# ISOLATION AND CHARACTERIZATION OF A THERMOPHILIC GLUCOKINASE FROM *BACILLUS STEAROTHERMOPHILUS*

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## 1. Introduction

Kinases (phosphotransferases), which catalyse the transformation of hexoses to hexose 6-phosphates, are distributed widely and differ greatly in their substrate specificities. Whereas the hexokinases isolated from, e.g. mammalian brain [1], and yeast [2,3] are unspecific (by definition) and achieve phosphorylation of D-glucose, D-fructose, D-mannose, 2-deoxy-D-glucose and other substrates, very specific glucokinases isolated from liver [4], *Aerobacter aerogenes* [5] and *Entamoeba histolytica* [6] have been described. *N*-Acetyl-D-glucosamine kinases from *Streptomyces pyrogenes* [7] and *E. coli* [8] seem to be related to hexokinase and glucokinase respectively.

The hexokinases and glucokinases found until now represent relatively labile species or alternatively are often found as mixtures of isoenzymes, which are very difficult to separate.

In contrast the glucokinase from the thermophilic *Bacillus stearothermophilus* described in this paper possesses considerably greater stability and, in addition, is present in only one molecular species. Hence this enzyme appears to be especially suitable for structural and functional studies.

## 2. Materials and methods

Alcohol dehydrogenase (horse liver), hexokinase (yeast), glucose 6-phosphate dehydrogenase (yeast) and glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle) were obtained from Boehringer, Mannheim, Germany; carbonic anhydrase (bovine erythrocytes) and lysozyme (hen egg white) from Serva, Heidelberg, Germany; horse myoglobin and bovine serum albumin from Pentex, Kaukakee, Ill., USA; β-lactoglobulin from Mann Research, New York, USA, and alkaline phosphatase (*E. coli*) from Miles Laboratories, Elkhart, Ind., USA. All reagents were of analytical purity. The Cellogel-electrophoresis strips came from Chemetron, Milan, Italy.

*B. stearothermophilus* (strain NCIB 8924) was cultured at 50°C by CIBA-Geigy, Basel, according to the procedure described by Sidler and Zuber [9]. Extraction and the first two purification steps ($(NH_4)_2SO_4$-fractionation, gel filtration on Sephadex G-150) were performed as described by Hengartner et al. [10]. The fractions from the Sephadex G-150 column which contained the kinase were combined and applied to a column of DEAE Sephadex A-50 which was equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$; the enzyme was eluted by a gradient of NaCl (0.15–0.30 M) in the same buffer. The fractions containing the kinase were collected and rebuffered in a Diaflo ultrafiltration cell (membrane: UM 20 E) by adding 0.05 M Tris–HCl buffer pH 9.0 (1mM $MgCl_2$ and 0.17 M NaCl). The enzyme solution was transferred to a QAE-Sephadex A-50 column in equilibrium with the same buffer and was eluted by a linear gradient of 0.17–0.3 M NaCl in the same buffer. The collected fractions of kinase were concentrated (Diaflo) and dialyzed against 0.05 M Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$. Final purification was performed by preparative polyacrylamide gel electrophoresis using 'Poly Prep' of Buchler Instruments, New Jersey, USA. In general the procedure was that of the 'instructions' as given by the manufacturer [11]. The upper buffer

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Table 1
Purification of glucokinase from *B. stearothermophilus*.

| Step no. | Fraction | Total protein [mg] | Total units | Yield per step [%] | Specific activity [units/mg] |
|---|---|---|---|---|---|
| 1 | Crude extract | 37 300[a] | 59 500 | | 1.6 |
| 2 | $(NH_4)$ $SO_4$-fractionation 50% saturation | 18 080[a] | 48 250 | 81 | 2.7 |
| 3 | Sephadex G-150 | 4 040[a] | 38 150 | 80 | 9.4 |
| 4 | DEAE-Sephadex | 1 320[a] | 28 850 | 76 | 22 |
| 5 | QAE-Sephadex | 500[a] | 26 320 | 91 | 52 |
| 6 | Preparative polyacrylamide gel electrophoresis | 29[b] | 11 800 | 45 | 304 |

Protein concentration was measured:
a) Spectrophotometrically by the method of Warburg and Christian [24] and according to Lowry et al. [25];
b) By amino acid analysis.

used was 0.03 M Tris–barbiturate, pH 7.5. Ammonium persulphate was removed from the gel by electrophoresis using 'Poly Prep' of Buchler Instruments, New Jersey, USA. In general the procedure was that of the 'instructions' as given by the manufacturer [11]. The upper buffer used was 0.03 M Tris–barbiturate, pH 7.5. Ammonium persulphate was removed from the gel by electrophoresis (prerun: 12 hr); elution was done with 0.05 M Tris–HCl pH 7.5 (1 mM $MgCl_2$).

