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A New Strategy for the Cloning, Overexpression and One Step Purification of Three DHAP-Dependent Aldolases: Rhamnulose-1-Phosphate Aldolase, Fuculose-1-Phosphate Aldolase and Tagatose-1,6-Diphosphate Aldolase[1]

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Abstract—Three DHAP-dependent aldolases, rhamnulose-1-phosphate aldolase (Rham-1PA), fuculose-1-phosphate aldolase (Fuc-1PA) and tagatose-1,6-diphosphate aldolase (TDPA) have been cloned and overexpressed in *Escherichia coli* using two different expression vectors: pTrcHis for the expression of Rham-1PA and Fuc-1PA and pRSET for the expression of TDPA. In each case the recombinant enzyme is synthesized as a fusion protein with a hexahistidine tag on the N-terminus. The three enzymes have been purified in only one step by chelation affinity chromatography. The effects of cultivation temperature and concentration of inducer have been studied in order to optimize the expression of the recombinant proteins and to avoid the formation of inclusion bodies.

Introduction

Rhamnulose-1-phosphate aldolase (Rham-1PA) from *Escherichia coli* is involved in the metabolism of L-rhamnose[2] and the gene is located in the L-rhamnose regulon. Four structural genes related to rhamnose metabolism have been described: *rha*A, encoding rhamnose isomerase; *rha*B, encoding rhamnulose kinase; *rha*D, encoding rhamnulose-1-phosphate aldolase;[3] and *rha*T, encoding the rhamnose transport system.[4] The sequences of these structural genes have recently been elucidated.[5] Fuculose-1-phosphate aldolase (Fuc-1PA, encoded by *fuc*A) is involved in the dissimilation of L-fucose by *E. coli* through an inducible pathway that requires the sequential action of fucose permease (encoded by *fuc*P), fucoisomerase (encoded by *fuc*I), fucokinase (encoded by *fuc*K) and Fuc-1PA. The sequence of the entire *fuc* region, including the regulatory gene *fuc*R, has been also elucidated.[6,7] These two enzymes belong to the class II aldolases that require a Zn^{2+} cofactor acting as a Lewis acid in the active site. Tagatose-1,6-diphosphate aldolase (TDPA) is involved in the galactose metabolism of *cocci*.[8,9] In *Lactococcus lactis* ssp. *lactis* MG1820 the gene that encodes TDPA (*lac*D) is located in the gene cluster (*lac*ABCD) of the lactose–PTS operon,[10] where *lac*AB encodes galactose-6-phosphate isomerase and *lac*C encodes tagatose-6-phosphate kinase. Different from the other two aldolases, TDPA from *L. lactis* belongs to the class I aldolases, which catalyze aldol reactions through a Schiff-base intermediate formed between a lysine residue in the active site and the donor substrate which in turn adds stereospecifically to the acceptor.

These three aldolases and fructose-1,6-diphosphate aldolase (FDPA) use dihydroxyacetone phosphate (DHAP) as donor substrate.[11] FDPA and TDPA use D-glyceraldehyde-3-phosphate (D-G3P) as an acceptor substrate to give respectively D-fructose-1,6-diphosphate and D-tagatose-1,6-diphosphate (Fig. 1). Rham-1PA and Fuc-1PA catalyze the reversible condensation of DHAP and L-lactaldehyde to give L-rhamnulose-1-phosphate and L-fuculose-1-phosphate respectively (Fig. 1). As can be observed in Figure 1, each one of these aldolase reactions yields a product whose stereochemistry at C3 and C4 is complementary to the other products. It has been found that in a kinetic process the selectivity of the reaction is completely controlled by the enzyme instead of the substrates. This implies that any one of the C3/C4 stereoisomers can be obtained from a variety of non-natural aldehydes using these enzymes. However, as with other aldolases these enzymes are quite specific for the donor substrate though they show a broad acceptor specificity.[11] In addition, TDPA has not been synthetically useful due to the lack of stereoselectivity.[12–14] In order to further investigate the synthetic utility of these aldolases and to alter their specificity, cloning and overexpression of these enzymes are required as FDPA from rabbit muscle is the only DHAP-dependent aldolase available to date. It is, however, noted that although the FDPA from rabbit muscle[15] and *E. coli*,[16] and the Rham-1PA[17] and Fuc-1PA[17,18] from *E. coli*, have been recently cloned and overexpressed in *E. coli*, only the recombinant *E. coli* FDPA is commercially available (ATCC # 77472).

Here we present a new strategy for the cloning and overexpression in *E. coli* of the Rham-1PA, Fuc-1PA and TDPA. The expression vectors used in this work

