Cloning, sequencing and biochemical characterization of xylose isomerase from *Thermoanaerobacterium saccharolyticum* strain B6A-RI

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The xylose isomerase gene from *Thermoanaerobacterium saccharolyticum* strain B6A-RI was cloned by complementation using *Escherichia coli* xyl-5 mutant strain HB101. One positive clone was detected and the recombinant plasmid, pZXI6, was isolated. The clone contained the vector pUC18 and an insert fragment of 4.5 kb. The cloned xylose isomerase gene (*xylA*) was expressed constitutively in *E. coli*. The gene contained one open reading frame (ORF) of 1317 bp, which corresponds to 439 amino acid residues. The molecular mass of the gene product was calculated to be 50474 Da from the deduced amino acid sequence. A putative promoter region (Pribnow box), TATAATATATAAT, which repeated twice at the –10 region in *E. coli*, was found 25 bp upstream of the ribosomal binding site. The deduced amino acid sequence of *T. saccharolyticum* strain B6A-RI xylose isomerase exhibited very high homology to those from *Thermoanaerobacterium thermosulfurigenes* 4B (formerly *Clostridium thermosulfurogenes* 4B) and *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E). Codon usage in *xynA*, *xynB* and *xylA* showed a clear propensity for AT-containing isocodons. The native molecular mass of the purified recombinant thermostable xylose isomerase was 200 kDa, and the enzyme was a tetramer comprised of identical subunits. The apparent temperature and pH optima for activity of the cloned xylose isomerase were 80 °C and 7.0 to 7.5, respectively.

Introduction

Thermoanaerobacterium saccharolyticum strain B6A-RI is a thermophilic anaerobic bacterium capable of actively degrading xylan. Endoxylanase and β -xylosidase degrade the polymer to D-xylose, which is isomerized to D-xylulose and further catabolized in the cell. The isomerization of D-xylose is catalysed by D-xylose isomerase (EC 5.3.1.5) which is also used industrially to convert D-glucose to D-fructose syrups (Antrim *et al.*, 1979; Bucke, 1980).

In *Bacillus subtilis*, the enzymes encoded by the xylose regulon consist of genes for xylan and xylose utilization,

which cooperate in the utilization of hemicelluloses (Hastup, 1988). These genes are negatively regulated by the repressor (*xylR*) and are induced by xylose (Kreuzer *et al.*, 1989). In *T. saccharolyticum* strain B6A-RI, the endoxylanase and β -xylosidase activities were induced during growth on xylan and xylose. The synthesis of xylose isomerase from *T. saccharolyticum* was also induced by either xylose or xylan (Y.-E. Lee, S. E. Lowe & J. G. Zeikus, unpublished data).

There is increasing interest in enzymes which are thermostable and active at high temperatures, and for this reason, thermophiles have received greater attention recently. Xylose isomerase has been purified and characterized from *Thermoanaerobacterium saccharolyticum* strain B6A (formerly *Thermoanaerobacter* strain B6A) (Lee & Zeikus, 1991) and cloned from *Thermoanaerobacterium thermosulfurigenes* 4B (formerly *Clostridium thermosulfurogenes* 4B) into *E. coli* and *B. subtilis* (Lee *et al.*, 1990). To date, there are no data regarding the genetic organization of the xylose isomerase (*xyl*) genes in *T. saccharolyticum* strain B6A-RI. We have isolated genes encoding endoxylanase (*xynA*) and β -xylosidase (*xynB*) from the genomic library of *T. saccharolyticum* strain B6A-RI, which were found to be organized in a

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cluster on the chromosome (Lee, 1992). In this report, we describe the cloning, sequencing of the *xylA* gene from *T. saccharolyticum* strain B6A-RI and discuss its relationships to endoxylanase and β -xylosidase.

