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Cloning and Expression of the Gene for Xylose Isomerase from *Thermus flavus* AT62 in *Escherichia coli*

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ABSTRACT

The gene encoding xylose isomerase (*xyl*A) was cloned from *Thermus flavus* AT62 and the DNA sequence was determined. The *xyl*A gene encodes the enzyme xylose isomerase (XI or *xyl*A) consisting of 387 amino acids (calculated Mr of 44,941). Also, there was a partial xylulose kinase gene that was 4 bp overlapped in the end of XI gene. The XI gene was stably expressed in *E. coli* under the control of *tac* promoter. XI produced in *E. coli* was simply purified by heat treatment at 90°C for 10 min and column chromatography of DEAE-Sephacel. The Mr of the purified enzyme was estimated to be 45 kDa on SDS-polyacrylamide gel electrophoresis. However, Mr of the cloned XI was 185 kDa on native condition, indicating that the XI consists of homomeric tetramer. The enzyme has an optimum temperature at 90°C. Thermostability tests revealed that half life at 85°C was 2 mo and 2 h at 95°C. The optimum pH is around 7.0, close to where by-product formation is minimal. The isomerization yield of the cloned XI was about 55% from glucose, indicating that the yield is higher than those of reported enzymes. The *K*m values for various sugar substrates were calculated as 106 m*M* for glucose. Divalent cations such

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as Mn^{2+}, Co^{2+}, and Mg^{2+} are required for the enzyme activity and 100 m*M* EDTA completely inhibited the enzyme activity.

Index Entries: *Thermus flavus* AT62; xylose isomerase; xylulose kinase; overlapping gene; gene expression; *tac* promoter.

INTRODUCTION

Xylose Isomerase (XI: glucose isomerase EC 5.3.1.5) is perhaps the single most widely used enzyme in the food industry. The enzyme, derived principally from *Bacillus coagulans (1)*, *Streptomyces* sp. *(2)*, and *Lactobacillus brevis (3)* is utilized in the annual conversion of 2.65 MMt (drybasis, 1981) of glucose (from hydrolyzed corn starch) into fructose *(4)*. Both immobilized whole cells and purified enzyme are employed in a number of reaction formats *(5,6)*. The true physiological substrate of this cytoplasmic enzyme is the pentose sugar xylose, which is isomerized to xylulose, hence the enzyme is correctly termed XI.

In all bacteria capable of utilizing xylose as a sole carbon source, xylose isomerization to xylulose is carried out by a single enzyme, XI *(1–3)*. Generally, the bacterial *xyl* operon has been reported to contain three inducible enzymes: xylose transport, XI, and xylulose kinase (XK) in addition to regulatory region that controls all of these functions *(7)*. Usually termed *xyl*A is XI gene and *xyl*B is XK except *Butyrivibrio fibrisosolvens* in which *xyl*B is β-xylosidase *(8)*. Industrially, XI is called glucose isomerase and actually, it has been used in glucose conversion to fructose *(4)*. Several groups have reported the cloning, and in most cases, the nucleotide sequence of the *xyl* operon genes from a number of bacteria including *Escherichia coli (9)*, *Bacillus subtilis (10)*, *Ampullariella* species *(11)*, *Actinoplanes missouriensis (12)*, *Clostridium* species *(13,14)*, *Thermus thermophilus* HB8 *(15)*, *Arthrobacter (16)*, *Streptomyces* species *(17,18)*, *Klebsiella pneumoniae* 1033 *(19)*, and *Lactobacillus brevis (20)*. Certain members of the *Lactobacillus* sp. have been reported to be able to catalyze xylose isomerization with a number of XI's having been purified and characterized *(7,20)*. There are also several reports in yeast *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae (21,22)*. The crystal structure of *Actinomycetes* XI, an $(\alpha/\beta)_8$ barrel, has been resolved *(23)*, and its reaction mechanism has been gradually unraveled *(14,24)*.

Since thermophiles such as *Thermus* species and *Thermatoga* species are known to produce highly thermostable enzymes *(11,12,37–39)*, it is interesting to analyze the structure and thermostability of XIs from thermophiles. Lehmacher and Biswanger *(25)* recently purified XI from *Thermus* species and showed that the XI is indeed exceptionally stable at elevated temperatures. After 30 d at 70°C, glutaraldehyde-crosslinked *Thermus* XI retained 100% activity, whereas similarly treated *Streptomyces* XI retained about 25% activity *(26)*. However, the amount of enzyme produced by *Thermus* species is too small.

In this study, XI gene from *Thermus flavus* AT62 was cloned in *E. coli* K12 and characterized. The amino acid sequence of this extremely stable enzyme is compared with sequences of other thermostable enzymes to see

if any amino acid substitutions could be found that might affect the irreversible inactivation at high temperatures *(9–22)*. XI is one of the frequently used enzyme for the study of thermostability *(26,27)*. And XIs from *Thermus* sp. are highly recommended enzymes for thermostabilty study *(25)*. After expression in mesophilic bacteria, like *E. coli*, purification of thermostable protein was carried out by simple boiling at 90°C for 10 min *(25,28)*. Therefore, it is adequate for industrial system.

