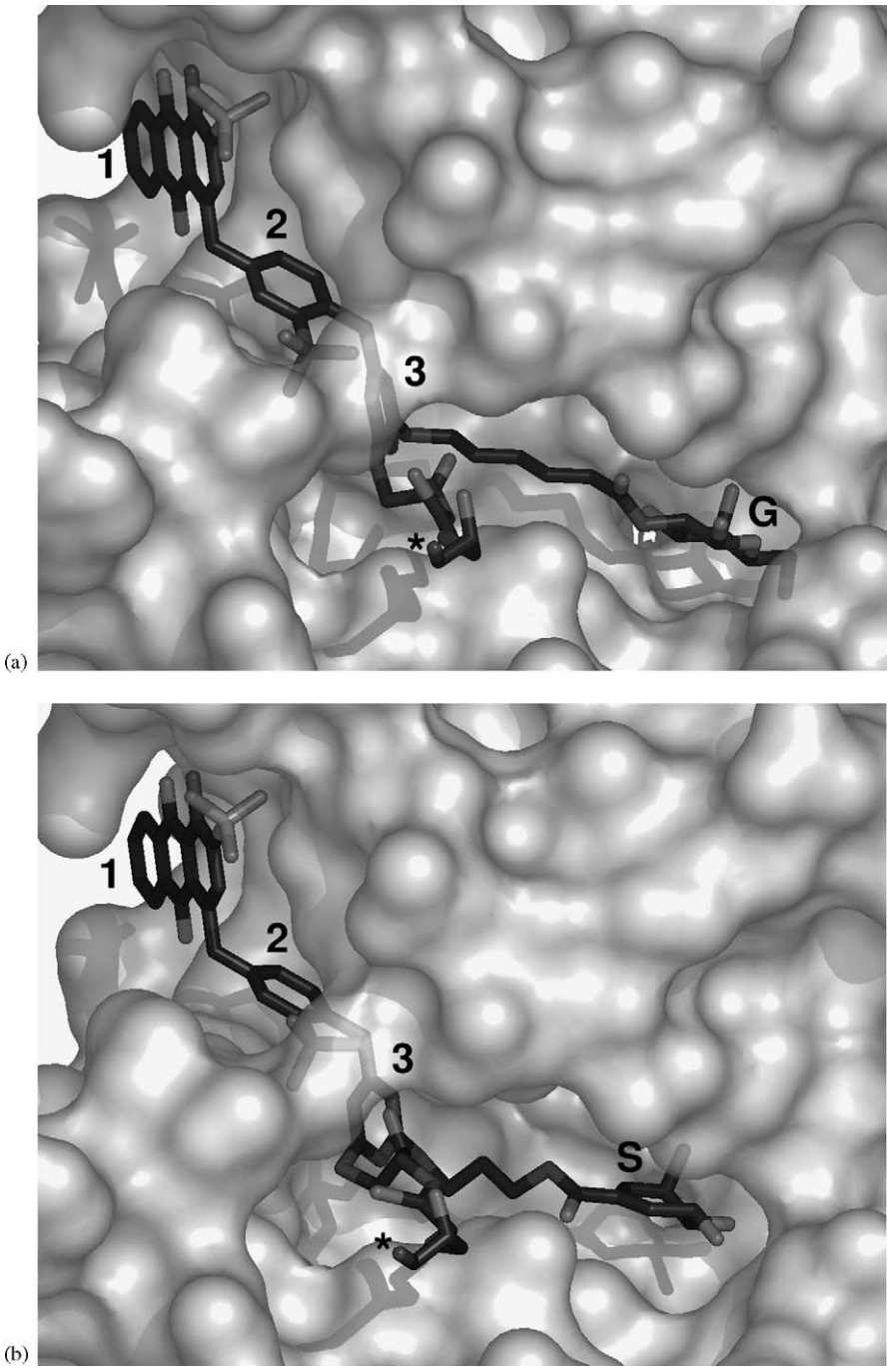

Fig. 2. Molecular graphics of the binding site of GaDH showing (a) the refined conformation of the biomimetic ligand BM1 and (b) the refined conformation of the biomimetic ligand BM2. In (a) and (b) 1–3 are used for ring systems anthraquinone (1), diaminobenzosulphonic (2) acid and triazine (3), G for the galactose moiety bearing the 'linker' molecule, S for the shikimate moiety bearing the 'linker' molecule, and (*) for the end of the connecting 'spacer' molecule (coming out of the page) which would be covalently bonded to the chromatographic matrix.

further studies were carried out. Fig. 3 depicts the elution profiles for analytical chromatography runs to determine the K_D values for the two immobilized ligands. These profiles were obtained by plotting eluting GaDH activity (expressed as %plateau) versus effluent volume, for BM1 and BM2 adsorbents, and are composed of an elution front and a plateau. The elution volume, V, of the front of the enzyme approximately equals the volume of effluent corresponding to the half-enzyme concentration in the starting material or at the plateau, $[E]_0/2$. In cases where the elution front of the enzyme is not symmetrical with regard to the midpoint, then, if fractions of constant volume are collected, V should

