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Purification of an L-arabinose isomerase from *Enterococcus faecium* DBFIQ E36 employing a biospecific affinity strategy

Pedro R. Torres[a,1], Ricardo M. Manzo[b,2], Amelia C. Rubiolo[b,2], Francisco D. Batista-Viera[a,1], Enrique J. Mammarella[b,*]

[a] *Cátedra de Bioquímica, Departamento de Biociencias, Facultad de Química, Universidad de la República, Avenida Gral. Flores 2124, CP 11800 Montevideo, Uruguay*

[b] *Grupo de Ingeniería de Alimentos y Biotecnología, Instituto de Desarrollo Tecnológico para la Industria Química, Universidad Nacional del Litoral (UNL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Colectora RN 168 Km 472 "Paraje El Pozo" S/N, S3000GLN Santa Fe, Argentina*

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ABSTRACT

L-Arabinose isomerase is an intracellular enzyme that can convert L-arabinose to L-ribulose and D-galactose to D-tagatose, a promising but rare nutraceutical. Most of L-arabinose isomerases purified up to date employed the combination between DNA recombinant technology and affinity chromatography based on poly-histidine tail recognition, but few of the enzymes were obtained and purified in a non-recombinant way. For these reasons, a specific affinity bioadsorbent containing L-arabitol as ligand, a competitive inhibitor of the enzyme, was designed and synthesized for achieving pure preparations of the enzyme L-arabinose isomerase from wild-type *Enterococcus faecium* DBFIQ E36 strain, isolated from raw cow milk.

The two-step purification procedure consisted in fractionation by ammonium sulphate precipitation followed by affinity chromatography with obtained bioadsorbent, allowing the purification, to electrophoretical homogeneity, of target enzyme. Characterization studies were performed with purified L-arabinose isomerase in order to increase knowledge of their physicochemical properties. In this sense, enzyme exhibited an optimum temperature of 50 °C and optimum pH of 7.0, maintaining good stability in the ranges 20–45 °C and pH 6.5–8. K_i were calculated, employing D-galactose as substrate, for L-arabitol and L-ribitol, achieving values of 7.9 mM and 183 mM, respectively. K_m and V_{max} values obtained were 35 mM and 81 U mg^{-1} at 50 °C, respectively. Mass spectrometry assay revealed a 48 kDa monomer whereas gel permeation chromatography achieved a 187 kDa molecular weight for native enzyme. Finally, 2D-electrophoresis and isoelectrofocusing analysis revealed an isoelectric point value of 3.80. Results have unveiled both an acidic nature and promising properties for L-arabinose isomerase isolated from *E. faecium* DBFIQ E36.

1. Introduction

L-Arabinose isomerase (EC 5.3.1.4) is an intracellular enzyme that catalyses, *in vivo*, the isomerization of L-arabinose to L-ribulose [7,31,45]. Besides, *in vitro* experiments have shown that this biocatalyst can also convert D-galactose to D-tagatose, a rare sugar with promising nutraceutical properties [3,14]. This ketohexose is of great interest for both technological and nutritional reasons, and the development of many alternative processes for obtaining it, apart from chemical synthesis [1], has been studied [10–13,28,29,38,40,46,47]. In this way, biological conversion of D-galactose to D-tagatose employing the enzyme L-arabinose isomerase is the most economically viable biological D-tagatose manufacturing process because D-galactose can be readily obtained from cheese whey, an industrial by-product produced during cheese elaboration [9,16,24,41]. Biotechnological processes for the production of D-tagatose requires the development of easy purification methods with potential application to obtain L-arabinose isomerases from safe sources.

Many L-arabinose isomerases have been characterized nowadays, from different bacterial species, mainly thermophilic

* Corresponding author. Tel.: +54 342 4511546x1077; fax: +54 342 4511170.
E-mail addresses: fbatista@fq.edu.uy (F.D. Batista-Viera), ejoma@intec.unl.edu.ar (E.J. Mammarella).
[1] Tel.: +598 2 9241806; fax: +598 2 9241906.
[2] Tel.: +54 342 4511546x1077; fax: +54 342 4511170.

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or hyperthermophilic organisms [4,15,17,18,20,23–26,35]. L-Arabinose isomerases from extremophiles, present better isomerization rate and conversion yield but, on the other hand, are difficult to obtain by using non-recombinant methods, mainly due to the intrinsic difficulties in culture of these microorganisms. Enzyme production by mesophilic bacteria was studied in lower extension [19,30,34,36,39,48,50], but recently the fact that most of them are GRAS organisms, which possess better technological applicability for manufacturing either food products or can be utilizing as food ingredients, increased the interest in these L-arabinose sources [21,37].

