Journal of Applied Life Sciences International
2(1): 1-8, 2015; Article no.JALSI.2015.001

SCIENCEDOMAIN *international*
www.sciencedomain.org

Expression in *Escherichia coli* and Purification of Microbial Enzymes for the Production of Enzymatic Analytical Kits to Measure Relevant Metabolites

Patrícia I. P. Ponte[1], Catarina I. P. D. Guerreiro[1], Vânia Fernandes[1], André T. Fernandes[1], Carlos M. G. A. Fontes[1,2], Luís M. A. Ferreira[1,2] and José A. M. Prates[1,2*]

[1]NZYTech - Genes and Enzymes, Estrada do Paço do Lumiar, Campus Lumiar, Edifício E, R/C, 1649-038 Lisboa, Portugal.
[2]CIISA, Faculdade de Medicina Veterinária, ULisboa, Avenida da Universidade Técnica, Pólo Universitário do Alto da Ajuda, 1300-477 Lisboa, Portugal.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI:10.9734/JALSI/2015/13113
Editor(s):
(1) Shahla Alalaf, Hawler Medical University, College of Medicine, Department of Obstetrics and Gynaecology.
Reviewers:
(1) Anonymous, Institute of Biophysics and Biomedical Engineering, Bulgaria.
(2) Subramanian Krishnan, Pediatrics and Infectious diseases, Children's Hospital Los Angeles, USA.
(3) Anonymous, the University of Samarra, Iraq.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=683&id=40&aid=6294

Original Research Article

Received 4th August 2014
Accepted 30th August 2014
Published 30th September 2014

ABSTRACT

Aim: The present study describes the procedure for the isolation, cloning, expression and purification of eight different microbial enzymes required for the assembly of a comprehensive range of enzymatic test kits.
Methodology: The genes encoding the enzymes were isolated through Polymerase Chain Reaction or synthesized *In vitro* and inserted into a variety of prokaryotic expression vectors. The resulting recombinant proteins were expressed at high levels in *Escherichia coli* cells and purified following different chromatographic approaches. The catalytic activity of each individual enzyme was determined.
Results: Eight enzymes (aspartate aminotransferase, EC 2.6.1.1; citrate synthase, EC 2.3.3.1; glucose-6-phosphate dehydrogenase, EC 1.1.1.49; hexokinase, EC 2.7.1.2; L-malate

**Corresponding author: Email: japrates@fmv.ulisboa.pt;*

dehydrogenase, EC 1.1.1.37; D-malate dehydrogenase, EC 1.1.1.83; and glucose-6-phosphate isomerase, EC 5.3.1.9) were engineered to contain a His_6-tag, cloned and expressed in *E. coli* BL21(DE3) strain, which was grown in auto-induced LB medium. Recombinant proteins expression, solubility and yields were analyzed. All recombinant proteins were expressed in soluble form with expression induction at 37°C, although the induction of expression of citrate synthase at 20°C resulted in higher levels of soluble protein. The recombinant proteins were captured by Immobilized Metal-Affinity Chromatography and polished by gel filtration. All proteins were purified from most *E. coli* contaminants (>95%) and were obtained at high concentrations (2-17 g/L). Specific activities varied from 2.1 to 1220 U/mg, depending on the enzyme.
Conclusion: The methodology presented here is suitable for the expression and recovery of highly purified recombinant enzymes for the development of enzymatic analytical kits, with relevant agricultural, biomedical and industrial applications.

Keywords: Recombinant enzymes; analytical kits; protein expression; Escherichia coli.

1. INTRODUCTION

Enzymes are biological catalysts responsible for all metabolic processes that sustain life. They are highly selective catalysts, greatly accelerating the rate of metabolic reactions, from the digestion of food to the synthesis of DNA [1]. For an enzyme to be used for analytical purposes, it must be of exceptional purity as individual enzymes are generally found along with many other molecules and these impurities must be removed before the enzyme is of any use [2,3]. Moreover, analytical enzymes are often required in large amounts, thus requiring cheap and rapid production and purification processes.

