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Preparation and biochemical characterization of magnetic CLEAs of recombinant L-arabinose isomerase from *Enterococcus faecium* DBFIQ E36 as a biocatalyst for D-Tagatose synthesis

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ABSTRACT

Magnetic cross-linked enzyme aggregates (mCLEAs) a novel carrier free immobilization method has recently gained vastly attention. In this work, the synthesis and characterization of a novel mCLEA of L-arabinose isomerase (m-CLEA-LAI) and its application in synthesis of D-Tagatose, a rare sugar of high commercial value, was evaluated. For that, recombinant L-arabinose isomerase from Enterococcus faecium DBFIQ E36 was used for m-CLEA-LAI preparations. The free and immobilized enzymes were characterized by kinetic and thermodynamic assays. The rL-AI immobilized showed a gain in thermal stability and there was an increase in the half-life at all temperatures analyzed. This greater thermal stability was also made clear through the calculation of thermodynamic parameters (ΔH*, ΔG* and ΔS*). ΔH* presented an increase of 1.38 folds compared to the free enzyme. Km values, 392.10 mM and 234.15 mM for free and immobilized enzymes, respectively, represented a reduction in substrate affinity after immobilization, possibly owing to stereo-conformational factors. Hence, the developed magnetic CLEAs of L-Arabinose isomerase shows promise for commercial production of the rare sugar D-Tagatose.

Keywords: Immobilization. Magnetic cross-linked enzyme aggregates. Thermodynamic parameters.

1 INTRODUCTION

D-Tagatose, a natural rare sugar, has been studied intensively as a sucrose substitute, not only because it provides a low caloric value of 1.5–2.4 kcal.g−1 with 92 % sweetness of sucrose[1], but also for many health benefits, including promotion of weight loss[2], prevention of oral disease[3], antidiabetic and prebiotic properties[4]. D-Tagatose can be produced from D-Galactose through chemical synthesis, a calcium catalyst, and biotechnological routes using L-Arabinose Isomerase (L-AI, EC 5.3.1.4)[5]. L-AI catalyzes L-Arabinose as the optimum substrate to the ketose isomer, L-Ribulose, and may also catalyze the isomerization of D-Galactose to D-Tagatose, based on the similarity in the configuration of the substrate[6].

However, soluble enzymes still present some challenges for industrial use, such as their high cost, difficult reuse, and low stability under operational conditions[7]. Enzyme immobilization is a technique that has been widely used to enhance enzyme properties, such as stability and specificity. The method of Cross-linked Enzyme Aggregates (CLEAs) involves protein precipitation and subsequent cross-linking using bifunctional agents for immobilization, without the need for a carrier[8]. Additionally, the CLEAs method combines both the purification and immobilization of enzymes in one-step, making it highly promising for commercialization[8]. To improve CLEAs stability for repeated usage in continuous processes and ease of CLEAs separation from the reaction media, CLEAs modification by adding magnetic nanoparticles and amino-functionalized magnetic nanoparticles could be performed[9].

In this work, magnetic CLEAS of a recombinant L-arabinose isomerase from Enterococcus faecium DBFIQ E36 (rL-AI) was prepared to increase thermotolerance and catalytic activity. Here, we characterize the activity and conformation of both soluble and immobilized enzymes in response to temperature, as well as to analyze their properties and kinetic parameters. The findings of this research will enable us to optimize their catalytic efficacy, thermal and operational stabilities, and recovery of the heterogeneous biocatalysts utilized in industrial processes.

2 MATERIAL & METHODS

Culture Medium and Assay Conditions: For Bacterial Strain, construction and conservation of the recombinant L-AI Escherichia coli BL21 (DE3) was transformed with the vector harboring the recombinant araA gene of *Enterococcus faecium* DBFIQ E36 by Manzo et al [10]. Transformed *E. coli* BL21 cells were reactivated in Luria–Bertani (LB_{amp}) in a rotary shaker at 37 °C overnight. The expression of rL-AI was conducted by auto-induction following the methodology proposed by Studier (Studier, 2005) using residual whey lactose as described on a previous work[11.]. At the end of assay, cultures were centrifuged at 5000 rpm at 4 °C for 30 min and cells were subsequently washed with 0.1 M NaCl solution followed by centrifugation at 5000 rpm at 4 °C for 30 min.

