A Stable Glucokinase from a Thermophilic Bacterium

I. Purification and Some Properties

Sachiko KAMEI,^{1,*} Kosuke TOMITA,² Kazuhiko NAGATA,²
Hiroshi OKUNO,² Takanari SHIRAISHI,² Akio MOTOYAMA,²
Akiyuki OHKUBO,¹ and Manabu YAMANAKA¹

¹Department of Laboratory Medicine, Faculty of Medicine,
The University of Tokyo, Hongo, Tokyo 113, Japan

²Research and Development Center,
UNITIKA Ltd., Uji 611, Japan

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Summary A glucokinase (EC 2.7.1.2) was purified from *Bacillus stearothermophilus*, a moderate thermophile. The native glucokinase, M_r about 68,000, consists of two identical subunits. This enzyme is relatively temperature- and pH-stable, the optimal pH range for the enzymatic activity being within the stable pH range. It selectively catalyzes the phosphorylation of glucose rather than other kinds of hexoses. The K_m values were estimated to be 1×10^{-4} M for glucose and 0.5×10^{-4} M for ATP. It shows no ATPase activity. The high stability, high substrate specificity, low K_m values for glucose and ATP, and the lack of ATPase activity make this enzyme advantageous for use in clinical chemical analysis.

Key Words: glucokinase, thermophilic bacterium, purification of glucokinase, properties of glucokinase

Clinical chemical analysis by use of enzymes has obvious advantages, but also some disadvantages, the most important of which is the poor stability of enzymes. Moreover, it is usually hard to obtain highly purified enzymes. Also, contaminating enzymes often cause errors in analysis.

Compared with common enzymes, enzymes obtained from thermophilic bacteria are known to be stable over a wide range of temperature, so they can be easily purified without loss of activity.

We have isolated some enzymes in a high state of purity from *Bacillus stearothermophilus*, a moderate thermophile, and investigated their application to clinical chemistry. Here we report the purification procedure for purification of a gluco-

*To whom correspondence should be addressed.

kinase (GK, EC 2.7.1.2) from the thermophilic bacterium, and some properties of the GK.

MATERIALS AND METHODS

Reagents. Diethylaminoethyl cellulose (DEAE cellulose, grade DE 52) was obtained from Whatman K.K., Tokyo; Phenyl-Sepharose CL-4B, from Pharmacia Japan K.K., Tokyo; Matrex Blue A from Amicon K.K., Tokyo; Ultrogel AcA₃₄, from LKB Japan K.K., Tokyo. Glucose, α -D-glucose, β -D-glucose, fructose, mannose, and glucosamine were obtained from Wako Pure Chemical Industries, Ltd., Osaka; xylose, 2-deoxyglucose, and galactose from Sigma Chemical Co., St. Louis. ATP, phosphoenolpyruvate (PEP), pyruvate kinase (PK, EC 2.7.1.40, pig heart), and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49, *Leuconostoc mesenteroides*) were purchased from Oriental Yeast Co., Ltd., Tokyo; and lactate dehydrogenase (LDH, EC 1.1.1.27, hog muscle) and hexokinase (HK, EC 2.7.1.1, yeast) from Boehringer Mannheim Yamanouchi, Tokyo. Other chemicals were of analytical grade.

Purification of GK. *B. stearothermophilus* UK 563 (FERM P-7275 strain, deposited at Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki, Japan, on September 29, 1983), was cultured as described by Suzuki and Imahori [1], and GK was isolated according to the procedure described hereafter. All steps were carried out at 0–4°C. The cells (500 g wet wt) were suspended in 1 liter of buffer A (100 mM potassium phosphate, 2 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.5), and disrupted by a cell mill (Willy A. Bachofen, Dyno-Mill, Basel). Cell debris was removed by centrifugation at 8,000 $\times g$ for 30 min. To the supernatant (1.5 liters) thus obtained, a 3.4% solution of protamine sulfate (265 ml) was added slowly with stirring. After 1 h the precipitate was removed by centrifugation, and solid ammonium sulfate was added to the supernatant. The fraction which precipitated from a 30–50% saturated solution of ammonium sulfate at 0°C was collected. The resulting precipitate was dissolved in buffer A, and then dialyzed against buffer B (25 mM potassium phosphate, 2 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.5). The dialyzed sample was applied to a column of DEAE-cellulose (4.4 $\times$ 90 cm) equilibrated with buffer B. The enzyme was eluted with a linear salt gradient composed of 4 liters each of buffer B and 500 mM KCl in the same buffer. GK activity was eluted over a KCl concentration range of 90 to 170 mM. The flow rate was 135 ml/h. The active fractions were pooled, and dialyzed against buffer A containing 15% ammonium sulfate. The dialyzed sample was applied to a column of Phenyl-Sepharose CL-4B (4.4 $\times$ 30 cm) equilibrated with the same buffer. Elution was carried out with a concave decreasing gradient composed of 1 liter of equilibration buffer and 4 liters of water. GK activity was eluted at ammonium sulfate concentrations between 6% and 3%. The flow rate was 220 ml/h. The fractions containing GK activity were pooled, and dialyzed against buffer B. Next the dialyzed sample was transferred to a Matrex