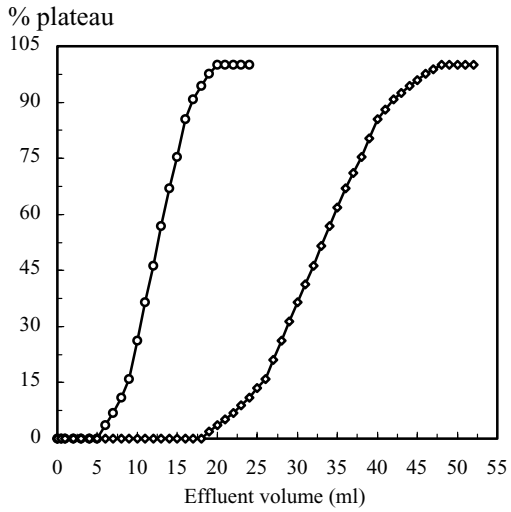

Fig. 3. Analytical affinity chromatography of *Pseudomonas fluorescens* GaDH for the determination of the K_D values of the immobilized BM1-GaDH (◇) and BM2-GaDH (○) complexes. The chromatography data collected are substituted in Eq. (2) ($V_0 = 0.2$ ml) and the results obtained are shown in Table 2.

be calculated from Eq. (1) [24,25]:

$$V = an - a\sum_{i=1}^{n}\frac{[E]_i}{[E]_0} \quad (1)$$

where a is the fraction volume, n the number of a plateau fraction, and $[E]_i$ is the enzyme concentration in fraction i. Because V is known from Eq. (1), the K_D value was calculated from Eq. (2) [24,25]:

$$K_D = \frac{L}{V - V_0} \quad (2)$$

where L is the amount of the immobilized dye-ligand, and V_0 is the elution volume of a substance which does not interact with the immobilized ligand (void volume).

The K_D values for the two complexes, immobilized ligand-GaDH, are equal to 5.9 μM (BM1) and 15.4 μM (BM2) (Table 2). From the profiles shown in Fig. 3, the capacities of the mimodye adsorbents were also calculated. The respective capacities for immobilised BM1 and BM2 are 2.88 U/0.16 ml adsorbent (18 U/ml adsorbent) and 0.96 U/0.16 ml adsorbent (6 U/ml adsorbent). Therefore, BM1 presents 2.6-fold higher affinity for GaDH than BM2, and three-fold higher binding capacity. These results are unsurprising since BM1 bears galactosamine as the biomimetic moiety which is structurally closer, compared to the shikimate of BM2, to the natural substrate galactose. These experimental findings can also be justified by the computationaly observed greater number of favourable interactions of 2-amino-2-deoxygalactose (BM1) with GaDH, compared to shikimic acid (BM2) [15].

Table 2
Data obtained from analytical affinity chromatography of GaDH (pH 7.0 for BM1 and pH 7.5 for BM2, 4 °C) and used in Eq. (2) for the determination of dissociation constants, K_D ($V_0 = 0.2$ ml)

Immobilized ligand	Adsorbent quantity (mg)	L (μmol)	V (ml)	K_D (μM)
BM1	100	0.19	32.2	5.9
BM2	100	0.18	11.9	15.4

3.3. The design of purification protocols for GaDH from *Pseudomonas fluorescens* and green peas extracts

Since GaDH specific activities higher than those in Table 1, have been reported [26,27], a step of ion-exchange chromatography was introduced prior to the affinity chromatography one. In general, ion-exchange chromatography when added as a preliminary step to the purification procedure [6,14] has proved useful to the purification improvement. Therefore, for the design of an effective purification protocol for GaDH, the ion-exchange chromatography conditions had first to be optimised. DEAE Sepharose CL-6B was chosen as the anion-exchanger, since *P. fluorescens* GaDH has an isoelectric point (p*I*) of 4.28 [26]. As it is evident from Table 3, at 20 mM potassium phosphate buffer, pH 5.5, containing 1 mM EDTA, the anion-exchanger displayed the highest purifying ability (54.1-fold), considerably higher than seen for the alternative conditions tested.

Therefore, a purification protocol for GaDH from *P. fluorescens* extract should feature, as a first step, anion-exchange chromatography (20 mM phosphate buffer, pH 5.5, including 1 mM EDTA) followed by affinity chromatography on immobilised mimodye BM1 or BM2. The results from such typical purification runs are summarised in Tables 4 and 5, respectively. The specific activity of the purified GaDH, using BM1, is equal to 1077 units/mg and is the highest reported so far (see below), while purified GaDH, using BM2, exhibits a specific activity of 854 units/mg. The enzyme preparations obtained from adsorbents BM1 and BM2 were analysed by SDS–PAGE and the BM1-derived material produced a single band, after Coomassie Blue R-250 staining (Fig. 4).