The purity of the enzyme was monitored with polyacrylamide disc electrophoresis according to the method of Davis [12] and Ornstein [13]. For gels at pH 7.5 in Tris–HCl buffer and at pH 5.5 in acetate–phosphate buffer the buffer system of Tris–barbiturate as described by Williams and Reisfield [14] was used. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) was performed according to the method of Weber and Osborn [15]. The enzyme was treated with 0.1% SDS. For denaturation by urea the enzyme was dissolved in a solution of 0.25 M sodium formate buffer, pH 4.3 (5 mM β-mercaptoethanol, 2.5 mM EDTA and 8 M urea). Electrophoresis on Cellogel strips of the urea-treated enzyme was done at pH 4.3 (0.25 M sodium acetate, 0.25 M formic acid, 6 M urea and 5 mM β-mercaptoethanol) and at pH 8.7

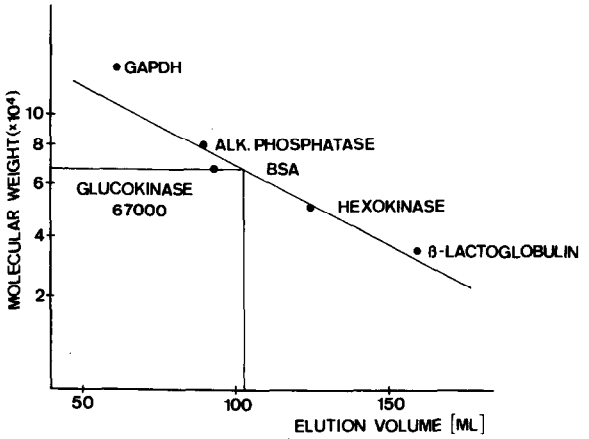


Fig. 1. Determination of the molecular weight of the native enzyme by gel filtration (Sephadex G-150).

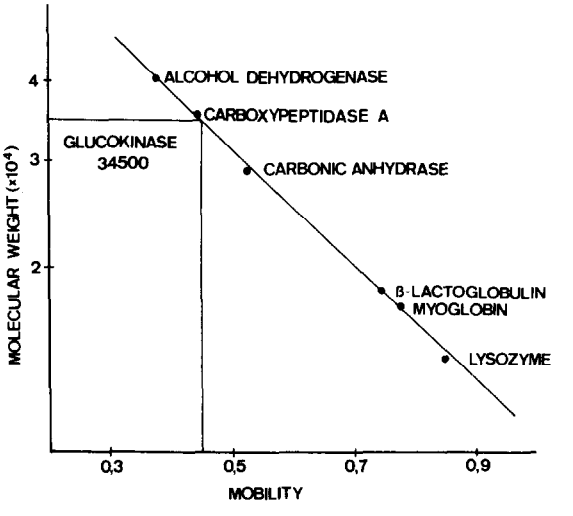


Fig. 2. Determination of the molecular weight of the subunits of glucokinase by gel electrophoresis in SDS.

Table 2
Amino acid composition of glucokinase.

| Residues | Amino acid residues per subunit[a] | Nearest integer | Residues per molecule (nearest integer) |
|---|---|---|---|
| Lysine | 7.09 | 7 | 14 |
| Histidine | 14.27 | 14 | 28 |
| Arginine | 14.76 | 15 | 30 |
| Aspartic acid | 24.36 | 24 | 48 |
| Threonine[b] | 21.90 | 22 | 44 |
| Serine[b] | 11.16 | 11 | 22 |
| Glutamic acid | 30.33 | 30 | 60 |
| Proline | 10.85 | 11 | 22 |
| Glycine | 45.33 | 45 | 90 |
| Alanine | 44.11 | 44 | 88 |
| Valine[c] | 30.03 | 30 | 60 |
| Methionine | 6.93 | 7 | 14 |
| Isoleucine[c] | 20.11 | 20 | 40 |
| Leucine | 30.13 | 30 | 60 |
| Tyrosine | 6.21 | 6 | 12 |
| Phenylalanine | 6.94 | 7 | 14 |
| Half-cystine | 4.10 | 4 | 8 |
| Tryptophan[d] | 3.24 | 3 | 6 |
| Total | | 330 | 660 |

a) Calculations were based on a molecular weight of 34 500.
b) Values for threonine and serine were extrapolated to zero time.
c) Values for valine and isoleucine were corrected to 120 hr hydrolysis.
d) Spectrophotometrically according to Beaven and Holiday [22].

Table 3
$K_M$ and $V_{max}$ of nucleotide triphosphates used as substrates by glucokinase.

| Nucleotide | $K_M$ [mM] | % of $V_{max}$ obtained with ATP |
|---|---|---|
| ATP | 0.06 | 100 |
| ITP | 0.60 | 75 |
| GTP | 1.2 | 30 |
| UTP | 4.5 | 32 |
| CTP | 3.6 | 10 |

(0.25 M ammonium carbonate buffer, 6 M urea and 5 mM β-mercaptoethanol). The Cellogel strips were stained as described by Heil and Zillig [16].

The molecular weight of the native enzyme was determined by gel filtration (Sephadex G-150 2.5 × 90 cm column, 0.05 M Tris–HCl buffer at pH 7.5, 1 mM $MgCl_2$, 0.1 M KCl) according to the method of Andrews [17]. The marker proteins were: β-lactoglobulin; hexokinase; bovine serum albumin; alkaline phosphatase and glyceraldehyde 3-phosphate dehydrogenase. The $NH_2$-terminal amino acid residue of the kinase was determined by the dansyl chloride procedure for proteins [18]. The dansyl amino acids were identified by thin-layer chromatography on polyamide sheet, as described by Woods and Wang [19].

