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Cloning and Overexpression of Rhamnose Isomerase and Fucose Isomerase†

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Abstract—Rhamnose isomerase and fucose isomerase were overexpressed in *E. coli*, purified and characterized. The rhamnose isomerase gene was ligated to the restriction sites of PstI and Hind III of vector pTrcHis and the fucose isomerase gene was ligated to the EcoRI and PstI sites of vector pKK223-3 for overexpression of the enzymes in *E. coli* XL1-Blue MRF'. Approximately 16,500 U of active fucose isomerase and 2400 U of rhamnose isomerase can be obtained per liter of culture from these expression systems.

Introduction

Rhamnose isomerase (RhamIso, EC 5.3.1.14) and fucose isomerase (FucIso, EC 5.3.1.3) from *Escherichia coli* are the first enzymes involved in the metabolic pathway of L-rhamnose and L-fucose, respectively.[1,2] The reactions catalyzed *in vivo* by these enzymes are shown in Figure 1. The L-rhamnose regulon is composed of four structural genes whose sequences have been recently determined.[3,4] The sequences of the four structural genes of the L-fucose regulon[5,6] are also known.

Here we report a new strategy for the cloning and overexpression in *E. coli* of the RhamIso and FucIso. Two different expression systems have been used through this work. RhamIso has been cloned in the vector pTrcHis, which produces the recombinant enzyme fused with a hexahistidine tag, allowing the purification of the enzymes by a single step. On the other hand, FucIso has been cloned in the vector pKK223-3. In this case, the preparation of the crude extract by freezing and thawing allows us to purify the enzyme also by a single chromatography step.

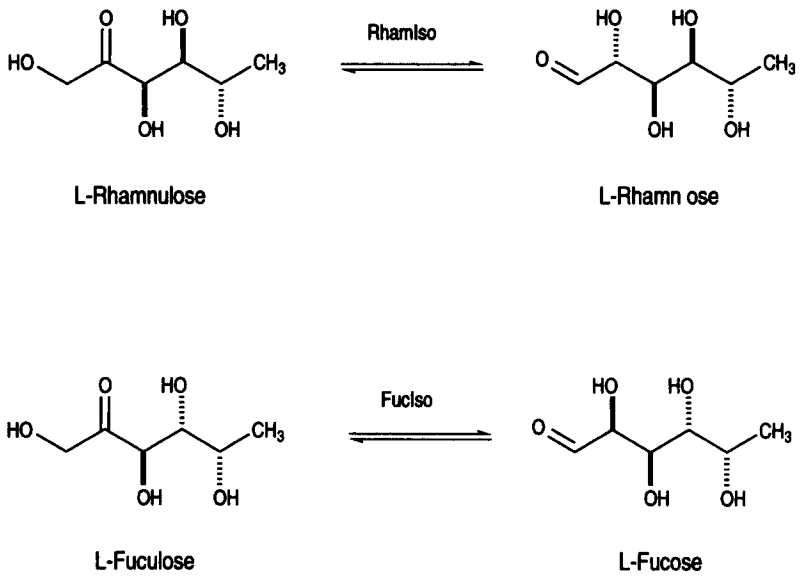

Figure 1. Reactions catalyzed *in vivo* by RhamIso and FucIso.

†The recombinant strains of *E. coli* encoding these enzymes have been deposited with the ATCC with the following numbers: 87023 for RhamIso and 87024 for FucIso.

Results and Discussion

Cloning of RhamIso and FucIso

For each target gene two primers were designed to specifically complement the C-terminal and N-terminal gene sequences of *rha*A[3] and *fuc*I.[5] 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 amplifications were quite specific, and only one band with a molecular weight consistent with that previously reported (1.3 and 1.8 kb, respectively for *rha*A, *fuc*I) was observed. The strategy for the cloning of these enzymes and the main characteristics of the plasmids constructed are shown in Figure 2. After the ligation of 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, seven to 10 colonies carried the desired insert. The Trc promoter present in the vector pTrcHis and the Tac promoter in the pKK223-3 vector, can be both induced by IPTG.