Methods

Bacterial strains and plasmids. *T. saccharolyticum* strain B6A-RI (Lee *et al.*, 1993) was used as the source of the xylose isomerase gene. *E. coli* *xyl*⁻ mutant strain HB101 (*F*⁻*mcrB mrr hsdS20*[r_B⁻m_B⁻]*recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20*[Sm^r]*xyl5 λ⁻ leu mtl1*) (Bachmann, 1972) was used as the host strain for cloning by complementation. Plasmid pUC18 was used as the cloning vector.

Chemicals, media and growth conditions. All chemicals were of analytical reagent grade and were of the highest purity available. *T. saccharolyticum* strain B6A-RI was grown under anaerobic conditions at 60 °C in TYE medium (Zeikus *et al.*, 1980) containing 0.5% xylose as a carbon source. For growth of *E. coli* strains, Luria broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per litre) was used for liquid culture, and solid medium contained 1.5% (w/v) agar (Difco). Ampicillin (50 µg ml⁻¹) was supplemented for the selection of transformants. MacConkey agar (Difco) plates containing 1% (w/v) xylose were used to detect xylose isomerase positive clones.

DNA preparation and cloning procedure. Chromosomal DNA of *T. saccharolyticum* strain B6A-RI was isolated by a modification of the method of Marmur (1961). Plasmid DNA was purified in large scale by the procedure described by Clewell (1972), followed by centrifugation in a caesium chloride/ethidium bromide density gradient. The alkaline denaturation method of Birnboim & Doly (1979) was used for rapid extraction of plasmid DNA.

Chromosomal DNA from *T. saccharolyticum* strain B6A-RI was partially digested with restriction endonuclease *AccI* and 4–10 kbp DNA fragments were isolated from an agarose gel by electroelution, using the elutrap (Schleicher & Schuell). Plasmid vector pUC18 was completely digested with *AccI* and dephosphorylated with calf intestinal alkaline phosphatase. Ligation was performed by the procedure of Sambrook *et al.* (1989) and transformed into *E. coli* HB101 competent cells prepared by the Hanahan method as described by Perbal (1988). Transformants were selected on MacConkey agar plates supplemented with 1% (w/v) xylose and ampicillin.

Sequence determination of the *xylA* gene. The recombinant plasmid was denatured by the method of Zhang *et al.* (1988), and the nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977), using a Sequenase version 2.0 kit (United States Biochemical). The sequence information was analysed using the GENEPRO software package (Hoefer Scientific Instruments) and the University of Wisconsin Genetics Computer Group GCG package (Devereux *et al.*, 1984).

Enzyme assay. Cell extracts prepared by sonication and purified preparations were used as enzyme sources, and glucose isomerase and xylose isomerase activities were measured as described by Lee *et al.* (1990b).

Purification of xylose isomerase. Xylose isomerase was purified from a 1 litre overnight culture of *E. coli* (pZXI6) cells as described by Lee *et al.* (1990b).

Protein determination and SDS-PAGE. Protein was measured by the method of Bradford (1976) using the Bio-Rad protein reagent and BSA (Sigma) as the standard. Enzyme fractions were analysed by SDS-PAGE in 12% (w/v) polyacrylamide gels in a Mini-PROTEAN II

system (Bio-Rad). Protein bands were visualized by Coomassie brilliant blue staining.

N-terminal amino acid sequence determination of xylose isomerase. The protein was prepared and analysed as described previously (Lee *et al.*, 1990b).

Results

Cloning of the xylose isomerase gene

The xylose isomerase gene from *T. saccharolyticum* strain B6A-RI was cloned by complementation using *E. coli* *xyl*-5 mutant strain HB101. Transformants were screened on MacConkey agar plates supplemented with 1% (w/v) xylose and ampicillin. One positive clone which gave a red colour on this plate was detected and the recombinant plasmid, pZXI6, was isolated. The clone contained vector pUC18 and an insert fragment of 4.5 kb (Fig. 1). The orientation of the fragment of *T. saccharolyticum* strain B6A-RI chromosomal DNA in the vector did not affect the level of xylose isomerase activity (data not shown), suggesting that the cloned *xylA* gene from *T. saccharolyticum* strain B6A-RI was expressed in *E. coli* by its own promoter. In *T. saccharolyticum* strain B6A-RI, xylose isomerase activity was induced by D-xylose, whereas in *E. coli* the cloned *xylA* was expressed constitutively and addition of D-xylose to the medium did not increase production (data not shown).

