# Cloning and Identification of a Novel Sugar Kinase from *Escherichia coli* K 12

by

***Mayada Hamed***

**2001**

# Cloning and Identification of a Novel Sugar Kinase from *Escherichia coli* K12

By

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Submitted in partial fulfillment of the requirements

for the degree of Master of Science

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September 2001

# LEBANESE AMERICAN UNIVERSITY

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## Dedication

I would like to dedicate my work to:

God who gave me the will

Mom and Dad who were always there for me

&

The soul of my brother, Khaled

## Acknowledgments

I would like to acknowledge my advisor Dr. Jamilah Borjac for all the attention she gave me and for all the time she devoted to me. I would like to acknowledge also my co-advisor Dr. Maha Khashab for encouraging me and for her valuable notifications. I do not know how to thank Mrs. Swassan Jabi, but I would like to say that I do highly appreciate your presence, your care and your attention whenever I needed you were a great supporter. Dr. Ahmad Kabbani, the spiritual father and brother for all members in the biology department, you gave me a great support thanks a lot, I am grateful for all the help and facilities you offered us. Dr. K. Bougharian, I was lucky for being one of your students, I learned lots from you during my BS as well as my MS studies I wish you could be always there for us. Dr. T. Nawaas I did enjoy your classes thanks for every thing. I am grateful also to Dr. J. Ousta, Dr. Sandra Rizk and Jihan Basbous for their technical assistance. I would like to thank all staff of the natural science division for all the help. Last but not least I would like to say to my

father Mohamed Hamed and my mother Amal Amash, you gave me a lot and without your I would have done nothing.

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# ABBREVIATIONS

| | |
|---|---|
| APS: | Ammonium persulfate |
| DAG : | Diacyl glycerol |
| DEAE: | Diethyl amino ethyl |
| DHAP: | Dihydroxyaceton phosphate |
| dNTP: | Deoxynuleotide triphosphate |
| DMSO: | Dimethyl sulfoxide |
| DTT: | Dithiothreitol |
| EDTA: | Ethylenediamine tetraacetic acid |
| gp: | Gene product |
| IPTG : | Isopropyl β-D-1- thiogalactoside |
| MOPS: | 3-N-morpholinopropane sulfonic acid |
| PAGE: | Polyacrylamide gel electrophoresis |
| PCR: | Polymerase chain reaction |
| PMSF: | Phenylmethanesulfonyl fluoride |
| SDS: | Sodium dodecyl sulfate |
| TEMED: | N, N, N', N'- tetramethylethylenediamine |
| X-gal: | 5-bromo-4-chloro-3-indolyl galactoside |

## ABSTRACT

The work done in this study aimed at cloning gene b1772 found at locus AAC74842 in the *E.coli* K12 genome followed by the identification of its product. Alignment with protein database had revealed that this protein is highly identical to sugar kinases from different species.

The gene product was purified and tested for its activity. Since the gene product is suspected to be a sugar kinase several sugars were tested for phosphorylation with this enzyme. The results had shown that this sugar kinase is more likely to be a hexokinase since the tests were mainly positive with glucose, fructose and galactose.

# I. INTRODUCTION

Phosphoryl transfer is one of the basic reactions in biochemistry that includes mainly phosphorylation reactions that regulate most of the steps of metabolic pathways[1]. Enzymes that catalyze this transfer of phosphoryl group from ATP to an acceptor are known as k*inases*. They are a subclass of transferases[2] that requires the presence of magnesium ions since the actual substrate of the enzyme is $MgATP^{2-}$ complex and not $ATP^{4-}$. Kinases are divided into 2 main classes: protein kinases and sugar kinases. Follows is a discussion of these two types and some of their characteristics.

## A. Protein kinases

Protein kinases are enzymes that are involved in many regulatory processes. They covalently modulate other substrate protein by phosphorylating them at a serine, threonine, or tyrosine residues. These enzymes play important roles in mitogenesis, cell cycle events and in many types of oncogenesis[2]. Some kinases require activation by phosphorylation while others do not. The activation segment of protein kinases and the control of its

conformation via phosphorylation are governed by the sequence specificity of the kinase and the sequence in the activation segment that surrounds the phosphorylated residue.