Localization and organization of *xyl* operons are diverse in many bacteria, affording its use in molecular evolutionary genetics *(17)*. Genome structure around *xyl*A shows taxonomic relationship with eubacteria and this comparison also shows the estimated time that diversity occurred. The comparison of DNA and amino acid between *Thermus* sp. shows taxonomic refinement. The two genes, *xyl*AB, are usually found in an operon, but overlapping *xyl*AB gene structure in bacterial strains is very rare *(17)*. In genomic structure of viruses, many cases of overlapping were reported, but only few cases of overlapping genes were reported from bacteria to human *(31–33)*. We found out that *xyl*A and *xyl*B coding regions in *Thermus flavus* AT62 is 4 bp overlapped. However, *xyl*B gene had only been partially cloned but this putative translational coding region of 65 amino acids clearly shows high amino acid homology with *N*-terminal amino acid of reported xylulose kinases. The translational regulation of overlapping *xyl*AB gene will be studied.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this work are given in Table 1. *E. coli* K12 strains and *T. flavus* AT62 were cultivated as by Dekker et al. *(28)*. Selective media were supplemented with 100 mg/L ampicillin. X-gal agar plates were prepared according to Experiments in Molecular Genetics *(32)*. For enzymatic assays, cells were grown to late exponential phase in 100 mL L broth with the appropriate antibiotic added and induced by 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for *tac* promoter.

Assay and Purification of Enzyme

After induction with IPTG, recombinant *E. coli* cells were harvested by centrifugation at 5000*g* for 10 min, and washed with buffer (10 m*M* Tris-HCl, 10 m*M* $MgCl_2$, pH 7.6). After sonication (2 × 30 s at 35–40 W) in an ice bath with a Branson sonifier B15 (Branson Sonic Power, Danbury, CT), cell debris were suspended in 50 mg/mL of streptomycin sulfate and boiled at 90°C or 10 min. The cell debris and host protein were spun down for 15 min at 20,000*g*, and the supernatant was dialyzed with 50 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid), pH 7.5. In some cases, the enzyme was further purified by fast protein liquid chromatography on a DEAE-Sephacel HR10/10 (Pharmacia, Upsala,

Table 1
Strains and Plasmids Used in the Present Work

Strains	Relevant genotype	Source of reference
Escherichia coli		
DH5	hsdR recA end gyr thi	Hanahan (*36*)
HB101	hsdS recA proA lacY galK xyl-5 mtl	Boyer (*41*)
XL1 blue		Amann (*42*)
Thermus flavus		
AT62		Oshima et al. (*43*)

Plasmid	Size, kb	Relevant markers	Source of reference
pUC18 & 19	2.7	*bla lacZ*	Messing et al. (*44*)
pKK223-3	3.3	*bla lacZ*	Amann (*42*)
pXI11	4.0	*bla xylA*	This study
pKX11	4.0	*bla tac-pro xylA*	This study
pXYLTf	5.5	*bla xylAB¢*	This study

Sweden). Protein contents were determined according to Bradford *(33)* with bovine serum albumin as standard. Molecular weight and purity of the enzyme were estimated by 10–14% SDS-PAGE *(34)*. XI activity was measured as by Dekker et al., using fructose or glucose as substrate with Carbopac PA1 column of High pH Anion Exchange Chromatography (HPAEC, Dionex) by pulsed-amperometric detector (PAD) or peroxidase-glucose oxidase (PGO) assay kit of Sigma (Sigma, Deisenhofen, Germany).

Polymerase Chain Reaction (PCR), Recombinant DNA and Electrophoretic Techniques

Preparation, restriction analysis, modification, and ligation of plasmid DNA were carried out following standard methods of Sambrook et al. *(35)*. Chromosomal DNA preparation, PCR, and electrophoresis of DNA fragments were carried out according to standard methods (Sambrook et al. 1992). DNA fragments were isolated using Geneclean BIO101 (Dianova, Hamburg, Germany). Transformation of competent cells was done according to Hanahan *(36)*.