Nucleotide sequence of the *xylA* gene

The nucleotide sequence and deduced amino acid sequences are shown in Fig. 2. The computer analysis of the nucleotides sequenced revealed one ORF of 1317 bp, which corresponds to 439 amino acid residues. This was confirmed as the correct reading frame for the *T. saccharolyticum* strain B6A-RI *xylA* gene by comparison of the N-terminal amino acid sequence (underlined in Fig. 2), with that determined for the cloned xylose isomerase purified from *E. coli* (pZXI6).

The ATG initiation codon is preceded, with a spacing of 7 bp, by a putative ribosomal binding sequence AAGGAGG which is complementary to the 3' end of

Fig. 1. The physical map of pZXI6 containing vector pUC18 with the insert fragment containing the xylose isomerase gene.

1	TATTTTAGTATCTTCCCAGATGTTGGCTAATAGTTGGTTTGACAGGGTCGGTTATTTTTT	60
61	TTAAAAATTTTATTATGAAGAAGGATTTTTTAAATTTGTGTAGAATAATATAATATATA	120
121	ATGAAAAGAAGGACAGACAAACGAATAGAAAGGAGGAAGCTTTATGAATAAAATATTTTGAGA SD MetAsnLysTyrPheGluA	180
181	ACGTATCTAAAAATAAAATATGAAGGACCAAAATCAAATAATCCTTATTCTTTAAATTTT snValSerLysIleLysTyrGluGlyProLysSerAsnAsnProTyrSerPheLysPheT	240
241	ACAATCCAGAGGAAGTAATCGATGGCAAGACGATGGAGGAGCATCTCCGCTTTTCTATAG yrAsnProGluGluValIleAspGlyLysThrMetGluGluHisLeuArgPheSerIleA	300
301	CTTATTGGCACACTTTTACTGCTGATGGAACAGATCAATTTGGCAAGGCTACTATGCAAA laTyrTrpHisThrPheThrAlaAspGlyThrAspGlnPheGlyLysAlaThrMetGlnA	360
361	GACCATGGAACCACTACACAGATCCTATGGATATAGCGAAACGAAGGGTAGAAGCAGCAT rgProTrpAsnHisTyrThrAspProMetAspIleAlaLysArgArgValGluAlaAlaP	420
421	TTGAGTTTTTTGATAAGATAAAATGCACCTTCTTCTGCTTCCATGATAGGGATATTGCC heGluPhePheAspLysIleAsnAlaProPhePheCysPheHisAspArgAspIleAlaP	480
481	CTGAAGGAGATACTCTTAGAGAGACAAACAAAACTTAGATACAATAGTTGCTATGATAA roGluGlyAspThrLeuArgGluThrAsnLysAsnLeuAspThrIleValAlaMetIleL	540
541	AGGATTACTTAAAGACCAGCAAGACAAAAGTTTTTGTGGGTACCGCAAATCTTTTCTCCA ysAspTyrLeuLysThrSerLysThrLysValLeuTrpGlyThrAlaAsnLeuPheSerA	600
601	ATCCGAGATTTGTACATGGTGCATCAACATCCTGCAATGCTGACGTTTTTGCATATTCTG snProArgPheValHisGlyAlaSerThrSerCysAsnAlaAspValPheAlaTyrSerA	660
661	CAGCGCAAGTCAAAAAAGCCCTTGAGATTACTAAGGAGCTTGGCCGCAAACTACGTAT laAlaGlnValLysLysAlaLeuGluIleThrLysGluLeuGlyArgGluAsnTyrValP	720
721	TTTGGGGTGGAAGAGAAGGGTACGAGACGCTTCTCAATACAGATATGGAGTTAGAGCTTG heTrpGlyGlyArgGluGlyTyrGluThrLeuLeuAsnThrAspMetGluLeuGluLeuA	780
781	ATAACTTTGCAAGATTTTTGCACATGGCTGTTGACTATGCAAAGGAAATCGGCTTTGAAG spAsnPheAlaArgPheLeuHisMetAlaValAspTyrAlaLysGluIleGlyPheGluG	840