Certain conventional protocols for purification of L-arabinose isomerases from non-recombinant sources have been studied in several mesophilic bacteria such as *Escherichia coli*, *Lactobacillus plantarum*, *Enterobacter aerogenes*, *Lactobacillus gayonii* and *Mycobacterium smegmatis*. These purification procedures have involved many sequential and time-consuming steps that include salting out, heat treatments and many chromatographic techniques such as ion-exchange, gel filtration, HPLC and hydrophobic interaction chromatography. However, most of the technologically relevant L-arabinose isomerases studied up to date were achieved as heterologous recombinant fusion proteins in which a tag sequence is introduced and acts as target for affinity support recognition and subsequent purification [15,24,37]. In that way, these affinity techniques employed to quickly purify L-arabinose isomerase did not use a specific property of the mentioned enzyme. Biospecific affinity purification procedures were not usually reported for L-arabinose isomerases, although other keto-isomerases (*e.g.* glucose isomerase and fucose isomerase) were successfully purified by this strategy. Finally, others L-arabinose isomerases were produced by recombinant technology but not as fusion proteins, so they were conventionally purified employing traditional purification techniques [5].

In a previous paper authors reported the screening of bacterial strains isolated from raw cow milk in relation to its potential to produce L-arabinose isomerase [27]. As a result, *Enterococcus faecium* DBFIQ E36 was selected as one of the probe strains for enzyme production attending to its characteristics. In this work, a fast and reliable two-step purification procedure employing a specially designed affinity chromatography adsorbent with L-arabitol as ligand, a competitive inhibitor of the enzyme L-arabinose isomerase, was developed for achieving pure preparations of this enzyme from a cell-free extract obtained from a GRAS and non-recombinant *E. faecium* DBFIQ E36 strain. Different physicochemical properties of the enzyme L-arabinose isomerase such as molecular weight, isoelectric point, effect of relevant putative inhibitors and activators and quaternary structure have been determined.

2. Materials and methods

2.1. Materials, sugars and culture media

MRS broth was provided by Difco Laboratories (Detroit, MI, USA). Sepharose 4B, PD-10 (Sephadex G 25) columns, and empty NAP-5 columns were purchased from GE Healthcare (Uppsala, Sweden). Epiclorhydrin, L-arabitol, D-galactose, D-tagatose L-ribitol, D-arabinose, L-arabinose, cysteine hydrocloride, carbazole and bovine serum albumine (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Mercaptoethanol was purchased from Bio-Rad Laboratories (Hercules, CA, USA), bicinchoninic acid (BCA) reagent from Pierce (Rockford, IL, USA), and all other chemicals were of analytical grade.

2.2. Enzyme production

2.2.1. Bacterial strain and culture preservation

E. faecium was chosen as L-arabinose source according to Manzo et al. report [27]. Long-term preservation was done by lyophilization, in order to obtain safe and stable stock culture. *Enterococcus* strain was frozen at −20 °C and −80 °C in MRS supplemented with 15% (*v*/*v*) glycerol [8].

2.2.2. Cell-free extract production

The initial culture was prepared by adding 0.2 mL of frozen stock culture to 5 mL of MRS broth and incubating for 24 h at 37 °C. This culture was transferred to 200 mL of modified MRS broth with the following composition in % (*w*/*v*): 1% meat extract; 0.5% yeast extract; 1% peptone; 0.2% K_2HPO_4; 0.02% $MgSO_4{\cdot}7H_2O$; 0.005% $MnSO_4{\cdot}4H_2O$; 0.2% triammonium citrate; 0.5% sodium acetate trihydrate; 0.108% polysorbate 80; 0.1% D-glucose and 0.5% L-arabinose and incubated at 37 °C for 24 h. The final culture was done by adding 180 mL of the last propagation culture in the late exponential growth phase to 6000 mL of modified MRS broth [3% (*v*/*v*) inocula] and allow growing at 37 °C for 24 h [27]. Cells obtained were harvested by centrifugation at 4000 × *g* for 20 min at 4 °C, resuspended in 500 mL of 50 mM phosphate buffer pH 7.0 (activity buffer) and treated with 1 mg/mL lysozyme for 3 h at 37 °C. Then, the cell suspension was disrupted with ultrasonic vibrations (60 W; 20 kHz; M.S.E.-Mullard Ultrasonic Disintegrator, Mullard Ltd., London, UK) for a period of 40 min at 4 °C and, afterwards, remaining cell debris was removed by centrifugation at 16,000 × *g* for 30 min at 4 °C. Cell-free extract was sterilized by filtration using 0.22 μm pore diameter membranes (Sartorius AG, Göttingen, Germany), alicuoted and stored at −20 °C.

2.2.3. Enzyme assays

L-Arabinose isomerase activity was determined by measurement of the amount of D-tagatose generated from D-galactose. The reaction mixture contained 1 mM $MnCl_2$, 500 mM of D-galactose, 200 μL of enzyme preparation properly diluted and 50 mM phosphate buffer pH 7.0 (activity buffer) to bring the final volume to 1 mL. The assay was done by incubating the reactive mixture and enzyme at 50 °C for 1 h. Subsequently, enzymatic reaction was stopped by cooling the samples on ice. The generated D-tagatose was determined spectrophotometrically at 560 nm by the cysteine carbazole sulfuric acid method [6]. One unit of L-arabinose isomerase activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of keto-sugar per min under the specified conditions. Protein concentration was determined by the bicinchoninic acid method employing BSA as standard [43]. Additionally, the K_i for L-arabitol and L-ribitol (substrate and product analogues, respectively) were determined in the range 0–250 mM performing the enzymatic assay with D-galactose as substrate in the presence of different concentrations of L-arabitol or L-ribitol in the reactive mixture.