The acceptors in protein phosphorylation reactions are situated inside cells where ATP is plentiful. In phosphorylation reactions the terminal (gamma) phosphoryl group of ATP is transfered to the corresponding amino acid residue[4]. Protein phosphatases reverse the effects of kinases by catalyzing the hydrolysis of protein phosphoryl groups. Phosphorylation alters the activity of the target protein since it adds two negative charges to the modified protein that may lead to structural changes. These changes alter the protein binding and catalytic activity. The rate of phosphorylation can be either fast or slow depending on physiological need. It may occur in less then a second or may take hours[5]. The phosphoryation reaction may lead to a cascade of reactions where many target proteins are also phosphorylated. In general, phosphorylation changes a protein from the inactive to the active form and in some cases from active into inactive form. Thus it is a mean of up-regulating or down-regulating process that is controlled by specific proteins.

## Types of Protein Kinases:

Protein kinases may be classified according to a variety of schemes based on their functional properties. In most general sense, these enzymes may be categorized based on the specific amino acid residue that serves as the phospho-acceptor. These include the serine/threonine protein kinase, the protein tyrosine kinase, and the dual specific protein kinases that transfer the phosphate group onto serine/threonine and tyrosine residues. Protein kinases can be also classified according to their dependence on second messenger (**table 1**). The three main groupings of this classification are: 1) cyclic-AMP dependent protein kinase, 2) protein kinase C and 3) calcium/calmodulin dependent protein kinase. These enzymes differ in their sizes, subcellular localization, structure of their subunits, and mechanism of activation. Almost all of the protein kinases are pleiotropic enzymes that phosphorylate different types of proteins. Therefore, each is a substrate-specific enzyme that recognizes definite consensus seguence[6] (**Table 2**). The recognition code is made of either basic or acidic amino acid residues located at the target site. Accordingly, the three types of protein kinases may be subdivided roughly into five categories

**Table 1**: Classification of protein kinases based on second messenger requirement.

| Messenger-dependent Protein Kinases | |
|---|---|
| Enzyme | Second Messenger |
| cAMP-dependcnt (PKA) | cAMP |
| cGMP-dependent (PKG) | cGMP |
| $Ca^{++}$ and phospholipid denpendent | $Ca^{++}$, DAC, and phospholipid |
| Calmodulin dependent PK | $Ca^{++}$, calmodulin |
| DNA-dependent PK | dsDNA |
| Ds RNA-dependent PK | dsRNA |
| Phosphorylase kinase | $Ca^{++}$, Calmodulin |
| Myosin light chain PK | $Ca^{++}$, Calmodulin |
| Cyclin dependent PK | Cyclin A,B,D,E |

| Messenger-Independent protein kinases | |
|---|---|
| Enzyme Class | Examples |
| Soluble Ser/Thr PK | Casein Kinase |
| Receptor Ser/Thr PK | Transforming growth Factor β |
| Receptor protein Tyrosine insulin-kinases | Insulin and like growth factor receptor |

Adapted from reference 46

**Table 2:** Consensus sequences for protein kinases

| Protein Kinase | Consensus Sequence |
|---|---|
| Protein kinase A | -X-R-(R/K)-(S/T)-X- |
| Protein kinase G | -X-$(R/K)_{2-3}$ -X-(S/T)-X- |
| Protein kinase C | -X-$(R/K_{1-3}, X_{0-2})$-(S/T)-$(X_{0-2}, R/K_{1-3})$- |
| X- | |
| $Ca^{++}$/calmodulin kinase II | -X-R-X-X-(S/T)-X- |
| Phosphorylase b kinase | -K-R-K-Q-I-(S/T)-V-R- |
| Insulin receptor kinase | -T-R-D-I-Y-E-T-D-Y-Y-R-K- |
| EGF receptor kinase | -T-A-E-N-A-E-A-YL-R-V-A-P- |