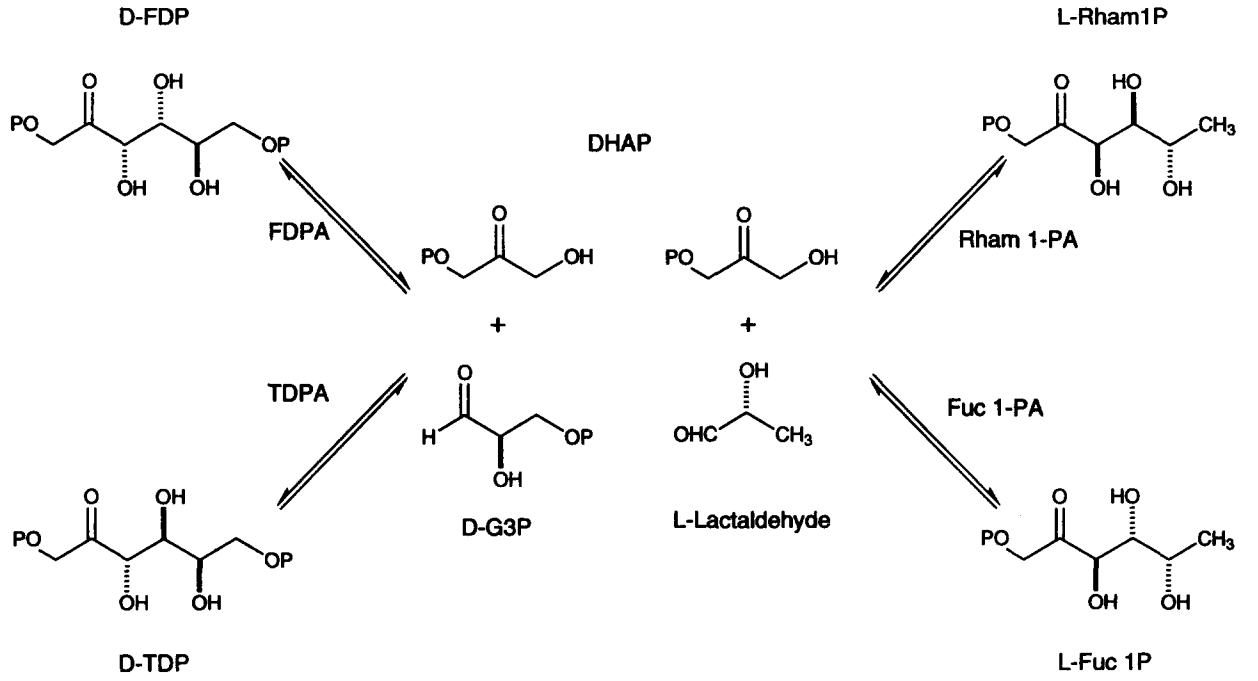

Figure 1. Reactions catalyzed *in vivo* by FDPA, TDPA, Rham-1PA and Fuc-1PA. The stereoselectivities of the products generated by the four DHAP-dependent aldolases are shown.

produce the recombinant enzyme fused with a hexahistidine tag, allowing purification of the enzymes by a single step. This work also illustrates that the formation of inclusion bodies can be avoided and the expression level can be optimized by controlling the culture conditions.

Results and Discussion

Cloning of Rham-1PA, Fuc-1PA and TDPA

For each target gene two primers were designed to specifically complement the C-terminal and N-terminal gene sequences of *rha*D,[5] *fuc*A[7] and *lac*D.[10] Each primer included the recognition sequence for the restriction enzymes chosen to clone the insert. The sequences of the primers and the cleavage sites for restriction enzymes used for each gene are shown in Table 1. The PCR's amplifications were quite specific, and only one band with a molecular weight consistent with that previously reported (820 bp, 650 bp and 1.0 kb respectively for *rha*D, *fuc*A and *lac*D) was observed. The strategy for the cloning of these enzymes and the main characteristics of the plasmids constructed are shown in Figure 2. After ligation of the digested vector and inserts, the DNA's were transformed into *E. coli* XL1-Blue MRF' strain and plated on LB-ampicillin plates. Out of 20–25 colonies selected for each gene, 7–10 colonies carried the desired insert. The transcriptions of the Rham-1PA and Fuc-1PA are controlled by Trc promoter and can be induced by IPTG. On the other hand, TDPA expression is controlled by the T7 promoter and is induced by M13/T7 phage. In both expression systems used in this work (pTrcHis and pRSET), the recombinant protein is expressed with a polyhistidine tag on the N-terminus.

Expression of cloned Rham-1PA and Fuc-1PA

The expressions of Rham-1PA and Fuc-1PA, with both enzymes cloned in the same expression vector, were studied in parallel. The clones were grown on LB medium containing 250 μg mL^{-1} ampicillin and induced with IPTG as described in the Experimental. The expression level of the enzyme was followed with time

Table 1. Primers and restriction sites used for the cloning of Rham-1PA, Fuc-1PA and TDPA

Aldolase	Restriction enzyme at 5'	Restriction enzyme at 3'	N-Terminal Primer (5' → 3')	C-Terminal Primer (5' → 3')
Rham-1PA	BamHI	Hind III	ATATTGGATCCCAAAACAT TACTCAG	TATTAAAGCTTTTACAGCG CCAGCGCACTGGC
Fuc-1PA	Pst I	EcoRI	ATATTCTGCAGGAACGAAA TAAACTT	GCGCGAATTCTTACTCTTC AATTCGTAACCC
TDPA	Xho I	EcoRI	ATATTCTCGAGGTACTTAC AGAACAGAAA	GCGCGAATTCTTATTATAT ACTTTATCAGTCCATGGA