Engineered affinity tags can facilitate very efficient purification of recombinant proteins, resulting in high yields and purities in a few standard steps. Polyhistidine tag, or His-tag, is undoubtedly the most common tag used for protein purification worldwide [4]. The primary advantage of this tag derives from its short sequence (6 to 10 histidine residues) that typically has minimal or no effect on the target protein structure or function. The small size of the tag allows it to be trivially added to either terminus of a given target protein. In addition, the His-tag does not require a specific fold in order to function, making it highly reliable in all major expression systems [5]. Finally, the simplicity associated with the purification of His-tagged proteins through Immobilized Metal-Affinity Chromatography (IMAC) usually results in high levels of protein purity.

Food is a complex type of sample and hence is difficult to analyze. The high specificity of enzymes enables the implementation of enzymatic analysis in complex sample matrixes without complicated sample preparation techniques [2]. This makes enzymatic food analysis a highly valuable tool because it saves time, reduces costs and gives reliable results independent of the sample matrix. Additionally, enzymatic methods use non-hazardous reagents, are environmentally friendly and can be automated for in-line process monitoring. In line with this, many enzymatic methods have been approved by European Community regulations and recommended by several Food and Beverages International Federations [3,6].

Analytical kits are usually performed using spectrophotometric methods, either in the ultraviolet region (UV tests) or in the visible region (colorimetric tests) [2,6]. UV tests are usually based on the $NAD(P)^+$/NAD(P)H system, with the production or consumption of NAD(P)H, which strongly absorbs the UV radiation at 340 nm (extinction coefficient of 6300 $M^{-1}cm^{-1}$). The principle of colorimetric tests is based on a chromogenic reaction with the formation of a colored compound, absorbing in the visible region of the electromagnetic spectrum. The colored compound results from the interaction between the product of a first enzymatic reaction and a chromogenic compound.

Here we describe the methodology used for the expression in *E. coli*, purification and biochemical activity of a large array of microbial enzymes required for the assembly of a comprehensive range of analytical kits. These enzymatic kits could be used for the determination of relevant metabolites in foodstuffs, such as wine, beer, bread, fruit and vegetable products, fruit juice, as well as in cosmetics, pharmaceuticals and biological samples.

2. MATERIALS AND METHODS

2.1 Isolation of Microbial Genes through PCR

The *Escherichia coli* genes encoding the enzymes aspartate aminotransferase (AST; EC 2.6.1.1), citrate synthase (CS; EC 2.3.3.1), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), hexokinase (HK; EC 2.7.1.2), L-malate dehydrogenase (LMDH; EC 1.1.1.37), D-malate dehydrogenase (DMDH; EC 1.1.1.83) and glucose-6-phosphate isomerase (PGI; EC 5.3.1.9) were isolated through polymerase chain reaction (PCR). *E. coli* genomic DNA was isolated using the NZY Tissue gDNA Isolation kit (NZYTech Ltd). Genes were amplified using the thermostable polymerase NZYProof (NZYTech Ltd) from *E. coli* genomic DNA and the primers listed in Table 1. PCR was performed as follows: preheating at 95ºC for 3 min, 25 cycles at 95ºC for 20 s, 55ºC for 30 s and 72ºC for 60 s, followed by a final elongation period at 72ºC for 10 min. The amplified products were recovered from a 1% agarose gel using the NZYGelpure Kit (NZYTech Ltd) and ligated into pNZY28 (NZYTech Ltd) to generate pPP1 to pPP7, respectively. The DNA inserts cloned in plasmids pPP1 to pPP7 were sequenced to ensure that no mutations occurred during the PCR. These plasmids were used for later sub-cloning into the prokaryotic expression vector.

2.2 Gene Synthesis of Microbial Genes

The gene encoding the acetyl-CoA synthetase (ACS; EC 6.2.1.1) of *Bacillus subtillis* was designed with codon utilization optimized for expression in *E. coli*. The gene, which included *Nhe*I and *Xho*I at its 5' and 3' ends, respectively, to facilitate the subsequent sub-cloning, was synthesized through oligo assembly by PCR following the protocol described by Young and Dong [7]. The resulting nucleic acid was separated through electrophoresis, eluted from agarose and cloned into pNZY28 following the protocol described above. Ten plasmids were sequenced to screen for a positive clone containing the integral Acetyl-CoA synthetase encoding gene. The data revealed that out of 10, two plasmids contained the required gene without any accumulated mutation. One of the positive clones was selected and the resulting plasmid was termed pPP8.