Cell Lysis and Extraction of rCA: Cell pellets were resuspended in 50 mM Tris HCl pH 8.0 and 150 mM NaCl buffer and cell lysis were conducted by sonication (Sonicator Qsonic, Q500 Sonicators, USA) at 30% of amplitude, for 4 min (working time and interval of 3 s) at 4 °C. Cell suspension was sonicated until a transparent pale yellow color was obtained. After cell disruption, samples were centrifuged at 5000xg, 4 °C for 30 min and supernatant (crude CA extract) was stored at − 20 °C and further use.

Protein Quantification: Protein concentration was determined by the Bradford method [12] using bovine serum albumin as standard for calibration curve construction.

Enzyme Purification: Purification of crude enzyme extracts was performed by immobilized metal affinity chromatography (IMAC) using Ni-Sepharose 6 Fast Flow matrix with a degree of activation of 15 µmol of Ni^{2+} ion per ml of wet resin (GE Healthcare as described previously[11].

Production of magnetic cross-linked enzyme aggregates (m-CLEA) of rL-AI: Magnetic cross-linked enzyme aggregates of L-arabinose isomerase (m-CLEA-L-AI) were prepared according to previously work with slightly modifications[13].

Enzymatic Activity Assay: The 0.1 g of m-CLEA-LAI was resuspended in 1 mL of 50 mM Tris-HCl pH 8.0 and 150 mM NaCl buffer. For assessing the catalytic activity, a reaction mixture containing 0.1 mL of rL-AI extract or immobilized rL-AI suspension and 0.4 mL of 625 mM D-Galactose in 50 mM sodium acetate buffer pH 5.6 supplemented with 1 mM $MnCl_2$ was prepared. Afterwards, the mixture was incubated at 50 °C for 60 min and D-Tagatose was determined by using the cysteine–carbazole–sulfuric acid method at 560 nm [14].

Protein melting temperature of rL-AI and m-CLEA-LAI: The protein melting temperature of free and immobilized rL-AI were determined using Tycho TM NT.6 instrument (NanoTemper Technologies, Munich).The capillaries were filled with 10µL sample and placed on the sample holder. A temperature gradient of 30°C.min—1 from 25 to 95 °C was applied and the intrinsic protein fluorescence at 330 and 350 nm was recorded. The brightness ratio 350 nm / 330 nm was used as a measure for a spectral shift in the emission profile of the Tryptophan (Trp) residues.

Thermodynamic parameters: The thermodynamics of the thermo-inactivation of soluble and immobilized L-arabinose isomerase was determined using the rate constants for deactivation (kd) at different temperatures (50–70 °C) and the energy barriers of the thermal inactivation (Ea_{in}) previously calculated. ΔH*, ΔG*, and ΔS* represent enthalpy, free energy, and entropy of inactivation respectively and were determined as proposed by Torabizadeh and colleagues[15].

Kinetic parameters: The kinetic parameters of rL-AI and m-CLEA-LAI were determined in 50 mM Tris HCl pH 8.0 and 150 mM NaCl buffer by varying the concentration of substrate (100–800 mM) at 50 °C. Kinetic results were obtained by fitting the data with a Michaelis-Menten equation using the software Origin 8.5.

3 RESULTS & DISCUSSION

Protein melting temperature of rL-AI and m-CLEA-LAI: Figure 1 shows the folding state transition of rLAI and m-CLEA-LAI by monitoring the ratio of fluorescence intensity at 330 nm and 350 nm as a function of temperature, where Tm (inflection temperature of thermal unfolding) were 68.2 °C and 83.9 °C for rLAI and m-CLEA-LAI, respectively. The average inflection temperature observed in the rLAI is slightly different from the melting temperature observed by the circular dichroism technique (74.19 °C), which it is due to the basic principle of each technique being different. This increase in resistance of temperature is a result of larger rigidity of the m-CLEA-LAI structure caused by additional linkage between enzyme molecules. It is known that the linkages formed by the reaction of glutaraldehyde and the amino group of the enzyme have good stability against high temperature and a wide range of pH values [16, 17].