Taken from reference 4

depending on their specificity to either serine/threonine or tyrosine at one side and on their preference to basic or acidic residues on the other side: 1) baso- philic - ser/thr specific protein kinases that recognize sites with basic surrounding; 2)acidophilic ser/thr specific protein kinase that recognize acidic residues[7];3)Ser/thr specific protein kinases that do net dependent on either acidic or basic residues[1]; 4) Tyrosine specific acidophilic protein kinases with acidic residues upstream the target tyrosine; 5) Tyrosine specific protein kinases with no preferences to neither acidic nor basic sites[8] **(Table 3).**

## 1- Cyclic -AMP Dependent Protein Kinase Known also as Protein Kinase A (PKA)

Cyclic 3'-5' adenosine monophosphate (cAMP) dependent protein kinase is an important enzyme in signal transduction in vertebrates and one of the first protein kinases to be discovered[9]. It plays an important role in the regulation of glycogen synthesis and breakdown, fatty acid synthesis, the oxidation of pyruvate to acteyl-COA, mobilization of triacylglycerols, and the reciprocal regulation of glycolysis and gluconeogenesis[10]. As the name

**Table3** Grouping of protein kinases according to their local structural determinants.

| Residues Affected | Specificity Determinants | Protein Kinases |
|---|---|---|
| Ser/Thr | Basic residues | PK-A |
| | | PK-G |
| | | PK-C |
| | | Ca/CaM-PKS |
| | | AMP-PK |
| | | PAKs |
| | | DsI |
| | | HRC |
| Ser/Thr | Acidie residues | CK~l |
| | | CK-2 GEF-CK |
| Ser/Thr | Neither acidic nor basic | Cdc2 |
| Tyr | Acidic residues | Most TPKs |
| Tyr | Neither basic nor acidic | Src-TPKs |

Taken form reference 1.

implies, the activity of the enzyme is dependent on the presence of cAMP[4]. Protein kinase A is classified as a member in the AGC group of protein kinases, because it phosohorylates at Ser and Thr residues, rather than Tyr. The enzyme occurs naturally as a 4-membered quaternary structure (R2C2), with two regulatory (R) and two catalytic (C) subunits[11] **(Fig.1)**. In the holoenzyme form, the regulatory subunits are bound to the active site of the catalytic subunits, inactivating them[5]. When cAMP is present, it binds to the regulatory subunit, causing a conformational change that releases and activates the two C subunits[13]. The holoenzyme form of protein kinase A, containing two regulatory subunits bound to two catalytic subunits, has not yet been crystallized. However, the structure of the catalytic subunit bound to a synthetic inhibitor has been resolved. The structure of this inhibitor gave insight into the natural regulatory domain of PKC and the substrate, because both occupy the active site of the catalytic subunit.

There are two known inhibitors of the C-subunits: The regulatory (R) subunits and the heat-stable protein kinase inhibitor (PKI)[14]. The mechanism of inhibition is almost similar among all catalytic subunits. Each of the inhibitors contains a region that occupies the peptide binding site preventing another substrate from binding.