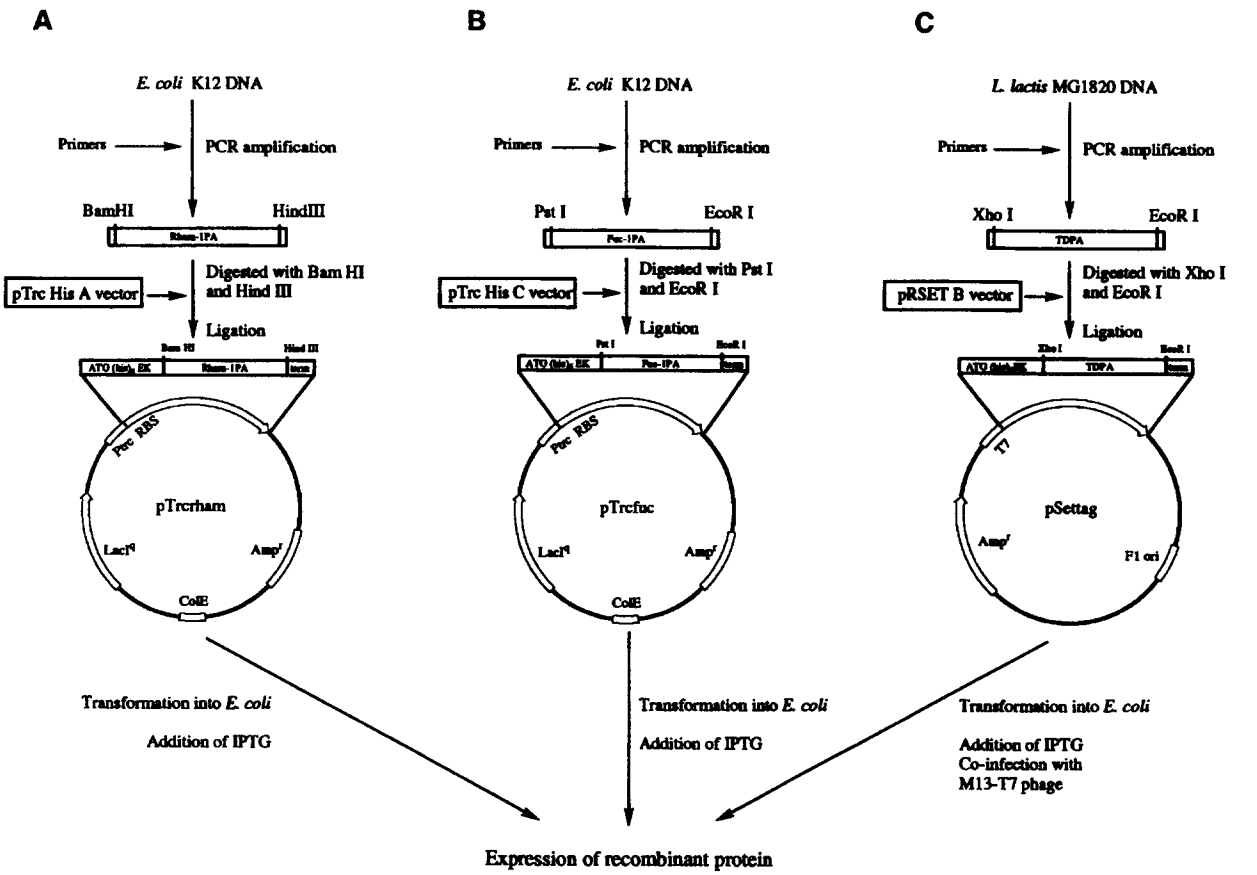

Figure 2. Strategies for the cloning of Rham-1PA, Fuc-1PA and TDPA. The main characteristics of the expression vectors are shown.

and examined with SDS-PAGE. In a typical experiment, the cells were grown at 37 °C after induction and only a light stained band of the expected molecular weight could be detected when the supernatants were analyzed. We examined the pellets from different samples to test if the enzymes were expressed as inclusion bodies. In both cases a main band matching the expected molecular weight was observed (data not shown), indicating that Rham-1PA and Fuc-1PA were indeed expressed as inclusion bodies. In order to obtain the enzymes in soluble form, the culture temperature was dropped to 19 °C after induction with IPTG. After 7 days of culture the transformed cells had grown to a final O.D. of about 1.75 and the enzyme was detected only in the soluble form. Increasing the temperature to 30 °C reduced the culture time required to reach the same O.D. to 8–10 h. Under these conditions, the recombinant proteins are expressed as soluble form, and in SDS–PAGE appear as major bands after 2 h of induction (Fig. 3). Also, we have observed that the proteins were stable in the cells even after 20 h of cultivation (Fig. 3). The influence of IPTG concentration on the expression level of the recombinant proteins and the viability of cells have also been studied. In the case of Rham-1PA the viability of the cells is strongly affected by the concentration of the IPTG. Thus, after 16 h of cultivation with 250 μM of IPTG the cells grow to reach an O.D. of 1.08. The final O.D. was doubled when the concentration of IPTG was decreased to 5 μM (Fig. 4). On the other hand, the amount of Rham-1PA obtained per gram of cells (about 36 U g^{-1}) remained constant for all the IPTG concentrations assayed; therefore, a 2.5-fold increase of the productivity per liter of the recombinant cell was observed by reducing the concentration of IPTG from 250 μM to 5 μM (Fig. 4). In a similar study the optimal concentration of IPTG for the expression of Fuc-1P was determined to be 50 μM, with a productivity of about 430 U L^{-1}.

