Thermostable glucose isomerase from psychrotolerant *Streptomyces* species

Bidur Dhungel¹, Manoj Subedi¹, Kiran Babu Tiwari¹,²,³, Upendra Thapa Shrestha², Subarna Pokhrel⁴ and Vishwanath Prasad Agrawal¹, ²

¹Universal science College, Pokhara University, Kathmandu, Nepal
²Research Laboratory for Biotechnology and Biochemistry, Kathmandu, Nepal
³Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
⁴Department of Enzyme Engineering, Seoul National University, Korea

**Corresponding Address:** Kiran Babu Tiwari, Research Scientist, Research Laboratory for Biotechnology and Biochemistry (RLABB), Maitidevi, Kathmandu, Nepal. Email: kiranbabu.babukiran@gmail.com

**ABSTRACT**

Glucose isomerase (EC 5.3.1.5) was extracted from *Streptomyces* spp., isolated from Mt. Everest soil sample, and purified by ammonium sulfate fractionation and Sepharose-4B chromatography. A 7.1 fold increase in specific activity of the purified enzyme over crude was observed. Using glucose as substrate, the Michaelis constant (K_M) and maximal velocity (V_max) were found to be 0.45M and 0.18U/mg, respectively. The optimum substrate (glucose) concentration, optimum enzyme concentration, optimum pH, optimum temperature, and optimum reaction time were 0.6M, 62.14μg/100μl, 6.9, 70ºC, and 30 minutes, respectively. Optimum concentrations of Mg²⁺ and Co²⁺ were 5mM and 0.5mM, respectively. The enzyme was thermostable with half-life 30 minutes at 100ºC.

**INTRODUCTION**

Glucose isomerase (EC 5.3.1.9), GI, is an intracellular bacterial enzyme (Chen, 1980a) that catalyzes the reversible isomerization of glucose to fructose or xylose to xylulose (Tsumura and Sato, 1961). Fructose is the sweetest of various naturally occurring sugars and there has long been a demand for it as alternative to sucrose. GI is one of the three highest tonnage value enzymes, amylases and proteases being the other two (Bhosale et al., 1996). The enzyme is used to produce high-fructose corn syrup from corn starch. This process involves several separate enzymatic steps, including liquefaction of corn starch by α-amylase, saccharification by glucoamylase, and isomerization by glucose isomerase (Lee and Zeikus, 1991).

Most commercially available GI has been isolated from mesophilic microorganisms, including *Streptomyces*, *Actinoplanes*, *Flavobacterium* and *Bacillus* spp (Bhosale et al., 1996). GIs are homotetramer with 45kDa or 49kDa, the former being more conservative (Kwon et al., 1987; Carrel et al., 1984; Farber et al., 1989; Dauter et al., 1989; Henrick et al., 1989). Most of the GIs are not highly thermostable (limited to 60ºC only) and less active at neutral. Thermostable GIs with neutral or slightly acidic pH optima have a potential for industrial applications. The thermo-acid-stable GI allow for faster reaction rates, higher fructose concentration at equilibrium, higher process stability, decreased viscosity of substrate and product streams, and reduced by-product formation (Chen et al., 1979). Owing to the industrial significance of the enzyme, GI from various microorganisms has been studied and their catalytic and physicochemical properties have been reviewed (Chen, 1980b; Chen, 1980c).
Thermophilic microorganisms produce industrially thermostable enzymes which have been evolved and adapted to the extreme environment of their natural habitats. As the Research Laboratory for Agricultural Biotechnology and Biochemistry (RLABB) has been studying and exploiting the actinomycetes’ population diversity from high altitude ecological niche, this work was done on D-glucose isomerase of the psychrotolerant *Streptomyces* spp. isolated from the soil sample collected from Khumbu, Mount Everest base camp.

**METHODOLOGY**

**Culture:** *Streptomyces* spp. Lob 15.4, isolated from soil samples collected from Lobuche, Mt. Everest base camp, was revived by inoculating spores into a 250 ml conical flask containing 50 ml of culture medium (1% tryptone, 0.7% yeast extract, 1% xylose and 0.1%MgSO₄.7H₂O, pH 7.0-7.2) followed by incubating at 28°C in a shaker waterbath (200rpm) for 4 days (Chou et al., 1976).

