XYLOSE (GLUCOSE) ISOMERASE GENE FROM THE THERMOPHILE CLOSTRIDIUM THERMOHYDROSULFURICUM; CLONING, SEQUENCING, AND EXPRESSION IN ESCHERICHIA COLI

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The xylose isomerase gene from the thermophile C. thermohydrosulfuricum has been cloned, using a fragment of the Bacillus subtilis gene as a probe. The complete nucleotide sequence of the gene was analyzed. C. thermohydrosulfuricum is the most thermophilic organism from which a xylose isomerase gene has been cloned and characterized. Comparison with amino acid sequences from other xylose isomerases showed that amino acids involved in substrate binding and isomerization are well conserved. Purification of the enzyme produced in E. coli was done by heating a cell-free extract at 85°C for 10 min, giving a 20-fold purified enzyme. The native enzyme is a homomeric tetramer with a molecular weight of 200,000.

XYLOSE ISOMERASE (EC 5.3.1.5) catalyzes the reversible isomerization of D-xylose to D-xylulose in the first step of xylose metabolism following the pentose phosphate cycle. It also catalyzes the isomerization of glucose to fructose. Therefore it is industrially used in the production of High Fructose Corn Syrup (HFCS) under the name glucose isomerase.1 The enzyme has been isolated from many microorganisms and is well studied.2,3 Genes from E. coli,4 Bacillus subtilis,5 Streptomyces violaceoniger,6 Ampullariella,7 and Actinoplanes missouriensis8 have been sequenced. The crystal structure of Actinomyces xylose isomerase, an (α/β)₈-barrel, has been resolved.9,10

Because the isomerization is reversible, the final fructose content depends on the reaction temperature. A higher temperature gives a higher fructose content. Recommended temperatures for commercially available xylose isomerases are about 60°C. Xylose isomerase with increased fructose production (per gram of enzyme) at higher temperatures might be

Materials and Methods

Strains and plasmids. E. coli MV118413 was grown in 2YT13 at 37°C and transformed by the method of Hanahan.14 C. thermohydrosulfuricum ATCC 3322312 was grown at 65°C by the method of Melasniemi15 with xylose as a carbon source and medium supplemented with 1 g/l NH₄Cl and 0.03 g/l CoCl₂. Plasmid pIW11 containing the B. subtilis xylose isomerase gene was kindly provided by Hollenberg.5

Cloning and sequencing. Genomic C. thermohydrosulfuricum DNA was extracted as follows: a frozen cell pellet (0.4 g) was dissolved in 4.5 ml of 50 mM Tris·HCl, pH 8, 200 mM NaCl, and 100 mM EDTA, and lysed with 1%
SDS (15 min, 60°C). The lysate was treated with RNase A (0.05 mg/ml, 30 min, 60°C) and protease K (0.05 mg/ml, 60 min, 60°C). Debris was removed by centrifugation and the clear lysate was extracted four times with phenol. After the addition of 0.8 volume of isopropanol, DNA was spooled out, dissolved in 50 mM Tris–HCl, pH 8, 150 mM NaCl, and 1 mM EDTA and purified on a DEAE column. Southern hybridization of DNA after electrotransfer to a nylon membrane was done at 58°C. For sequencing, overlapping fragments were subcloned in pUC118 or pUC119. Single-stranded DNA was prepared by the method of Vieira13) and sequenced with modified T7 DNA polymerase.

**Homology search.** Comparisons of nucleotide and amino acid sequences were made with Genetyx 6.0 (Software Development Co.) programs HOMOGAPN and HOMOGAPP. These programs make small gaps for optimal alignment.

**Enzyme assay.** Enzyme activity was measured by incubation of 0.1–0.4 U xylose isomerase in 1 ml of 100 mM Hepes, pH 7, 400 mM fructose, and 10 mM MgCl₂ at 85°C. Glucose production was followed by analysis of 20-μl samples with a glucose oxidase based assay (glucose B-test, Wako). One unit was defined as the amount of enzyme that produces 1 μmol of glucose per minute under these conditions.