Table 1. Primers and restriction sites used for the cloning of RhamIso and FucIso

Isomerase	Restriction enzyme at 5′	Restriction enzyme at 3′	N-Terminal Primer (5′ → 3′)	C-Terminal Primer (5′ → 3′)
RhamIso	PstI	HindIII	ATATTCTGCAGACCACTCA ACTGGAA	GGCGCAAGCTTTTACCTGC TGGCGTATCGTGT
FucIso	EcoRI	PstI	ATATTGAATTCAACATGAA AAAAATCAGCTTACCG	GCGCCTGCAGTTAAACGCT TGTACAACGGA

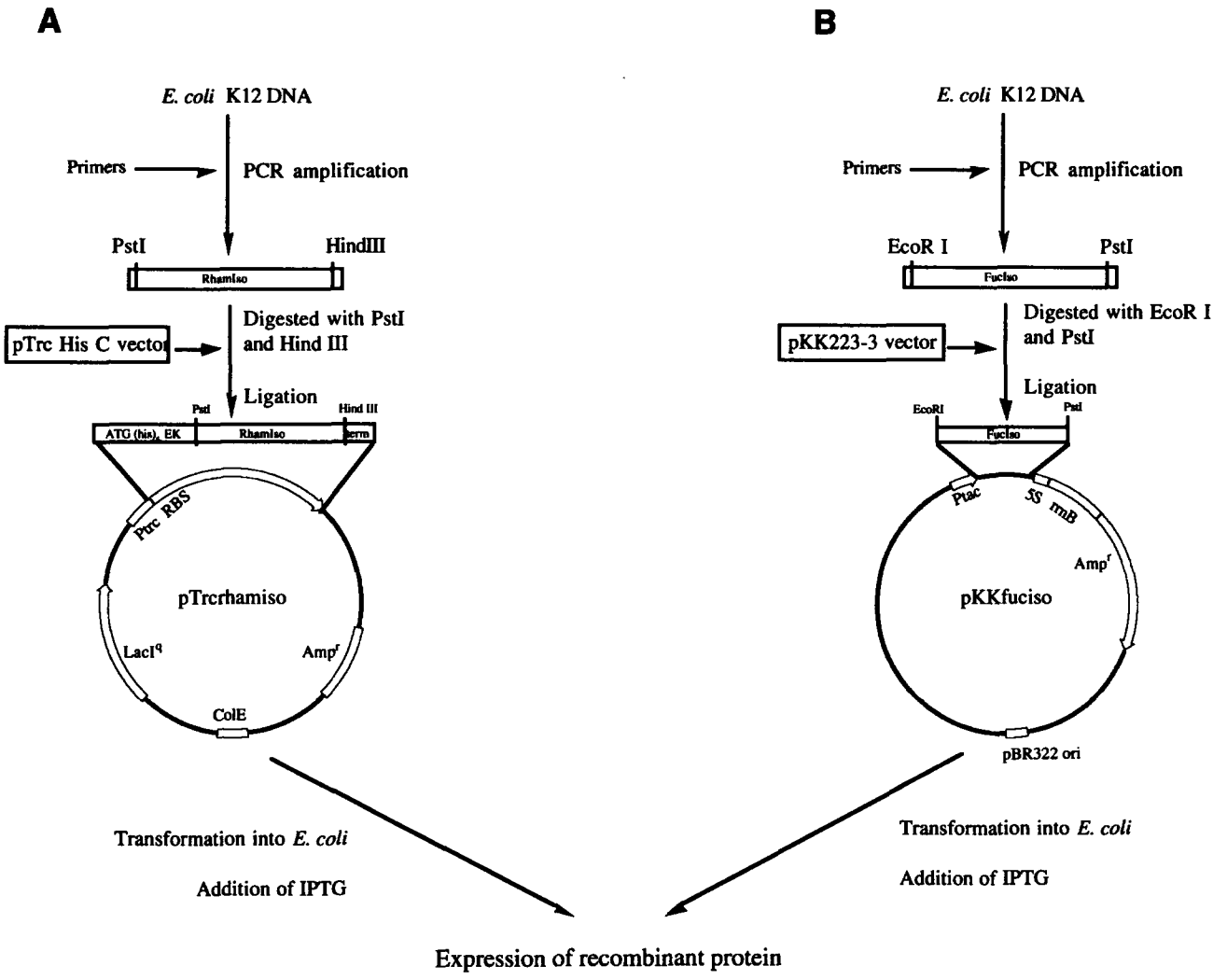

Figure 2. Strategy for the cloning of RhamIso and FucIso. The main characteristics of the expression vectors are shown.