2.3 Cloning of the DNA Sequences into Prokaryotic Expression Vectors

The sequences of the primers used in this study and the gene synthesized in vitro included *Nhe*I and *Xho*I restriction sites (see Table 1; sequences in bold). Plasmids pPP1 to pPP8 were digested with NheI and XhoI, separated through agarose gel electrophoresis and the excised genes were purified as described above. The isolated genes were cloned into the expression vector pET21a (Novagen), previously digested with the same restriction enzymes. This vector carries a T7*lac* promoter and the resulting recombinant proteins contained a C-terminal His_6-tag to facilitate purification. The resulting plasmids were termed pET21a_1 to pET21a_8, respectively.

Table 1. Primers used in Polymerase Chain Reactions (PCR). The nucleotides in bold represent engineered restriction sites, which were used to clone the amplified DNA sequences into the expression vector pET21a

Enzyme	Primers
Aspartateaminotransferase	*5´*- CTC**GCTAGC**TTTGAGAACATTACC *-3´*
	5´- CAC**CTCGAG**CAGCACTGCCACAATC -3´
Citratesynthase	*5'*- CTC**GCTAGC**GCTGATACAAAAGCAAAAC *-3'*
	5'- CAC**CTCGAG**ACGCTTGATATCGCTTTTA *-3'*
Glucose-6-phosphate dehydrogenase	*5´*- CTC**GCTAGC**GCGGTAACGCAAACAG *-3´*
	5´- CAC**CTCGAG**CTCAAACTCATTCCAG -3´
Hexokinase	*5´*- CTC**GCTAGC**AAAATCAGTCGGGAAACAC *-3´*
	5´- CAC**CTCGAG**TTGCGCAAAGGGATTTTTC -3´
L-Malatedehydrogenase	*5´*- CTC**GCTAGC**AAAGTCGCAGTCCTC *-3´*
	5´- CAC**CTCGAG**CTTATTAACGAACTC -3´
D-Malatedehydrogenase	*5´*- CTC**GCTAGC**ATGAAAACGATGCGTATTGC *-3´*
	5´- CAC**CTCGAG**ACGCAAAATAATTTTGCAAATC -3´
Glucose-6-phosphate isomerase	*5´*- CTC**GCTAGC**AAAAACATCAATCCAACGC *-3´*
	5´- CAC**CTCGAG**ACCGCGCCACGCTTTATAG -3´

2.4 Expression of Recombinant Enzymes

To optimize expression of the various recombinant proteins, different growing and induction conditions were tested for a variety of recombinant *E. coli* strains. The *E. coli* strains used in this study were BL21(DE3), BL21(DE3)pLysS, Tuner™(DE3) and Origami™(DE3) (Novagen). In addition, levels of recombinant gene expression were tested when cell were grown in two different culture media: LB-broth and NZY Auto-Induction LB medium (NZYTech Ltd). The different *E. coli* strains were transformed with the 8 recombinant plasmids following standard procedures. Cells cultivated in Luria broth (LB) medium were grown at 37ºC to mid-exponential phase (OD_{595}=0.6) and the medium was supplemented with 100 µg/mL ampicillin. At this point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM (Tuner cells) or 1 mM IPTG for the other strains and the cultures were incubated for different periods at different temperatures to identify the best expression conditions. Cells cultivated in auto-induction medium were grown to mid-exponential phase (OD_{595}=3.0) at 37ºC. From this point cells were further incubated for 16 h at different temperatures to identify the best expression conditions.