**Enzyme isolation.** Clostridium cells (5 l culture) were harvested in late logarithmic phase. E. coli cells (2 l culture) were induced at mid-log phase by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside, grown for three more hours, and harvested in stationary phase. Washed cells were resuspended in 100 mM Hepes, pH 7, 400 mM fructose, 10 mM MnCl₂, and 0.05 mM phenyl-methyl-sulfonyl-fluoride (a protease inhibitor), disrupted by sonication or French press, and the debris spun down. The supernatant was heated for 10 min at 85°C and allowed to cool. Denatured proteins were removed by centrifugation (15,000 rpm, 10 min, 4°C). The supernatant was dialyzed three times against 500 volumes of 50 mM Hepes, pH 7, and 5 mM EDTA, and two times against 500 volumes of buffer without EDTA. Further purification of *C. thermohydrosulfuricum* enzyme was done by FPLC anion exchange chromatography and gel filtration on Superose 12 (Pharmacia, Sweden). The molecular weight of the native enzyme was estimated from Superose 12 data.

**Results**

**Cloning and sequencing**

Genomic DNA from *C. thermohydrosulfuricum* was digested with *PstI*, *HindIII*, or *EcoRI* and separated on a 0.6% agarose gel. Southern hybridization with a 490 nucleotide *BbaI–BgII* fragment from the *B. subtilis* xylose isomerase gene⁵ (corresponding to nucleotides 647–1135 in *C. thermohydrosulfuricum*) gave a single positive band for *PstI* (3.6 kb), *HindIII* (2.2 kb) and *EcoRI* (8.7 kb) digests. Thus only one copy was present in the *C. thermohydrosulfuricum* genome. Genomic *PstI* fragments between 3.3 kb and 3.9 kb were isolated from agarose and ligated with *PstI* digested pUC119. Single-stranded DNA was prepared by the method of Vieira¹³ and sequenced with modified T7 DNA polymerase.

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Fig. 1. Nucleotide and Deduced Amino Acid Sequence of the *C. thermohydrosulphuricum* Glucose Isomerase Gene. rbs, the possible ribosome binding site; -10 and -35 indicate consensus sequences from procaryotic promoters.
Fig. 2. Comparison of the Xylose Isomerase Amino Acid Sequences of: (1) B. subtilis, (2) C. thermohydrosulfuricium, (3) E. coli, (4) S. violaceoniger, (5) Ampullariella.

Numbers are for the C. thermohydrosulfuricium enzyme and correspond to the numbers in Fig. 3. Boxes indicate active site amino acid residues. Dots under the sequence mark amino acids which are conserved in all five sequences compared.
Collyer\textsuperscript{10} are well conserved. This suggests that both models indicate amino acids important for activity, irrespective of the orientation of the sugar molecule. These conserved active site residues are not situated in one domain, but are spread out along the primary structure. Hence few regions of homology are found.

**Expression of the cloned gene**

The gene was cloned into plasmid pUC118\textsuperscript{13} for expression in *E. coli* under control of the inducible lac promoter. A 2.2 kb *PstI–HindIII* fragment, with 100 nucleotides upstream and 800 nucleotides downstream the coding region, was inserted in the *SmaI* site of the *lacZ* gene. After transformation, xylose isomerase levels reached 40 U/l during stationary phase, roughly a 4-fold increase compared with that of *C. thermohydrosulfuricum* (Table II). Endogenous activity of *E. coli* was not detected. Thus the xylose isomerase produced by *E. coli* appeared to be fully active.

**Table I. Relative Homology between Nucleotide and Amino Acid Sequences from Several Microorganisms**

<table>
<thead>
<tr>
<th></th>
<th>Amino acids (%)</th>
<th>C.t.</th>
<th>E.c.</th>
<th>B.s.</th>
<th>S.v.</th>
<th>A.</th>
<th>A.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. thermohydrosulfuricum</em></td>
<td>52</td>
<td>71</td>
<td>27</td>
<td>27</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>57</td>
<td>51</td>
<td>25</td>
<td>26</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>69</td>
<td>59</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. violaceoniger</em></td>
<td>42</td>
<td>47</td>
<td>44</td>
<td>67</td>
<td>67</td>
<td></td>
<td></td>
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<tr>
<td><em>Amphictyona</em></td>
<td>44</td>
<td>45</td>
<td>47</td>
<td>75</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. missouriensis</em></td>
<td>44</td>
<td>46</td>
<td>45</td>
<td>75</td>
<td>92</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3.** Models of the Active Site of *Actinomycetes* Xylose Isomerase from Carrell (a) or Collyer (b). Numbers indicate the position in the *C. thermohydrosulfuricum* amino acid sequence and correspond to the numbers in Fig. 2. The xylose molecule is drawn in thick lines, with numbered carbon atoms. Dashed lines indicate possible molecular interactions.
Table II. Production and Activity of Partially Purified Glucose Isomerase from C. thermohydrosulfuricum and E. coli
For conditions, see Materials and Methods.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Activity (U/mg protein)</th>
<th>Production (U/l culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. thermohydrosulfuricum</td>
<td>0.03</td>
<td>10</td>
</tr>
<tr>
<td>E. coli, no induction</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>E. coli, induced</td>
<td>0.09</td>
<td>40</td>
</tr>
</tbody>
</table>

Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of the Cell-free Extract from E. coli Harboring the C. thermohydrosulfuricum Xylose Isomerase Gene.
Lanes 1 and 2 contain sample from cells grown without inducer. Lanes 3 and 4 contain sample from cells grown in the presence of inducer (IPTG, see Materials and Methods). Lanes 1 and 4 contain the same volume of sample as 2 and 3 respectively, but after heat treatment. The small arrow indicates the xylose isomerase band.

Purification of the xylose isomerase
Purification of the enzyme produced by E. coli was rather straightforward. Heat treatment at 85°C for 10 min of cell-free extract in the presence of fructose and Mn²⁺ gave a 20-fold purified enzyme as judged by specific activities of the enzyme before and after heat treatment (Fig. 4). The xylose isomerase is stable under these conditions. Estimation of the subunit molecular weight by SDS-polyacrylamide gel electrophoresis indicated 51,000. Calculation from the deduced amino acid sequence predicts a molecular weight of 50,200. Gel filtration showed a molecular weight of about 200,000. This suggests that the native enzyme is a homomeric tetramer.

Discussion
Xylose isomerase from C. thermohydrosulfuricum has a high optimum temperature, around 85°C, suggesting potential for production at high temperatures of High Fructose Corn Syrup with increased fructose content. The xylose isomerase gene from C. thermohydrosulfuricum has been cloned and sequenced. Amino acids that have been identified as constituting the active site are found in similar positions in all five sequences from different microorganisms compared. The amino acid sequence of the C. thermohydrosulfuricum enzyme shows a high homology (71%) with the B. subtilis enzyme. Hydropathy plots of xylose isomerase from C. thermohydrosulfuricum and B. subtilis are almost the same, except for the 30 NH₂-terminal amino acids (data not shown). In this region, the enzyme from B. subtilis is more hydrophobic, while the enzyme from C. thermohydrosulfuricum has more charged residues. The glycine content of the C. thermohydrosulfuricum xylose isomerase was lower than in any of the other organisms. Low glycine contents may be related to increased protein stability.¹⁶

Expression in E. coli under control of the lac promoter gave a 4-fold increase in yield and easy purification. Heat treatment can easily be done on a large scale, and is thus suitable for the purification of cheap industrial enzymes.

Application of C. thermohydrosulfuricum xylose isomerase in solvents other than water might be attractive to shift the fructose/glucose equilibrium.¹⁷ Enzymes from thermophilic organisms sometimes have increased stability in solvents.¹⁸ Use of solvents might also increase the activity. Crystallography of Actinomycetes xylose isomerase⁹,¹⁶ has shown that the open aldehyde form of the sugar is
bound in the active site. Of the five possible glucose configurations, \(\alpha\)-\(D\)-glucopyranose, \(\beta\)-\(D\)-glucopyranose, \(\alpha\)-\(D\)-glucofuranose, \(\beta\)-\(D\)-glucofuranose, and open aldehyde, the aldehyde constitutes only 1%. On the other hand, open aldehyde of xylose amounts to 7%. The lower \(K_m\) for xylose\(^{3,19}\) probably reflect in part this difference in substrate concentration. If the percentage of the open aldehyde can be increased by changing the solvent, the real substrate concentration and thus the activity should increase.

Lee et al. have cloned and sequenced the xylose isomerase gene from \textit{C. thermosulfurogenes} (personal communication). This enzyme had an optimum temperature around 80°C. Comparison of the probably highly homologous genes might be interesting to see if amino acids responsible for the difference in optimum temperature can be located.

Further experiments are under way to study the stability and usefulness of \textit{C. thermohydrosulfuricum} xylose isomerase for fructose production.

References