2.3. L-Arabinose isomerase purification

2.3.1. Fractionation with ammonium sulphate

The protein of cell-free extract was fractionated by adding solid $(NH_4)_2SO_4$ at 85% saturation, followed by overnight incubation at 4 °C. This fraction was centrifuged at 12,000 × *g* during 30 min at 4 °C and the pellet was dissolved in activity buffer and gel filtered employing a Sephadex G-25 PD-10 column (GE Healthcare, Uppsala, Sweden).

2.3.2. Affinity purification

For the application of this technique, the affinity adsorbent was developed. For activation of the support, 12 g of Sepharose 4B beads

were suspended in 2 M NaOH. Immediately, 0.2 g $NaBH_4$ and 2 mL epichlorohydrin were added to the mixture and incubated during 24 h at room temperature with continuous stirring. Afterwards, suspension was washed sequentially with 2 M NaOH and water until pH 7.0 was achieved. In order to determine the epoxy group content of activated gel, 4 g of epoxy-activated Sepharose was suspended in 10 mL of deionized water and pH was adjusted to 7.0. Then, 15 mL of 1 M $Na_2S_2O_3$ were added and allowed to react for 1 h with continuous stirring. Finally, suspension was back-titrated with 60 mM HCl employing phenolphthalein as indicator. The amount of epoxy groups present is calculated from the amount of HCl required to reach pH 7.0 [42]. Coupling of ligands (L-arabitol, L-ribitol and D-arabinose) was carried out by incubating overnight at room temperature, with permanent stirring, 8 g of epoxy-activated gel in 25 mL of 10% (*w*/*v*) L-arabitol dissolved in 0.5 M NaOH. The gel derivative was filtered in sintered glass filter and washed with 0.5 M NaOH. Then, remaining epoxy groups were blocked by adding 200 μL of 2-mercaptoethanol in 25 mL of 0.5 M NaOH and incubated for 1 h at room temperature. Affinity adsorbent was washed with 0.5 M NaOH and water until neutrality and stored at 4 °C in 100 mM, pH 7.5 phosphate buffer.

Subsequently, L-arabitol-agarose affinity adsorbent was employed for L-arabinose isomerase purification. For this purpose, 2 mL of affinity derivative was packed in a NAP-5 column and equilibrated with 100 mM phosphate buffer pH 7.5. Gel filtered dissolved pellet was applied to column either manually (protocol 1, discontinuous mode) or with a peristaltic pump (protocol 2, continuous mode) and incubated (protocol 1) or recycled at 0.5 mL/min (protocol 2) during 2 h at room temperature. Then, the pass-through fraction was reserved and the column was washed with 2 volumes of the same activity buffer. Elution was performed with 30 mM of L-arabitol in activity buffer. Finally, samples were gel filtered, in order to eliminate competitive inhibitor. All samples achieved after each purification step were monitored by determining absorbance at 280 nm; activity and protein assays of relevant fractions were performed.

2.4. Primary characterization

2.4.1. Electrophoresis and isoelectric focusing

Native polyacrylamide gel electrophoresis (PAGE) was performed on an homogeneous 12.5% Polyacrylamide PhastGel™ gel. Besides, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was done on a 8–25% T polyacrylamide PhastGel™ gradient gel and on an homogeneous 12.5% T polyacrylamide PhastGel™ gel employing the PhastSystem equipment (Pharmacia LKB, Uppsala, Sweden). For molecular weight determination, low-molecular-weight protein markers (14.4–94 kDa) for SDS-PAGE from GE Healthcare was used. PAGE and SDS-PAGE were made following the protocols described by [22]. Isoelectric focusing (IEF) was also performed on Polyacrylamide PhastGelIEF media (gradient, pH 3–10) employing the broad pI calibration kit (3.5–9.3) from GE Healthcare, according to the manufacturer's instructions. For 2D-PAGE, IEF was performed as previously described. Prior to the second dimension, the IEF strips were equilibrated by incubation for 15 min in a solution containing 2% (*w*/*v*) SDS, 50 mmol/L Tris–HCl (pH 8.8), 30% (*v*/*v*) glycerol, and 1% (*w*/*v*) 2-mercaptoethanol. Following equilibration, the strips were placed on 12.5% T SDS-PAGE gels and electrophoresis was made using the PhastSystem apparatus according to manufacturer's instructions. The gels were visualized by silver staining.