Expression of recombinant RhamIso and FucIso

The expressions of RhamIso and FucIso 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 and examined with SDS-PAGE. The expression of these enzymes was strongly dependent on the cultivation temperature. When the cells were grown at 37 °C after induction with 150 μM of IPTG, the enzymes were expressed as inclusion bodies (data not shown). We have shown previously[7] that the formation of inclusion bodies can be avoided by dropping the cultivation temperature. In the expression of FucIso, when the temperature was dropped to 30 °C the enzyme was obtained in a soluble form and was the major band detected in the gel (Fig. 3). The expression of FucIso reached a maximum 3 h after the induction, and the enzyme remained stable in a long period of cultivation (Fig. 3). However, accumulation of the enzyme in overnight culture did not differ significantly from that found in the first 3 h of cultivation (Fig. 3). The production of FucIso in these conditions is about 16,500 U L^{-1} (≈ 2640 U g^{-1} of cell). On the other hand, the pattern of expression for RhamIso (Fig. 4) differed significantly from that found for FucIso. At 30 °C, after induction the enzyme is expressed almost exclusively as inclusion bodies. By dropping the temperature to 25 °C an increase in the proportion of soluble enzyme was observed. The activity found in this case in the soluble fraction was 227.5 U L^{-1}. The percentage of soluble enzyme expressed could not be greatly improved by dropping the temperature to 20 °C, and at this temperature a decrease in the total amount of RhamIso was observed. Because of these results, we attempted solubilization of the inclusion bodies. The pellet, obtained after disruption of the cells harvested from 1 L culture at 30 °C, was resuspended in 10 mL of distilled water. Urea was then added to the suspension to a final concentration of 6 M. After 1 h of incubation at room temperature, the suspension was centrifuged at 12,000 *g* for 30 min. The solubilization of the inclusion bodies was checked by SDS-PAGE of the supernatant (data not shown). The urea was removed slowly by dialysis against a total of 12 L of Tris–HCl buffer (50 mM, pH 7.6) with three changes during 36 h. After dialysis the sample was again centrifuged as above, and the supernatant analyzed by SDS-PAGE (data not shown) to check the presence of the soluble protein. The activity of the soluble enzyme from the inclusion bodies was determined by the cysteine–carbazol method[8] and was found to be about 2400 U L^{-1} of culture and the specific activity shown by RhamIso in this condition was 108.6 U mg^{-1} of protein. This method of preparing active enzyme is simple and can be applied to a larger volume of sample, with the advantage of obtaining the enzyme in a relatively pure form (Fig. 4) to be used directly in organic synthesis.

