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Some Properties of the Thermostable Xylose Isomerase of *Saccharococcus caldoxylosilyticus* No. 31

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Abstract: The xylose/glucose isomerase of *Saccharococcus caldoxylosilyticus* No. 31, a Gram-positive, motile, facultatively anaerobic, thermophilic rod is described in the continuing search for novel enzymes needed to improve industrial production of high fructose corn syrup. The organism was grown in a xylose medium and purified using four stages of chromatography. Yield of enzyme was 17.83% and specific activity, 4.62 U mg^{-1} protein. Native molecular weight was 160 kDa. Km and Vmax were 111 mM and 2.02 mg min^{-1} mg^{-1} protein and 334 mM and 0.92 mg min^{-1} mg^{-1} protein, respectively for xylose and glucose substrates at 60°C. Maximal enzyme activity was observed at 60°C. Activation energy was 60.35 kJ mol^{-1} K^{-1} and half-life at 70°C was 20 min. This enzyme showed maximal activity at pH 6.4 and stability at pH 7. It required divalent metals, Mn^{2+}>Mg^{2+}>Co^{2+} for activity and stability. Cu^{2+} inhibited enzyme activity but Ca^{2+} did not. The pH profile and non-inhibition by Ca^{2+} distinguish this enzyme from most glucose isomerases so far characterized.

Key words: Xylose/glucose isomerase *Saccharococcus caldoxylosilyticus* No. 31, purification, properties

INTRODUCTION

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5), also known as glucose isomerase, catalyzes the reversible isomerization of D-xylose and D-glucose to their respective ketoses, D-xylulose and D-fructose, in the presence of divalent metal ions. The most important application of these enzymes is in the industrial production of High Fructose Corn Syrup (HFCS), used as a sweetener in the food industry. In view of their high industrial significance, glucose isomerases from various microorganisms have been studied and their catalytic and physicochemical attributes reviewed by Wong (1995), Boshale *et al.* (1996) and Hartely *et al.* (2000) among others. Silva *et al.* (2006) have also, noted that the glucose isomerases have enjoyed the largest application of the technology of immobilized enzymes over the last 40 years.

In the industrial process, enzymes in current use yield HFCS comprising 39- 42% fructose because of limitations, which include excessive time required to attain equilibrium as well as the presence of oligosaccharides in the substrate stream. Expensive chromatographic methods are still employed to obtain syrups with higher levels of fructose needed in some applications (Silva *et al.*, 2006). However, Hartely *et al.* (2000), have theorized that if isomerization is carried out at 90-95°C, then 55% fructose syrups will be achieved. In addition, glucose isomerase enzymes with lower pH optima of about 4.5-5.5 and resistance to Ca^{2+} inhibition will eliminate the differences in operational conditions between HFCS production and enzymatic saccharification of starch. This will enable a one step conversion of liquefied starch to HCFS. Finally, elimination of the requirement for divalent metal ions is desirable from the environmental point of view.

It is therefore apparent that a new set of attributes is required for glucose isomerase enzymes that will catalyze the ideal isomerization process. A potent route towards achievement of the desired set of attributes is the search for novel enzymes. For example, Brown *et al.* (1993) have purified a xylose isomerase having an optimum temperature of 105-110°C from a species of *Thermotoga maritima.* Liu *et al.* (1996) also described an enzyme with a pH optimum of 6.4 at 60°C purified from *Thermoanaerobacterium* strain JW/SL-YS 489. Kaneko *et al.* (2000) also reported a glucose isomerase purified from *Streptomyces olivaceoviridis* E-86 strain showing no loss in enzyme activity at pH 5.0 after incubation at 60°C for 30 h. In this study, we describe another xylose isomerase isolated from *Saccharococcus caldoxylosilyticus* No. 31, which has an acidic pH optimum and is not inhibited by the calcium ion.

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MATERIALS AND METHODS

All chromatography matrix materials were obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden. Dehydrated media were from Difco Laboratories. Sigma Chemical Co. Germany was the source of sugars, sugar-alcohols, L-histidine base, Phenyl Methyl Sulphonyl Fluoride (PMSF), bovine albumin, EDTA and standard proteins. Coomassie brilliant blue R 250 was from Serva GmbH. Carbazole was from Aldrich Germany and cysteine hydrochloride was obtained from BDH, Poole, England. Other reagents and buffers were prepared from analytical grade quality chemicals purchased from May and Baker, Chemical Co. England. This study was carried out in Professor Odibo's Biotechnology laboratory at the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria.