DNA Sequence Analysis

DNA sequence determination was done using inserts in plasmids pUC18 by dideoxy chain-termination method and the T7 sequencing system (Pharmacia LKB, Freiburg, Germany) or Taq DyeDeoxy Terminator Cycle Sequencing Kit with ABI 373A Automatic DNA Sequencer (Applied Biosystems Inc., CA). α-[^{35}S]-dATP (Amersham, Braunshweig, Germany) and synthetic primers (Korea Biotech, Taejon, Korea) were used. DNA and

Table 2
Oligo Primers Used for PCR Amplification and Southern Hybridization

Primer designation	The 5′-3′ sequence[a]	Description
XIN-1[b]	CATATGTACGAACCCAAACCGGAG CACAGG	NdeI site,[c] and nucleic acid no. 1–27[d]
XIN-2	TTCGGSYTBTGGACSGTBGAC	Conserved region aa 12–17[e]
XIN-3	AAGYTBGCSGAGYTBGGSGCSGCS TACGG	Conserved region aa 41–49[e]
XIN-4	GAGCCBAAGCCBAACGAGCC	Conserved region aa 210–216[e]
XIC-1	TGATCACCCCCGCACCCCCAGGAG GTACTC	Nucleic acid no. 1138–1164[d] and BclI site[c]
XIC-2	TCANCCNCKNACNCCNARNARRTA	C-terminal conserved region aa 382–387[c] and TGA codon
XIC-3	TCNGCNCKNACYTTDATDATNA	Conserved region aa 310–316[e]
XIC-4	GTTVARGTCRATGTGGAAVARCTT	Conserved region aa 239–246[e]

[a] These sequences were designed with PRIMERGEN program *(45)*, R = A or G; Y = C or T; S = G or C; K = G or T; D = A or T or G; V = A or C or G; B = C or G or T; N = A or C or G or T.

[b] XIN-1 has substitution GTG to ATG for expression in *E. coli*.

[c] *Nde*I site was designed for the subcloning. All numbering based on *T. flavus* AT62 sequence as presented in Fig. 1.

[d] Based on nucleic acid sequence of the reported *Thermus thermophilus* HB8.

[e] Complementary nucleotide sequence deduced for conserved aa sequence present in all XI's isolated from prokaryotes (Feldman et al., 1992) especially *Ampulariella* sp. and *Streptomyces* sp.

protein sequence analysis were performed using MicroGenie program (Beckman Instruments, Palo Alto, CA) and PC/GENE program (IntelliGenetics, San Rafael, CA).

RESULTS AND DISCUSSIONS

Cloning and Characterization of the *xylA* Gene of *T. flavus* AT62

In order to clone and investigate the *xyl*A gene from the chromosome of *T. flavus* AT62, probe DNA fragments was made by polymerase chain reaction. A set of primers with only a limited degeneracy could be synthesized that were deduced from conserved amino acid sequences. The following amino acid sequences FGLWTVD, KLAELGAYGV, EPKPNEP, KLFHIDLN, and LLGXRXU, which appears to be conserved in all reported XIs, were selected from the amino acid sequences of XIs from phylogenetically closed strain *Actinoplanes missouriensis*, *Streptomyces*, and *Ampulariella* species (Table 2).

Initial attempts to amplify the intervening sequences failed. Recently, Dekker et al. *(15)* reported *xyl*A sequence from *T. thermophilus* HB8 that allowed synthesis of less degenerate N-terminal and C-terminal primers. These primers, in conjunction with primer set mentioned above were used in amplification of *xyl*A. Several DNA fragments of PCR product could be detected after 30 cycles of amplification with total chromosomal DNA. These PCR products were cloned into the *Sma*I site of pUC18/19 and its nucleotide sequences were determined. The sequence of each PCR product showed the expected internal primer sequences but XIC-3 and XIN-3 were different. Genomic DNA from *T. flavus* AT62 was digested with *Sac*I-*Xho*I and *Hin*dIII and electrotransferred to a nylon membrane. Southern hybridization was carried out at 60°C with a 1.2 Kb nucleotide PCR product that was established in pUC18. About 1350 bp region with *Sac*I-*Xho*I digest and about 900 bp and 650 bp region with *Hin*dIII digest, showed a positive band (data not shown). Thus, these were cloned into a *Sac*I-*Xho*I or *Hin*dIII digested pUC18. Colony hybridization of transformed *E. coli* identified more than 100 positive colonies in each plates. These fragments were sequenced in both directions and *Sac*I-*Xho*I or *Hin*dIII fragments sequences overlapped with each other. Finally, the constructed *Sac*I-*Hin*dIII fragment of 1635 bp sequence showed a putative *xyl*A coding region and partial *xyl*B coding region (Fig. 1). The *xyl*A ORF codes a 387 amino acid protein and putative *xyl*B ORF codes 65 amino acids, starting from 4 bp upstream of *xyl*A termination codon. Translation of this ORF except *xyl*B clearly shows homology of the amino acids sequences to that reported by Dekker et al. *(15)*. The possible ribosome binding site was located 8 base upstream of the translation start. The -35 and -10 regions are not obvious, but putative AT-rich and -35 regions are located around 30–70 base upstream of the translation start of *xyl*A. The obviously overlapped *xyl*A and *xyl*B ORFs are unusual in bacterial strains. In genomic structure of viruses, many cases of overlapping were reported, but only several cases of overlapping genes were reported from bacteria to human *(31–33)*. The putative translational coding region of *xyl*B clearly shows high amino acid homology with *N*-terminal amino acid of reported xylulose kinases (data not shown). The translational regulation of overlapping *xyl*AB gene will be studied.