blue A column (3.2×60 cm) in equilibrium with the same buffer. GK was eluted at a flow rate of 80 ml/h with a linear gradient composed of 2 liters each of equilibration buffer and 1 M KCl in the same buffer. The GK active fractions, which were eluted at KCl concentrations between 200 and 400 mM, were collected and concentrated to 3 ml. The concentrated sample was finally applied to an Ultrogel AcA₃₄ column (1.6×120 cm) in equilibrium with buffer B. The column was developed with the same buffer at a flow rate of 8 ml/h, and the active fractions were collected.

Assaying of GK. GK activity was measured by two methods.

Method 1, GK-G6PD-system: the formation of glucose 6-phosphate (G6P) from glucose and ATP was determined in a coupled assay with G6PD. The reaction is as follows:

The reaction mixture contained Tris-HCl buffer (60 mM, pH 9.0), glucose (12 mM), ATP (4.0 mM), NADP⁺ (0.9 mM), magnesium chloride (20 mM), and G6PD (700 U/liter). The assay was started by addition of an appropriate amount of GK solution to a cuvette thermostatted at 30°C. The change in absorbance at 340 nm was recorded on a Gilford System 2600 spectrophotometer (Amco, Tokyo). If not mentioned otherwise, Method 1 was used for GK assays.

Method 2, GK-PK-LDH system: The formation of ADP from ATP and glucose was determined in a coupled assay with PK and LDH. The reaction is as follows:

The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.0), glucose (12 mM), ATP (4.0 mM), PEP (5.0 mM), NADH (0.2 mM), potassium chloride (75 mM), magnesium chloride (20 mM), PK (10,000 U/liter), and LDH (10,000 U/liter). Method 2 was used for the determination of the substrate specificity.

One unit of GK was defined as the amount of the enzyme required to catalyze the formation of 1 μ mol of NADPH (Method 1), or the consumption of 1 μ mol of NADH (Method 2), per minute at 30°C.

Measurement of ATP. The ATP concentration was measured by the same principle underlying Method 1 of the GK assay. In the reaction mixture of Method 1, GK (600 U/liter) was used in the place of ATP.

Measurement of protein. The protein concentration was determined by the absorbance at 280 nm using A^{0.1%}. The A^{0.1%}_{280nm} value for the purified enzyme was 0.95 determined by direct weighing (dry weight). For the crude enzyme during purification, the enzyme concentration was calculated from the absorbance at 280 nm assuming A^{0.1%} = 1.0.

Electrophoretic analysis. Polyacrylamide gel electrophoresis was carried out according to Davis [2] using 10% gels. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [3]. Gels were stained with Coomassie Brilliant Blue.

Gel filtration. Estimation of the molecular mass of GK was performed on a column of Ultrogel AcA₃₄ (1.2 × 100 cm). The protein was eluted at a flow rate of 4 ml/h with 25 mM Tris-HCl buffer, pH 8.0, containing 100 mM KCl.

RESULTS

Purification of GK. Typical results of the purification procedure for GK are summarized in Table 1. The final preparation yielded about 30% activity and had a specific activity of about 350 U/mg protein. The purified enzyme showed a single band by polyacrylamide gel electrophoresis in the absence or presence of SDS, even when 30 µg of GK was loaded onto the 5 × 90 mm disc gel to pick up contaminants. Figure 1 shows the pattern obtained by gel scanning at 600 nm with a Gilford Model 2520 gel scanner.