Microorganism: The microorganism was a thermophilic Gram positive, motile, facultatively anaerobic rod isolated from the soil in Awka, Nigeria. It was identified as *Saccharococcus caldoxylosilyticus* No. 31 by the Deutsche Sammlung fur Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Culture method and preparation of crude extract: Culture was carried out by the method of Chou *et al*. (1976) in a medium of composition: tryptone, 1%; yeast extract, 0.7%; xylose, 1%; $MgSO_4.7H_2O$, 0.1%; pH 7.0-7.2 and incubated at 60°C in a shaker incubator at a speed of 160 rpm for 48 h. A total of I L of culture was produced from which about 6 g wet cells were harvested by centrifugation (3.5 x 1000 g for 10 min). The cells were washed three times with deionized water before resuspending in a minimal amount of 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF buffer. This suspension was frozen and the cells lysed in an ice bath using a sonic cell disrupter (Virsonic, Model, 16-850) for 3 min. The cell debris was removed by centrifugation at 3.5 x 1000 g for 20 min. The crude enzyme extract, (about 60 mL), was concentrated by dialysis against 6 M sucrose solution at 4°C and kept at same temperature pending purification.

Enzyme and protein assays: D-xylose isomerase activity was determined from the formation of D-xylulose using the colorimetric assay of Dische and Borenfreund (1951) with D-xylulose as standard. Test mixture, in 50 mM phosphate buffer pH 7.0, which, contained 0.2 M D-xylose and 0.4 mM $MnSO_4$ was reacted with enzyme for 10 min at 60°C, in a water bath. Absorbance was measured at 546 nm. One unit of enzyme activity was defined as the quantity of enzyme, which converted one micromole of xylose to xylulose per minute under these assay conditions. In the assay for the D-glucose isomerase activity, xylose was replaced with 1 M D-glucose and $MnSO_4$ by 2 mM $CoCl_2$ in the test mixture. Protein concentration was measured by the method of Bradford (1976). Results reported during these assays for the various parameters studied are means of three determinations.

Purification of enzyme: Purification was carried out using chromatographic methods at room temperature (about 30°C). Enzyme samples were usually concentrated by dialysis against 6 M sucrose solution at 4°C before each stage.

The crude extract was applied to a 14.5×2 cm anion-exchange column of Q-Sepharose (Fast Flow), pre-equilibrated with several volumes of 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF. The enzyme was eluted in 5 mL fractions using a 450 mL linear gradient (zero-0.8 M) of NaCl in 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF at a flow rate of 0.6 mL min^{-1}.

Fractions showing xylose isomerase activity were subjected to Hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose 6-Fast Flow (High sub) column of dimensions 14×2 cm. The sample was made hydrophobic by the addition of $(NH_4)_2SO_4$ to a concentration of 1.5 M and column by equilibrating with 50 mL of 1.5 M $(NH_4)_2SO_4$ in 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF. The column was eluted in fractions of 5 mL with 300 mL of a linear gradient (1.5-0 M) of $(NH_4)_2SO_4$ in buffer at a flow rate of 0.7 mL min^{-1}. Enzyme fractions were desalted by dialysis against 1 L of 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF buffer for 3 h.

The active fractions from HIC were then subjected to gel filtration on a Sephadex G-100 column (37×1.5 cm). The column was equilibrated with two volumes of 10 mM histidine/HCL pH 6.0, 0.05 mM PMSF buffer and then eluted in 5 mL fractions with 250 mL of same buffer. Flow rate was 0.27 mL min^{-1}. Active fractions were subjected to a second gel filtration on a Sephadex G-200 column of dimensions 42×1.2 cm at a flow rate of 10 mL h^{-1} and fraction volume of 2 mL. Purified enzyme was stored at 4°C during the duration of the work.

Determination of molecular mass of the native enzyme: The molecular weight of the native enzyme was estimated by the gel filtration method of Andrews (1970) using a 52×1.2 cm column of Sephadex G-200. Three standard protein markers were used (potato β-amylase, 200,000, amyloglucosidase, 97,000 and bovine albumin, 66,000, Daltons, respectively).