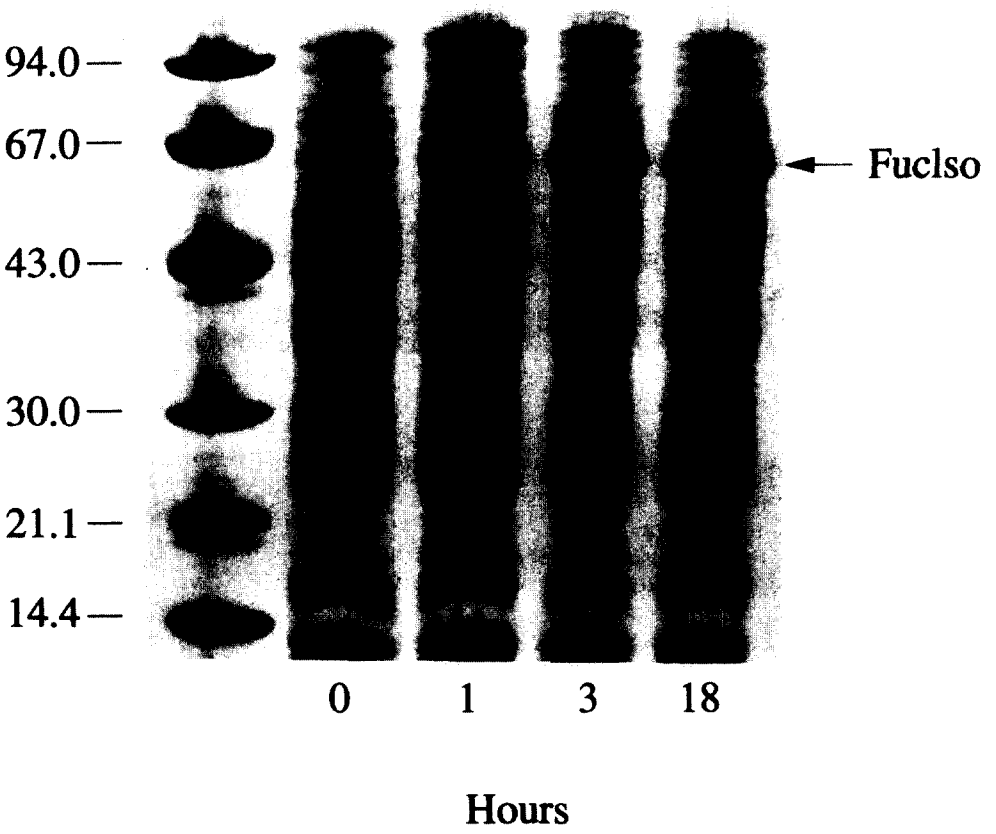

Figure 3. Monitoring of FucIso expression during the culture time. Aliquots were taken at different times after induction and analyzed by SDS-PAGE. The maximum accumulation is observed 3 h after induction. The enzyme remained stable after 18 h of culture.

