Isolation and characterization of an extremely thermostable D-xylose isomerase from *Thermus aquaticus* HB 8

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The extremely thermophilic organism *Thermus aquaticus* possesses high activities of enzymes catalysing the degradation of xylans and metabolizing D-xylose via the pentose phosphate pathway. The D-xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5), an important enzyme of this process, is efficiently induced by its substrate D-xylose, and, to a lesser extent, by related pentoses and some derivatives of D-xylose. The D-xylose isomerase from *T. aquaticus* has been purified by anion-exchange chromatography, chromatography on D-xylose agarose and gel filtration. A single band migrating according to an M_r of 50 000 was obtained by SDS-PAGE. An M_r of 196 000 for the native enzyme, determined by gel filtration and ultracentrifugation in a glycerol gradient, suggested that the D-xylose isomerase is a homomeric tetramer. Arrhenius plots of the enzyme activity of the D-xylose isomerase were linear up to a temperature of 85 °C. At 70 °C the enzyme was inactivated in the absence of divalent cations, with a half-life of 4 d, while in the presence of Mn^{2+} or Co^{2+} it remained fully active for at least 1 month. The enzyme had an isoelectric point at 4.4 and showed a broad optimum in the pH range from 5.5 to 8.5. No significant differences in the pH and temperature behaviour could be observed when D-xylose was compared with D-glucose as substrate. Different methods of immobilization of the enzyme to solid supports as well as inclusion into nylon beads were studied. Attachment of the enzyme to epoxy-activated agarose and its co-aggregation with bovine serum albumin gave immobilized preparations with the same stability as free enzyme supplemented with Mn^{2+} or Co^{2+} .

Introduction

Various micro-organisms are able to grow on hemicelluloses, which amount to 40% of the plant biomass (Rehm & Reed, 1982), as sole carbon source. Exoenzymes degrade the polymer to D-xylose, which is transported into the cell, isomerized to D-xylulose and phosphorylated to xylulose 5-phosphate which enters either the pentose phosphate pathway (Doelle, 1975; Barnett, 1976; Dekker & Richards, 1976; Lam *et al.*, 1980; Kersters-Hilderson *et al.*, 1982; Deleyn *et al.*, 1982; Jeffries, 1983) or the phosphoketolase pathway (Jeffries, 1983). The isomerization of D-xylose is carried out by D-xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5). This enzyme has been used commercially because of its capacity to produce a fructose-enriched syrup by converting D-glucose into fructose (Antrim *et al.*, 1979; Rehm & Reed, 1982). D-Xylose isomerase is also of industrial interest for the fermentation of

hemicelluloses to ethanol, together with yeasts (Jeffries, 1983).

The catalytic mechanism of the isomerization reaction is not completely understood. It is not clear whether the enzyme accepts D-glucose solely because of its structural similarity to its physiological substrate D-xylose due to a broad specificity of the substrate-binding site or whether D-glucose is isomerized by a different catalytic mechanism (Noltman, 1972). The enzyme has a requirement for divalent metal ions as essential cofactors. Mn^{2+} or Mg^{2+} ions are most efficient with D-xylose while the glucose isomerization reaction is more active with Co^{2+} or Mg^{2+} ions (Young *et al.*, 1975; Antrim *et al.*, 1979). This observation argues in favour of distinct catalytic mechanisms for the two substrates.

In the work described here we have studied D-xylose isomerase in the extremely thermophilic bacterium *Thermus aquaticus*. We report the isolation of this enzyme and its characterization, with particular reference to a comparison between the two types of isomerase reactions. The temperature behaviour of the free enzyme is compared with that of the immobilized one.