This strong correlation between cultivation temperature and IPTG concentration with the expression level and solubility of the recombinant protein has also been found in the study of the heterologous expression of the mannosyltransferase from *Saccharomyces cerevisiae* in *E. coli*.[19,20] These results indicate that a slow expression of the desired protein could lead to a productive folding to form the active soluble enzyme.

Expression of cloned TDPA

The expression of TDPA was studied in a 1-L culture containing 250 μg mL^{-1} ampicillin and induced by infection with phage M13/T7 which contains a T7 polymerase gene, as described in the Experimental. The accumulation of recombinant enzyme reached the highest level 3 h after the infection (Fig. 5). However, in contrast to the cases of Rham-1PA and Fuc-1PA, TDPA was degraded in the cell if the culture was incubated overnight (Fig. 5). Since TDPA from *L. lactis*

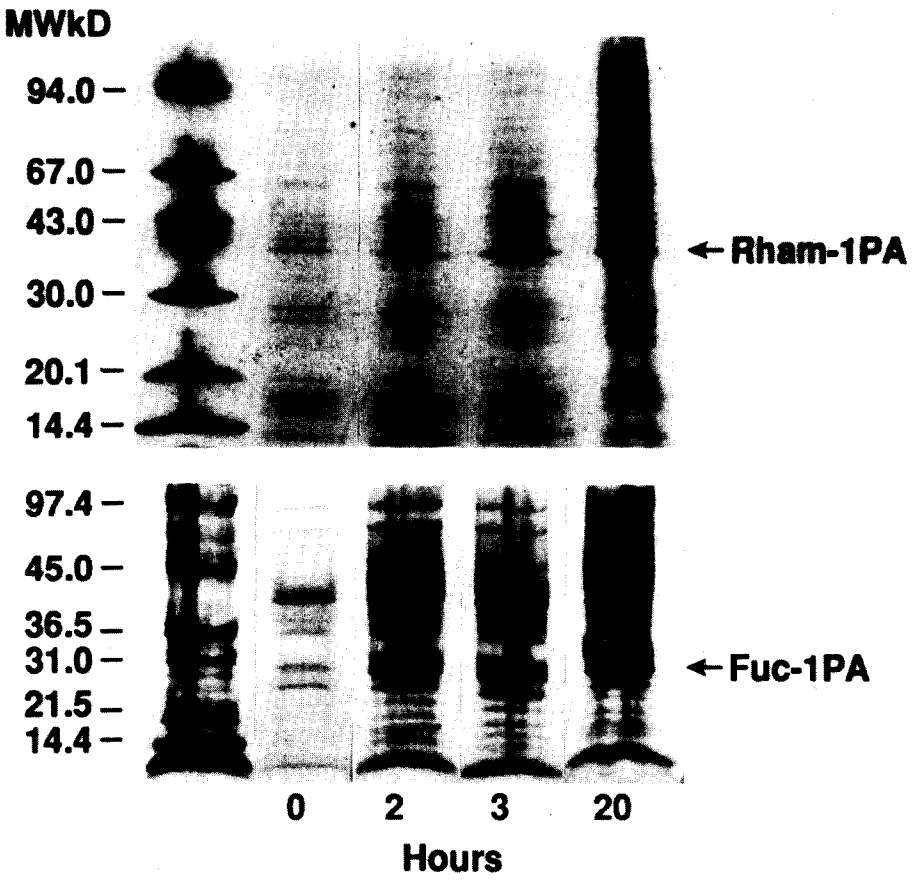

Figure 3. Monitoring of the Rham-1PA and Fuc-1PA expression during the culture time. Aliquots were taken at different times after induction and the soluble fraction of the cells was analyzed by SDS-PAGE.

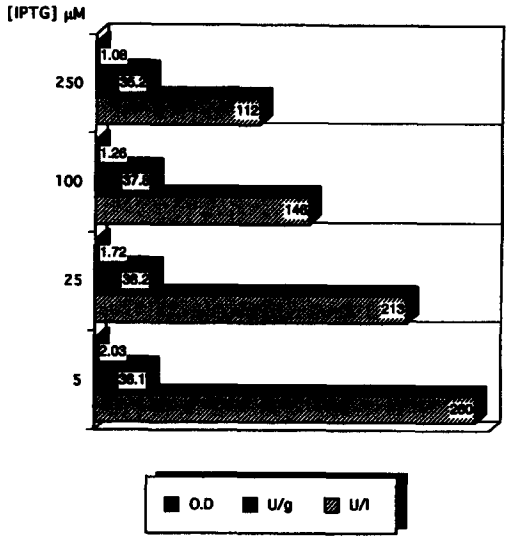

Figure 4. Influence of the IPTG concentration on the cell viability and productivity of Rham 1PA.