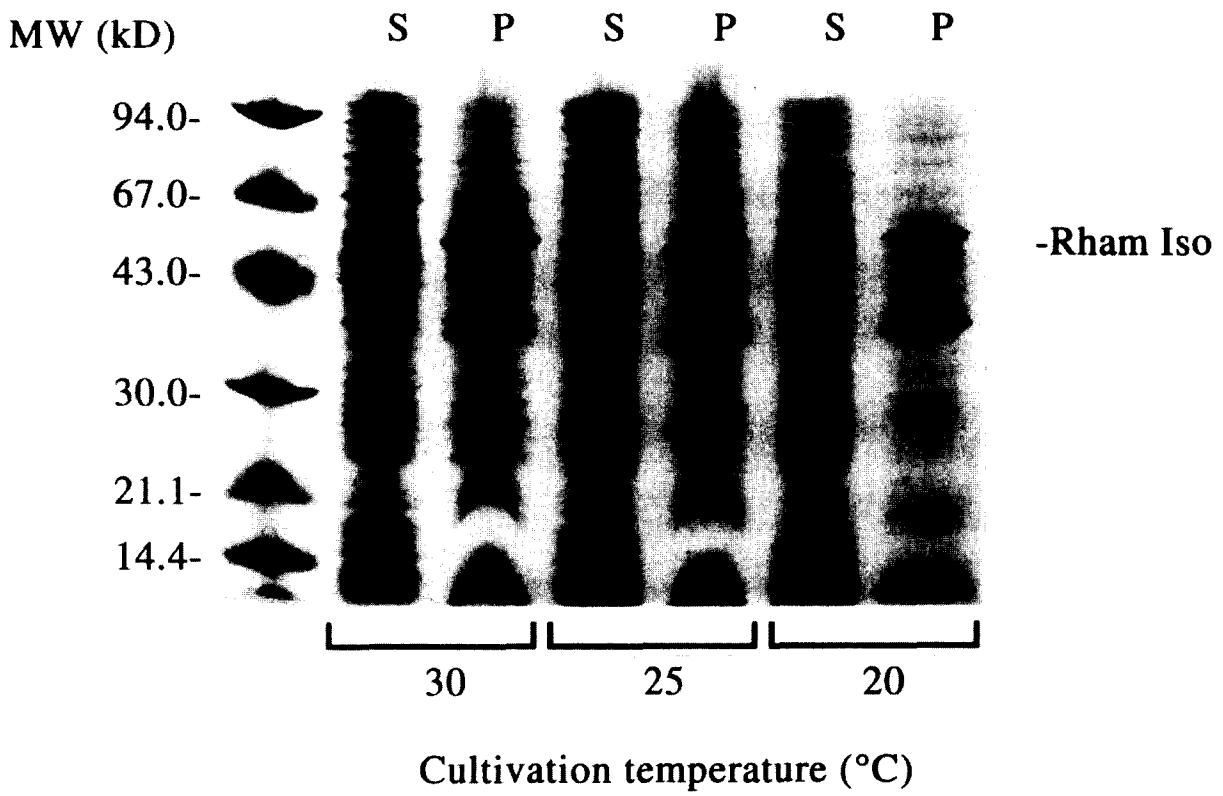

Figure 4. Expression analysis of RhamIso at different temperatures. Lanes S, supernatant; Lanes P, pellets. For details see the Experimental.

One step purification of FucIso

Recently it has been reported that recombinant proteins can be purified by repeating cycles of freezing and thawing.[9] We had applied this method to the purification of FucIso, but in our case the protein was not obtained in a completely pure form (Fig. 5, Lane 1). Further purification by FPLC using a Mono Q column (anion exchange) with a gradient between 0.2 and 0.5 M of NaCl provided the enzyme in a completely pure form (Fig. 5, Lane 2). The specific activity calculated from this preparation is 746.9 U mg^{-1} of protein.

Conclusion

In summary we have developed a new and efficient expression system for the overproduction of RhamIso and FucIso. We have optimized the culture conditions for the expression of the soluble FucIso. Also, we have developed a simple procedure for the solubilization of the inclusion bodies obtained in the case of RhamIso. We have also developed a new method for the one-step purification of FucIso.

Experimental

Vectors

The vector pTrcHis was obtained from Invitrogen Co. (San Diego, CA) and pKK223-3 was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). The vector pTrcHis (used to clone RhamIso) contains Trc promoter to allow a high expression of the required protein when induced with IPTG. The Trc promoter has the –35 region of the Trp promoter and the –10 region of the lac promoter. The Trc promotor is repressed by the lac repressor, which is encoded by the $lacI^q$ gene in the vector. The vector pKK223-3 (used to clone FucIso) contains the strong Tac promoter which is also regulated by the lac repressor and induced by the addition of IPTG to the medium. In addition, pTrcHis has a sequence that encodes an N-terminal peptide that includes a hexahistidine tag to function as a metal binding domain in the recombinant protein, and an enterokinase cleavage recognition sequence.

Microorganism

Escherichia coli K12 (ATCC 10798) was 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, the 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 of *E. coli* K12 was extracted according to the method described by Maniatis *et al.*[10] PCR amplifica-