Comparison of *T. flavus* AT62 XI Amino Acid Sequence with Other XIs

A number of XIs has been purified and several *xyl*A genes have been sequenced. Their translated amino acid sequences revealed that a number of XI's could be grouped into two as exemplified by *E. coli, B. subtilis, C. thermosulfurogenes, L. brevis*, and *L. pentosus*, and *S. violaceonniger, S. griseofuscus, A. missouriensis*, and *Ampullariella* sp. 3876 since they are significantly homologous to each other (Fig. 2). The two groups are reported to posses differences in size as 440 aa and 390 aa each. XI of *Thermus* falls into this latter

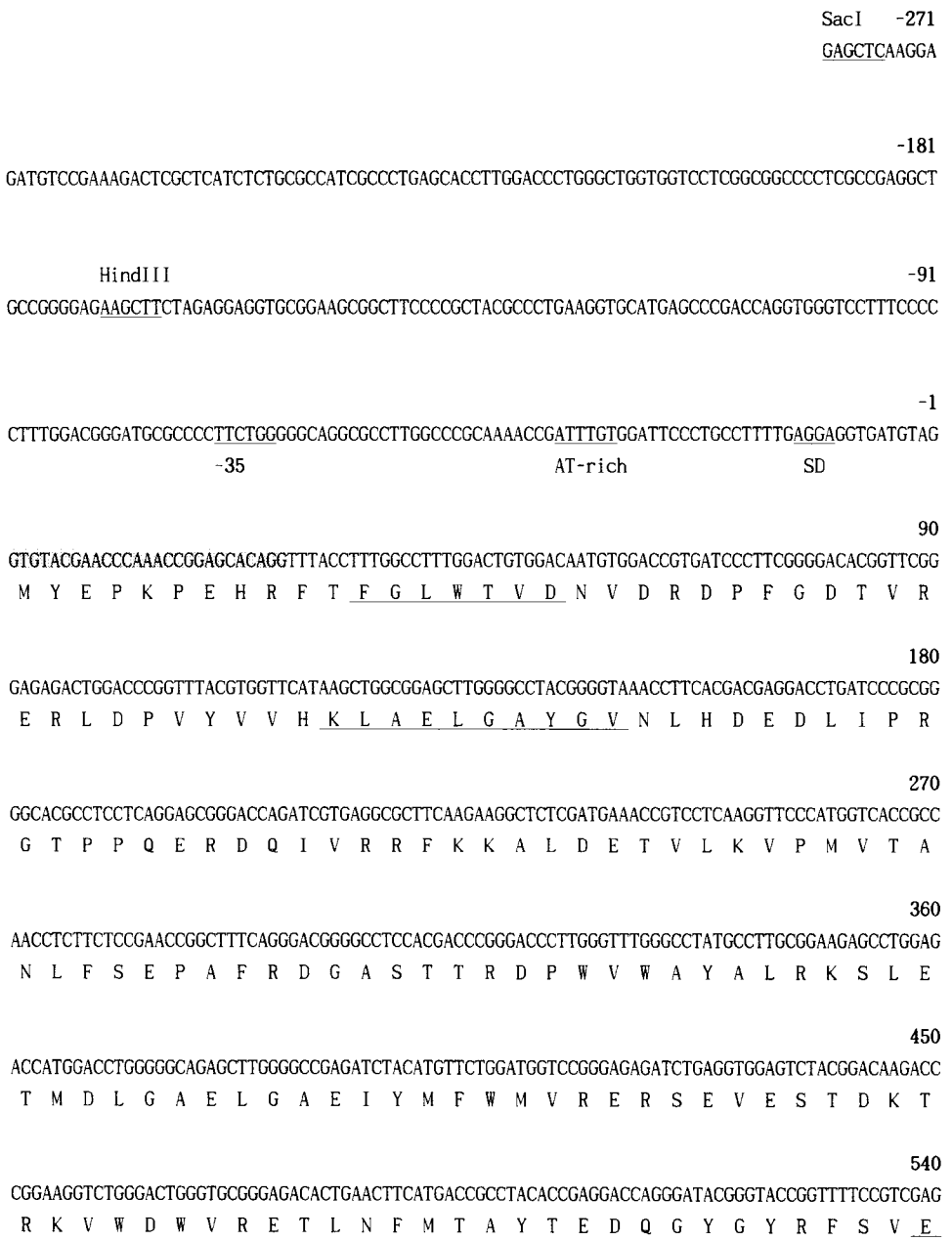

Fig. 1. DNA sequences of *T. flavus* AT62 *xyl*AB′ genes and their putative promoter region. SD, Shine–Dalgano sequence (ribosome binding site). The AT-rich and -35 regions are indicated, conserved amino acid regions for deducing primer were underlined, *N*-terminal and C-terminal priming sites for expression PCR were shadowed. A possible intergenic stem-loop structure in *xyl*B coding region is underlined.