2.4.2. Mass spectrometry

Mass spectrum of whole protein was acquired in a 4800 MALDI TOF/TOF (Ab Sciex, Framingham, Massachusetts, USA) in lineal mode (lineal High) and using sinapinic acid as matrix from 10,000 to 150,000 *m*/*z* lineal range using a 200 Hz laser and a wavelength of 355 nm. Peptide mass fingerprinting of protein selected spots was carried out by in-gel sequencing-grade modified trypsin treatment (Promega, Fitchburg, WI, USA) overnight at 37 °C. Peptides were extracted from the gels using 60% (*v*/*v*) acetonitrile in 0.2% (*v*/*v*) trifluoroacetic acid (TFA), concentrated by vacuum drying and desalted using C_{18} reverse phase micro-columns (OMIX Pippete tips, Varian, Palo Alto, CA, USA). Peptide elution from micro-column was performed directly into the mass spectrometer sample plate with 3 μL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 60% (*v*/*v*) aqueous acetonitrile containing 0.2% (*v*/*v*) TFA.

Similarly, mass spectra of digestion mixtures were acquired in a 4800 MALDI-TOF/TOF instrument in reflector mode and were externally calibrated using a mixture of peptide standards (Applied Biosystems, Foster City, CA, USA) including des-Arg1-Bradykinin (*m*/*z* 904), Angiotensin I (*m*/*z* 1296), Glu1-fibrinopeptide B (*m*/*z* 1570), ACTH (1–17, *m*/*z* 2093), ACTH (18–39, *m*/*z* 2465) and ACTH (7–38, *m*/*z* 3657). Collision-induced dissociation MS/MS experiments of selected peptides were performed. Resulting MS and MS/MS spectres were searched with MASCOT software and compared against the available protein sequence database [33].

2.4.3. Gel-filtration chromatography for quaternary structure determination

In order to estimate the molecular weight of the native enzyme, 6 mL of the purified preparation were applied to a preparative column containing the resin Superdex 200, previously equilibrated with 50 mM phosphate buffer pH 7.0. Column was previously calibrated employing high-molecular-weight standard kit (HMW kit, GE Healthcare), where elution volumes of each calibration protein were determined. Elution of the enzyme was performed in the same equilibration buffer and followed measuring the absorbance at 280 nm. Partition coefficient was calculated and molecular weight of the sample was determined from calibration curve.

2.4.4. Properties of soluble enzyme

The influence of pH and temperature on the activity and stability of enzyme was studied. Determination of kinetic parameters (K_m and V_{max}) in the range 30–50 °C was also performed.

2.4.5. Effect of temperature on activity

The influence of temperature on the activity of enzyme was determined in the range of 20–70 °C in activity buffer using D-galactose as substrate.

2.4.6. Thermal stability

The thermostability assays were performed at different temperatures in the range 20–70 °C in activity buffer, by incubating enzyme in a shaking bath. The inactivation kinetics of the enzyme was monitored for 1–24 h by monitoring residual activity employing the enzyme assay described above. Half-lives were calculated according to Arrhenius model.

2.4.7. Optimum pH and pH stability

The influence of pH on the activity of enzyme was studied in the range of pH 4–9 at 25 °C, using D-galactose as substrate. The effect of pH on the stability of the enzyme was determined in the same range by measuring the residual activity after incubating the biocatalysts for 1–10 h at 25 °C.

2.4.8. Determination of kinetic parameters

The kinetic parameters of enzyme were determined with L-arabinose (at 50 °C) and D-galactose (at 30–50 °C) 0–500 mM in activity buffer (pH 7.0). The parameters were calculated according

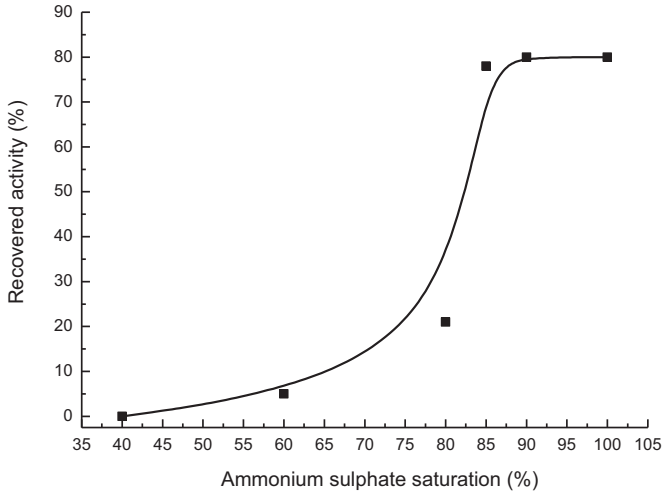

Fig. 1. Enzymatic activity recovery after the precipitation of the protein contained in cell-free extract with different ammonium sulphate concentrations. The results are the average of the assays performed in triplicate.