Temperature dependent behavior: The optimum temperature for activity was determined by assay of activity (Dische and Borenfreund, 1951) at various temperatures (40-90°C). Arrhenius plots of the enzyme activities over the temperature range 40-65°C were used to calculate activation energy. To determine temperature stability, residual activity was measured after purified enzyme samples, were pre-incubated at various temperatures (40-80°C) for 1 h. Decay rate was determined by incubating the purified enzyme sample in the presence of 0.4 mM $MnSO_4$ at temperatures between 50 and 100°C. Over a period of 1 h, aliquots were removed for enzyme assay at 10 min intervals. Half-life of enzyme was estimated from a plot of logarithm of enzyme activity against time.

pH dependent behavior: The optimum pH for enzyme activity was determined by performing the normal isomerisation reaction and enzyme assay in buffer systems of different pH values. For pH stability, enzyme samples were pre-incubated in the various buffer systems for 1 h at 30°C and thereafter, residual enzyme activity measured under normal test conditions at pH 7. Buffer systems used were 50 mM acetic acid/sodium acetate buffer for pH 4 and 5, 50 mM phosphate buffer for pH 6- 8 and 50 mM Tris/HCL buffer for pH 9-10.

Effect of substrate concentration on enzyme activity: The effect of substrate concentration on enzyme activity, was studied using test mixtures containing different concentrations of D-xylose (0-200 mM) in 50 mM phosphate buffer pH 7 containing 0.4 mM $MnSO_4$. For D-glucose, (0-500 mM) was used in reaction mixtures containing 2 mM $CoCl_2$. The kinetic parameters, (Km and Vmax) were calculated by plotting the Lineweaver-Burk diagram.

Effect of divalent metals on enzyme activity: Before the test, it was necessary to obtain a metal-free enzyme. To achieve this the purified xylose isomerase (0.36 mg in 0.5 mL 50 mM phosphate buffer, pH 7) was incubated for 12 h in 0.5 mM EDTA at 4°C and dialyzed for 24 h at the same temperature against 1 L of same buffer. The dialysis buffer solution was changed every 8 h. The following metals, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+} were examined for ability to reactivate enzyme activity. Kinetic studies were also carried out using various concentrations (0-50 mM) of Mg^{2+}, Mn^{2+} and Co^{2+}, ions which were found to be activators of this xylose isomerase. The kinetic constants for these divalent metal ions were deduced from Eadie-Hofstee plots.

Inhibition of enzyme activity by sugar-alcohols: This was determined by measurement of enzyme activity after incubation in test mixtures containing 40 mM D-xylose, 0.4 mM $MnSO_4$ in 50 mM phosphate buffer, pH 7 and different concentrations (0-0.6 M) of the various sugar-alcohols; D-sorbitol, D-inositol and D-mannitol. Inhibition constant, Ki was determined by the graphical method for sorbitol, which showed most potent inhibitory activity against this enzyme.

RESULTS AND DISCUSSION

Purification of enzyme and molecular properties: A summary of purification achieved during the four stages of chromatography employed during this study is given in Table 1. The molecular weight of the *Saccharococcus caldoxylosilyticus* enzyme determined by the gel filtration method of Andrews (1970) was 160 kDa. On this basis, this enzyme probably belongs to the shorter xylose isomerase class and therefore would be a homotetramer with four subunits of approximately 40 kDa. Similar xylose isomerases have been purified from *Ampullariella*, *Streptomyces*, *Arthrobacter* and *Actinoplanes* (Boshale *et al*., 1996). These enzymes are more homogeneous in their amino acid sequences than the longer chain class enzymes and have identical amino acid termini. Hartley *et al*. (2000) have further suggested that the enzymes in this class are identical in catalytic mechanism, structure and thermostabilities.

Temperature dependent behavior of enzyme: Xylose isomerases are generally heat stable. This is an important attribute of these enzymes because the higher the temperature of isomerization, the higher the percentage concentration of D-fructose in HFCS produced as noted by Hartley *et al*. (2000). Maximal enzyme activity was observed for this enzyme at 60°C with both xylose and glucose as substrates. Between 40 and 60°C a linear dependence was observed in the Arrhenius plot and activation energy of 60.35 kJ mol^{-1} K^{-1} was derived for D-xylose. The enzyme also demonstrated good heat stability suffering no loss in activity after incubation for 1 h at 60°C. At 70°C however, a half-life of 20 min was observed (Fig. 1). Hartley *et al*. (2000), reviewed the thermostabilities of some shorter class enzymes. From this review, it was suggested that inactivation of these enzymes may be due to reversible conformational changes in the apoenzyme, followed by an irreversible change to an ordered and more stable inactive tetramer that is incapable of binding metal ions. According to these authors heating over a period of 1 h at temperatures