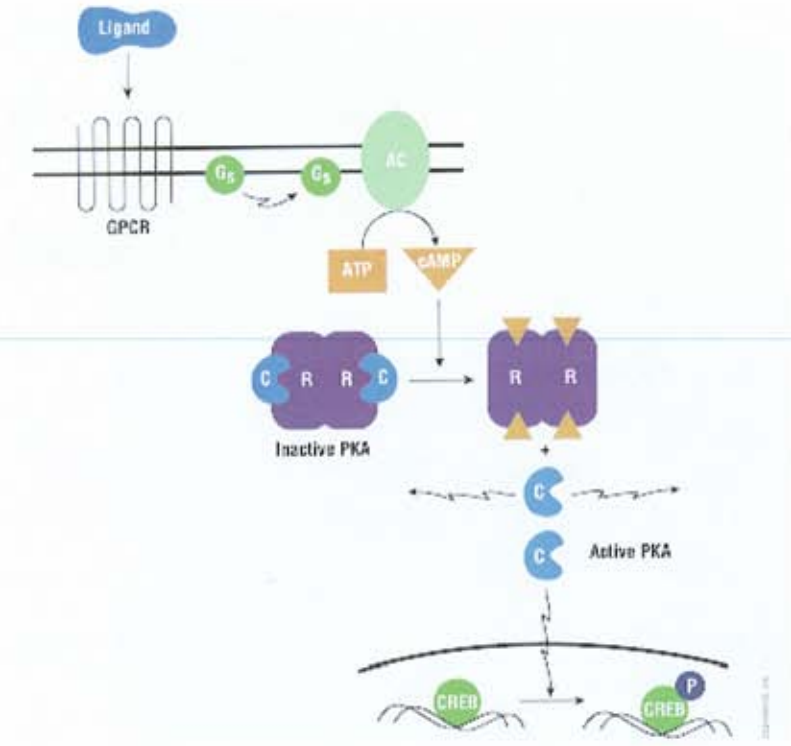


**Fig.1** cAMP-dependent protein kinase (PKA). Active catalytic subunits are released following cAMP binding to the regulatory subunits. The active catalytic subunits phosphorylate targets in both the cytoplasm and in the nucleus. cAMP Response Element Binding Protein (CREB) is one target protein phosphorylated by PKA.

Adapted from reference 25.

However, the inhibition by the R-subunit is reversible by the binding of cAMP, where the inhibition by PKI is irreversible[4].

Once separate from the R subunit, the C subunit can perform its function, which is the phosphorylation of a Ser or Thr residue on the substrate. The phosphorylation causes a change in activity of the substrate, and the substrate can go on to continue the message by performing a function on yet another substrate[13]. The kinase domain of the catalytic subunit is particularly well conserved among members of the protein kinase superfamily and serves as a framework for the entire family. Because of its relative simplicity, cAMP dependent Protein Kinase is used as the basic structure for comparison and nomenclature for the catalytic parts of the protein kinase superfamily. This kinase domain is divided into 12 subdomains that are very similar among the various other related protein kinases. There may be some insertions or deletions, but all of the members of the family are along the same basic lines.

## 2- Protein Kinase C (PKC):

The function of PKC was initially defined due to its ability to phophorylate histone h1 which is lysine rich depending on calcium ions and phospholipid[1].

Members of the protein kinase C family are single polypeptides that comprise 4 conserved domains. The catalytic core of this enzyme is similar to that of the protein kinase A[16]. Similarly, this kinase requires MgATP complex as a substrate. PKC catalyzes the phosphorylation of serine or threonine basic sequences residues in some proteins. It is able to undergo autophosphorylation by an intramolecular mechanism at the amino and carboxy termini[5]. In addition to phosphorylation PKC has both an ATPase and a phosphatase activity. Protein kinase C requires the presence of calcium and a phospholipid for activation as well as diacylglycerol (DAG) as an endogenous second messenger[7] (**fig.2**). The proteins that are phosphorylated by PKC are usually found on biological membranes, particularly the plasma membrane and nuclear envelope[13].

Besides the catalytic domain, all isoforms of PKC contain regulatory domains, where diacylglycerol, calcium ions, and phospholipid bind[3]. The catalytic domain contains