Fig. 2. For legend see p. 1230.

841	GTCAGTTCTTGATTGAGCCGAAGCCAAAGGAGCCTACAAAACATCAATACGACTTTGACG lyGlnPheLeuIleGluProLysProLysGluProThrLysHisGlnTyrAspPheAspV	900
901	TGGCAAATGTATTGGCATTCTTGAGAAAATACGACCTTGACAAATATTTCAAAGTAAATA alAlaAsnValLeuAlaPheLeuArgLysTyrAspLeuAspLysTyrPheLysValAsnI	960
961	TCGAAGCAAACCATGCGACATTGGCATTCCACGACTTCCAACATGAGCTAAGATACGCCA leGluAlaAsnHisAlaThrLeuAlaPheHisAspPheGlnHisGluLeuArgTyrAlaA	1020
1021	GAATAAACGGTGTATTAGGATCAATTGACGCAAATACAGGCGACATGCTTTTGGGATGGG rgIleAsnGlyValLeuGlySerIleAspAlaAsnThrGlyAspMetLeuLeuGlyTrpA	1080
1081	ATACGGACCAGTTCCCTACAGATATACGCATGACAACGCTTGCTATGTATGAAGTCATAA spThrAspGlnPheProThrAspIleArgMetThrThrLeuAlaMetTyrGluValIleL	1140
1141	AGATGGGTGGATTTGACAAAGGTGGCCTTAACTTTGATGCAAAAGTAAGACGTGCTTCAT ysMetGlyGlyPheAspLysGlyGlyLeuAsnPheAspAlaLysValArgArgAlaSerP	1200
1201	TTGAGCCAGAAGATCTTTTCTTAGGTACATAGCAGGAATGGATGCTTTTGCAAAAGGCT heGluProGluAspLeuPheLeuGlyHisIleAlaGlyMetAspAlaPheAlaLysGlyP	1260
1261	TTAAAGTTGCTTACAAGCTTGTGAAAGATGGCGTATTTGACAAGTTCATCGAAGAAAGAT heLysValAlaTyrLysLeuValLysAspGlyValPheAspLysPheIleGluGluArgT	1320
1321	ACGCAAGCTACAAAGAAGGCATTGGCGCTGATATTGTAAGCGGTAAAGCTGACTTCAAGA yrAlaSerTyrLysGluGlyIleGlyAlaAspIleValSerGlyLysAlaAspPheLysS	1380
1381	GCCTTGAAAAGTATGCATTAGAGCACAGCCAGATTGTAAACAAATCAGGCAGACAAGAGC erLeuGluLysTyrAlaLeuGluHisSerGlnIleValAsnLysSerGlyArgGlnGluL	1440
1441	TATTAGAATCAATCCTAAATCAGTATTTGTTGCAGAATAATGAAACATGAGGGCGGCTT euLeuGluSerIleLeuAsnGlnTyrLeuPheAlaGluEndEnd	1500
1501	CATGCTTCATTAAGGCTGCCCTCAACAAAAATCATGGAGGTAAATGTATGTATTTTTTAG	1560
1561	GGATAGATTTAGGGACATCATCAGTTAAGATAATACTGATG	1601