Actinomycetes group (Family I). Some amino acids are preferred and some amino acids are less frequently found than in *Actinomycetes* group. Homology of the two aa sequences between *T. flavus* AT62 and *T.*

630
CCCAAGCCTAACGAGCCCCGGGGGGACATTTACTTCACCACCGTGGGGAGCATGCTCGCCCTTATTCATACCCTGGACCGGCCCGAGCGC
P K P N E P R G D I Y F T T V G S M L A L I H T L D R P E R

HindIII 720
TTCGGCCTGAACCCCGAGTTCGCCCACGAGACCATGGCCGGGCTTAACTTCGACCACGCCGTGGCCCAGGCTGTCGACGCCGGGAAGCTT
F G L N P E F A H E T M A G L N F D H A V A Q A V D A G K L

810
TTCCACATTGACCTCAACGACCAACGGATGAGCCGGTTTGACCAGGACCTCCGCTTCGGCTCGGAGAACCTCAAGGCGGGCTTTTTCCTG
F H I D L N D Q R M S R F D Q D L R F G S E N L K A G F F L

900
GTGGACCTCCTGGAAAGCTCCGGTTACCAGGGGCCCCGCCACTTCGAGGCCCACGCCCTGCGTACCGAGGACGAAGAAGGGGTTTGGACC
V D L L E S S G Y Q G P R H F E A H A L R T E D E E G V W T

990
TTCGTACGAGTCTGCATGCGTACCTACCTGATCATAAAGGTAAGGGCTGAAACCTTCCGCGAGGATCCCGAGGTCAAGGAGCTTCTTGCC
F V R V C M R T Y L I I K V R A E T F R E D P E V K E L L A

1080
GCTTACTATCAAGAAGATCCTGCGACCTTGGCCCTTTTGGACCCCTACTCCCGCGAGAAGGCCGAAGCCCTCAAGCGGGCGGAGCTTCCC
A Y Y Q E D P A T L A L L D P Y S R E K A E A L K R A E L P

XhoI 1170
CTCGAGACCAAGCGGCGCCGGGGTTATGCCCTGGAACGCCTGGACCAGCTGGCGGTGGAGTACCTCCTGGGGGTGCGGGGGTGAGGGCGG
L E T K R R R G Y A L E R L D Q L A V E Y L L G V R G U
M R A

1260
CCATCGGCTTGGACCTGGGAACGAGCGGGCTCAAGGCCCTGGTGCTGGACGAGGAGGGTAGAAAGCGCGCTGAGGCCCGGGCCGGTTACC
A I G L D L G T S G L K A L V L D E E G R K R A E A R A G Y

1350
CCCTTCACACCCCGAGGCCGGGCTGGACGGAGCAGGACCCCCAGGACTGGGCCCGGGCCCTGAAGGAGGTGTTCCGGGCCCTGGCGCCGA
P L H T P R P G W T E Q D P Q D W A R A L K E V F R A L A P

HindIII
AGCTT
K L

Fig. 1. *(continued)*

thermophilus HB8 is 92%, but differences are observed through the entire ORF. This difference occurs primarily within the *N*-terminal sequence where 10 out of the first 50 aa are different. The authors proposed this difference did not affect thermostability and optimum temperature, because

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Tf  M-YEPKPEHRFTFGLWTVDNVDRDPFGDTVRERLDPVYVVHKLAELGAYGVNLHDEDLIPRGTPPQERDQIVRRFKKALDETVLKVPMVTANLFSEPAFR  99
Tt  M-YEPKPEHRFTFGLWTVGNVGRDPFGDAVRERLDPVYVVHKLAELGAYGVNLHDEDLIPRGTPPQERDQIVRRFKKALDETGLKVPMVTANLFSDPAFK  99
Ar  MSVQPTPADHFTFGLWTVGWTGADPFGVATRKNLDPVEAVHKLAELGAYGITFHDNDLIPFDATEAEREKILGDFNQALKDTGLKVPMVTTNLFSHPVFK 100
Ap  MSLQATPDDKFSFGLWTVGWQARDAFGDATRPVLDPIEAVHKLAEIGAYGVTFHDDDLVPFGADAATRDGIVAGFSKALDETGLIVPMVTTNLFTHPVFK 100
Am  MSVQATREDKFSFGLWTVGWQARDAFGDATRTALDPVEAVHKLAEIGAYGITFHDDDLVPFGSDAQTRDGIIAGFKKALDETGLIVPMVTTNLFTHPVFK 100
Sd  MSYQPTPEDKFTFGLWTVGWQGRDPFGDATRGALDPAESVRRLAELGAHGVTFHDDDLIPFGATDSERAEHIKRFRQGLDETGMKVPMATTNLFTHPVFK 100
Sv  MSFQPTPEDKFTFGLWTVGWQGRDPFGDATRPALDPVETVQRLAELGAYGVTFHDDDLIPFGSSDTERESHIKRFRQALDATGMTVPMATTNLFTHPVFK 100
Sr  MNYQPTPEDRFTFGLWTVGWQGRDPFGDATRRALDPVESVRRLAELGAHGVTFHDDDLIPFGSSDSEREEHVKRFRQALDDTGMKVPMATTNLFTHPVFK 100
    *  ..   . *.******.    *.** . *  ***   *..***.** *. .**.**.* ..    *   .  * ..*  * . *** *.***..* *.