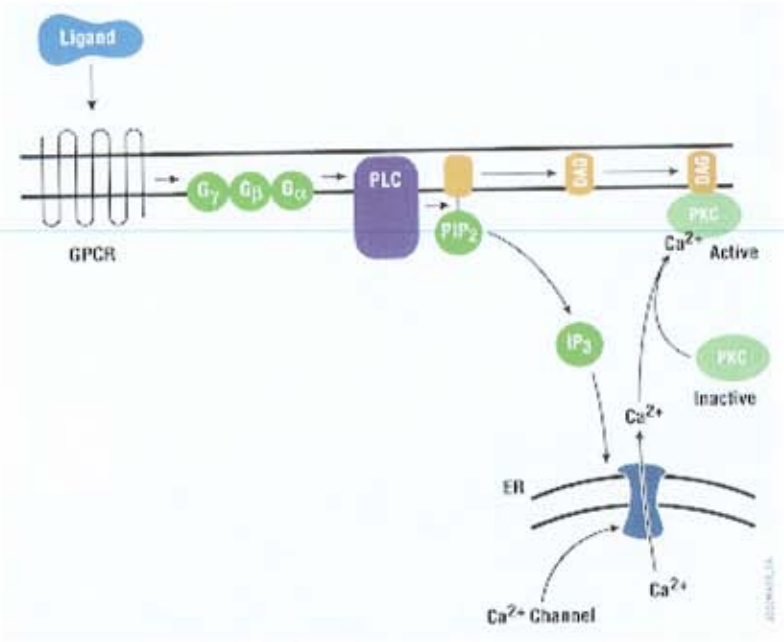


**Fig.2** Activation of a conventional protein kinase C (PKC).Activated G-protein leads to the activation of phospholipase C (PLC) that cleaves phosphoinositol-4,5-bisphosphate ($PIP_2$)into 1,2 diacylglycerol and inositol-1,4,5-triphosphate ($IP_3$). The $IP_3$ leads to the increase in calcium level in the cytoplasm. This increase in the amount of calcium activates PKC, Which translocates to the membrane, anchoring to DAG and phosphatidylserine.

Adapted from reference 46.

an ATP-binding site and a site where the protein substrate binds. Interesting region in the regulatory domain of these kinases is a sequence that resembles the phosphorylation site on the protein substrate, termed as pseudosubstrate sequence. In the resting form, this pseudosubstrate region binds to the enzyme and blocks the function of the catalytic domain upon activation by binding to diacylglycerol, calcium ions and phospholipids to the regulatory domain, a conformational change takes place and liberates the catalytic domain from inhibition by the pseudosubstrate[3]. The pseudosubstrate becomes highly sensitive to proteolysis by trypsin or endoproteinase upon activation of the enzyme. Phosphorylation renders the enzyme active through the alignment of the residues in a way to localize protein kinase to the cytosol[18].

DAG and phorbol esters anchor PKC to the membranes and increase their affinity to the substrate. Membrane anchorage occurs by capping the hydrophilic ligand groove, the binding of phorbol ester alters the surface hydrophobicity of the catalytic domain[19]. DAG doubles the catalytic efficiency of the enzyme that is bound to phophatidylserine and stimulates the activation promoted by fatty acids and short chained phosphatidylcholines.

Calcium ions increase the affinity of conventional protein kinase C for negatively charged lipids with no selectivity for the head groups other than the requirements for negative charge[20].

PKC tends to phosphorylate a number of arginine rich proteins that are able to displace the pseudosubstrate from the core of the enzyme. It is possible that these arginine rich peptides tend to neutralize the acidic patch that maintains the pseudosubstrate in the active site[21].

Therefore, PKC is regulated by two different mechanisms: 1) phosphorylation regulating the active site and localization of the enzyme, and 2) by second messengers resulting in pseudosubstrate exposure.

**3-Calcium/calmodulin Dependent Protein Kinases (CPK)**

CPK are serine/threonine protein kinases with calmodulin like domains in their C-termini. CPK potentially play important roles in mediating calcium signaling in a variety of cellular processes. The common denominator of calcium' s action is its ability to bind to various cytosolic calcium-

binding proteins. One of the most important of these proteins is calmodulin. Once it binds to calcium, calmodulin undergoes conformational changes that allow it to bind to several types of enzymes either inhibiting them or activating them[21]. One mole of calmodulin is able to bind four moles of calcium ions[22].