2.5 Purification of Recombinant Enzymes

Recombinant *E. coli* cells were harvested by centrifugation at 7,000 g at 4ºC for 15 min, ressuspended in 20 mM Tris-HCl pH 7.0, 20 mMNaCl, 5 mM CaCl2 ($2H_2O$) (Buffer A) and disrupted by sonication on ice. The resulting cell-free extracts were collected by centrifugation at 20,440 g at 4ºC for 30 min and the His_6-tagged recombinant proteins purified by IMAC, using 5 mL Niquel Hi-Trap Columns (GE Health). The column charged with a 0.1 M $NiSO_4$ was equilibrated with 20 mM Na_3PO_4, 500 mMNaCl, 40 mM Imidazole, pH 7.4. The cell extracts were loaded into the column which was washed with the equilibration buffer. Finally the recombinant proteins were eluted with 20 mM Na_3PO_4, 500 mMNaCl, 300 mM Imidazole, pH 7.4. Following IMAC, fractions containing the purified proteins were buffer exchanged, using PD-10 Sephadex G-25M gel-filtration columns (GE Healthcare), into 50 mM HEPES-Na buffer, pH 7.5, containing 200 mMNaCl and 5 mM $CaCl_2$, and proteins then subjected to gel filtration using a HiLoad 16/60 Superdex 75 column (GE Healthcare) at a flow rate of 1 ml/min. After purification, proteins were analyzed for purity in 14% (w/v) SDS-PAGE gels, with Coomassie Brilliant Blue staining. Predicted sizes for the recombinant proteins are: aspartate aminotransferase, 43.6 kDa; citrate synthase, 48 kDa; glucose-6-phosphate dehydrogenase, 55.7 kDa; hexokinase, 35.9 kDa; L-malate dehydrogenase, 32.3 kDa; D-malate dehydrogenase, 40.3 kDa; glucose-6-phosphate isomerase, 61.5 kDa; and acetyl-CoA synthetase, 64.9 kDa.

2.6 Enzyme Activity Assays

Enzyme assays were performed under the optimal incubation conditions for each enzyme, as described in Bergmeyer [1], unless otherwise stated. The assays were monitored in an UltroSpec 2100 Pro (Amersham Biosciences) spectrophotometer, with controlled temperature (at 25ºC), with the SWIFT II Applications Software. Catalytic activities (U/ml) were determined and expressed as specific activity (U/mg protein).

3. RESULTS AND DISCUSSION

3.1 Cloning and Expression of Recombinant Enzymes

Eight proteins were cloned and expressed as described above. The recombinant enzymes were engineered to contain a C-terminal His_6-tag. All recombinant enzymes were expressed in *E. coli*. To identify the best expression yields for each recombinant protein, different growing and induction conditions for a variety of recombinant *E. coli* strains, as well as two different culture media, were assessed. For each protein, higher expression rates were obtained when *E. coli* BL21(DE3) strain was grown in auto-induction LB medium. Similar results have been described by several authors for LB medium with auto-induction [8,9,10]. In general, this represents an improvement in cell densities rather than an improvement in the levels of protein expressed per gram of cell paste.

The optimized expression conditions and the yield of recombinant protein obtained per liter of medium (mg of purified protein per liter; mg/L) are presented in Table 2. For all enzymes, high protein yields were obtained in the soluble fraction of *E. coli* growth at 37ºC. However, the highest yield of citrate synthase in the soluble

fraction was obtained at 20ºC, not at 37ºC, which resulted from a higher level of inclusion bodies when the protein is produced at higher temperatures (data not shown). In fact, the aggregation reaction is in general favoured at higher temperatures due to the strong temperature dependence of hydrophobic interactions that determine the aggregation reaction [11]. A well-known technique to limit the *in vivo* aggregation of recombinant proteins consists of cultivation at reduced temperatures [12,13].

The highest induced yields were observed for L-malate dehydrogenase, aspartate aminotransferase and glucose-6-phosphate dehydrogenase, with 266 mg, 194 mg and 187 mg per liter of growth medium, respectively. In contrast, glucose-6-phosphate isomerase, citrate synthase and acetyl-CoA synthetase had the lowest induced yields, with 59 mg/L, 56 mg/L and 50 mg/L, respectively. For all enzymes, the protein expression yield obtained is high enough to set the basis for the assembling and production of enzymatic test kits [2].