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Methods

Growth of the bacteria. *Thermus aquaticus* HB 8 (ATCC 27634) was obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, FRG. The bacteria were grown in 20 l of the minimal salt medium of Yoshida *et al.* (1984) with 9.5 mM-ammonium chloride as nitrogen source and 0.5% D-xylose as carbon source and inducer of the enzyme. Growth was for 14 h at 70 °C with 0.2 vol. vol.⁻¹ min.⁻¹ air and stirring (1000 r.p.m.) in a 20 l 'Intensor b-20' fermenter (Giovanna Frères). Before each fermentation run the fermenter pot was treated with 25% (v/v) nitric acid for 16 h in order to passivate its steel surface (Sonnleitner *et al.*, 1982). The cells were harvested by centrifugation. From one fermentation run about 90 g wet weight of cells were obtained. For experiments other than the isolation of D-xylose isomerase, crude extracts were obtained by suspension of the cells in 0.1 M-histidine/HCl, pH 6.0 [2 ml (g cells)⁻¹], followed by sonication using a type B-12 sonicator (Branson Ultrasonics) for 2 min. The cell debris was removed by centrifugation at 38000 g for 30 min.

Enzyme assays. (a) *D-Xylose isomerase.* The formation of D-xylulose from D-xylose was measured using the colorimetric assay of Dische & Borenfreund (1951) in which 20 µl of a test mixture, containing 0.1 M-TES/NaOH pH 7.0, 0.2 M-D-xylose and 0.4 mM-MnSO₄, was incubated together with 20 µl of the enzyme solution for 10 min at 70 °C. After this period the samples were put on ice to stop the enzyme reaction and 40 µl 1.5% (w/v) cysteine hydrochloride in water, 40 µl 0.12% (w/v) carbazole in ethanol, and 1.2 ml 70% (v/v) sulphuric acid were added. After standing for 10 min the absorbance at 540 nm was determined. The concentration of D-xylulose was determined from a standard curve of D-xylulose. One unit (U) of enzyme activity is equal to the formation of one µmol D-xylulose per min at 70 °C.

(b) *D-Glucose isomerase.* The test for the D-glucose isomerase activity was the same as that for D-xylose isomerase, with the exception that the test mixture contained in 20 µl 1 M-D-glucose and 0.4 mM-CoCl₂ in 0.1 M-TES/NaOH, pH 7.0. The incubation period for the development of the colour with carbazole was extended to 30 min and the absorbance was measured at 560 nm. The enzyme activity was calculated on the basis of a standard curve obtained with D-fructose.

(c) *Other assays.* The activity of xylanase was tested using the method of Panbangred *et al.* (1983a) at 70 °C following the release of reducing sugars from xylans from oat spelt at 750 nm by the colorimetric assay of Somogyi (1952). β-Xylosidase was assayed according to Panbangred *et al.* (1983b), D-xylose dehydrogenase after Scher & Horecker (1966) and transketolase according to Brin (1970). Transaldolase was measured by the coupled assay procedure of Brand (1970), and the method of Goldberg *et al.* (1966) was used to determine the activity of phosphoketolase. The temperature in all these tests was 70 °C. The decrease in the absorbance of 10.5 mM-hydrogen peroxide in 50 mM-potassium phosphate, pH 7.0, at 25 °C was followed spectrophotometrically to determine the activity of catalase (Bergmeyer, 1970).

Protein concentration. Protein concentration was determined according to Bradford (1976). The method of Ahmud & Saleemuddin (1978) was used to measure the protein content of the immobilized enzyme samples.

Purification of the D-xylose isomerase. Crude extract and DEAE-cellulose chromatography. Cells (10 g wet weight) were suspended in 20 ml of 10 mM-histidine/HCl, pH 6.0, 0.5 mM-PMSF and treated with 10 mg lysozyme at 37 °C for 45 min. The disintegrated cells were centrifuged for 10 min at 38000 g and the resultant supernatant was applied to a 2 × 8 cm DEAE-cellulose column and equilibrated with 10 mM-histidine/HCl, pH 6.0, 0.5 mM-PMSF. The column was washed with the same buffer, which also contained 0.5 mM-PMSF, 100 mM-NaCl, at a flow rate of 35 ml h⁻¹. The D-xylose isomerase was eluted

with a 60 ml linear gradient of 100 to 500 mM-NaCl in 10 mM-histidine/HCl, pH 6.0, 0.5 mM-PMSF. The active fractions were pooled and dialysed for 14 h against 10 mM-histidine/HCl, pH 6.0, 0.5 mM-PMSF.