The enzyme has a broad substrate specificity, suggesting that it may play a role in many cellular functions [19]. An external stimulus increases intracellular calcium levels and increases the amount of calcium bound calmodulin. Calcium/calmodulin binds to the inhibitory domain of the α subunit, activating CPK by dissociating the catalytic subunit from the auto-inhibitory domain. Active CPK auto-phophorylates at threonine 286. This auto- phosphorylation increases the affinity of CPK for calcium/calmodulin making the enzyme resistant to fluctuation in calcium ion levels[20] (**fig.3**).

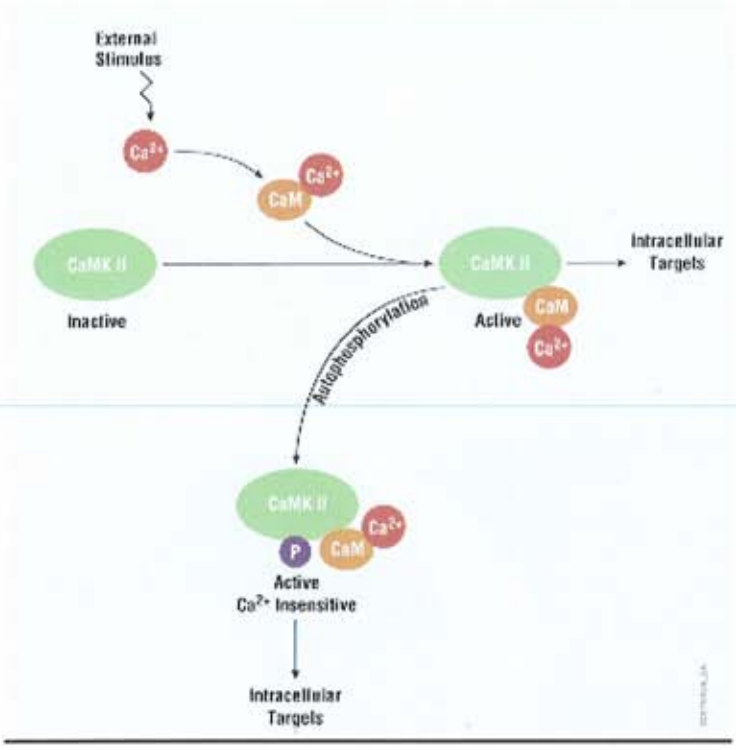


**Fig.3** Activation of calcium/calmodulin dependent protein kinase.

Taken from reference 46.

## B. Sugar kinases

Sugar phosphorylation is an important step in sugar metabolism since all interconversions between sugars, with exception of the synthesis of fructose from glucose by the male in the seminal fluid, proceed via phosphorylated derivatives[23]. These derivatives are either simple phosphates, such as the formation of ribose-5-Phosphate from glucose 6P in the pentose phosphate pathway or nucleoside diphosphate sugars which are high energy carbohydrates analogues of nucleoside triphosphate e.g. uridine diphosphate glucose.

Kinases that phosphorylate sugars are called sugar kinases. They are divided into three distinct families. The first is the **hexokinase** family; it contains several prokaryotic and eukaryotic sugar kinases with diverse specificities. The second is the **ribokinase** family of unknown three-dimensional structure. It comprises prokaryotic and eukaryotic ribokinases, bacterial fructokinase, and phosphofructokinase. The third family includes several bacterial and yeast galactokinases. Each family catalyzes reactions on similar or identical substrates[24].

## 1- Hexokinases

Hexokinases catalyze the transfer of a phosphoryl group from ATP to a variety of hexoses. This takes place by the condensation of phosphoric acid with one of the hydroxyl group of a sugar forming a phosphate ester[26]. Sugar phosphates are relatively stable at neutral pH, and bear a negative charge[24,26]. One effect of the sugar phosphorylation within the cell is to prevent the diffusion of the sugar out of the cell. In general, highly charged molecules do not cross biological membranes without specific transport system[4]. phosphorylation acts as an activator for subsequent steps in sugar metabolism[4]. Like all other kinases, hexokinases require the presence of magnesium ions for their activities.