Table 1: Purification summary of *S. caldoxylosilyticus* No. 31 xylose isomerase

Purification step	Volume (mL)	Total activity (U)	Protein (mg mL^{-1})	Specific activity (U mg^{-1} protein)	Purification factor (Fold)	Yield (%)
Cell free extract	28.0	42.00	58.80	0.714	1.00	100.00
Q-Sepharose (Fast flow)	14.0	24.62	14.00	1.760	2.46	58.66
Phenyl sepharose 6-Fast flow (High sub)	7.0	19.60	5.60	3.500	4.90	46.66
Sephadex G-100	4.0	13.44	3.60	3.700	5.18	31.90
Sephadex G-200	1.8	7.48	1.62	4.620	6.47	17.83

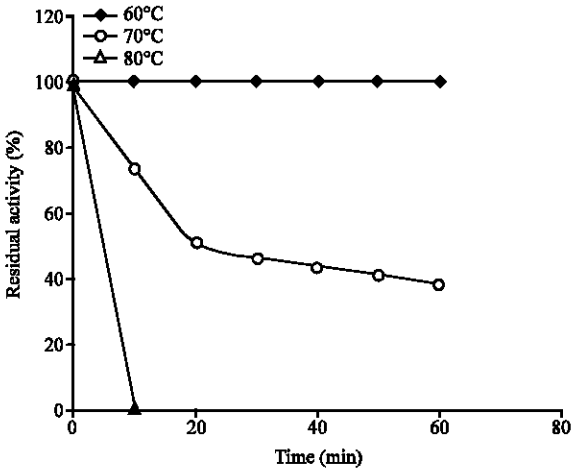

Fig. 1: Decay of *S. caldoxylosilyticus* No. 31 enzyme over 60 min at various temperatures (1 μg of purified enzyme sample was withdrawn at intervals of 10 min and assayed for residual xylose isomerase activity)

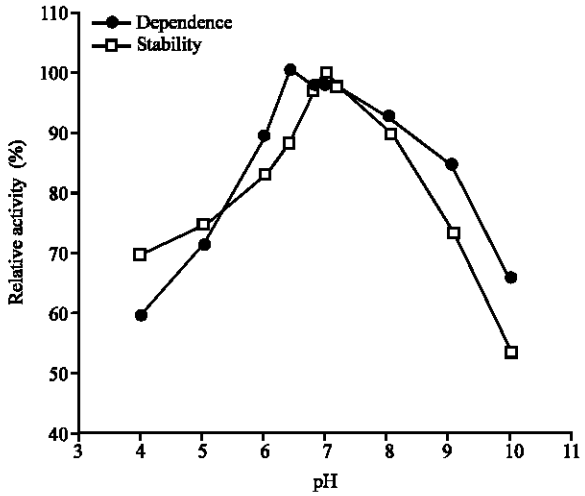

Fig. 2: Influence of pH on activity and stability of *S. caldoxylosilyticus* No. 31 enzyme

indicated caused enzymes from the following organisms to lose 50% of their activities: *Actinoplanes missousriensis*, 76°C; *Arthrobacter* B 3728, 67°C and *Thermus thermophilus*, 67°C. These results compare favourably with observations about the heat stability of the *Saccharococcus caldoxylosilyticus* enzyme. These enzymes are suitable for current processes operating at 60°C.

pH dependent behavior of enzyme: The pH optimum for xylose isomerases is generally in the alkaline range (Boshale *et al*., 1996). Commercial applications under acidic pH conditions are however, desirable as stated by Antrim *et al*. (1979). This will minimize the production of unwanted compounds like psicose and ultimately make for the development of a single step process for both starch liquefaction and glucose isomerization. The xylose isomerase of *Saccharococcus caldoxylosilyticus* was maximally active at pH 6.4 and demonstrated remarkable stability within the acidic pH range (Fig. 2). This enzyme is comparable with only a few enzymes so far characterized. These include the enzymes of *Thermoanaerobacterium* strain JW/SL-YS 489 with optimum pH of 6.4 at 60°C (Liu *et al*., 1996) and *S. olivaceoviridis* (Kaneko *et al*., 2000) reported to show no losses in enzyme activity at pH 5 after incubation at 60°C for 30 h. Liu *et al*. (1996) suggest that enzymes showing acidic pH optima may arise from one or more amino acid changes in the amino acid sequences of these enzymes. The xylose isomerase of *Saccharococcus caldoxylosilyticus* therefore has great potentials for the production of HFCS under acidic conditions of pH and ultimately, realization of the desirable single step starch to fructose process.