Fig. 2. The nucleotide sequence and deduced amino acid sequence of the cloned xylose isomerase. The computer analysis of the nucleotides sequenced revealed one open reading frame (ORF) of 1317 bp which corresponds to 439 amino acid residues. The underlined region denotes the correct reading frame for the *T. saccharolyticum* strain B6A-RI *xylA* gene by comparison of the N-terminal amino acid sequence determined from the xylose isomerase purified from *T. saccharolyticum* strain B6A (formerly *Thermoanaerobacter* strain B6A) (Lee & Zeikus, 1991).

both the *E. coli* and *B. subtilis* 16S ribosomal RNAs (McLaughlin *et al.*, 1981; Stormo *et al.*, 1982). A putative -10 promoter region (Pribnow box),

TATAATATATAAT, which repeated twice at the -10 region in *E. coli*, was found 25 bp upstream of the ribosomal binding site.

Table 1. Relative homology between xylose isomerase from *T. saccharolyticum* strain B6A-RI and other xylose isomerases

Strain	Similarity (%)	Identity (%)
<i>B. subtilis</i>	82.5%	69.6%
<i>E. coli</i>	64.0%	45.9%
<i>Thermus thermophilus</i>	51.1%	30.5%
<i>S. rubiginosus</i>	49.9%	26.5%
<i>S. violaceoniger</i>	48.3%	26.4%
<i>T. thermosulfurigenes</i> 4B (<i>C. thermosulfurogenes</i>)	99.1%	98.2%
<i>Thermoanaerobacter ethanolicus</i> 39E (<i>C. thermohydrosulfuricum</i>)	ND	90.4%

ND, Not determined.

Comparison of amino acid sequences

The deduced amino acid sequence of xylose isomerase from *T. saccharolyticum* strain B6A-RI was compared to those of *T. thermosulfurigenes* 4B (Lee *et al.*, 1990b), *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) (Dekker *et al.*, 1991b), *E. coli* (Schellenberg *et al.*, 1984), *B. subtilis* (Wilhelm & Hollenberg, 1985), *Streptomyces violaceoniger* (Drocourt *et al.*, 1988), *Streptomyces rubiginosus* (Wong *et al.*, 1991) and *Thermus thermophilus* (Dekker *et al.*, 1991a) and the results are shown in Table 1.

The length of the polypeptide chain encoded by the *xylA* gene in *T. saccharolyticum* strain B6A-RI was identical to that of *T. thermosulfurigenes* 4B (439 amino