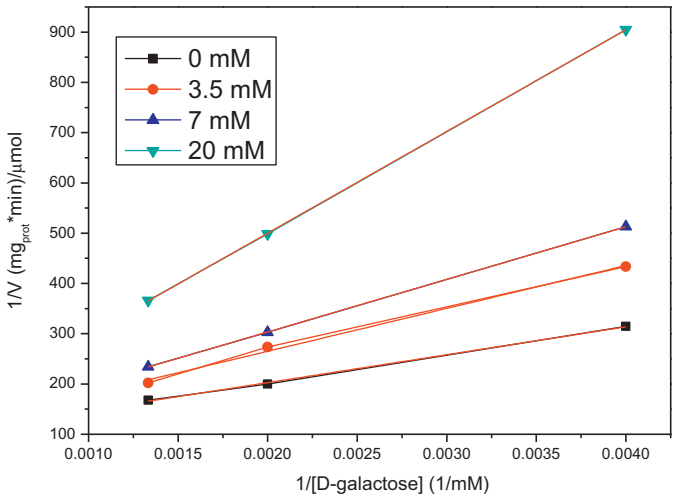

Fig. 2. Lineweaver–burk plots for L-arabitol K_i determination. L-Ribitol graphic is not shown. The results are the average of the assays performed in triplicate.

to Michaelis–Menten model, applying the Eadie–Hofstee linearization procedure.

3. Results and discussion

3.1. Fractionation by ammonium precipitation

As seen in Fig. 1, salting out screening study was performed employing ammonium sulphate saturation percentages from 40 to 100%. After salting out of crude enzyme extracts from *E. faecium* DBFIQ E36 with ammonium sulphate at 60 and 80% saturation, low L-arabinose isomerase recoveries were achieved (9 and 21% respectively). A dramatic increase in enzyme recovery up to 78% was reached at saturation percentages equal or above 85%. However, an increase of this percentage, even at 100% of salt saturation, did not increase this recovery, probably because part of the enzyme activity was present, as enzymatic aggregates, in the foam produced during protein precipitation so, unfortunately, this enzyme could not be recovered completely. In addition, these results clearly reveal the hydrophilic nature of target enzyme owe to the need of a high ammonium sulphate concentration for L-arabinose isomerase salting-out, in concordance with the acidic isoelectric point determined by means of isoelectric focusing. Furthermore, crude enzyme extract had a poor enzyme amount so, salting out step, has allowed concentrating it, which represents an additional advantage when this methodology was applied.

3.2. L-Arabinose isomerase bioadsorbents

Affinity chromatography uses specific binding interactions between biomolecules and immobilized ligands. In that way, for a given research purpose, each specific affinity system will require their own set of conditions revealing its own peculiar challenges.

The inhibitory effects of L-arabitol (substrate analogue) and L-ribitol (product analogue) were studied. The two polyols produced the competitive inhibition of the enzyme. However, L-ribitol showed a very high value of K_i (183 mM) compared with L-arabitol K_i (7.9 mM) when D-galactose was used as substrate, indicating a high affinity that could be exploited for rational affinity bioadsorbent design (Fig. 2).

In that way, a bioadsorbent based on covalent linkage of L-arabitol to an epoxy-activated agarose matrix was developed (Fig. 3). The agarose matrix (Sepharose 4B) had undergone an activation degree of 43 μmol of epoxy groups per gram of filter-dried gel. The support activation degree was selected in order to achieve an L-arabitol concentration that has assured an adequate amount of available ligand for L-arabinose isomerase interaction with the bioadsorbent and, simultaneously reduced undesirable steric restrictions when enzyme is being purified by affinity chromatography.

3.3. L-Arabinose isomerase two-step purification

To study an enzyme, a highly purified native form of the protein must be achieved. Although, a major protein is not so difficult to be purified, a minor one may need several purification steps and high skills on the manipulation of the techniques. In that sense, the methods of purification vary from protein to protein, making impossible to design a general purification strategy valid for all cases. However, in this particular case, most of L-arabinose isomerases reported far are strongly inhibited by L-arabitol [21].

In the adopted strategy, L-arabinose isomerase was purified to homogeneity from a crude extract of *E. faecium* DBFIQ E36 through a two-step procedure including ammonium sulphate precipitation followed by biospecific affinity chromatography using an L-arabitol-agarose adsorbent and employing two different purification protocols. As shown in Table 1, protocol included fractionation by ammonium sulphate precipitation at 85% saturation followed by the affinity chromatography step. An increase of only 5% in $(NH_4)_2SO_4$ saturation (85%) allowed the recovery of 78 of total activity % (against the 21% recovery when precipitation was carried out with 80% saturation) with a slight increase in protein quantity. The selected protocol has made possible to achieve a 4.4 purification fold value, as seen in Table 1. Activity recovery results from salting out step was one of the highest reported up to date, being only surpassed by those reported by [7,48] which obtained an 83% and a 79% yield, respectively, from conventional purification of L-arabinose isomerase from *L. plantarum*. None of the scientific reports which purified the enzyme from recombinant sources and employed salting out as part of their purification protocols achieved values as high as described above [5,17,35].