Tf  DGASTTRDPWVWAYALRKSLETMDLGAELGAEIYMFWMVRERSEVESTDKTRKVWDWVRETLNFMTAYTEDQGYGYRFSVEPKPNEPRGDIYFTTVGSML 199
Tt  DGAFTSPDPWVRAYALRKSLETMDLGAELGAEIYVVWPGREGAEVEATGKARKVWDWVREALNFMAAYAEDQGYGYRFALEPKPNEPRGDIYFATVGSML 199
Ar  DGGFTSNDRSIRRFALAKVLHNIDLAAEMGAETFVMWGGREGSEYDGSKDLAAALDRMREGVDTAAGYIKDKGYNLRIALEPKPNEPRGDIFLPTVGHGL 200
Ap  DGGFTSNDRSVRRYAIRKVLRQMDLGAELGAKTLVLWGGREGAEYDSAKDVGAALDRYREALNLLAQYSEDQGYGLPFAIEPKPNEPRGDILLPTAGHAI 200
Am  DGGFTSNDRSVRRYAIRKVLRQMDLGAELGAKTLVLWGGREGAEYDSAKDVSAALDRYREALNLLAQYSEDRGYGLRFAIEPKPNEPRGDILLPTAGHAI 200
Sd  DGGFTANDRDVRRYAVRKTIRNIDLAVELGAQTYVAWGGREGAESGAAKDVRVALDRMKEAFDLLGEYVTSQGYDIPFAIEPKPNEPRGDILLPTIGHAL 200
Sv  DGGFTANDRDVRRYALRKTIRNIDLAAELGAKTYVAWGGREGAESGGAKDVRDALDRMKEAFDLLGEYVTAQGYDLRFAIEPKPNEPRGDILLPTVGHAL 200
Sr  DGGFTANDRDVRRYALRKTIRNIDLAVELGAETYVAWGGREGAESGGAKDVRDALDRMKEAFDLLGEYVTSQGYDIRFAIEPKPNEPRGDILLPTVGHAL 200
    **. *. *  .. .*. * .  .**. *.**   . *  ** .*  ..       *. .*  .    *  .**  ...********** . * *  .

Tf  ALIHTLDRPERFGLNPEFAHETMAGLNFDHAVAQAVDAGKLFHIDLNDQRMSRFDQDLRFGSENLKAGFFLVDLLESS------GYQGPRHFEAHALRTE 293
Tt  AFIHTLDRPERFGLNPEFAHETMAGLNFVHAVAQALDAGKLFHIDLNDQRMSRFDQDLRFGSENLKAAFFLVDLLESS------GYQGPRHFDAHALRTE 293
Ar  AFIEQLEHGDIVGLNPETGHEQMAGLNFTHGIAQALWAEKLFHIDLNGQRGIKYDQDLVFGHGDLTSAFFTVDLLENGFPNGGPKYTGPRHFDYKPSRTD 300
Ap  AFVQELERPELFGINRETGHEQMSNLNFTQGIAQALWHKKLFHIDLNGQHGPKFDQDLVFGHGDLLNAFSLVDLLENG-PDGGPAYDGPRHFDYKPSRTE 299
Am  AFVQELERPELFGINPETGHEQMSNLNFTQGIAQALWHKKLFHIDLNGQHGPKFDQDLVFGHGDLLNAFSLVDLLENG-PDGAPAYDGPRHFDYKPSRTE 299
Sd  AFIDGLERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKLFHIDLNGQSGIKYDQDLRFGPGDLAAAFWLVDLLES------AGYEGPRHFDFKPPRTE 294
Sv  AFIERLERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKLFHIDLNGQSGIKYDQDLRFGAGDLRAAFWLVDLLES------AGYEGPRHFDFKPPRTE 294
Sr  AFIERLERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKLFHIDLNGQNGIKYDQDLRFGAGDLRAAFWLVDLLES------AGYSGPRHFDFKPPRTE 294
    *... *.. .  *.* * .** *. *** ...***.   ********.*   ..**** **  .*  .*  *****.         * *****.  . **.