Dependence of enzyme activity on substrate concentration: The enzyme of *Saccharococcus caldoxylosilyticus* showed normal Michaelis Menten behavior with both glucose and xylose substrates, which gave linear dependencies in the Lineweaver-Burk plots (not shown). D-xylose was a better substrate than D-glucose. This is evident from the lower Km and higher Vmax (111 mM and 2.02 mg min^{-1} mg^{-1} protein) for xylose compared with (334 mM and 0.92 mg min^{-1} mg^{-1} protein), respectively for glucose at 60°C and pH 7. This enzyme therefore is a true xylose isomerase.

Dependence of enzyme activity on divalent metals: The role of divalent metals in the stability and activity of this xylose isomerase is confirmed by the absence of activity

Table 2: Reactivation of metal-free *S. caldoxylosilyticus* No. 31 xylose isomerase

Metal ion	Relative activity (%)
Control	0.0
$MnSO_4.H_2O$	100.0
$MgSO_4.7H_2O$	73.8
$CoCl_2.6H_2O$	55.8
$ZnSO_4.7H_2O$	5.4
$CuSO_4.5H_2O$	0.0
$CaCl_2$	8.0

(Activity with Mn^{2+} was taken as 100%)

in enzyme samples treated with EDTA. This is characteristic of all xylose isomerases so far characterized (Boshale *et al*., 1996). Among the divalent metals studied, efficiency in reactivation of enzyme activity was in the order: Mn^{2+}> Mg^{2+} > Co^{2+} (Table 2). The natural occurrence of preference for Mn^{2+} and low pH optima in the enzyme of *Saccharococcus caldoxylosilyticus* is similar to characteristics observed by Tilbeurgh *et al*. (1992) with a mutated *A. missourienses* enzyme. These workers described experiments in which a single amino acid substitution lowered optimum pH and altered preference for metal ions from Mg^{2+} to Mn^{2+}. Further studies of the *Saccharococcus caldoxylosilyticus* enzyme may reveal information necessary for the understanding of the catalytic mechanism and techniques for the improvement of these enzymes. Although Ca^{2+} reactivated this enzyme to only a low level (Table 2), this observation is significant because it suggests that this metal ion is not inhibitory to its activity. This attribute is technologically relevant for the development of a single step process to convert starch to fructose. Current processes require removal of Ca^{2+} from the glucose syrup before isomerization because of its inhibitory activity against most xylose isomerases.

Increasing concentrations within the range, zero-20 mM of metal ions, Mn^{2+}, Mg^{2+}, Co^{2+}, as co-substrates with xylose resulted in the increased activity of metal-free enzyme. Eadie-Hofstee plots (not shown) of these data reveal biphasic behavior in all cases. Linear portions of these curves obtained in the higher metal ion concentration ranges were used to calculate the kinetic constants, maximum velocity (Vmax) and binding constants (Kd), respectively as follows: Mn^{2+}, 0.036 $\mu mole^{-1}$ and 0.023 mM; Mg^{2+}, 0.018 $\mu mole^{-1}$ and 0.018 mM; Co^{2+}, 0.008 $\mu mole^{-1}$ and 0.016 mM. These results indicate the existence of two non-identical metal binding sites per enzyme subunit as has been reported for most D-xylose isomerases (Hartley *et al*., 2000).

Inhibition by sugars alcohols (D-sorbitol, D-inositol and D-mannitol): The sugar alcohols, D-xylitol, D-sorbitol and D-mannitol are known to inhibit xylose isomerase activity (Wong, 1995). At 0.1 M concentration, inositol, mannitol and sorbitol reduced enzyme activity to 83, 84 and 88%, respectively. Further studies using Lineweaver-Burk plots on the mechanism of inhibition by D-sorbitol revealed that this substrate exhibited a mixture of competitive and non-competitive types when D-xylose was used as substrate. Inhibitory activity was weak as revealed by its high Ki of 370 mM. Smith *et al*. (1991) and Danno (1970) reported similar observations with *Arthrobacter* strain NRRL B3728 and *Bacillus coagulans* strain HN-68 xylose isomerases, respectively. Smith *et al*. (1991) explained that inhibition resulted from steric constraints imposed on the enzyme molecule in accommodating the additional-CH_2OH group in D-sorbitol. This produces adverse catalytic activity on adjacent active sites on other enzyme subunits.

CONCLUSIONS

It has been shown that *Saccharococcus caldoxylosilyticus* No. 31 produced a D-xylose isomerase possessing some properties desired for improvement of the efficiency of high fructose corn syrup production. These include an acidic pH activity/stability optima and non-inhibition by Ca^{2+}.

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