Besides, a 44% of total activity was achieved when applying a continuous elution mode which depicts more than twice activity yield percentage in contrast with batch elution mode protocol. However, activity recovery after affinity chromatography step decayed at 56% when comparing with ammonium sulphate step which could be attributed to partial loss of activity by enzyme

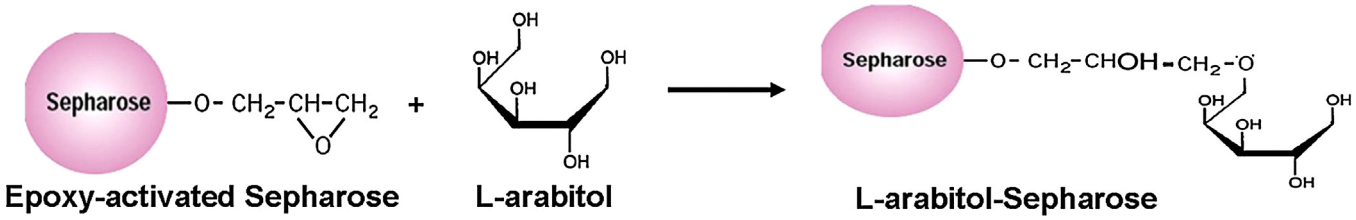

Fig. 3. Graphic summary of the synthesis of L-arabitol-agarose bioadsorbent (specific position of linkage in L-arabitol-agarose is uncertain).

inactivation, probably due to the demanding conditions at which the enzyme was exposed when interaction with the bioadsorbent had occurred.

Furthermore, Table 1 shows that L-arabinose isomerase was purified between 609 and 720 folds in relation with cell-free extract, respectively. A single active fraction was obtained after elution with 35 mM L-arabitol which means that the enzyme was found at low concentrations and was purified to homogeneity from a complex cell-free extract containing many undesirable proteins and other substances, which are present in bigger quantities. These purification steps were only managed from cell-free extracts obtained from wild-type bacteria and purified to homogeneity with conventional techniques as described by [7] and [48]. Besides, the purification factors reported were the highest obtained up to date and this could be possible by means of employing a highly specific interaction between the enzyme and its natural competitive inhibitor, L-arabitol. This also reveals that cell-free extract enzyme concentration is low being necessary to be increased either by improving lytic methods or by culture medium optimization. Moreover, the developed bioadsorbent was regenerated after each purification procedure and reused efficiently for a long time.

Fractions containing L-arabinose isomerase activity obtained by ammonium sulphate precipitation were also applied onto columns containing D-arabinose-agarose and L-ribitol-agarose bioadsorbents based on the covalent immobilization of these ligands, representing similar structures to L-arabinose and L-ribulose, respectively (data not shown). Results have showed that only the L-arabitol-agarose bioadsorbent allowed a successful purification of the enzyme. Furthermore, preliminary kinetics studies indicated that enzyme was not inhibited by its product, fact that could explain the weak bioaffinity adsorption on L-ribitol-agarose, owe to the similarities in their chemical structures.

3.4. Native and SDS-PAGE analysis

Native PAGE electrophoresis was performed on purified L-arabinose isomerase. A single band was seen and the presence of higher molecular weight aggregates was not detected (data not shown). In their native state, this enzyme has been characterized as tetramer [32] and hexamer [44] also being found more rarely as a dimer [37]. Furthermore, L-arabinose isomerase was purified to electrophoretical homogeneity with a single band of 56 kDa on 12.5% T SDS-PAGE gels by affinity chromatography using the synthesized adsorbent, as shown in Fig. 4A. All L-arabinose isomerases from different organisms previously characterized as monomers, were found in the molecular weight range from 52 to 60 kDa [21]. Besides, it can be seen that this band is not present in the pass-through fraction.

3.5. Isoelectrofocusing and 2D-electrophoresis

The pI value turned out to be 3.8 (Fig. 4B), being the lowest of the L-arabinose isomerases reported up to date [25,37]. The acidic pI, generated by the presence of a great number of acidic residues, was in accordance with the high ammonium sulphate concentration needed for salting out of enzyme.

The 2D-electrophoresis has proved that two-step purification method allowed obtaining a single band around 56 kDa with an isoelectric point of 3.80. As previously mentioned, IEF also has shown same isoelectric point as 2D.

3.6. Molecular weight determinations

MALDI-TOF pattern showed a putative monomer of about 48 kDa. The peaks visualized up to 150,000 Da in molecular weight were assigned to monomer, dimer and trimer structure (Fig. 5). The difference between mass values estimated by electrophoretic analysis and mass spectrometry (about 8 kDa) could be due to distortion in electrophoretic behaviour of protein, as reported in literature also for other enzymes and proteins [49]. The presence of a single peak in gel permeation chromatography on Superdex 200 has shown the homogeneity of the purified enzyme obtained by affinity chromatography. The mass of the native enzyme determined by this procedure was 187 kDa, in congruence to that calculated from MALDI-TOF, supposing a homotetrameric structure (Fig. 5). Results of MALDI-MS rendered no match in MASCOT database.