is a class I aldolase,[12] the enzyme does not require a divalent metal ion for its activity. One can therefore measure the recombinant enzyme activity without interference from the wild-type class I aldolases present in *E. coli* which can be inhibited by EDTA. The cells harvested 3 h after the infection showed an activity of more than 3,600 U L^{-1}, with a productivity of about 850 U per gram of cells. Unlike other aldolases the stereoselectivity of TDPA is lower than that of the other DHAP dependent aldolases.[12-14] TDPA from *E. coli* showed a very high stereoselectivity (TDP/FDP aldolase ratio 100:1) at least in the cleavage pathway,[21] but when the enzyme was used in synthesis with unnatural acceptor substrates, it was found that more than 90% of products contained the D-*threo* configuration (FDP type) at C-3/C-4.[14] In the case of the enzyme from *Staphylococcus aureus*,[13] the ratio of TDP to FDP aldolase activity is 2.1:1 and is similar to the ratio (1.99:1) found for the enzyme from *L. lactis* ssp. *cremoris* E8.[12] Interestingly, although the enzyme from *L. lactis* ssp. *lactis* MG1820 reported here is virtually identical to the TDPA purified from *L. lactis* E8[10] regarding the amino acid composition, and very similar to the E8 enzyme with regard to K_m, V_{max} and optimum pH[12], it shows a higher stereoselectivity with a 48:1 ratio of TDP to FDP aldolase activities. Work is in progress to test the specificity and stereoselectivity of this enzyme in synthetic reactions.

One step purification of Rham-1PA, Fuc-1PA and TDPA

The vectors pTrcHis and pRSET used in this work contain a hexahistidine tag that is expressed in the N-

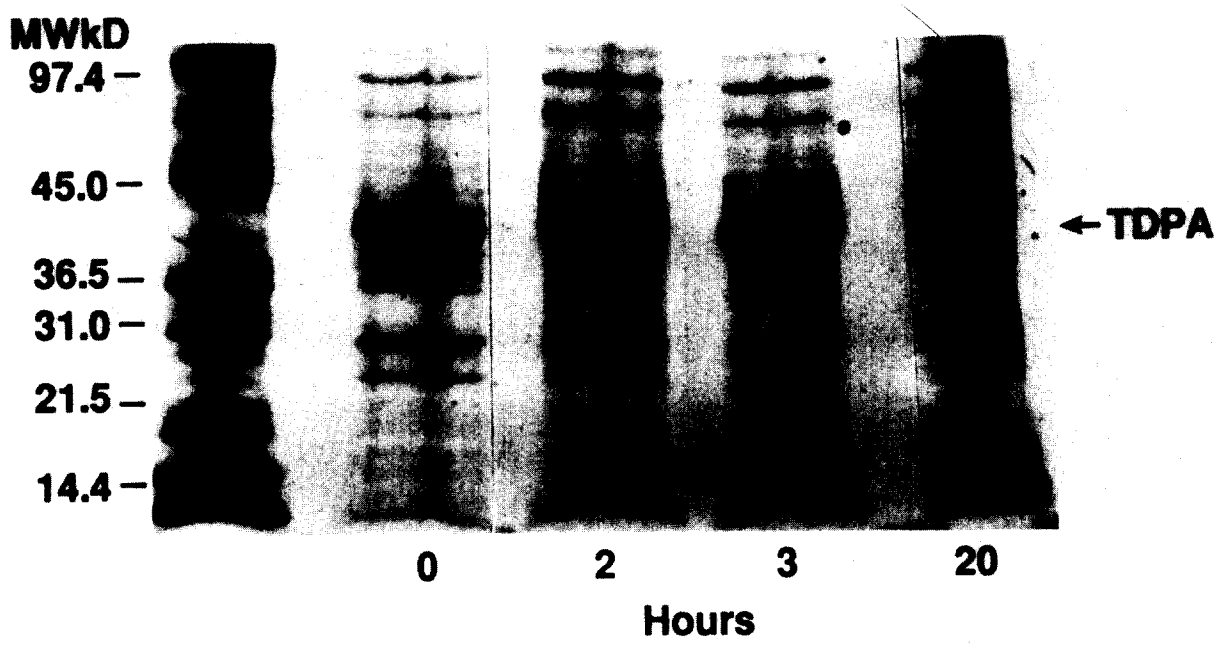

Figure 5. Monitoring of the TDPA expression during the culture time. The expression of the enzyme is at maximum 3 h after the induction and its accumulation decreases dramatically if the cell culture is prolonged overnight.

terminal region of the cloned protein. This His tag allows purification of the recombinant protein using a Ni^{2+}-column. For the one step purification, the crude extract obtained from 100 mL culture was concentrated to 2 mL, in order to exchange the Tris–HCl buffer with the native binding buffer, and loaded on a 2 mL column. The column was then washed 20 times with 7 mL each of the low stringency buffer and then 20 times with 7 mL each of the high stringency buffer. The recombinant proteins were recovered from the column by elution with the low pH buffer. However, in the cases of Rham-1PA and Fuc-1PA, the yield of the activity recovered in this step was much lower than that obtained in the case of the TDPA. This result may indicate that the class II aldolases may form an additional interaction with Ni^{2+} through the active center. Interestingly, an additional elution step with low pH buffer containing 50 mM of EDTA results in a more than 50% increase of the activity. The purity of the proteins after the metal affinity chromatography was analyzed by SDS-PAGE. In each of the three cases only one band could be detected when the gel was stained with Coomasie Blue (Fig. 6). This procedure of purification can be scaled up with similar results when keeping the same ratios between crude extract, column and washing volumes. The specific activities calculated from these preparations were 6.9 U mg^{-1} for Rham-1PA, 6.5 U mg^{-1} for Fuc-1PA and 16.4 U mg^{-1} for TDPA. The values obtained for TDPA and Fuc-1P are in the same range as those previously reported for the TDPA purified from *L. lactis* E8 and the wild type Fuc-1PA from *E. coli* (23.2 and 4 U mg^{-1} of protein respectively).[12,22] On the other hand, the value obtained for the Rham-1PA was significantly lower than that reported for the wild type enzyme[2] (17.2 U mg^{-1} of protein). However, it is not clear if this difference is due to the presence of the His tag or the different methods used to measure the protein concentration.