3.2 Purification and Activity of Recombinant Enzymes

After optimizing expression in *E. coli*, the recombinant proteins were captured by IMAC and polished by gel filtration. All proteins were purified from most *E. coli* contaminants and were obtained at high concentrations. As an example, purification chromatograms and SDS-PAGE analysis of aspartate aminotransferase and L-malate dehydrogenase are presented in Fig. 1. Although the levels of purified protein obtained are high (Table 2) a considerable percentage of the protein is produced in the form of inclusion bodies (Fig. 1). The levels of insoluble recombinant produced varied between 10-60% depending on the protein (data not shown). The gel filtration chromatograms of the same proteins are displayed in Fig. 2. At the end of the purification process, highly purified proteins (>95%) were obtained, as shown in Figs. 2 and 3. These results confirm the adequacy of the His_6-tag based purification strategy to generate extremely pure proteins appropriate to the assembly of enzymatic test kits. The final concentration of recombinant proteins ranged from 2 to 17 g/L, being remarkably high for most of the enzymes (Table 2).

To evaluate biological activity of each one of the enzymes, in order to evaluate the suitability of each recombinant biocatalyst to the assembly of enzymatic test kits, enzyme catalytic activity was evaluated at 25ºC, as described in materials and methods section. The specific activity of each recombinant protein is presented in Table 3. The highest specific activities were obtained for glucose-6-phosphate isomerase and L-malate dehydrogenase, with 1220 U and 1100 U per mg of protein, respectively. In contrast, D-malate dehydrogenase and hexokinase had the lowest specific activities, with 2.1 U/mg and 5.8 U/mg, respectively. Nevertheless, the specific activity obtained is high enough for all enzymes to set the basis for the production of rapid and specific enzymatic test kits [14].

Table 2. Optimized protein expression conditions in *E. coli* for 8 analytical enzymes. Different cell strains and induction conditions were tested. The final protein concentration, after purification, is also presented

	Cell strain	Growth medium	Induction		Protein yield (mg/L)	Final protein concentration (g/L)
			Temp	Time		
Aspartate aminotransferase	BL21(DE3)	AI LB	37 ºC	16 h	194	2
Citrate synthase	BL21(DE3)	AI LB	20 ºC	16 h	56	11
Glucose-6-phosphate dehydrogenase	BL21(DE3)	AI LB	37 ºC	16 h	187	6
Hexokinase	BL21(DE3)	AI LB	37 ºC	16 h	97	17
L-Malate dehydrogenase	BL21(DE3)	AI LB	37 ºC	16 h	266	4
D-Malate dehydrogenase	BL21(DE3)	AI LB	37 ºC	16 h	103	13
Glucose-6-phosphate isomerase	BL21(DE3)	AI LB	37 ºC	16 h	59	2
Acetyl-CoA synthetase	BL21(DE3)	AI LB	37 ºC	16 h	50	8