Of the remaining three sources, the purification of GaDH was examined only from green peas, since the other two sources gave discouraging results. As it was mentioned be-

Table 3
DEAE anion-exchange chromatography conditions for GaDH *Pseudomonas fluorescens*

Equilibration buffer potassium phosphate (containing 1 mM EDTA) (pH)	Equilibration buffer potassium phosphate (containing 1 mM EDTA) (mM)	Specific activity (units/mg)	Purification (fold)
5.5	20	59.5	54.1
5.5	10	45.9	41.7
6.0	10	25.0	22.7
6.5	10	21.2	19.3

Washing and elution steps were performed at 100 mM KCl and 120 mM KCl, respectively.

Table 4
Purification protocol of GaDH from *Pseudomonas fluorescens* extract on immobilised mimodye BM1[a]

Step	Volume (ml)	Activity (units)	Protein (μg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	1	0.72	630	1.1	1	100
Anion exchange chromatography (elution with 120 mM KCl)	8	0.70	11.7	60	54.6	97.2
BM1 affinity chromatography (elution with 0.5 mM NAD^+/5 mM Na_2SO_3)	5	0.70	0.65	1077	979.1	97.2

[a] Procedures were performed at 4 °C. For details see text.

Table 5
Purification protocol of GaDH from *Pseudomonas fluorescens* extract on immobilised BM2[a]

Step	Volume (ml)	Activity (units)	Protein (μg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	1	0.74	640	1.1	1	100
Anion exchange chromatography (elution with 120 mM KCl)	8	0.72	12	60	54.6	97.3
BM2 affinity chromatography (elution with 0.8 mM NAD^+/8 mM Na_2SO_3)	5	0.70	0.82	854	776.4	94.6

[a] Procedures were performed at 4 °C. For details see text.

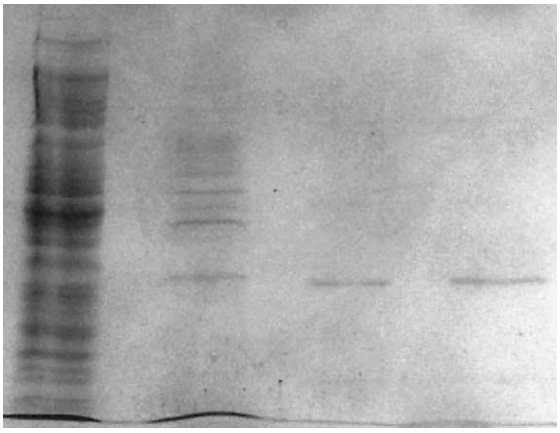

Fig. 4. SDS–PAGE analysis. Performed on a 1.0 mm-thick vertical slab gel containing 10.0% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Blue R-250. Lanes: 1, *Pseudomonas fluorescens* extract (20 μg protein); 2, after DEAE anion-exchange chromatography (5 μg protein); 3, after affinity chromatography on immobilised BM1 (2 μg protein); 4, after affinity chromatography on immobilised BM2 (2 μg protein).

fore (Table 1), GaDH from rabbit liver presented negligible binding to both adsorbents. GaDH from baker's yeast bound to a certain degree on BM1, but when a preliminary DEAE anion-exchange chromatography step was introduced, a significant loss of GaDH activity was observed. When employing a CM cation-exchanger, the purification was marginally improved, but with only moderate recovery of enzyme activity. The results obtained with GaDH from green peas were more encouraging, and allowed for the incorporation of an anion-exchange chromatography step. At pH 7.0, DEAE displayed the higher purifying ability (2.6-fold), whereas considerable fall is observed for the rest of the conditions applied. The results of a typical purification run for GaDH from green peas are summarised in Table 6. This procedure led to 21.9-fold overall purification and 32.1% enzyme recovery.

Galactose dehydrogenase from *P. fluorescens* has been purified in the past using multistep and laborious procedures, for example, after successive steps comprising ammonium sulfate fractionation, bulk separation on CM-Sephadex cation-exchanger and DEAE-Sephadex anion-exchange, hydroxyapatite chromatography, and preparative

Table 6
Purification protocol of GaDH from green peas extract on immobilised BM1[a]

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	20	0.28	66.7	0.0042	1	100
Anion exchange chromatography	20	0.27	24.6	0.011	2.6	96.4
BM1 affinity chromatography (elution with 0.5 mM NAD^+/5 mM Na_2SO_3)	2	0.09	0.98	0.092	21.9	32.1

[a] Procedures were performed at 4 °C. For details see text.

disc electrophoresis, in a five-step protocol [26]. In that study, the specific activity of the starting extract was equal to 1.13 units/mg, while that of the purified enzyme 849 units/mg (750-fold purification, 37% yield). Another multistep purification protocol is essentially the same to the previous one, except that at the final step comprised DEAE-cellulose anion-exchange chromatography [27]. In this case, the starting specific activity was 1.39 U/mg, while that of the purified enzyme 849 U/mg (611-fold purification, 30% yield) [27]. In both cases above, GaDH activity was assayed at 30 °C, whereas in the present study 25 °C was used.

In conclusion, we have described the application of two galactosyl–mimodye-ligands for the affinity chromatographic purification of galactose dehydrogenase. The purification protocols proposed are simple, effective and produce good yields of GaDH of the highest specific activity reported so far.

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