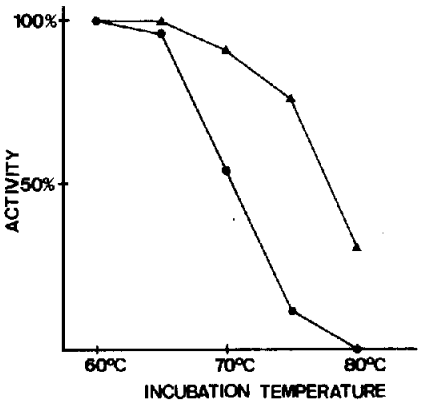


Fig. 3. Heat inactivation of glucokinase from *Bacillus stearothermophilus* (incubation of the enzyme solution (18 Units/ml) in 0.05 M *N*, *N'*-bis-(2-hydroxyethyl)-glycine buffer, pH 9.1, containing: 1 mM $MgCl_2$, 0.01 M glucose with (▲) and without (●) 0.2 M NaCl at 60°, 65°, 70°, 75° and 80°C for 30 min).

Amino acid analyses of acid hydrolysates (5.7 N HCl, 110°C, 24, 48 and 72 hr) were performed with a Beckman amino acid analyzer 121 C [20]. Half cysteine was determined as cysteic acid after performic acid oxidation as described by Hirs [21]. Tryptophan content was estimated spectrophotometrically by the method of Beaven and Holiday [22].

The thermostability of the kinase was measured by preincubation of the enzyme solution (18 units per ml) at different temperatures and determination of the residual activity (conditions as in fig. 3). As a suspended precipitate in a 60% saturated solution of ammonium sulphate the enzyme can be stored at a temperature of 4°C for at least 3 months.

The activity of kinase was assayed in 2 ways (incubation temperature 37°C):

*Method 1* [2]: The formation of glucose 6-phosphate was followed indirectly by measuring enzymatically the amount of NADPH formed (absorbance

at 340 nm, $E^{340}_{1\,cm} = 6.2 \times 10^3$) using excess $NADP^+$ and glucose 6-phosphate dehydrogenase. The reaction mixture was as follows: 0.01 M glucose; 2 mM ATP; 3 mM $MgCl_2$; 1 mM $NADP^+$ and 5 $\mu$g per ml glucose 6-phosphate dehydrogenase in 0.05 M Tris–HCl buffer pH 9.0. Tris–HCl buffer can be replaced by 0.05 M $NaHCO_3$ or by *N*,*N'*-bis-(2-hydroxyethyl)-glycine.

*Method 2*: For determination of substrate specifity the formation of ADP was measured by incubating the reaction mixture (glucose, ATP, $MgCl_2$, conditions see *Method 1*) with excess of both pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH (NADH absorbance) as described by Parry and Walker [23]. One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mole of glucose 6-phosphate per min at 37°C.

## 3. Results and Discussion

The glucokinase was isolated following the purification steps as indicated in table 1. Five-hundred grams of bacteria were extracted.

Purified glucokinase (after preparative gel electrophoresis) showed a single band on polyacrylamide disc electrophoresis in the absence and in presence of sodium dodecyl sulfate. The enzyme also showed no evidence of heterogeneity upon Cellogel electrophoresis in urea (single band). The molecular weight of the kinase estimated by gel filtration on Sephadex G-150 is 67 000 (fig. 1), whereas as measured by gel electrophoresis in sodium dodecyl sulphate is 34 500 (fig. 2).

Only on $NH_2$-terminal amino acid residue was found in the glucokinase of *B. stearothermophilus*. The amino acid was identified as phenylalanine by end group analysis using dansyl chloride. The amino acid composition was estimated from 5.7 N HCl hydrolysates at 110°C for 24, 48, 72 hr (4 analyses each, table 2).

The glucokinase followed simple Michaelis–Menton kinetics with respect to glucose and ATP. With regard to substrate specifity it was shown (using *Method 2*) that at concentrations of monosacharides of 0.01 M and of ATP of 2 mM the kinase phosphorylates only glucose and *N*-acetyl-glucosamine; no phosphorylation of D-mannose, D-galactose, D-fructose, 2-deoxy-D-glucose, glucosamine-HCl or D-xylose was observed, hence the enzyme was designated as glucokinase. $K_M$ for glucose in the presence of 2 mM ATP is 0.52 mM. With *Method 1* the nucleotide specificity was examined at glucose concentration of 0.01 M. The values of $K_M$ and $V_{max}$ obtained from a Lineweaver–Burke plot are given in table 3.

ATP and ITP were good substrates. These results show that the enzyme is very specific toward the monosacharide substrate, but less specific for the nucleotide triphosphate. This is in contrast to the known hexokinases. The kinase showed maximum activity at pH 9.0 at 37°C and is stable up to 65°C. The thermostability, which is already quite high in the presence of NaCl, is further increased by the addition of glucose (fig. 3).

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