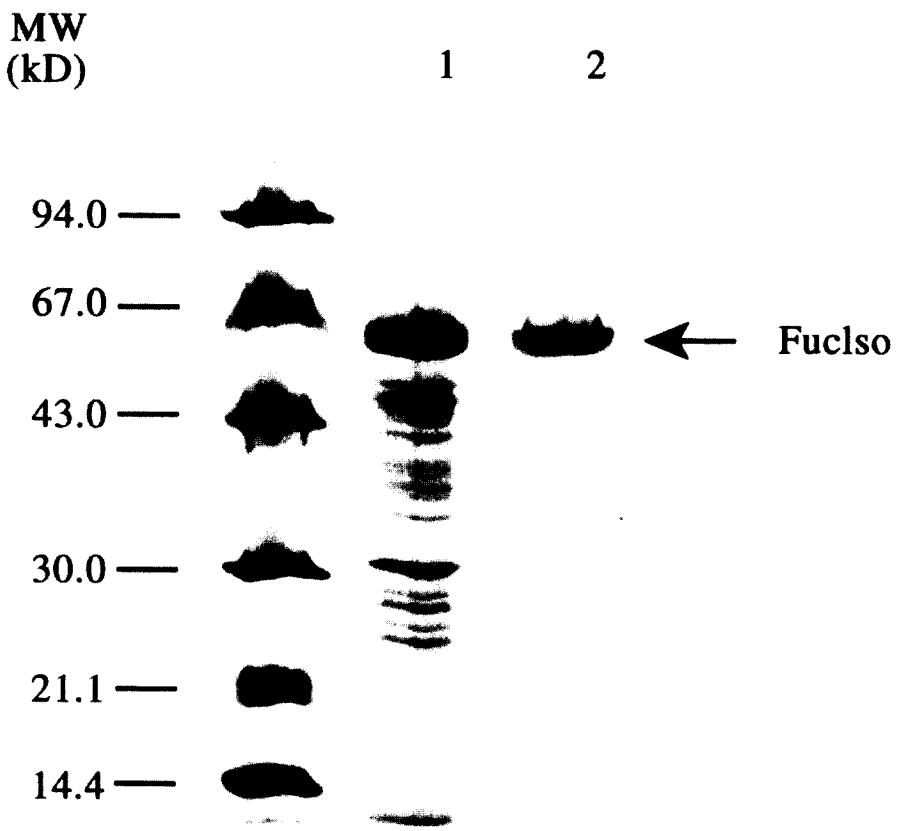

Figure 5. SDS-PAGE of the FucIso purification. Lane 1, crude extract after four cycles of freezing and thawing; Lane 2, FucIso after anion exchange chromatography.

tion 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 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 the 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 corresponding to the RhamIso gene was digested with PstI and Hind III, and the one corresponding to FucIso was digested with EcoRI and PstI (all the restriction enzymes were from 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 M NaOAc, pH 5.2), and purified by agarose (0.8%) gel electrophoresis as above. This DNA was used as insert. The vector pTrcHis was digested with PstI and Hind III and the vector pKK223-3 was digested with EcoRI and PstI. Then both vectors were recovered with ethanol precipitation after extraction with phenol: chloroform. The restriction enzyme-digested vectors were 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

Between 20 and 25 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 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 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 a 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 reach a final concentration of 150 μM to induce the expression of the target protein. Different temperatures of culture after induction were applied in order to optimize the conditions for the expression of the recombinant protein. The expression level of FucIso was analyzed at different times after induction until a total of 18 h of culture. In the case of the RhamIso the cells were harvested when the OD_{600} was about 2.0 (3 h when the cells were grown at 30 °C after induction, 4 h when the temperature was 25 °C and 18 h at 20 °C).

Analysis of inclusion bodies

The presence of inclusion bodies was analyzed according to the procedure describe by Maniatis *et al.*[10,11] Cells from 1 L culture were centrifuged (10,000 *g*, 30 min, 4 °C), and the pellet was suspended in Tris–HCl buffer (50 mM, pH 7.5) containing 8 μL of 50 mM phenylmethylsulfonylfluoride (PMSF) and 4 mg of deoxycholic acid per g of cell. The cells were disrupted by a French press at 16,000 lb in^{-2} and centrifuged at 16,000 *g* for 30 min. The pellet was resuspended in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM EDTA and 0.5% of Triton X-100. After incubation for 5 min at room temperature, the sample was centrifuged at 12,000 *g* for 15 min. Finally, the pellet obtained in this way was resuspended in 10 mL of distilled water. The presence of the recombinant protein in the supernatants and/or pellet was checked by SDS-PAGE in a Phast-system (Pharmacia Co.) using precast gels with a 10–15% gradient of polyacrylamide in the separation zone.

Solubilization of inclusion bodies

The inclusion bodies present in the pellet obtained as described above were solubilized by addition of urea to a final concentration of 6 M. After 1 h incubation at room temperature, the suspension was centrifuged at 12,000 *g* for 30 min. The urea was removed slowly by dialysis against a total of 12 L of Tris–HCl buffer (50 mM, pH 7.6) with three changes in 36 h. After dialysis the sample was again centrifuged as above.

Purification of the FucIso

The crude extract of the FucIso was prepared, with slight modifications, according to the procedure of Johnson and Hecht.[9] The cells obtained from 1 L cultures were frozen in a dry-ice:EtOH bath for 3 min, and then the sample was thawed by immersion in an ice:H_2O bath for 10 min. These steps were repeated three more times. Then the cells were gently resuspended in Tris–HCl buffer (50 mM, pH 7.6) and kept in an ice:H_2O bath for another 30 min. Finally the sample was centrifuged at 10,000 *g* for 4 min. Further purification of the FucIso was done on FPLC using an anion exchange Mono Q column. The sample was eluted with a gradient of NaCl between 0.2 and 0.5 M in Tris–HCl (50 mM, pH 7.6). Fractions containing proteins were detected by absorbance at 280 nm. The fractions containing FucIso were pooled and the NaCl removed using Centripep tubes (Amicon Co.) with M_r cut off of 10,000. The enzyme purity was determined by SDS-PAGE using precast gels with a gradient of polyacrylamide in the separation zone of 10–15%.