Chromatography on D-xylose/agarose. A column 2 cm in diameter and 4 cm in height was filled with D-xylose/agarose, equilibrated with 10 mM-histidine/HCl, pH 6.0, 0.5 mM-PMSF and run at a flow rate of 20 ml h⁻¹. The dialysed DEAE-cellulose eluate was applied to the column and washed with 120 ml buffer. To elute the enzyme a 20 ml gradient from 0 to 150 mM-NaCl in the same buffer was used. The D-xylose isomerase activity eluted at 50 mM-NaCl.

Chromatography on Ultrogel Ac A 34. The fractions from the D-xylose/agarose column containing D-xylose isomerase activity were collected and applied to a 2.5 × 80 cm Ultrogel Ac A 34 column. Equilibration and elution of this column employed the histidine buffer at a flow rate of 20 ml h⁻¹. The active fractions of the D-xylose isomerase were pooled and concentrated on a ω-aminoethyl-Sepharose 4B column to 5 mg ml⁻¹ by the method of Schmincke-Ott & Bisswanger (1980).

Electrophoresis and isoelectric focusing. The SDS-PAGE (Laemmli, 1970) employed a gradient of 7 to 14% (w/v) acrylamide and a ratio of acrylamide to N,N'-methylene-bis-acrylamide of 100:2.68. Staining of protein bands was done with Coomassie brilliant blue R-250, according to Fairbanks *et al.* (1971).

Isoelectric focusing was carried out at 4 °C in a Multiphor unit (Pharmacia) on 125 × 125 mm polyacrylamide layers (Servalyt Pre-cotes) at 5 mA, and 1.2 kV for 2 h. The solution for the anode chamber contained 3.3 g L-aspartic acid l⁻¹ and 3.7 g L-glutamic acid l⁻¹, that for the cathode chamber 20 g glycine l⁻¹. After focusing, the layer was transferred into 20% (w/v) trichloroacetic acid for 15 min, and washed thereafter in methanol/acetic acid/water (40:10:50, by vol.) for 30 s. Staining was done for 1 h with 0.08% Coomassie brilliant blue G-250 and destaining with the methanol/acetic acid/water mixture. The layer was dried at 100 °C for 5 min.

Immobilization of D-xylose isomerase. (a) *Hollow-fibre entrapment.* The method of Chambers *et al.* (1976) was used to immobilize 2 mg purified D-xylose isomerase ml⁻¹ in 10 mM-histidine/HCl, pH 6.0, to 50000 M_r cut-off fibres of the hollow-fibre cartridge BPR 500006 (Berghof).

(b) *Inclusion into nylon beads.* Using the method of Chang (1976), 1 ml 10 mM-histidine/HCl, pH 6.0, containing 2 mg of purified D-xylose isomerase was mixed with the same volume of a solution of 44 mg 1,6-diaminohexane, 16 mg sodium hydrogen carbonate and 66 mg sodium carbonate in water. To this mixture a freshly prepared solution of 18 mM-sebacoyl chloride (0.4 ml sebacoyl chloride in 20 ml chloroform and 80 ml cyclohexane) was added drop-wise with a microlitre syringe so that the beads were formed without touching one another. After 5 min the organic phase was decanted and the solute was allowed to evaporate completely. The beads were then washed with 0.9% sodium chloride in 50 mM-potassium phosphate, pH 7.0.

(c) *Fixation on cyanogen-bromide-activated Sepharose 4B.* D-Xylose isomerase was immobilized on cyanogen-bromide-activated Sepharose 4B according to Cuatrecasas *et al.* (1968). Gel (1 g) was washed with 200 ml 1 M-HCl and suspended in 1 ml 0.1 M-sodium bicarbonate, pH 8.3, that contained 0.5 M-sodium chloride and 2 mg of the purified enzyme. The suspension was shaken at room temperature for 5 h. The supernatant was discarded, 20 ml of 0.2 M-glycine/NaOH, pH 8.0 were added, and the mixture was shaken for another 2 h in order to block free cyanogen bromide groups.