Influence of Zn^{2+} on the Rham-1PA and Fuc-1PA activities

The class II aldolases exist in an equilibrium between the apoenzyme and the holoenzyme formed by the complex of the protein and the zinc ion. We have attempted to find the optimal concentration of Zn^{2+} to shift this equilibrium to the formation of the holoenzyme. The enzymes purified as described above, were incubated in 100 mM of EDTA for 1 h in order to chelate all the divalent cation present in the extracts. The EDTA was then removed using Centricon tubes and the sample equilibrated with Tris–HCl buffer (50 mM pH 7.6). Aliquots (0.1 mL) of the solution were then incubated for 15 min with increasing concentrations of Zn^{2+} from 2.0 nM to 10.0 mM. The profiles of the activity change at different concentrations of Zn^{2+} for both enzymes are shown in Figure 7. The Rham-1PA activity reached its maximum after incubation with 0.5 mM of Zn^{2+} (Figure 7A) and reduced as the concentration of zinc increased. Fuc-1PA showed a similar behavior (Figure 7B), but the maximum activity is reached with only 10 μM of zinc. It is not clear why higher concentrations of Zn^{2+} reduced the activity of the enzyme.

Conclusion

In summary we have developed an efficient expression system for overproduction of the three not readily available DHAP-dependent aldolases (Rham-1PA, Fuc-1PA and TDPA). In addition, we have optimized the

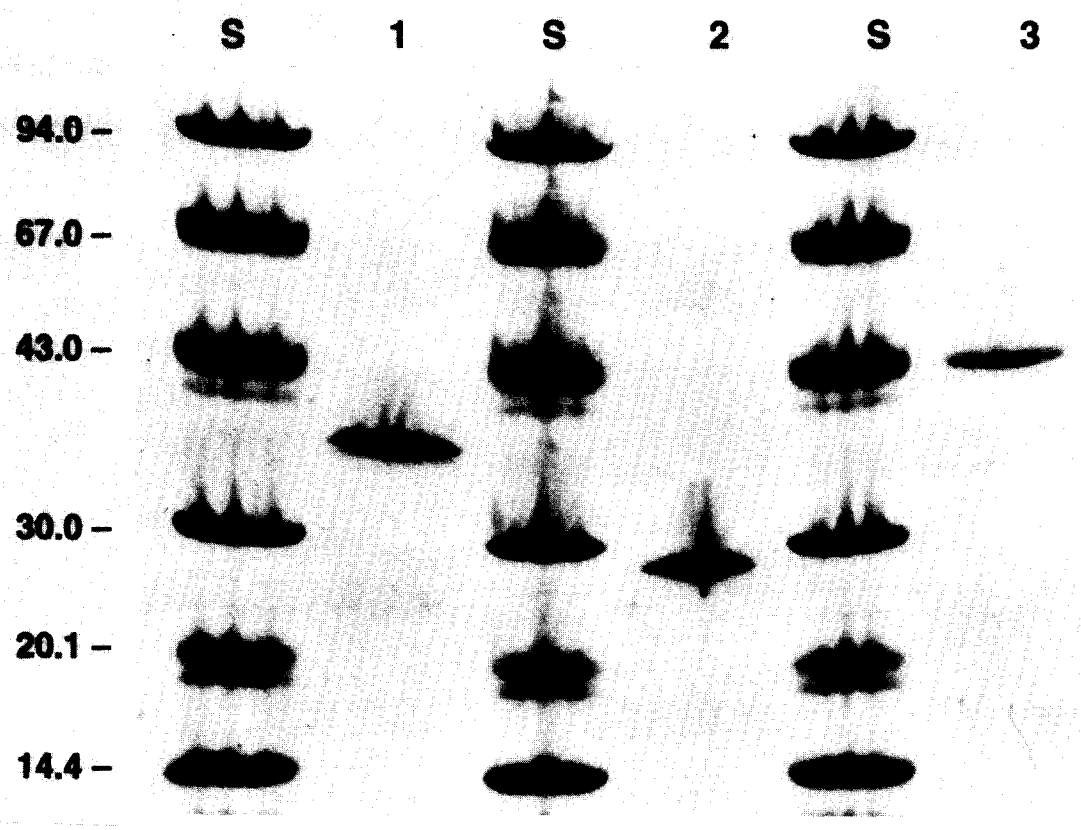

Figure 6. SDS-PAGE of the three DHAP dependent aldolases after one-step purification. Lane S, molecular weight markers. Lane 1, rhamnulose-1-phosphate aldolase. Lane 2, fuculose-1-phosphate aldolase. Lane 3, tagatose-1,6-diphosphate aldolase.