**Enzyme preparation:** *Streptomyces* cells were collected by centrifugation, washed several times with deionised water and homogenized in vortexer in 0.1M phosphate buffer (pH 7.0) containing 5mM MgSO₄, 0.5mM CoCl₂ and 1mM PMSF. Cells were disrupted in a bath sonicator for 30 min with ice and centrifuged at 10000 rpm for 20 min at 4°C to obtain enzyme supernatant (Chou et al., 1976).

**Enzyme assay:** A 100 μl of the enzyme was incubated in 900 μl phosphate buffer (pH 7.0) containing 5mM MgSO₄, 0.5mM CoCl₂ and 0.8M glucose at 37°C for 40 minutes, followed by keeping the tubes in an ice bath. The amount of the product, fructose, was determined by Seliwanoff’s method (Chen et al., 1979).

**Protein determination:** Protein content in the supernatant was determined by Bradford assay (Bradford, 1976).

**Enzyme purification:** Ammonium sulphate was added to the crude enzyme extract to 45% saturation, incubated for an hour at 4°C with gentle mixing. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4°C and dissolved in 0.1M phosphate buffer (pH 7.0) containing 5mM MgSO₄, 0.5mM CoCl₂. The ammonium sulphate concentration was increased stepwise to 60%, 75% and finally to 90% saturation; and the precipitates were harvested accordingly. The fraction containing glucose isomerase activity was pooled and dialyzed overnight against 0.1M phosphate buffer (pH 7.0) (Liu et al., 1996). Then, a Sepharose 4B column (3.2 by 38.5cm) was prepared and equilibrated with 0.05M phosphates buffer containing 0.15M NaCl. The dialyzed enzyme was applied to the column and eluted with the phosphate buffer. Fraction containing glucose isomerase activity was collected, concentrated with ammonium sulphate and dialyzed against 0.1M phosphate buffer (pH 7.0) (Liu et al., 1996). The purification steps were monitored by SDS-PAGE (Laemmli, 1970) and Native-PAGE (Blackshear, 1984).

**Optimization:** Optimization was done in phosphate buffer (pH 7.0) containing 5mM MgSO₄, 0.5mM CoCl₂ at 37°C for 40 min. unless mentioned otherwise. Glucose (substrate) from 0.1-1.0M and enzyme from 6-124μg were mixed in the respective optimization reaction. Phosphate buffers from pH 4-10 were used to optimize pH of the respective reaction with 0.6M glucose. Time and temperature were optimized by incubation the respective reaction mixtures for 10 to 60 minutes and 30 to 90°C in phosphate buffer with pH 6.9. To optimize Mg²⁺ and Co²⁺ concentrations, 0.05-10mM ions were mixed in the respective reaction buffer. Amount of product (fructose) produced was determined by Seliwanoff’s method (Chen et al., 1979).
Half-life and Thermal stability were determined by measuring residual activity under optimum assay condition after pre-incubation of the enzyme- at 100ºC for 5-30 minutes for half life and at 40-90ºC for 30 to 150 minutes for thermal stability (Liu et al., 1996).

**Result**

A maximum enzyme activity of broth was obtained after 96h (4days) of cultivation in media. Fraction collected during 90% saturation of ammonium sulphate showed glucose isomerase activity and was further purified by column chromatography to 7.1 fold increase in activity (Fig. 1). The purified fraction had specific activity 0.490U/mg (Fig. 2). Native-PAGE revealed that the enzyme had mol. wt. about 200kD. The Michaelis constant (K_M) and maximal velocity (V_max) were found to be 0.45M and 0.18U/mg (Fig. 3), respectively. The optimum substrate (glucose) concentration, optimum enzyme concentration, optimum pH, optimum temperature, and optimum reaction time were 0.6M (Fig. 4), 62.14μg/100μl (Fig. 5), 6.9 (Fig. 6), 70ºC (Fig. 7), and 30 minutes (Fig. 8), respectively. Optimum concentrations of Mg^{2+} and Co^{2+} were 5mM (Fig. 9) and 0.5mM (Fig. 10), respectively. The enzyme had half life 30 minutes at 100ºC (Fig. 11). The enzyme was quite thermostable (Fig. 12).