(d) *Immobilization on epoxy-activated agarose.* Epoxy-activated agarose (1 g) was washed with 100 ml distilled water and allowed to react with 1 ml of a solution of 2 mg purified D-xylose isomerase in 0.1 M-

sodium bicarbonate, 0.5 M-sodium chloride, pH 8.5, at room temperature for 5 h with constant shaking. Unreacted epoxy groups were inactivated by shaking for 2 h in 0.2 M-glycine/NaOH, pH 8.0.

(e) *Co-immobilization with bovine serum albumin (BSA) and glutaraldehyde*. D-Xylose isomerase and BSA were co-immobilized according to the method of Broun (1976). Purified D-xylose isomerase (2 mg), 60 mg BSA and 2 mg glutaraldehyde in 1 ml 50 mM-potassium phosphate, pH 7.0, were frozen at -30°C and allowed to thaw slowly in a refrigerator at 4°C for 4 h. The co-polymer was washed successively with 20 ml volumes of distilled water, with 0.1 M-L-lysine, and finally with 50 mM-potassium phosphate, pH 7.0.

Chemicals. Yeast extract and Bacto-Tryptone were purchased from Difco; catalase from bovine liver, BSA and phenylmethylsulphonyl fluoride (PMSF) were obtained from Boehringer Mannheim; D-xylose and sebacoyl chloride were from Fluka AG; DEAE-cellulose (DE-52) was from Whatman; D-xylitol, lysozyme from chicken egg white, acrylamide, Coomassie brilliant blue R-250 and G-250, Servalyte Precotes, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine and SDS were from Serva; D-glucose, D-arabinose, D-fructose, D-galactose, D-lyxose, D-ribose, D-xylose 1-phosphate, D-xylulose, methyl α -D-xyloside, methyl β -D-xyloside, 2-O-methyl-D-xylose, D-xylose tetraacetate, xylan from oat spelts, ω -aminohexyl-Sepharose 4B, cyanogen-bromide-activated Sepharose 4B, epoxy-activated agarose [1,4-bis-(2,3-epoxypropoxyl)-butane attached to cross-linked agarose], TES, Tris, M_r markers MW-SDS-200 and isoelectric focusing markers IEF-M1 were from Sigma; Ultrogel Ac A 34 was from LKB; carbazole was from Aldrich. All other chemicals were obtained from Merck.

D-Xylose-agarose was synthesized from epoxy-activated agarose according to Sundberg & Porath (1974). D-Xylonate was prepared according to Moore & Link (1940). D-Xylose 5-phosphate was produced by the method of Levene & Raymond (1933).

Results

D-Xylose metabolism of T. aquaticus HB 8

High- M_r xylans are the main source of D-xylose. Therefore it should be advantageous to any micro-organism able to metabolize D-xylose to also possess xylanase activity. Accordingly, *T. aquaticus* was grown at 70°C in minimal medium with 0.5% D-xylose as carbon source. After 20 h the cells were collected by centrifugation and disrupted by sonication. The crude cell-extract and the culture filtrate were tested for xylanase activity. High levels of activity (986 U l^{-1}) were found in the culture filtrate, while no xylanase activity could be detected in the crude extract. However, activities of D-xylose isomerase (25 U l^{-1}) and D-xylulokinase (3.8 U l^{-1}) (which feeds D-xylose into the pentose phosphate pathway) as well as transketolase (71 U l^{-1}) and transaldolase (13 U l^{-1}) (which catalyse important reactions of this pathway) were found in this fraction. Enzyme activities for alternative pathways such as D-xylose dehydrogenase, or phosphoketolase, were not detected in the crude extract of the cells of *T. aquaticus*, nor was β -xylosidase activity detected.

