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Response surface methodology to optimize partition and purification of two recombinant oxidoreductase enzymes, glucose dehydrogenase and D-galactose dehydrogenase in aqueous two-phase systems

Hamid Shahbaz Mohammadi [a,b,1], Seyede Samaneh Mostafavi [b,1], Saeideh Soleimani [b], Sajad Bozorgian [b,*], Maryam Pooraskari [c,*], Anvarsadat Kianmehr [d,*]

[a] Department of Biochemistry, Genetic and Metabolism Research Group, Pasteur Institute of Iran, Tehran 13164, Iran
[b] Hormoz Zist Enzyme Company, Bandar Abas, Hormozgan, Iran
[c] Department of Microbiology, Islamic Azad University, East Tehran Branch, Tehran, Iran
[d] Department of Medical Biotechnology, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Oxidoreductase are an important family of enzymes that are used in many biotechnological processes. An experimental design was applied to optimize partition and purification of two recombinant oxidoreductase, glucose dehydrogenase (GDH) from *Bacillus subtilis* and D-galactose dehydrogenase (GalDH) from *Pseudomonas fluorescens* AK92 in aqueous two-phase systems (ATPS). Response surface methodology (RSM) with a central composite rotatable design (CCRD) was performed to optimize critical factors like polyethylene glycol (PEG) concentration, concentration of salt and pH value. The best partitioning conditions was achieved in an ATPS composed of 12% PEG-6000, 15% K_2HPO_4 with pH 7.5 at 25 °C, which ensured partition coefficient (K_E) of 66.6 and 45.7 for GDH and GalDH, respectively. Under these experimental conditions, the activity of GDH and GalDH was 569.5 U/ml and 673.7 U/ml, respectively. It was found that these enzymes preferentially partitioned into the top PEG-rich phase and appeared as single bands on SDS–PAGE gel. Meanwhile the validity of the response model was confirmed by a good agreement between predicted and experimental results. Collectively, according to the obtained data it can be inferred that the ATPS optimization using RSM approach can be applied for recovery and purification of any enzyme of oxidoreductase family.

Introduction

Oxidoreductases are a family of enzymes that catalyze transfer of electrons from a donor to an acceptor molecule, generally using nicotinamide adenine dinucleotide phosphate (NADP) or nicotinamide adenine dinucleotide (NAD^+) as cofactors [1]. Among this group, NAD-harboring enzymes such as glucose dehydrogenase and D-galactose dehydrogenase are the most industrially attractive. These cofactor-dependent enzymes catalyze the oxidation of their substrates by transferring electrons to an oxidized NAD^+ [2]. D-Galactose dehydrogenase (GalDH; EC 1.1.1.48) catalyzes the dehydrogenation reaction of β-D-galactopyranose in the presence of NAD^+ to D-galacto-1,5-lactone and NADH. It has been identified in plants (e.g. green peas and *Arabidopsis thaliana*), algae (e.g. *Iridophycus flaccidum*), bacteria and mammals [3]. GalDH has been received much attention for the measurement of β-D-galactose, α-D-galactose and lactose as well. The enzyme has been used in diagnostic kits to screen blood serum of neonates for galactosemia diseases [4,5]. Galactosemia is an inborn metabolic disorder that without strict dietary control results in mental retardation, microcephaly and seizures. Newborn screening using GalDH is a simple method which has proved sensitive, reliable, rapid and cheap compared to other methodologies [6]. Glucose dehydrogenase (GDH, EC 1.1.1.118) is the first enzyme in a variant of the Entner–Doudoroff pathway, involving nonphosphorylated intermediates, which is utilized as the central hexose catabolic pathway. It catalyses the oxidation of D-glucose to D-glucono-1,5-lactone and NADH via NAD+ as cofactor [7]. GDH has been identified from different sources such as *Sulfolobus solfataricus*, *Thermoplasma acidophilum*, and *Bacillus* species [8]. The important application of this biocatalyst includes enzymatic determination of blood glucose level,

* Corresponding authors. Tel.: +98 9183139324 (A. Kianmehr).
E-mail addresses: sajad_bozorgian@yahoo.com (S. Bozorgian), maryam_porasgari@yahoo.com (M. Pooraskari), kiabiotpro@yahoo.com (A. Kianmehr).
[1] These authors contributed equally to this work.

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cofactor regeneration as well as enzymatic production of gluconic acid [9,10]. Due to the facts that GalDH and GDH are important enzymes for diagnostic applications, different efforts have been done to recovery and purification of these biocatalysts [5,8].

However, these techniques that generally are chromatography methods present some disadvantages including high costs and low yield. Therefore, developing the efficient and scalable alternative methods to perform high yield separation of these enzymes is of great interest. Liquid–liquid extraction using aqueous two-phase systems (ATPS) has been employed for recovery and purification of many industrial enzymes [11,12]. When two aqueous solutions of certain incompatible substances, such polyethylene glycol (PEG) and dextran or PEG and salt, are mixed above a critical concentration, two phase separation occurs [13]. Separation techniques based on the two-phase partitioning have proved to be suitable tools for recovery of bio-molecules. Some successful applications of ATPS on industrial scales have also been demonstrated [14,15]. Compared with the traditional techniques, ATPS has the advantages such as high biocompatibility, high resolution, and easy to scale-up [16]. However, the partition of compounds in ATPS is very complex due to the several factors including the characteristics of proteins and environmental conditions of system [17]. The classical optimization approach varying the level of one parameter at a time, while keeping the rest of the variables constant, is generally time-consuming. For this reason, mathematical modeling that can predict the protein partition behavior and provides insights into the protein partitioning mechanism is of critical importance [18]. An effective statistical technique is the response surface methodology (RSM) which is a useful statistical tool where several independent variables influence the responses [19]. The main advantage of RSM is the reduced number of tests needed to calculate multiple factors and their interactions [20]. In this work, we used two recombinant enzymes of oxidoreductase family; glucose dehydrogenase (GDH) from *Bacillus subtilis* and D-galactose dehydrogenase (GalDH) from *Pseudomonas fluorescens* AK92 to evaluate the RSM method for partition optimization of theses biocatalysts in ATPS. This is the first report studying the partitioning behavior of GDH and GalDH in ATPS.