(1)	MNKFENVSKIKEYEGPKSNNPYSEKFYNPEEVIDGKTMEEHLRFSIAYWH	50
(2)	MNKFENVSKIKEYEGPKSNNPYSEKFYNPEEVIDGKTMEEHLRFSIAYWH	50
(3)	-MEYFKNVPQIKYEGPKSNNPYAFKFYNPDEIIDGKPLKEHLRFSVAYWH	49
	*** * ** * * * * *	
(1)	TFTADGTDQFGKATMQRPWNHYTDPMDIAKRRVEAAFEFFDKINAPFFCF	100
(2)	TFTADGTDQFGKATMQRPWNHYTDPMDIAKARVEAAFEFFDKINAPYFCF	100
(3)	TFTANGTDPFGAPTMQRPWDHFTDPMDIAKARVEAAFELEKLDVPPFFCF	99
	* * * * * * * * * *	
(1)	HDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRF	150
(2)	HDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRF	150
(3)	HDRDIAPEGETLRETNKNLDTIVAMIKDYLKTSKYKVLWGTANLFSNPRF	149
	* *	
(1)	VHGAATSCNADVFAYSAAQVKKALEITKELGRENIVFWGGREGYETLLNT	200
(2)	VHGAATSCNADVFAYSAAQVKKALEITKELGGENIVFWGGREGYETLLNT	200
(3)	VHGAATSCNADVFAYSAAQVKKALEITKELGGQNYVFWGGREGYETLLNT	199
	* * * *	
(1)	DMELELDNFARFLHMAVDYAKEIGFEGQFLIEPKPKPTKHKQYDFDVANV	250
(2)	DMEFELDNFARFLHMAVDYAKEIGKEGQFLIEPKPKPTKHKQYDFDVANV	250
(3)	DMELELDNLFARFLHMAVEYAQEIIGFEGQFLIEPKPKPTKHKQYDFDAASV	249
	* * * * * *	
(1)	LAFLRKDYLDKYFKVNIENHATLAFHDFQHELRYARINGVLGSIDANTG	300
(2)	LAFLRKDYLDKYFKVNIENHATLAFHDFQHELRYARINGVLGSIDANTG	300
(3)	HAFLKKDYLDKYFKLNIEANHATLAGHDFQHELRYARINNMVLSIDANMG	299
	* * * * * ** *	
(1)	DMLLGWDTQFPTDIRMTTLAMYEVIKMGGFDKGGNLFDAKVRASFEPE	350
(2)	DMLLGWDTQFPTDIRMTTLAMYEVIKMGGFDKGGNLFHAKVRASFEPE	350
(3)	DMLLGWDTQYPTDIRMTTLAMYEVIKMGGFNKGGNLFDAKVRASFEPE	349
	* * *	
(1)	DLFLGHIAGMDAFAGFKVAYKLVKDGVDKFIERYASYKEGIGADIVS	400
(2)	DLFLGHIAGMDAFAGFKVAYKLVKDRVDFKFIERYASYKDGIGADIVS	400
(3)	DLFLGHIAGMDAFAGFKVAYKLVKDGVDKFIERYKSYKEGIGAEIVS	399
	* * * *	
(1)	GKADFKSLEKYALEHSQIVNKSQRQELLESILNQYLF AE	439
(2)	GKADFKSLEKYALERSQIVNKSQRQELLESILNQYLF AE	439
(3)	GKANFKTLEEYALNNPKIENKSGKQELLESILNQYLF SE	438
	* * * * * * * *	

Fig. 3. Comparison of the predicted xylose isomerase amino acid sequence of: (1) *T. saccharolyticum* strain B6A-RI, (2) *T. thermosulfurigenes* 4B (*C. thermosulfurogenes* 4B), (3) *Thermoanaerobacter ethanolicus* 39E (*C. thermohydrosulfuricum* 39E). Bars under the sequences indicate amino acids which are different in the sequence from *T. saccharolyticum* strain B6A-RI and *T. thermosulfurigenes* 4B, and the stars indicate differences between the sequence of *T. saccharolyticum* and *Thermoanaerobacter ethanolicus* 39E.

acid residues) and was similar to those of *Thermoanaerobacter ethanolicus* 39E (438 residues), and *E. coli* and *B. subtilis* (440 residues), whereas the enzymes from *S. violaceoniger*, *Ampullariella* and *Arthrobacter* have polypeptides which are shorter by 47 residues at the N-terminal. The deduced amino acid sequence of *T. saccharolyticum* B6A-RI xylose isomerase exhibits very high homology to those from *T. thermosulfurigenes* 4B and *Thermoanaerobacter ethanolicus* 39E (98 % and 89 % identity, respectively) and their sequence alignment is shown in Fig. 3.

Codon usage in *xynA*, *xynB* and *xylA*

Table 2 summarizes codon usage in the three genes from

Table 2. A comparison of the codon usage frequency in the three genes from *T. saccharolyticum* strain B6A-RI which are involved in xylan degradation