Relative molecular mass and subunit structure. The M_r of GK was estimated to be $68,000 \pm 2,000$ (3 determinations) by gel filtration on Ultrogel AcA₃₄ (Fig. 2). Next the M_r of enzyme subunit was determined by SDS-polyacrylamide gel electrophoresis. GK showed a single band, and as shown in Fig. 3 the subunit M_r was $33,000 \pm 2,000$ (3 determinations). These results suggest that the native GK consists of two identical subunits.

pH stability. GK (0.08 mg protein/ml concentration) was dissolved in buffer solutions of various pH values and then stored at 4°C. After 24 h the remaining activity was measured. As shown in Fig. 4A, GK retained full activity over a wide range of pH above 8. In contrast the stability of HK measured in a similar manner as for GK was stable only over a narrow pH range around neutrality.

pH dependency of the reaction. Figure 4B shows the GK activity at various pH values compared with corresponding HK activity. The optimal pH for GK activity was 8.5, similar to that of HK.

Thermal stability. GK was dissolved in 100 mM Tris-HCl buffer, pH 8.5, and

Table 1. Purification of GK from *Bacillus stearothermophilus*.

Step	Total protein ^a (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	263,000	0.06	100
Protamine treatment	76,000	0.18	87
Ammonium sulfate fraction (30–50%)	23,800	0.55	83
DEAE-cellulose	540	19.4	66
Phenyl-Sepharose CL-4B	130	58	47
Matrex blue A	30	195	37
Ultrogel AcA ₃₄	13.5	350	30

^a Protein concentration was estimated from absorbance at 280 nm assuming $A_{280\text{nm}}^{0.1\%} = 1.0$.

Fig. 1. Polyacrylamide disc gel electrophoresis of purified GK in the absence of SDS. This pattern was obtained by gel scanning at 600 nm with a Gilford Model 2,520 gel scanner.

Fig. 2. Determination of the M_r of native GK by gel filtration. V_0 and V_e are the void volume of Ultrogel AcA₃₄ column and the elution volume of each protein, respectively. The marker proteins used were: 1, PK (M_r , 237,000); 2, aldolase (M_r , 158,000); 3, LDH (M_r , 140,000); 4, egg albumin (M_r , 45,000); 5, cytochrome c (M_r , 12,500). The position of GK is indicated by the arrow.

Fig. 3. Determination of the M_r of GK subunit by polyacrylamide gel electrophoresis in the presence of SDS. The marker proteins used were: 1, phosphorylase b (M_r , 97,400); 2, bovine serum albumin (M_r , 68,000); 3, ovalbumin (M_r , 43,000); 4, carbonic anhydrase (M_r , 30,000). The position of GK is indicated by the arrow.

Fig. 4. Comparison of GK and HK in terms of pH stability and pH dependency. A, pH stability of GK and HK. GK and HK were dissolved in 100 mM buffer solutions of various pH and stored at 4°C. After 24 h of storage the remaining activity was measured. Buffers were acetate (pH 4.5–6.0), phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.5), and carbonate (pH 9.5–11.0). B, pH dependency of GK and HK activities. Enzyme activities were determined by Method 1 using 60 mM buffer solutions of various pH. The same buffer as for experiment A was used.

then stored for 15 min at various temperatures (Fig. 5A) or for various periods of time at 60, 70, or 80°C (Fig. 5B). The remaining activity after heating was measured. GK retained full activity for at least 60 min at 60°C. As a comparative study, HK was dissolved in 100 mM potassium phosphate buffer, pH 7.0, which is the optimum value for the pH stability of HK, and then stored for 15 min at various temperatures (Fig. 5A). These results show that GK from *B. stearothermophilus* is more stable than HK from yeast.

Fig. 5. Comparison of GK and HK in terms of thermal stability. A, Thermal stability of GK and HK. GK was dissolved in a 100 mM Tris-HCl buffer, pH 8.5 (the optimal pH for the GK activity), and HK was dissolved in a 100 mM phosphate buffer, pH 7.0 (a stable pH for HK). After incubation for 15 min at various temperatures, the remaining enzymatic activity was measured. The protein concentrations used were 0.08 mg/ml. B, Thermal stability of GK. GK in the same buffer solution as in experiment A was kept at 60, 70, or 80°C. After various periods of time, the remaining enzymatic activity was measured.