Table 1. *Induction of synthesis of D-xylose isomerase*

The cells were cultivated in minimal medium with 0.5% glycerol as carbon source. After reaching an optical density of 0.8 the cells were collected by centrifugation, washed with a sterile solution of 0.9% sodium chloride in 50 mM-potassium phosphate, pH 7.0, and re-suspended in 10 mM-potassium phosphate, pH 7.0, containing 0.5 mM of the inducer. The suspension was shaken for 20 min at 70°C , centrifuged and the cells disrupted by sonication. After further centrifugation for 10 min at $38000g$ the activity of the D-xylose isomerase and the protein content were measured in the crude extract. The purity of the compounds was checked by thin-layer chromatography.

Inducer	Specific activity [U (mg protein) $^{-1}$]
D-Xylose	0.109
D-Xylonate	0.104
D-Xylitol	0.080
Methyl α -D-xyloside	0.045
Methyl β -D-xyloside	0.041
2-O-Methyl-D-xylose	0.056
D-Xylosetetraacetate	0.009
D-Xylose 1-phosphate	0.009
D-Xylose 5-phosphate	0.010
D-Lyxose	0.063
D-Ribose	0.022
D-Arabinose	0.008
D-Glucose	0.008
D-Galactose	0.007
D-Fructose	0.010
Glycerol	0.009

Induction of the D-xylose isomerase activity

In many organisms it has been shown that D-xylose isomerase is induced by its substrate D-xylose (Shamanna & Sanderson, 1979; Briggs *et al.*, 1984; Lawlis *et al.*, 1984; Wilhelm & Hollenberg, 1985; Marcel *et al.*, 1987). Induction of the enzyme by a range of compounds was tested in *T. aquaticus*. The specific activities obtained are shown in Table 1. D-Xylose was the most potent inducer of the D-xylose isomerase. Compounds structurally related to D-xylose, like D-xylonate, D-xylitol, 2-O-methyl-D-xylose, methyl α -D-xyloside and methyl β -D-xyloside were also able to stimulate the synthesis of the enzyme. All hexoses tested were ineffective, while, of the pentoses, D-ribose was a weak and D-lyxose a fairly good inducer. The metal ions Mg^{2+} , Mn^{2+} and Co^{2+} , which are essential cofactors of the enzyme (Antrim *et al.*, 1979), were ineffective as inducers.

The rate of induction of D-xylose isomerase activity by D-xylose is shown in Fig 1(a). A steep increase in the D-xylose isomerase activity was observed for 20 min, followed by a decline to reach a constant level of about half of the maximum activity after 1 h. In Fig. 1(b) the dependence of the induction on the concentration of D-xylose is presented. The D-xylose isomerase activity, measured after an induction period of 20 min, was

Fig. 1. Induction of D-xylose isomerase activity. *T. aquaticus* cultures were prepared as described in Table 1 and the rate of induction of the enzyme by D-xylose measured (a). The extent of induction was also assayed after incubation of washed cell suspension for 20 min in the presence of different concentrations of D-xylose (b).

Fig. 2. SDS-PAGE of purified D-xylose isomerase from *T. aquaticus*. Lanes 1 and 2 contain 4 µg and 2 µg D-xylose isomerase, respectively. Lane 3 contains M_r marker proteins (4 µg each) added to 3 µg D-xylose isomerase. M_r marker proteins were: phosphorylase b (97400), BSA (66000), pyruvate kinase (57000), fumarase (49000), creatine kinase (40500), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (31000), chymotrypsinogen A (25000), trypsin inhibitor (20100), β -lactoglobulin (18400), lysozyme (14300). XI marks position of D-xylose isomerase.

Table 2. Purification of D-xylose isomerase

All fractions were assayed for both D-xylose isomerase (XI) and D-glucose isomerase (GI) activities.