Tf  DEEGVWTFVRVCMRTYLIIKVRAETFREDPEVKELLAA--YYQEDPATLALLDPYSREKAEALKRAELPLETKRRRG-YALERLDQLAVEYLLGVRG  387
Tt  DEEGVWAFARGCMRTYLILKERAEAFREDPEVKELLAA--YYQEDPAALALLGPYSREKAEALKRAELPLEAKRRRG-YALERLDQLAVEYLLGVRG  387
Ar  GYDGVWDSAKANMSMYLLLKERALAFRADPEVQEAMKTSGVFELGETTLNAGESAADLMNDSASFAGFDAEAAAERN-FAFIRLNQLAIEHLLGSR-  395
Ap  DFDGVWESAKDNIRMYLLLKERAKAFRADPEVQAALAESKVDELRTPTLNPGETYADLLADRSAFEDYDADAVGAKG-YGFVKLNQLAIDHLLGAR-  394
Am  DYDGVWESAKANIRMYLLLKERAKAFRADPEVQEALAASKVAELKTPTLNPGEGYAELLADRSAFEDYDADAVGAKG-FGFVKLNQLAIEHLLGAR-  394
Sd  DFDGVWASAAGCMRNYLILKERAAAFRADPEVQEALRAARLDELAQPT--AGDGLQALLPDRSAFEDFDPDAAAARGM-AFERLDQLAMDHLLGARG  388
Sv  DFDGVWASAEGCMRNYLILKERAAAFRADPEVQEALRAARLDQLAQPT--AADGLEALLADRTAFEDFDVEAAAARAAWPFERLDQLAMDHLLGARG  389
Sr  DFDGVWASAAGCMRNYLILKERAAAFRADPEVQEALRASRLDELARPT--AADGLQALLDDRSAFEEFDVDAAAARGM-AFERLDQLAMDHLLGARG  388
    . .***      .  **..* ** .** ****.  .      .    .                .      ..   .  . .*.***.. *** *
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Fig. 2. Alignments of xylose isomerase amino acid sequences reported from different bacterial origins. Asterisks below the sequences indicate identical amino acid residues in all sequences, and dots indicate similar amino acid residues. Am, *Actinoplanes missouriensis*; Ar, *Arthrobacter* sp.; Ap, *Ampulariella* sp. 3876; Sv, *Streptomyces violaceoniger*.; Tf, *T. flavus* AT62 (in this work); Tt, *T. thermophilus* HB8; Bs; *B. subtilis* ; Bp, *Bacillus* sp.; Sr, *S. rubinginoosus* ; *Sd S. diastaticus*.

characteristics between two *Thermus* Xis are not so different. It was in agreement with other XI's case even in the members of first group *L. brevis* and *L. pentosus*. Within the XI's aa sequences of the *T. thermophilus* HB8 and *T. flavus* AT62, a number of regions that are conserved in other XIs are present, but some sites are changed. The WGGREG (aa 136–141) was changed to

Table 3
Activation and Inhibition of *T. flavus* AT62 Xylose Isomerase by Divalent Cations[a]

Addition	Relative activity, %
None	10
$CaCl_2$	20.1
$CuCl_2$	16.7
$CoCl_2$	100
$MgCl_2$	100
$MnCl_2$	100
$FeCl_2$	11.1
$NiCl_2$	20.6
$ZnCl_2$	18.0

[a] The enzyme was freed of metal ions by exhaustive dialysis against 10 m*M* Tris-HCl, pH 7.5, and assayed as by Dekker et al. and as indicated in Materials and Methods. The final concentration of metal ions was 1.0 m*M*. Enzyme activity in the case of $MgCl_2$ was taken to be 100%.

WMVRER but W, the substrate binding site, that was elucidated by Carrell et al. *(23)* was conserved. Also, His53, (H101 in *E. coli*) catalytic and ring opening residue, was conserved. A second His residue is located at aa 221 (aa 273 in *E. coli*) that appears to serve as a ligand for the distal metal ion in the active site. Probably they are conserved on a structural level. Recently, these were also reported for extremely thermophilic XI from *Thermatoga maritima* and *Thermatoga neopolitana (37–39)*. These are a different type (type II) of xylose isomerases, but it could give possible insight to the thermostable nature.

Expression in *E. coli*

The simplest construct for expression in *E. coli* was the high-copy-number plasmid pUC18 carrying the XI coding rigions from *T. flavus* AT62 under control of the *lac*UV5-promoter. This plasmid was named pXI11. *xyl*A gene in pXI11 was cut with *Sal*I-*Eco*RI restriction enzymes and ligated to pKK223-3 and the space between ribosome binding site and translation start was enlarged 9 to 15 nucleotide for elimination of inclusion body formation. The GTG translation start codon in *Thermus* strains was changed to ATG for expression in *E. coli*. Expression levels differ from *xyl*A gene source, but the codon usage was not the limiting factor for expression in *E. coli*. Table 3 showed the yields obtained under conditions optimal for production systems described above. As can be seen, expression of the *Thermus xyl*A genes in *E. coli* under control of *lac*UV5-promoter was not efficient. Replacement by the diminished *tac*-promoter (pKXI11) resulted in a more than five times higher production level.