Storage stability. The storage stability of GK was examined at 0.08 mg protein/ml concentration in 100 mM Tris-HCl buffer, pH 8.5, containing 10 mM NaN_3 . GK samples retained about 90% activity on storage for one month at room temperature or six months at 4°C.

Michaelis constants. GK activities were measured at various concentrations of glucose (0.02–12.0 mM) or ATP (0.01–5.0 mM) and a fixed concentration of ATP (4.0 mM) or glucose (12.0 mM), respectively. The K_m values obtained from Lineweaver-Burk plots were 1.0×10^{-4} M for glucose and 0.5×10^{-4} M for ATP (3 determinations).

ATPase activity. GK (10,000 U/liter), ATP (2.0 mM) and magnesium chloride (10 mM) were dissolved in three different buffer solutions (50 mM; potassium phosphate, pH 7.0, 7.5; and Tris-HCl, pH 8.0) and were incubated for 48 h at 37°C. Then ATP remaining in each buffer solution was measured by the method described in "Materials and Methods." The ATP concentrations did not change on incubation. Thus the GK preparation showed no contamination with ATPase, and further, GK from *B. stearothermophilus* does not have an intrinsic ATPase activity, which is observed for HK from yeast [4–6].

Substrate specificity. The substrate specificity of GK was determined by Method 2, in which various monosaccharides were substituted for glucose. As shown in Table 2, no activity was observed when fructose, mannose, and glucosamine were used as phosphoryl acceptors, even at concentrations as high as 12 mM. However, 2-deoxyglucose, xylose, and galactose were slightly active, and their respective activities were 13, 0.3, and 0.9% (3 determinations) of the glucose con-

Table 2. Substrate specificities of GK and HK.

Substrate	Relative activity	
	GK	HK ^a
Glucose	100	100
Fructose	0	180
Mannose	0	80
Glucosamine	0	70
2-Deoxyglucose	13	100
Xylose	0.3	0
Galactose	0.9	0.2

n = 3 throughout. ^a Taken from ref. [7].

trol. For comparison, the relative activities of HK, which are the values reported by Sols *et al.* [7], are also shown in Table 2. Thus GK from *B. stearothermophilus* is highly specific for glucose, but HK utilizes various monosaccharides such as fructose, mannose, glucosamine, and 2-deoxyglucose as well as glucose.

D-Glucose anomeric preference. GK catalyzes the phosphorylation of both α -D-glucose and β -D-glucose. The ratio of the $V_{\max}$ for the α -D-anomer to that for the β -D-anomer (V_{α}/V_{β}) was 0.92 (single determination).

DISCUSSION

B. stearothermophilus UK 563 produces a glucokinase but not a hexokinase. The purification of GK from this bacterium necessitated four different column chromatographies. However, due to its stability, a highly purified enzyme was obtained with an adequate yield of 30% (Table 1). We studied some properties of the GK which are thought to be necessary for its application in clinical chemical analysis, such as pH dependency of the reaction, stability in various environments, K_m for glucose and ATP, and so on. We compared some of its properties with those of HK, since HK and GK can be used for the same purposes in clinical chemistry.

The present GK, we believe, is more useful than the HK from yeast which is currently used in clinical chemistry in the following points: Firstly, the purification of GK is much easier, due to its stability, than that of HK. Secondly, the GK is stable not only over a wide temperature range (Fig. 5) but also over a wide pH range (pH above 8) (Fig. 4A), which includes its optimal pH (pH 9) (Fig. 4B). On the other hand, HK is only stable over a narrow acidic pH range (pH 6–7) (Fig. 4A), whereas its optimal pH is alkaline (pH 9) (Fig. 4B). Thirdly, GK shows no ATPase activity. So, in a working solution containing GK, ATP is stable. On the other hand, even a highly purified preparation of yeast HK was reported to show ATPase activity [4–6]. Fourthly, the enzymatic reaction of GK is more specific than that of HK. On the other hand, the K_m for glucose of some mammalian GK is known to be much higher than those of HK, and those GK's act preferably on

α -D-glucose among glucose anomers [8]. However, the GK from the thermophilic bacterium shows a K_m for glucose that is as low as that of HK from yeast, and its K_m for ATP was lower than that of the HK, and it reacts with α -D-glucose as well as β -D-glucose.

From the above facts, we conclude that the GK from *B. stearothermophilus* is an excellent tool for enzymatic analysis in the field of clinical chemistry.

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