Enzyme activity assay

The activity of the isomerase was assayed using the ketose as substrate and measuring its disappearance by the cysteine–carbazol method.[8] The reaction mixture in the case of the FucIso contained 1.2 μmol of glycine buffer (pH 9.3), 0.05 μmol of $MnCl_2$, 5 μmol of L-fuculose and a certain amount of the enzyme in a total volume of 100 μL. For the RhamIso assay the reaction mixture contained 1.2 μmol of Tris–HCl buffer (pH 7.6), 1 μmol of $MnCl_2$, 1 μmol of L-rhamnulose and the enzyme also in a total volume of 100 μL. The mixture was incubated at 37 °C and aliquots of 5 μL were taken at different times in a period of 10 min. The calibration curve for the ketoses was done using L-fuculose and L-rhamnulose synthesized as described below. One unit of enzyme activity is defined as the quantity that catalyzes the formation of 1 μmol of aldose min^{-1} under the condition assayed. The protein concentration was determined using the BCA kit from Pierce. The samples were incubated during 30 min at 37 °C and the absorbance measured at 562 nm. The calibration curve was obtained using the concentrations of BSA between 25 and 2000 μg mL^{-1}.

Synthesis of L-rhamnulose and L-fuculose

L-Rhamnulose and L-fuculose were synthesized via aldolase-catalyzed condensation between dihydroxyacetone phosphate (DHAP) and L-lactaldehyde, followed by phosphatase-catalyzed cleavage of the phosphate group. DHAP[12] and L-lactaldehyde[13] were prepared as previously described. Aldolases (rhamnulose and fuculose-1-phosphate aldolases) were used as crude extract, which was prepared as previously reported.[7]

L-Hexulose-1-phosphate barium salt. Freshly prepared aqueous solutions of DHAP (6.0 mmol) and L-lactaldehyde (8.4 mmol) were combined and the resulting solution (70 mL) was adjusted to pH 6.8 with 6 M NaOH. Aldolase (rhamnulose-1-phosphate aldolase 60 U; fuculose-1-phosphate aldolase 80 U) was added and the reaction mixture incubated at 25 °C until the consumption of DHAP was higher than 90%, then the solution was adjusted to pH 7.0 with 6.0 M NaOH. $BaCl_2 \cdot 2H_2O$ (2 equiv.) was added and the mixture was refrigerated at 4 °C for 1 h. The precipitate was filtered off through Celite, acetone (200 mL) was added to the solution and the mixture was allowed to stand at 4 °C overnight. The precipitate was collected by centrifugation, washed with EtOH:Et_2O (1:1, 30 mL) and dried under vacuum. Yield: 80% (1.82 g) for rhamnulose-1-phosphate and 86% (1.96 g) for fuculose-

1-phosphate. The crude barium salt was used directly without characterization for the next reaction.[14]

L-Rhamnulose and L-fuculose. The crude barium salt of both hexuloses-1-phosphate was powdered, suspended in H_2O (35 mL) and treated with Dowex 50W-X8 (H^+ form, 200–400 mesh) for 30 min. The resin was filtered off and washed with H_2O (2 × 8 mL) and the solution was adjusted to pH 4.7 with 6 M NaOH. Acid phosphatase (80 U) was added and the mixture was stirred at 25 °C for 36 h. Then the solution was adjusted to pH 7.0 with $Ba(OH)_2 \cdot 8H_2O$ solution followed by addition of MeOH (2 vol). The precipitate was filtered off and the solution concentrated under reduced pressure (bath temperature <25 °C) for silica gel chromatography with $CHCl_3$:MeOH (2:1) to give L-rhamnulose (279 mg; 1.70 mmol; yield 40%) or L-fuculose (321 mg; 1.97 mmol; yield 46%) as slightly yellowish oils.

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