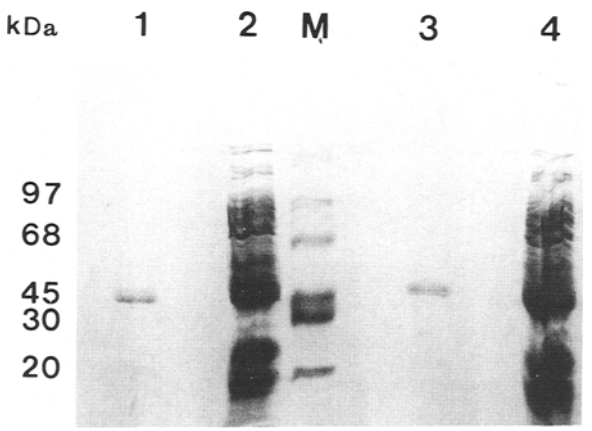

Fig. 3. Coomasie Brilliant Blue stained SDS-PAGE of: lane 1, *E. coli* (pKXI11) cell extract after heat treatment at 90°C for 10 min; lane 2, cell extract of *E. coli* (pKXI11); lane 3, *E. coli* (pXI11) cell extract after heat treatment at 90°C for 10 min; lane 4, cell extract of *E. coli* (pXI11). The amount of samples was equivalent to 10 µl of culture. The position of relative molecular mass markers (kDa) is indicated on the left side.

Purification of XIs

Purification of cloned enzyme was simple. Heat treatments of cell extract at 90°C for 10 min followed by centrifugation resulted in a fairly pure enzyme (Fig. 3). More severe heat treatment gave more purity but also gives lower yields of recovery. The enzyme was further purified by fast protein liquid chromatography on a DEAE-Sephacel HR10/10 to study biochemical characteristics on native condition.

Biochemical Characteristics of XI

Some physico-chemical properties of isomerase from all expression systems and between gene sources were compared. Temperature profiles and isoelectric points were similar and the specific activity of the *Thermus* isomerases at 85°C were about 20 U/mg protein each. Xylose isomerases have two metal ions bound in the active site of each monomer *(24)*. These metals are required for enzyme activity, but XIs from different organisms require different metals for optimum activity *(40)*. XI from *Thermus flavus* AT62 requires the addition of Mg^{2+}, Co^{2+}, or Mn^{2+} for 100% activity (Table 3), whereas XI from *Thermus thermophilus* HB8 required Co^{2+} to a lesser extent *(28)*. Other metal ions, including Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+} were not effective (about 20% activity). A very important characteristic of any xylose isomerase is its thermostability at high temperatures. The stability is also strongly influenced by the addition of divalent cations. The addition of Mn^{2+} gave higher thermostability than Mg^{2+}, whereas other metals had

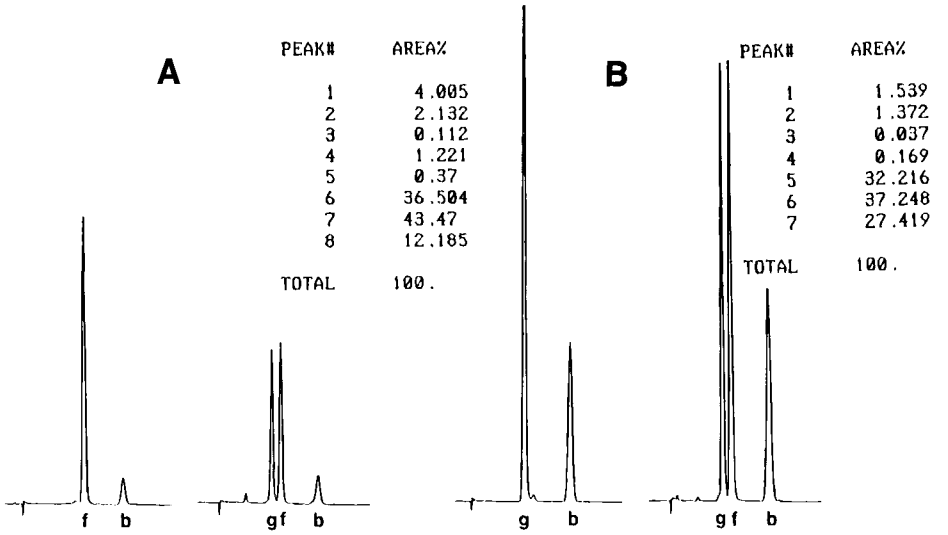

Fig. 4. Chromatogram of glucose reaction sample. Analysis was carried with Carbopac PA1 column of High pH Anion Exchange Chromatography (HPAEC, Dionex) by pulsed-amperometric detector (PAD). **A**, reaction with frucose; **B**, reaction with glucose; g, glucose; f, fructose; b, HEPES buffer.

no effect. The *Thermus* isomerases have an optimum pH around 7.0 in HEPES at 90°C.

The K_m value was calculated with glucose (106 m*M*). In Fig. 4, The calculation of peak area of fructose in the chromatogram was 55%. Such a high conversion rate of glucose to fructose by the thermostable XI, which is attributable to reaction temperature, is useful for industrial application. Thus, this system obviate the concentration step in making high fructose corn syrup after isomerization reaction.

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