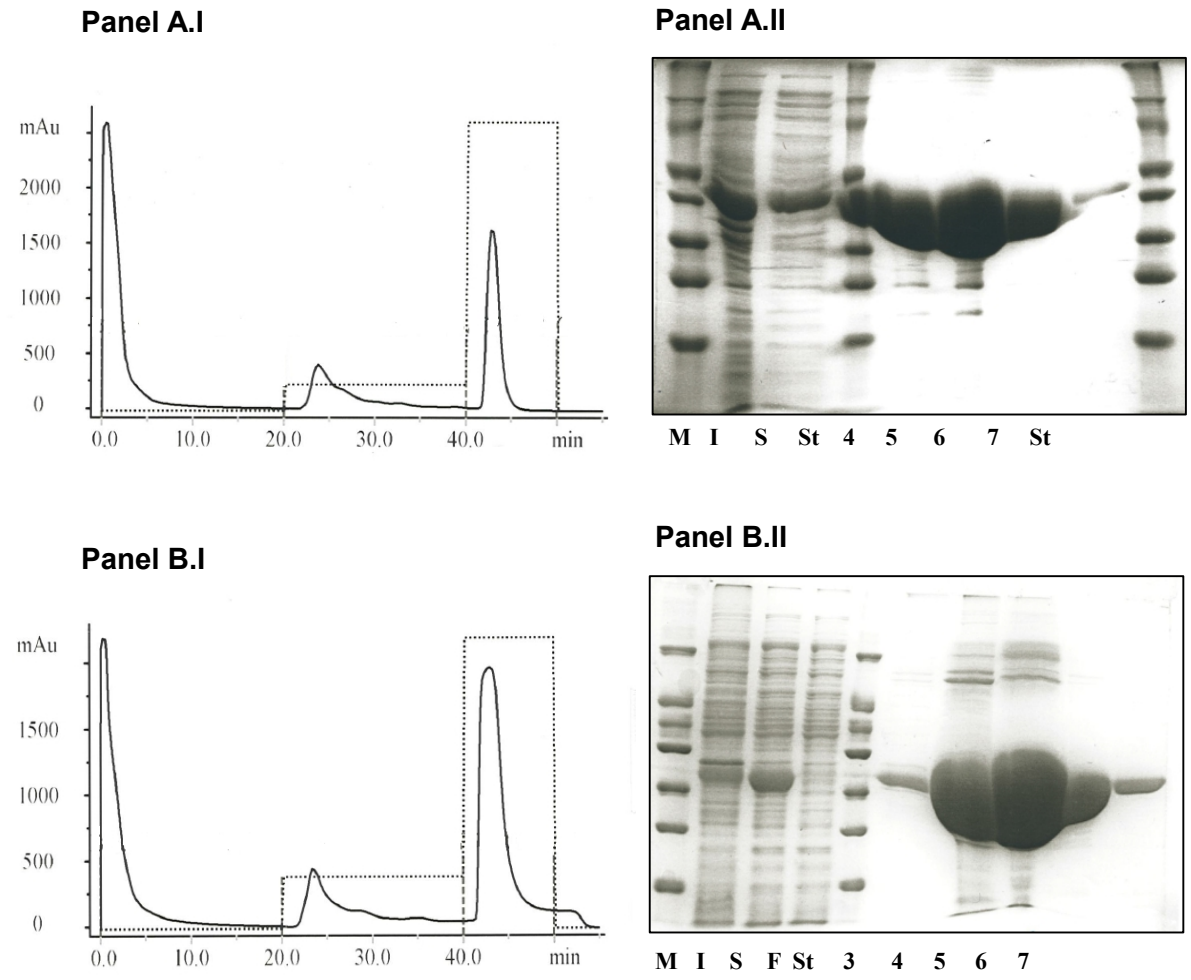

Fig. 1. Purification of the recombinant proteins expressed in *E. coli* as exemplified by the purification of aspartate aminotransferase (Panel A) and L-malate dehydrogenase (Panel B) by IMAC. In sections I: Purification chromatograms. In sections II: SDS-PAGE analysis. Abbreviations: I, insoluble cell pellet; S, soluble cell-free extract; 3 to 7, purified protein fractions; M, molecular mass protein standards

Table 3. Catalytic activity of 8 recombinant enzymes after expression in *E. coli*. Enzyme assays were performed at 25ºC at the specified pH

	Temperature	pH	Specific activity
Aspartate aminotransferase	25 ºC	8.5	268 U/mg
Citrate synthase	25 ºC	8.0	16.9 U/mg
Glucose-6-phosphate dehydrogenase	25 ºC	7.5	175 U/mg
Hexokinase	25 ºC	7.5	5.8 U/mg
L-Malate dehydrogenase	25 ºC	7.5	1100 U/mg
D-Malate dehydrogenase	25 ºC	8.0	2.1 U/mg
Glucose-6-phosphate isomerase	25 ºC	7.5	1220 U/mg
Acetyl-CoA synthetase	25 ºC	8.4	39 U/mg