Purification step	Volume (ml)	Protein (mg ml ⁻¹)	Total activity (U)		Specific activity (U mg ⁻¹)		Yield (%)		Purification factor	
			XI	GI	XI	GI	XI	GI	XI	GI
Crude extract	10	6.9	34.1	20.2	0.5	0.3	100	100	1	1
DEAE-Cellulose	23.5	1.4	36.2	21.0	1.1	0.66	106	104	2.2	2.3
D-Xylose/agarose	10	1.9	34.9	20.0	1.9	1.1	102	99	3.8	3.7
Ultrogel Ac A 34	31	0.1	35.4	20.2	11.4	6.5	104	100	22.8	21.7

greatest with 0.2–1 mM-D-xylose. Above 1 mM-D-xylose the activity declined slightly.

Purification of the D-xylose isomerase from *T. aquaticus* HB 8

The purification of D-xylose isomerase is summarized in Table 2. Three chromatographic steps, anion-exchange

chromatography on DEAE-cellulose, chromatography with D-xylose/agarose and gel filtration on Ultrogel Ac A 34, resulted in a highly purified preparation of high yield. D-Glucose isomerase activity co-purified exactly with the D-xylose isomerase activity. One predominant protein band was found in the SDS-PAGE (Fig. 2). From the data an apparent M_r of 50000 was determined for the enzyme band. Isoelectric focusing of the purified D-

xylose isomerase gave a single protein band corresponding to an isoelectric point of 4.4 (not shown).

The M_r of the native D-xylose isomerase from *T. aquaticus* was determined by gel filtration. The purified enzyme was passed through a Sephacryl S-300 column together with standard proteins of known M_r ; an M_r of 191 000 was determined.

The M_r of native D-xylose isomerase was also determined by the technique of gradient ultracentrifugation according to Martin & Ames (1961). In order to avoid interactions of sugar with the enzyme, glycerol was used instead of sucrose to form a linear gradient from 10 to 50% (v/v). The D-xylose isomerase was centrifuged in the gradient together with malate dehydrogenase and catalase as standards of known M_r . An estimate of the M_r of the D-xylose isomerase of 196 000 was obtained based on the assumption that the ratio of the distances travelled by standard (s) and sample (x) enzymes in the gradient corresponds to the ratio of their respective sedimentation coefficients, $s_{20,w(s)}/s_{20,w(x)}$ (Martin & Ames, 1961).

Effects of temperature on D-xylose isomerase

The enzymic activity of D-xylose isomerase was measured at various temperatures utilizing D-xylose and D-glucose, respectively, as substrates. For both substrates the Arrhenius plots yielded straight lines up to a temperature of 85 °C and declined thereafter, probably because of thermal inactivation (not shown). From the slopes of the linear part of these plots activation energies of 64.8 kJ mol⁻¹ and of 98.3 kJ mol⁻¹ were calculated for D-xylose and for D-glucose, respectively.

To test its stability with respect to temperature the D-xylose isomerase was incubated at 4 °C, 25 °C and 70 °C. After different intervals samples were taken to determine the D-xylose isomerase activity (Fig. 3). The enzyme was stable at 4 °C and 25 °C for more than 1 month, while at 70 °C its activity declined with a half-life of 4 d. Since the D-xylose isomerase requires divalent cations for its catalytic activity (Antrim *et al.*, 1979), the stability of the enzyme at 70 °C was tested in the absence and presence of Mg²⁺, Mn²⁺ and Co²⁺. Mn²⁺ and Co²⁺ individually protected the enzyme from thermal inactivation for at least 1 month, while stabilization was not observed with Mg²⁺ (Fig. 3).

Effects of pH on D-xylose isomerase

The activity of the D-xylose isomerase increased between pH 3 and 5, remained nearly constant in the pH range from 5.5 to 8.5, and declined at pH 10 (Fig. 4a). The glucose isomerase reaction showed nearly the same dependence on pH, though the flanks of the curve were less steep. This pH profile indicates more than one

Fig. 3. Thermal stability of the D-xylose isomerase. The purified enzyme was incubated at a concentration of 2 mg ml⁻¹ in 50 mM-TES/NaOH, pH 7.0, at 4 °C (●), 25 °C (○), and at 70 °C in the absence (■) and in the presence of 0.5 mM-magnesium chloride (△), 0.5 mM-manganese chloride (▲) and 0.5 mM-cobalt chloride (□).