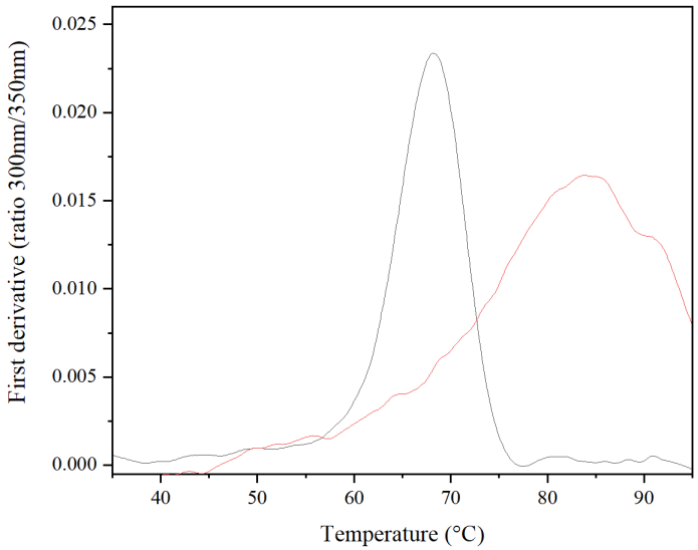

Figure 1 First derivative of fluorescence intensity ratio of (—) rL-AI and (—) m-CLEA-LAI

Thermodynamic parameters: The thermodynamic parameters are presented in Table 1. These results indicated that immobilization process increases about 38.04% in ΔH* and a slightly improvement of 2.64% in the Gibbs free energy (ΔG*). The large positive enthalpic term is associated with a more stable enzyme, as a larger amount of energy is necessary for the process of inactivation to occur, which is confirm by the higher ΔG* that is associated with more tolerance toward heat inactivation [18]. These results shows higher thermal stability for the immobilized enzyme. One possible explanation for the increasing

thermostability of rL-arabinose isomerase after immobilization could be the formation of new bonds by the Schiff base formation between lysine residue and glutaraldehyde forming strong covalent bonds, which imparts rigidity to the enzyme molecule [9, 15].

Table 1 Thermodynamic parameters of soluble and immobilized L-arabinose isomerase calculated at 50 °C.

Biocatalyst	E_a (KJ/mol)	ΔH^* (KJ/mol)	ΔG^* (KJ/mol)	ΔS^* (KJ/mol.K)
Soluble	149.96 ± 0.65	147.27 ± 2.04	78.20 ± 0.47	0.21 ± 0.01
m-CLEA-LAI	205.98 ± 0.73	203.29 ± 1.96	80.26 ± 0.89	0.38 ± 0.01

Kinetic parameters: The apparent Km value of rL-AI was decreased from 392.11 to 234.15 mM upon immobilization in mCLEA, suggesting a higher affinity of immobilized L-AI for the substrate (Table 2). This decrease in the Km value after immobilization by m-CLEAs method can confirm the claim that, this kind of enzyme immobilization should stabilize the quaternary structure of multimeric enzyme, such as L-arabinose isomerase, may due to appropriate subunit assembly of the enzyme[19]. However, the apparent V_{max}, K_{cat} and K_{cat}/K_m values of rL-AI was observed to be decreased upon immobilization, indicating reduced catalytic potential of the immobilized catalyst. These results can be explained because immobilization in most cases will produce slight distortions in the enzymes structure, and this may alter the final properties of the enzyme[20]. According to Daniel there is an interrelationship of both conformational stability and enzymatic activity, thus excessive stability may result in lower activity[21].

Table 2 Kinetic parameters of soluble and immobilized L-arabinose isomerase.

Biocatalyst	$[E_0]$ (μmol)	V_{max} (U/mg)	K_m (mM)	K_{cat} (1/min)	K_{cat}/K_m (1/min.mM)
Soluble	0.002	0.71 ± 0.13	392.11 ± 147.88	198.41 ± 15.81	0.51 ± 0.11
m-CLEA-LAI	0.0071	0.47 ± 0.03	234.15 ± 47.64	52.65 ± 4.23	0.22 ± 0.09

4 CONCLUSION

Magnetic CLEAs of recombinant L-Arabinose isomerase was successfully prepared and demonstrated a high recovery activity. The thermodynamic studies revealed that, the energy barriers of the thermal inactivation also enthalpy of the inactivation were raised about 1.4 folds which represents an increase in thermostability of the enzyme after immobilization. Hence, magnetic cross-linked L-Arabinose isomerase aggregates are an efficient and simple promising biocatalyst for the industrial manufacturing of D-Tagatose.

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