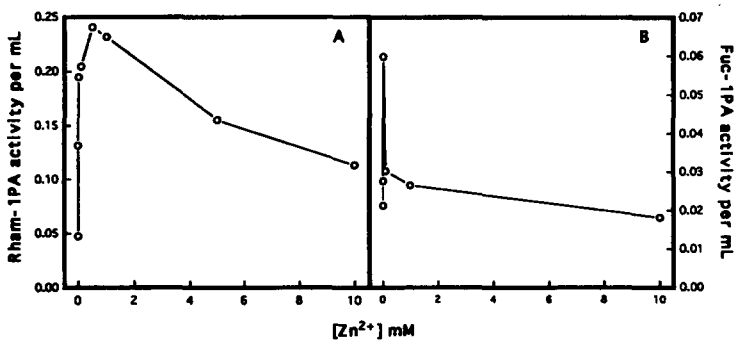

Figure 7. Effect of Zn^{2+} on the activity of (A) rhamnulose-1-phosphate aldolase and (B) fuculose-1-phosphate aldolase.

procedure for the one-step purification by chelation affinity chromatography of the enzymes. Optimization of the culture conditions allows us to obtain the recombinant proteins in a soluble form, avoiding tedious procedures for the resolubilization of the inclusion bodies. In the case of the TDPA the recombinant enzyme shows a high degree of stereoselectivity that may make this enzyme useful for organic synthesis.

Experimental

Vectors

The vectors used throughout this work, pTrcHis and pRSET, were obtained from Invitrogen Co. (San Diego, CA). The vector pTrcHis contains Trc promotor to allow a high expression of the required protein. The Trc promotor has the –35 region of the Trp promotor and the –10 region of the lac promotor. The Trc promotor is repressed by the lac repressor, which is encoded by the $lacI^q$ gene in the vector. The vector pRSET contains the T7 promotor. In addition, both vectors have a sequence that encodes an N-terminal peptide containing a hexahistidine tag to function as a metal binding domain in the recombinant protein, and an enterokinase cleavage recognition sequence. Three different versions of these vectors were used in this study in order to maintain the proper coding frame between the 5' end of the fusion sequence and the DNA insert. The pTrc His A vector was used to clone the Rham-1PA and pTrcHis C was used in the case of the Fuc-1PA. TDPA was cloned using the vector pRSET B.

Microorganism

Escherichia coli K12 (ATCC 10798) and *Lactococcus lactis* ssp. *lactis* (ATCC 11454) were obtained from American Type Culture Collection. The host strain *E. coli* XL1-Blue MRF' was purchased from Stratagene Co. (San Diego, CA). The microorganisms were maintained on LB (Luria–Bertani) medium. When host strains harbored with plasmids, LB medium containing 250 μg mL^{-1} of ampicillin was used. Stock cultures were kept as cell suspension at –70 °C in 30% glycerol solution.

PCR amplification

The DNA's of *E. coli* K12 and *L. lactis* were extracted according to the method described by Maniatis *et al.*[23] PCR amplification was performed in a 100 μL reaction mixture containing 1 μL (1.5 μg) of DNA template, 300 nmol of the corresponding primers, 200 μM of dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 units of *Thermus aquaticus* DNA polymerase (Stratagene Co.). The reaction mixture was overlayed with mineral oil and subjected to 30 cycles of amplifications. The cycle conditions were set as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min and elongation at 72 °C for 1.5 min.

Construction of the expression vectors

The DNA insert obtained from PCR amplification was purified on 0.8% agarose gel. The DNA band corresponding to the target gene was cut and purified with QIAEX gel extraction kit (Qiagen Co., Chatworth, CA) and eluted with TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5). The DNA was twice digested with the corresponding restriction enzymes (Boehringer Mannheim Biochemical Co.) for 2 h at 37 °C. The digested DNA was then recovered by phenol/chloroform extraction and ethanol precipitation (70% of final ethanol concentration containing 10% of 3 N Na-acetate, pH 5.2) and purified by agarose (0.8%) gel electrophoresis as above. This DNA was used as insert. The vectors were also digested and recovered with ethanol precipitation after extraction with phenol/chloroform. The restriction enzyme-digested vector was further purified on agarose gel as described above. The insert was then ligated with the vector with T4 DNA ligase. The expression plasmids constructed in this way were then transformed into *E. coli* XL1-Blue MRF' strain and plated on LB agar plates containing 250 μg mL^{-1} ampicillin.

Screening for positive clones

Twenty to twenty five colonies were randomly selected for each gene. The plasmids were isolated using the QIAprep-spin Plasmid Kit (Qiagen Inc.). The isolated plasmids were digested with the corresponding restriction enzymes (see Table 1) and analyzed on agarose gel to confirm the presence of the gene insert. The positive clones were selected and used for protein expression.