Fig. 4. Dependence of the enzyme activity and stability of D-xylose isomerase on the pH value. (a) The D-xylose isomerase reaction (●, ▲, ■) was tested with 4 µg and the D-glucose isomerase reaction (○, △, □) with 2 µg of the purified D-xylose isomerase in buffer systems of different pH values: pH 2.9–5.65, 50 mM-acetic acid/sodium acetate (●, ○); pH 5.1–8.0, 50 mM-TES/NaOH (▲, △); pH 7.55–10.0, 50 mM-glycine/NaOH (■, □). For the D-glucose isomerase reaction the ordinate scale must be multiplied by a factor of 0.2. (b) Samples of purified D-xylose isomerase (2 mg ml⁻¹) were preincubated for 1 h at 25 °C in the above-mentioned buffer system at the respective pH value given at the abscissa. After this time the activities of D-xylose isomerase (□) and D-glucose isomerase (●) were tested under normal test conditions at pH 7.0. The respective activities of the untreated enzyme are defined as 100%. The buffer systems are the same as those described for (a).

functional group involved in the pH-dependent mechanism.

The stability of the D-xylose isomerase as a function of pH was tested by preincubation of the enzyme at 25 °C at different pH values for 1 h. After the incubation the activities of D-xylose isomerase and D-glucose isomerase were measured at pH 7.0. The stability curves obtained by this experiment (Fig. 4b) resembled the pH optimum curves (Fig. 4a) and no difference was observed with D-xylose or with D-glucose as substrate.

Table 3. Immobilization of the D-xylose isomerase from *T. aquaticus*

Immobilization technique	Xylose isomerase activity		
	Yield (%)	Sp. act. (U mg ⁻¹)	Ratio Sp. act. _{immobilized} / Sp. act. _{native}
Hollow fibre	100	5.43	1.0
Nylon beads	56	2.93	0.54
Epoxy-activated agarose	71	4.58	0.84
Cyanogen bromide-activated agarose	65	5.03	0.92
Glutaraldehyde/BSA	37	ND	ND

ND, Not determined

Immobilization of the D-xylose isomerase

Different techniques of immobilization of the purified D-xylose isomerase from *T. aquaticus* were tested. These included fixation on solid supports by epoxy- and cyanogen-bromide-activated agarose, inclusion into nylon beads and hollow fibres and co-aggregation with BSA by cross-linking with glutaraldehyde (Table 3). The best results were obtained with hollow-fibre entrapment, which was done without loss of enzyme activity. About two-thirds of the activity was retained after fixation of the enzyme on agarose. All five immobilized enzyme preparations remained completely active for at least 45 d when stored at 4 °C or at 25 °C. The activity disappeared, however, with a half-life of about 4 d, upon incubation at 70 °C, with the exception of the enzyme attached to epoxy-activated agarose and that co-aggregated with BSA (Fig. 5).

Discussion

The D-xylose isomerases from *Salmonella typhimurium* (Shamanna & Sanderson, 1979), *Escherichia coli* (Briggs *et al.*, 1984; Lawlis *et al.*, 1984), *Bacillus subtilis* (Wilhelm & Hollenberg, 1985) and *Streptomyces violaceoniger* (Marcel *et al.*, 1987) are under positive genetic control. D-Xylose binds to a regulator protein, which activates the promoter of the D-xylose operon. A similar mechanism may occur in *T. aquaticus*, where the enzyme is induced 14-fold by D-xylose. Analogues of D-xylose, methyl α -D-xyloside and methyl β -D-xyloside, 2-O-methyl-D-xylose, D-lyxose, D-xylonate and D-xylitol, induce the enzyme substantially while other sugars are ineffective.