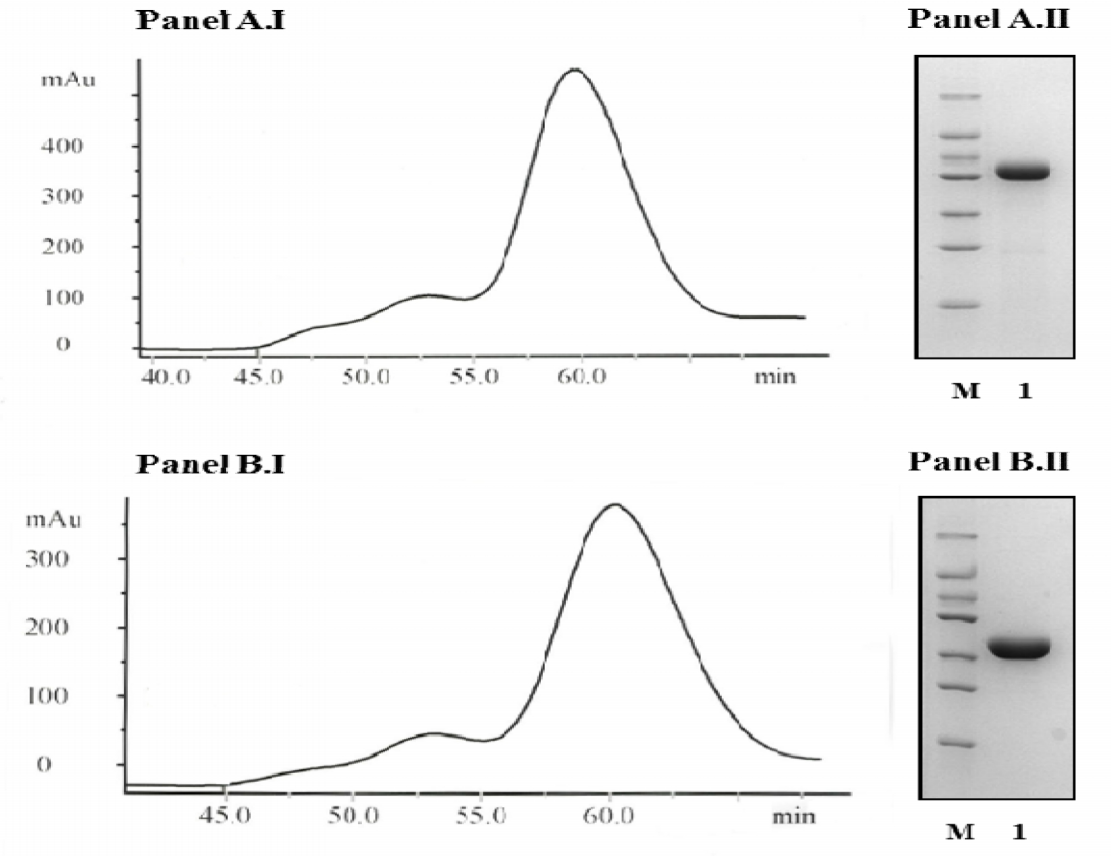

Fig. 2. Purification of the recombinant proteins aspartate aminotransferase (Panel A) and L-malate dehydrogenase (Panel B) by gel filtration. In section I, the respective chromatograms are presented. In section II, the purified proteins were analyzed through SDS-PAGE