Experimental

Materials

Polyethylene glycols (PEGs) with different molecular weights (MWs) were purchased from Merck (Darmstadt, Germany). D-Galactose, glucose and NAD^+ were obtained from Sigma–Aldrich (St. Louis, MO, USA) and utilized in the enzyme activity assay. The salts and all other chemicals applied were of analytical grade. Restriction endonucleases, DNA modifying enzymes and molecular mass markers for electrophoresis were purchased from Fermentas (Germany).

Production of recombinant B. subtilis GDH

Genomic DNA from *B. subtilis* was used as a template for PCR amplification of *gdh* gene using the following primers: sense, 5′-GTCGACATGTATCCGGATTT-′3 and antisense, 5′-AAGCTTT-TAACCGCGGCC-′3. The introduced restriction sites *Sal*I (forward) and *Hind*III (reverse) are underlined. PCR was performed under program: 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 51 °C, 40 s at 72 °C with a final elongation step at 72 °C for 10 min. The obtained PCR product was purified from an agarose gel, digested with *Sal*I and *Hind*III and cloned into the pET-28a (+) vector (Invitrogen). The resulting expression vector, named pET-28aGDH, was introduced into *Escherichia coli* BL-21 (DE3), and the recombinant cells were cultured in Luria–Bertani (LB) medium containing 40 μg/ml kanamycin at 37 °C. Expression of GDH was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD600 = 0.6–0.8 for 5 h at 37 °C. The cells were harvested by centrifugation (4000 rpm 1 h), washed with 50 mM Tris–HCl buffer (pH 8.0) and lysed by sonication [9].

Production of recombinant P. fluorescens AK92 GalDH

Primers for PCR amplification were designed based on the available nucleotide sequence of GalDH from the *P. fluorescens* genome using DNASIS MAX software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The coding gene *gdh* (accession number KF358694) was amplified by PCR with the forward primer GDHFw (5′-T*GGATCC*ATGCAACCGATTCGTCTCG-′3) and the reverse primer GDHRev (5′-GCG*AAGCTT* TTAATCGTAGAACGGC-′3), which contained the restriction sites for *Bam*HI and *Hind*III, respectively. PCR amplification was performed under condition: preincubation at 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 61 °C, 1 min at 72 °C with a final elongation step at 72 °C for 10 min. The PCR reaction product was cut with *Bam*HI and *Hind*III, and ligated into the pET-28a (+) expression vector. The construct bearing the *gdh* gene was named pET28aGDH and transformed into the *E. coli* BL-21 (DE3). A recombinant strain of *E. coli* BL21 (DE3) was grown overnight in LB medium containing 40 μg/ml of kanamycin at 37 °C and 150 rpm until an cell density of OD600 = 0.6–0.8 was reached. GalDH expression was induced by the addition of 0.7 mM sterile IPTG. After 5 h induction at 37 °C, cells were harvested and stored at −20 °C for further use. Pelleted *E. coli* cells were suspended in lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0), mechanically broken by sonication using a pulse sequence of 15 s on and 10 s off and clarified by centrifugation at 4000 rpm for 1 h. The supernatant were employed as a crude enzyme in the partition experiments [21].

ATPS preparation

ATPS were prepared by mixing required amounts of PEG-6000, K_2HPO_4, and cell culture supernatant. A final weight of 10 g system was obtained by adding a sufficient amount of 0.1 M potassium phosphate buffer (pH 7.4). Systems were agitated for 30 min at room temperature and then centrifuged at 2000×*g* rpm at 25 °C for 40 min to speed up the phase separation. The volumes of the phases were determined, and the samples from the two phases were carefully tested for enzyme activity and total protein concentration. To avoid interference of the phase components, samples were analyzed against blanks containing the same compositions,

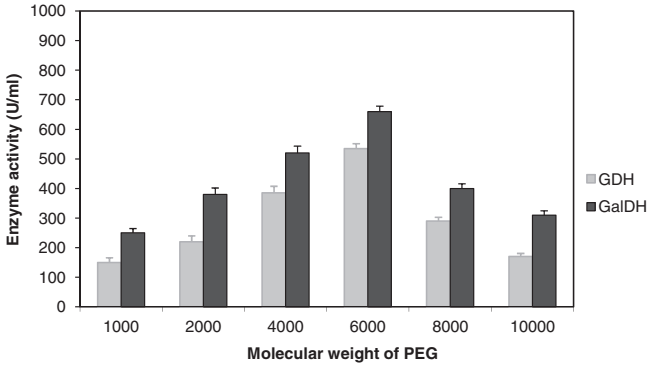

Fig. 1. Influence of PEG MW on the activity of GDH and GalDH in ATPS composed of 11.5% (w/w) PEG-4000 and 14% (w/w) K_2HPO_4 at pH 7.5. The experiments were done in triplicate to estimate experimental errors.

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