**DISCUSSION:**

The production of glucose isomerase from *Streptomyces* species has been documented by several investigators (Takasaki, 1966; Strandberg and Smiley, 1971). The sample sonicated for 20minutes showed maximum enzyme activity (Chou et al., 1976).

Chou and Anderson also found glucose isomerase activity in 90% ammonium sulfate saturation (Antrim et al., 1979). To get more purified form of the enzyme, the chromatography has to be done several times. Chen and Anderson reported that the enzyme was purified using DEAE –Sephadex A-50 and the purification was about 12.6 fold over the crude (Antrim et al., 1979). The enzyme activity was stable at 37ºC; therefore, all steps of purification were performed at that temperature. The purified fraction had specific activity 0.490U/mg and crude enzyme had 0.069 U/mg, suggesting a 7.1 fold increase in specific activity of the purified enzyme over crude was observed.

Homogeneity of the purified enzyme was determined by Sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme was homogeneous by the detection of a single protein band on SDS-PAGE and Native-PAGE. Approximately, mol. wt. of 200kD protein band on Native-PAGE and about 50kD on SDS-PAGE was determined, suggesting that the enzyme to be tetramer.

The enzyme also called as Xylose isomerase (XI) as it converts xylose to xylulose besides converting glucose to fructose. Hence, xylose was used as the inducer of the enzyme in the culture medium. The enzyme was, then, optimized using glucose as a substrate. The optimum glucose concentration was 0.6M. The Michaelis constant (K_M) and maximal velocity (V_max) were found to be 0.45M and 0.18U/mg, respectively. In the other studies the K_M value upto 0.2M (Chen et al., 1979) to 0.167M (Lama et al., 2001) was also been reported. Lama et al. reported V_max of 6.3U/mg (Lama et al., 2001). The enzyme with lower K_M and higher V_max towards substrate is more preferred for exploitation of enzyme behavior, suggesting the enzyme extract in the work may not be as competent to that obtained from mesophilic or thermophilic bacteria which are more suitably adapted to higher temperature.
The optimum pH is the ranges between pH 7.0 to 9.0 (Lee and Zeikus, 1991). The optimum pH of the glucose isomerase is slightly acidic, pH 6.9. It was apparently lower than that of enzymes from other *Streptomyces* species (Bucke, 1997). Therefore, a low pH optimum is an attractive property for enzyme application because the use of the enzyme at neutral or low pH prevents the formation of by-product, psicose.

Most of glucose isomerase isolated to date showed an optimum temperature around 80ºC (Chou et al., 1976). Most of the industrially exploitation of the enzyme is done at 60ºC, as Hodge indicated that degradation of ketoses occurs at high temperatures, characterized by pronounced discoloration of an aqueous sugar solution. Interestingly, in this study, the optimum temperature of the enzyme from the cold tolerant bacteria was 70ºC. This may be due to conservation of the gene in bacterial population (Carrel et al., 1984; Farber et al., 1989; Dauter et al., 1989; Henrick et al., 1989). Lama et al. (2001) also documented that the kinetic characteristics for XI or GI were similar to XI from distantly related bacteria. The optimum temperature of the GI explored that psychrotolerant organisms may have thermostable proteins.

The optimum reaction time of the enzyme was 30min, similar to most of the enzymes from diversified bacteria (Chen, 1980a; Lee and Zeikus, 1991; Antrim et al., 1979). Compared to the half life reported by Chou et al. (1976), 120h at 70ºC, the half life of the GI in this work was 30 minute at 100ºC, suggesting to be a quite thermostable one.

Glucose isomerases typically require the presence of divalent metal cations such Mg$^{2+}$ or Co$^{2+}$ as essential cofactors for their catalytic activity (Whitlow et al., 1991). Treatment of purified enzyme with EDTA resulted in an almost complete loss of enzyme activity. However, the activity could be restored by the addition of metal ions. In particular, increasing amounts of Mg$^{2+}$ or Co$^{2+}$ (each up to 10mM) were able to restore only 60-80% of the original xylose isomerase activity. Lama et al. reported, for glucose isomerase, 10mM Mg$^{2+}$ was required to restore 80% of the original activity (Lama et al., 2001). As is common with other isomerases, 10mM Mg$^{2+}$ plus 1mM Co$^{2+}$ restored total glucose isomerase activity. However, the lower values of the cations observed in this study, suggested that the enzyme might be adapted in the ecological niche.

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References


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