Amino acid	Codon	Frequency of codon usage (mol %)			
		<i>T. saccharolyticum</i> strain B6A-RI			<i>E. coli</i>
		<i>xynA</i>	<i>xynB</i>	<i>xylA</i>	
Phe	TTT	2.8	5.6	5.2	1.3
	TTC	0.4	1.0	3.0	2.2
Leu	TTA	2.1	1.4	1.6	0.7
	TTG	1.7	2.0	1.8	0.9
	CTT	1.4	2.0	3.0	0.8
	CTC	0	0.4	0.5	0.8
	CTA	0.1	0.4	0.7	0.2
Ile	CTG	0.6	0.2	0	6.8
	ATT	3.3	2.4	1.6	2.2
	ATC	0.6	0.8	1.1	3.7
Met	ATA	3.4	3.4	2.3	0.2
	ATG	1.8	2.2	2.7	2.8
Val	GTT	3.1	2.2	1.1	2.9
	GTC	0.3	1.0	0.5	1.2
	GTA	2.8	2.4	2.7	1.8
	GTG	1.0	1.8	0.5	2.2
Ser	TCT	2.4	1.4	0.7	1.3
	TCC	0.2	0.4	0.7	1.5
	TCA	2.0	1.0	1.4	0.4
	TCG	0.4	0.2	0	0.6
	AGT	1.5	0.4	0	0.3
Pro	AGC	1.2	1.2	1.1	1.4
	CCT	1.2	2.0	1.4	0.5
	CCC	0.2	0.2	0	0.3
	CCA	1.9	2.4	1.1	0.7
Thr	CCG	0.4	0.8	0.5	2.5
	ACT	2.9	1.2	1.1	1.1
	ACC	0.4	0.6	0.5	2.4
	ACA	3.6	2.0	2.7	0.3
Ala	ACG	1.1	1.0	0.9	0.8
	GCT	2.0	1.6	2.7	2.6
	GCC	0.4	0.4	0.7	2.2
	GCA	3.1	1.2	4.6	2.3
	GCG	0.4	0.6	0.7	3.2

Fig. 4. SDS-PAGE analysis of the thermostable *T. saccharolyticum* strain B6A-RI xylose isomerase expressed in *E. coli* (pZXI6). Lane M, molecular mass markers; lane 1, crude extract; lane 2, after heat treatment; lane 3, after anion exchange chromatography; lane 4, after gel filtration.

T. saccharolyticum strain B6A-RI which are involved in xylan degradation. *xynA* and *xynB* were closely linked on a chromosome of *T. saccharolyticum* strain B6A-RI (Lee, 1992). Average codon usage for *E. coli* (Allf-Steinberger, 1984) is included for comparison. The patterns of codon usage among the three genes were very similar to each other. There is a clear propensity toward AT-containing isocodons, and this probably results from the low G + C content (36 mol %) of this organism.

Biochemical characterization of cloned xylose isomerase

The thermostable *T. saccharolyticum* strain B6A-RI xylose isomerase expressed in *E. coli* was purified to homogeneity (Fig. 4). Heat treatment of cell extracts at 85 °C for 15 min was a very efficient purification step for the enzyme from *E. coli*, as approximately 90 % of *E. coli* proteins could be removed by this procedure. After heat treatment, the purification yield increased 18-fold. After additional purification by anion-exchange chromatography and gel filtration, preparations of homogeneous recombinant xylose isomerase were obtained and their purity was demonstrated by SDS-PAGE (Fig. 4). The purified enzyme consisted of one type of subunit with a molecular mass of 50 kDa. The molecular mass of the purified recombinant enzyme was 200 kDa, indicating that the enzyme is a tetramer composed of identical subunits.

The apparent temperature and pH optima for activity of the cloned xylose isomerase were 80 °C and 7.0 to 7.5,

respectively. The enzyme was stable at 80 °C for 60 min, and 0.5 mM-Co²⁺ and 5 mM-Mg²⁺ were required for optimum enzyme activity and thermostability.

Discussion

Xylose isomerase is an important industrial enzyme (Antrim *et al.*, 1979; Bucke, 1980) because of its ability to catalyse the conversion of glucose to fructose. Xylose isomerases from thermophilic bacteria have high temperature optima and are thermostable, indicating their potential use for the commercial conversion of starch to high-fructose corn syrup at high temperature with increased fructose yield.