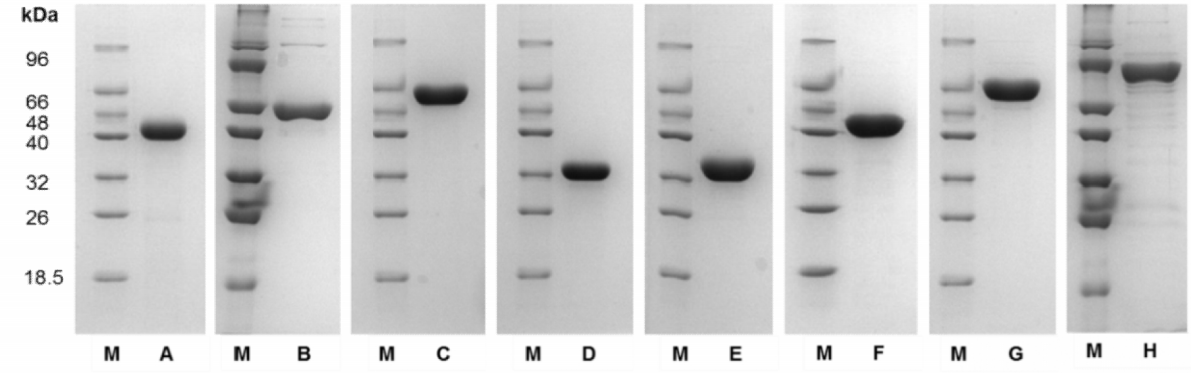

Fig. 3. SDS-PAGE analysis of recombinant proteins after purification through IMAC and gel filtration. Lanes M, molecular mass protein standards; Lane A, purified AST; Lane B, purified CS, Lane C, purified G6PDH, Lane D, purified HK, Lane E, purified LMDH, Lane F, purified DMDH, Lane G, purified PGI and Lane H, purified ACS

4. CONCLUSION

Eight analytical enzymes were cloned and the expression conditions optimized in *E. coli*. Recombinant proteins were obtained in high yields and protocols for purifying the recombinant proteins by IMAC and gel filtration were developed. Highly purified enzymes were obtained and thus the resulting specific activities of each enzyme were also consistently high.

Data presented here confirm the suitability of this methodology to produce recombinant enzymes for the development of enzymatic test kits.

Further characterization of each one of the individual recombinant enzymes is currently in progress, aiming the development and validation of enzymatic test kits to quantify relevant metabolites, such as D-glucose, D-fructose, L-malic acid, D-malic acid and acetic acid in a wide range of matrices. Therefore, the major applications of these enzymatic kits will be explored in the food, clinical chemistry and biochemistry sectors.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

This work was supported by project QREN 5282/2009.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nelson DL, Cox MM, editors. Lehninger Principles of Biochemistry. 5th edition. New York: W. H. Freeman and Company. 2008;183-233.
2. Bergmeyer HU, editor. Methods of Enzymatic Analysis (3rd edition). Volumes I. VCH Publishers, Cambridge, UK; 1985.
3. Boheringer Mannheim, editor. Methods of enzymatic bioanalysis and food analysis. Boehringer Mannheim, Biochemica; 1997.
4. Kuo WH, Chase HA. Exploiting the interactions between poly-histidine fusion tags and immobilized metal ions. Biotechnol Lett. 2011;33:1075-1084.
5. Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S. Comparison of affinity tags for protein purification. Protein Expr Purif. 2005;41:98-105.
6. Latimer GW. Official Methods of Analysis of AOAC International (19th edition). Two volumes. AOAC International, Gaithersburg, MD, USA; 2012.
7. Young L, Dong Q. Two-step total gene synthesis method. Nucleic Acids Res. 2004;15:32-37.
8. Studier FW. Protein production by auto-induction in high density shaking cultures. Protein Expr Purif. 2005;41(1):207-234.
9. Li Z, Kessler W, Van Den Heuvel J, Rinas U. Simple defined autoinduction medium for high-level recombinant protein production using T7-based *Escherichia coli* expression systems. Appl Microbiol Biotechnol. 2011;91(4):1203-1213.
10. Sivashanmugam A, Murray V, Cui C, Zhang Y, Wang J, Li Q. Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*. Protein Sci. 2009;18:936-948.
11. Kiefhaber T, Rudolph R, Kohler HH, Buchner J. Protein aggregation *in vitro* and *in vivo*: A quantitative model of the kinetic competition between folding and aggregation. Biotechnol. 1991;9:825-829.
12. Schein CH. Production of soluble recombinant proteins in bacteria. Biotechnol. 1989;7:1141-1148.
13. Vasina JA, Baneyx F. Expression of aggregation prone recombinant proteins at low temperatures: a comparative study of the *Escherichia coli* csp A and tac promoter systems. Protein Expr Purif. 1997;9:211-218.
14. Bergmeyer HU, Bergmeyer J, Grassl M. Methods of Enzymatic Analysis: Enzymes 1: Oxidoreductases, Transferases. VCH Verlag Chemie. 1995;3.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=683&id=40&aid=6294