3.7. Enzyme properties

3.7.1. Effects of pH on activity and stability

The effects of pH on activity and stability were determined. L-Arabinose isomerase showed an optimum at pH 7.0, and activity at acidic pH (70% of maximal activity at pH 4.5) was better than activity at alkaline pH (data not shown). The pH stability profile for the enzyme was similar in the pH range 4.0–6.0, with maximum stability at pH 7.0–7.5. The stability decreased moderately at alkaline pH (data not shown). Enzyme retained more than 60% of its activity when incubated 6 h at pH 6.5–8.5. Thus, enzyme presented in this work showed good activity and stability at pH near neutrality and slightly acidic pH, which is technologically important because by-product formation was minimized at acidic pH [5].

Table 1
Two-step purification of L-arabinose isomerase from *E. faecium* DBFIQ E36.

Fraction	Volume (mL)	Total protein (mg)	Total activity (U)[a]	Specific Activity (U mg^{-1})	Purification fold	Yield (%)
Cell-free extract	73	56	14	0.25	1	100
85% ammonium sulphate precipitation	16	10	11	1.1	4.4	78
Affinity chromatography	1	0.034	6.2	180	720	44

[a] Total activity was multiplied per 1×10^3. The results are the average of the assays performed in triplicate.

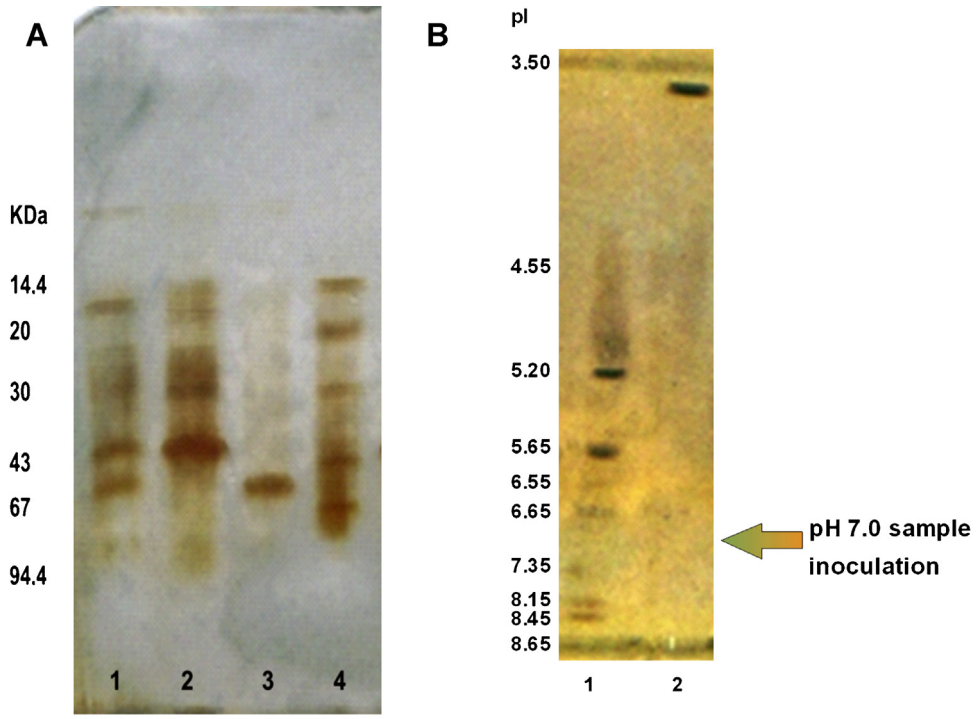

Fig. 4. (A) SDS-PAGE analysis on a 12.5% T polyacrylamide precast gel of different fractions obtained after enzyme purification. Lane 1: redissolved 85% ammonium sulphate precipitate; lane 2: pass-through fraction from affinity chromatography; lane 3: eluted fraction with 30 mM L-arabitol solution; lane 4: molecular mass standards (LMW Marker, GE Healthcare). (B) Isoelectric focusing of the purified *E. faecium* DBFIQ E36 L-arabinose isomerase. Lane 1, standard pI calibration kit; lane 2, affinity chromatography purified enzyme. For assays, 4 μL of each sample were employed. Gels were silver stained according to Blum protocol [2].