Xylose isomerases have been isolated and studied from many micro-organisms. *xyl* genes from *E. coli* (Schellenberg *et al.*, 1984), *B. subtilis* (Wilhelm & Hollenberg, 1984, 1985), *Thermus thermophilus* (Dekker *et al.*, 1991a), *S. violaceoniger* (Marcel *et al.*, 1987), *Thermoanaerobacter ethanolicus* 39E (Dekker *et al.*, 1991b) and *T. thermosulfurigenes* 4B (Lee *et al.*, 1990b) have been cloned and sequenced. Comparison of the primary structure of xylose isomerases from three thermophilic anaerobic bacteria, *T. saccharolyticum* strain B6A-RI, *T. thermosulfurigenes* 4B and *Thermoanaerobacter ethanolicus* 39E, indicate extensive similarities between the enzymes from these organisms. Because these strains have evolved in thermal hotspots in Yellowstone National Park (Lee *et al.*, 1993; Schink & Zeikus, 1983) these organisms might have a common phylogenetic origin with conservation of xylose isomerase, or alternatively, gene transfer might have occurred between them.

In several bacteria in which the genetic organization of the *xyl* genes has been examined, the xylose isomerase and xylulose kinase genes were found to be part of one operon (Ghangas & Wilson, 1984; Lawlis *et al.*, 1984; Wilhelm & Hollenberg, 1984). Another ORF with the same transcriptional orientation was found downstream of *xylA* from the cloned DNA fragment from *T. saccharolyticum* strain B6A-RI (data not shown) and this may be a xylulose kinase gene (*xylB*).

The predicted molecular mass of 50474 Da, calculated from the deduced amino acid sequence, is in good agreement with the subunit molecular mass of 50 kDa reported for xylose isomerase purified from *T. saccharolyticum* strain B6A (formerly *Thermoanaerobacter* strain B6A) (Lee & Zeikus, 1991). The recombinant protein was purified to homogeneity and its physical and biochemical properties were determined. The molecular mass of the purified recombinant xylose isomerase was 200 kDa, indicating that the enzyme is a tetramer composed of identical subunits, and both enzymes had the same N-terminal sequence. This result is similar to that for the native xylose isomerase from *T. saccharo-*

lyticum strain B6A (Lee & Zeikus, 1991), and the two xylose isomerases also displayed identical pH and temperature optima, thermostability, and metal ion requirements.

The base composition of the coding region of *xylA* is 39.7 mol% G+C, a value that is close to the G+C content (36 mol%) of this organism (Lee *et al.*, 1993). The low G+C content of *T. saccharolyticum* strain B6A-RI *xylA* results in an extremely biased usage of the bases, A or T, which were present in the third position of all dominant codons examined in this organism.

The high thermostability of *T. saccharolyticum* strain B6A-RI xylose isomerase made it possible to use heat treatment as a very efficient step in purifying the cloned enzyme produced in the mesophilic host *E. coli*. The presence of metal ions (Mg²⁺ and Co²⁺) and a high protein concentration in cell extracts were essential for optimal recovery of the enzyme during heat treatment. The thermostable xylose isomerase produced by the recombinant plasmid pZXI6 was one of the most abundant proteins in *E. coli*.

The catalytic mechanism for xylose isomerase was originally believed to involve histidine-directed general base catalysis (Rose *et al.*, 1969). Lee *et al.* (1990b) identified the active site histidine residues of xylose isomerase from *T. thermosulfurigenes* 4B by site-directed mutagenesis, and found that His¹⁰¹ was the only essential histidine residue involved directly in catalysis. The rate-limiting step in the isomerization reaction was found to be hydrogen transfer and not substrate ring opening.

At present, the molecular mechanism underlying the high thermophilicity (i.e., temperature optimum for activity at 80 °C and thermostability at 85 °C) of this enzyme is not clear. This problem could be addressed through the use of nested deletion mutants to identify gene regions which are responsible for activity and thermostability.

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