Expression of the recombinant proteins

To express the desired protein, the positive clone was grown on 100 mL of LB medium containing 250 μg mL^{-1} ampicillin at 37 °C with shaking (300 rpm). After the cell growth reached a point where the turbidity was about 0.5 as measured by the absorbance at 600 nm (OD_{600}), this culture was transferred to fresh LB medium (1 L) containing 250 μg mL^{-1} ampicillin and incubated until OD_{600} = 0.4–0.5 then IPTG was added to induce the expression of the target protein. In the case of TDPA, the T7 RNA polymerase was introduced after 1 h of shaking by coinfection of 10 mL of M13/T7 phage solution (5–10 pfu/cell, supplied by Invitrogen Co.) containing the T7 RNA polymerase gene.[24] After induction the expression level of the targeted protein was analyzed at different times (until a total of 20 h of culture) by SDS-PAGE in a Phastsystem (Pharmacia Co.) using precast gels with a 8–25% gradient of polyacrylamide in the separation zone. Different concentrations of IPTG were used in the cultivation of cells containing the Rham-1PA and Fuc-1PA, and different temperatures of culture after induction were applied in order to optimize the conditions for the expression of the recombinant proteins.

Preparation of cell free extract

The culture broth was centrifuged (10,000 *g*, 30 min, 4 °C), and the cell pellets were suspended in Tris–HCl buffer (50 mM pH 7.5). The cells were disrupted by a French press cell at 16,000 lb in^{-2} and centrifuged at 16,000 *g* for 30 min.

TDPA stereoselectivity study

The stereoselectivity of TDPA was studied by measuring its activity against tagatose-1,6-diphosphate and fructose-1,6-diphosphate (for details on the enzymatic assay see below). TDPA from *L. lactis* is a class I aldolase but the naturally occurring TDPA and FDPA in *E. coli* are class II aldolases, which can be inhibited by adding 10 mM of EDTA to the reaction mixture. This allows us to perform this study directly using the crude extract, without crossreaction from the enzyme naturally present in *E. coli*.

Purification of the tagged proteins

The cells obtained from 100-mL cultures were resuspended in Tris–HCl buffer (50 mM, pH 7.6). The cell free extract was prepared as described above. The extracts were concentrated to 2 mL and the buffer exchanged with the native-binding buffer (20 mM sodium phosphate, pH 7.8, containing 500 mM of NaCl) using Centicons tubes (Amicon Co.) with a M_r cut off of 10,000. The samples were loaded on a 2-mL column filled with ProBond resin (Invitrogen Co.) and equilibrated with the same buffer. The column was

washed with native-binding buffer until the O.D. at 280 nm dropped to 0.01. After that, a second wash was performed with the high stringency buffer (20 mM sodium phosphate, pH 6.0, containing 500 mM of NaCl) until the O.D at 280 nm dropped again to 0.01. Finally elution of the proteins was performed with the low pH elution buffer (20 mM sodium phosphate, pH 4.0, containing 500 mM of NaCl), and fractions of 1 mL were collected and analyzed by SDS-PAGE. For the purification of Rham-1PA and Fuc-1PA an additional elution step was necessary. This was performed with the low pH elution buffer containing 50 mM of EDTA. The enzyme purity was determined by SDS-PAGE using precast gels with a gradient of polyacrylamide in the separation zone of 10–15%.

Influence of Zn^{2+} concentration on the Rham-1PA and Fuc-1PA activities

The influence of Zn^{2+} on the Rham-1PA and Fuc-1PA activities was studied using the pure enzyme. First the enzymes were incubated in 100 mM of EDTA for 1 h at 4 °C, then the EDTA was removed using Centricon tubes (Amicon Co.) with a M_r cut off of 10,000. The samples were washed three times with Tris–HCl (50 mM, pH 7.6). Finally aliquots of 100 μL were incubated for 15 min with different concentrations of Zn^{2+} (from 2.0 nM to 10.0 mM), and the activity was assayed as described below.

Synthesis of rhamnulose-1-phosphate, fuculose-1-phosphate and tagatose-1,6-diphosphate

The two ketose-1-phosphates were synthesized by aldol condensation of DHAP and L-lactaldehyde according to the procedure previously reported,[25] except that the recombinant enzyme was used. L-Lactaldehyde was prepared from D-threonine by reaction with ninhydrin.[26] Tagatose-1,6-diphosphate was synthesized following the chemoenzymatic strategy described by Eyrisch *et al.*[21]

Enzyme activity assay

The three enzymatic activities were assayed with a coupled enzymatic system where 2 mM of the corresponding substrate (rhamnulose-1-phosphate, fuculose-1-phosphate, tagatose-1,6-diphosphate and fructose-1,6-diphosphate), 0.12 mM NADH, and a mixture of glycerolphosphate dehydrogenase and triose phosphate dehydrogenase were incubated in 50 mM triethanolamine buffer, pH 7.5 at 25 °C. The assay was initiated upon addition of the corresponding aldolase, and the decrease in the absorbance at 340 nm was monitored. When the crude extract of TDPA was assayed, 10 mM of EDTA was added. One unit of enzyme activity is defined as the quantity that catalyzes the formation of 1 μmol of DHAP per minute under the assay conditions. The protein concentration was determined using the BCA kit from Pierce. The samples were incubated during 30 min at 60 °C and the absorbance measured at 562 nm. The calibration curve was obtained for concentrations of BSA between 5 and 200 $\mu g\ mL^{-1}$.

Acknowledgment

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References and Notes

1. The recombinant strains of *E. coli* encoding for these enzymes have been deposited with the ATCC with the following numbers: 86983 for Rham-1PA, 86984 for Fuc-1PA and 87025 for TDPA

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