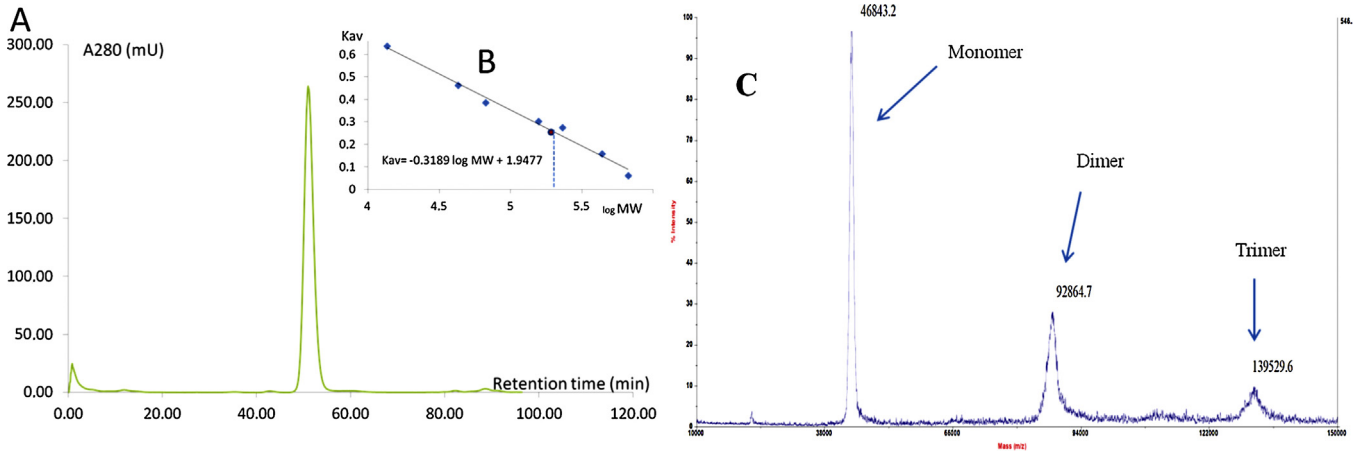

Fig. 5. (A) Elution chromatogram obtained after application of two-step purified L-arabinose isomerase to a Superdex 200 exclusion chromatography column. (B) Gel filtration selectivity curve (red circle: L-arabinose isomerase). Molecular weight standards employed were: ribonuclease (13.7 kDa), egg white albumine (43 kDa), bovine serum albumine (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobuline (669 kDa). (C) Mass spectra of pure L-arabinose isomerase.

3.7.2. Optimum temperature and thermal stability

L-Arabinose isomerase activity showed an optimum at 50 °C, and activity declined over 60 °C (data not shown). The thermal stability profile exhibited that enzyme possesses good stability at 45–50 °C, with half-life of 10 h at 50 °C. The stability strongly decreased in the range 55–70 °C (data not shown).

3.7.3. Kinetic parameters

The K_m for the enzyme was determined at 30 °C, 40 °C and 50 °C using D-galactose as substrate (Table 2). As expected, K_m decreased when temperature increased following the general tendency. Besides, when temperature was increased from 30 °C to 50 °C, decreased almost 3-fold, thus improving the affinity for the substrate, an advantage considering the applications of these biocatalysts. The low value of K_m for D-galactose reinforces the technological interest of this enzyme. Significant differences were observed also in V_{max} (V_{max} for D-galactose at 30 °C was 8 times

Table 2
Kinetic parameters of L-arabinose isomerase from *E. faecium* DBFIQ E36 at different temperatures and employing L-arabinose and D-galactose as substrate.

Substrate	Temperature (°C)	K_m (mM)	V_{max} (U mg^{-1})
L-Arabinose	50	42	30
D-Galactose	30	101	11
D-Galactose	40	71	41
D-Galactose	50	35	81

The results are the average of the assays performed in triplicate.

lower than V_{max} at 50 °C). In a comparative way, kinetic parameters for L-arabinose (the natural substrate of enzyme) were also determined at 50 °C utilizing the same methodology (Table 2). Results revealed that K_m for D-galactose was approximately 3-fold higher than the corresponding K_m for L-arabinose. Nevertheless, the enzyme from *E. faecium* exhibited kinetic advantages with respect to other L-arabinose isomerases reported far in terms of D-galactose biotransformation [4].

4. Conclusion

In this work, an L-arabinose isomerase from wild-type strain *E. faecium* DBFIQ E36 was successfully purified by affinity chromatography using a specific L-arabitol-agarose bioadsorbent conveniently designed. The enzyme (present in low amount in sample applied to affinity column) was purified 720-fold in relation to cell-free extract. Purification factor reported was the highest obtained up to date and this could be possible by means of employing a highly specific interaction between the enzyme and its immobilized natural competitive inhibitor, L-arabitol. This methodology, greatly employed for other industrially relevant keto-isomerases, has several advantages compared with conventional purification techniques or recombinant production of enzyme, regarding reusability of affinity bioadsorbent, time/equipment requirements and safety, respectively.

The purified enzyme exhibited a more acidic pI compared with other L-arabinose isomerases explaining the charged and hydrophilic nature which, in turns resulted in a high salt concentration necessary for enzyme salting-out. Mass spectrometry technique revealed a molecular weight of 48 kDa for monomer whereas molecular mass of native enzyme (tetrameric) determined by gel-filtration was 187 kDa. The enzyme exhibited an optimum pH of 7.0 and optimum temperature of 50 °C, and showed good pH and temperature stability. Preliminary kinetics studies indicated that the enzyme was not inhibited by its product, a technologically attractive characteristic, explaining the weak bioaffinity adsorption on L-ribitol-agarose bioadsorbent.

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