Since the D-xylose isomerase from *T. aquaticus* becomes liberated after sonication or incubation of the

Fig. 5. Thermal stability of immobilized D-xylose isomerase. The immobilized enzyme preparations were incubated in 50 mM-TES buffer at 70 °C, and samples corresponding to 2 mg ml⁻¹ were taken at different times and the D-glucose isomerase activity was tested. The activity of the native enzyme (○) was defined as 100% at $t = 0$; △, hollow fibre entrapment; ■, inclusion into nylon beads; ●, epoxy-activated agarose; ▲, cyanogen bromide-activated agarose; □, co-immobilization with BSA by glutaraldehyde cross-linking.

cells with lysozyme, it is apparently cytosolic. No enzyme activity is released after osmotic shock treatment (not shown), as would be expected for an enzyme located in the periplasm.

The enzyme appears to be relatively stable against denaturation and, therefore, high yields could be obtained during the purification procedure. As D-xylose isomerase eluted from xylose/agarose in the presence of salt, but was not displaced by D-xylose (data not shown), this column was apparently acting as an ion-exchanger rather than as a true affinity ligand for this enzyme. The co-purification of both D-xylose and D-glucose isomerase activities strongly suggested that these are activities of the same enzyme molecule. No evidence was found for the existence of separate enzyme molecules for the two activities in *T. aquaticus*.

An M_r of about 196000 for the native D-xylose isomerase from *T. aquaticus* and of 50000 for its subunits suggests that the enzyme is a homomeric tetramer. These results are consistent with X-ray crystallography data from *Streptomyces rubiginosus* and *Arthrobacter* (Carrell *et al.*, 1984; Henrick *et al.*, 1987) and also with the M_r determinations of the enzyme from *Streptomyces griseofuscus* (Kasumi *et al.*, 1981), *Streptomyces violaceoruber* (Callens *et al.*, 1985), *Bacillus coagulans* (Danno, 1970), *Lactobacillus brevis* and *Lactobacillus xylosus* (Yamanaka & Takahara, 1977), where values around 180000 for the native enzyme and 43000 for the identical subunits were reported. In contrast to these results, the D-xylose isomerases from *Streptomyces olivochromogenes* (Suekane *et al.*, 1978) appear to be homomeric dimers of subunits

with an M_r of 2×56000 and 2×40000 , respectively, while the enzyme from *Bacillus stearothermophilus* was described as a monomer (Suekane *et al.*, 1978) with an M_r of 127000.

The D-xylose isomerase from *T. aquaticus* has a broad pH optimum sharply declining on both sides, giving half-maximal pH values of 4.5 and 9.4. D-Xylose isomerase from *Streptomyces* has a less broad optimum with half-maximal values of 6.8 and 8.4. For this enzyme as well as for the enzyme from *Lactobacillus* an essential histidine residue was found (Gaikwad *et al.*, 1988; Vangrype *et al.*, 1988). In addition these enzymes contain an active-site carboxylate residue (Vangrype *et al.*, 1989). Recently it was reported that conserved histidine at position 219 and glutamic acid at position 180 appear to be involved in the binding of a divalent metal ion in the enzyme from *Streptomyces albus* (Dauter *et al.*, 1989). D-Xylose isomerase has significant similarity to the triose-phosphate isomerase which is also an aldose-ketose isomerase. In the latter enzyme the nucleophilic glutamic acid 165, electrophilic lysine 13, and histidine 95 bind to the substrate (Straus *et al.*, 1985; Raines & Knowles, 1986). The half-maximal pH values of the D-xylose isomerase from *T. aquaticus* suggest that a similar situation may exist for the catalytic mechanism of this enzyme.

D-Xylose isomerase from *T. aquaticus* proved to be very stable. It loses no activity for at least 1 month when stored at 4 °C as well as at 25 °C, irrespective of the presence of cofactors, and even at 70 °C a half-life of 4 d is remarkable. Mn^{2+} and Co^{2+} ions, which act as cofactors of the enzyme, cause a dramatic enhancement of the enzyme stability.

The high stability of the D-xylose isomerase is further illustrated by the fact that the enzyme could be immobilized by different techniques without severe losses of enzyme activity. In the absence of divalent cations the immobilized enzyme was more stable than the native one.

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