X-ray crystallographic studies of yeast hexokinase had revealed that the binding of a hexose to hexokinase induces a conformational change that makes the environment around the enzyme more nonpolar encouraging the transfer of phosphoryl from ATP[27]. This conformational change occurs only when glucose and ATP are bound together. Hexokinase consists of two lobes, which come together when glucose is bound. Besides, this structural change makes hexokinase

active only when it binds a hexose and prevents ATPase activity. The two lobes of the enzyme that form its active site cleft swing together by up to 8 Å so as to engulf the glucose like closing jaws[4] (**fig.4**). The cleft between the lobes closes and the bound glucose becomes surrounded by protein except for its hydroxymethyl group[4]. This *substrate -induced cleft closing* is a general feature of kinases. Hexokinases form a ternary complex with glucose and Mg-ATP complex before the reaction occurs. In this way, the phosphorus atom becomes accessible for the nucleophilic attack of the $C_6$-OH group of glucose. This movement places the ATP in close proximity to the $-C_6H_2OH$ group of glucose and excludes water from the active site. If hexokinase were rigid, a water molecule occupying the binding site for the $-CH_2OH$ of glucose would attack the α-phosphate of ATP[28].

Hexokinase is capable of binding 2 ligands glucose and glucose-6-phosphate. Glucose binds so that glycolysis can occur and glucose-6 phosphate binds as an allosteric inhibitor. This mechanism controls the influx of substrate into glycolytic pathway[26].

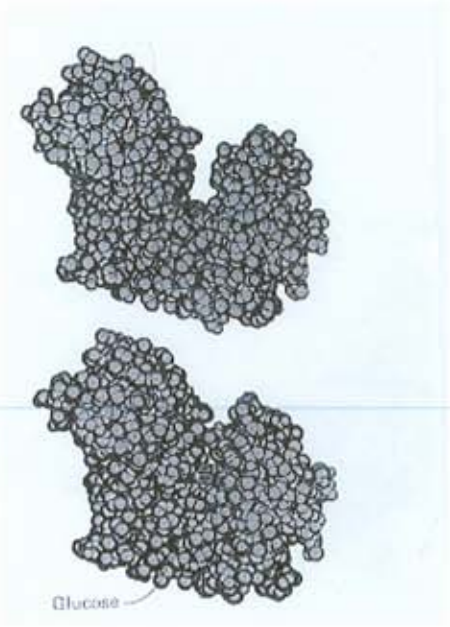


**Fig.4** Hexokinase conformational change . The two lobes of the enzyme come together and surround the substrate.

Taken from reference 25.

## 2- Ribokinase family

Ribokinase catalyzes the first step in ribose metabolism, i.e.the phosphorylation of ribose to 5-ribose-phosphate. Thus, ribokinase is an obligatory enzyme for the utilization of ribose. Ribose-5-phosophate is the key metabolic intermediate in biosynthetic pathway that interconverts 5- and 7-carbon sugars for anabolic and catabolic purposes[29].

As mentioned earlier, one member of ribokinase family is fructokinase. This enzyme, which is lacking in the muscle, is found in the liver. It catalyzes the phosphorylation of fructose to yield fructose-1-phosphate (F1P). Fructose that is found within the muscles is usually phosphorylated by hexokinase yielding fructose-6-phosphate (F6P). Fructokinase is a non-specific enzyme since it utilizes L-sarbose, D-xylose, L-galactose, and 5-Keto-D- fructose. Thus, it is considered as Keto -hexokinase[31].

Three inherited abnormalities in fructose metabolism have been identified. The first abnormality is *Essential Fructosuria* or *Hepatic fructokinase deficiency*[34]. It is a benign metabolic disorder caused

by the lack of fructokinase. This disease, which depends on the time and amount of sucrose intake, is asymptomatic and harmless and may go without diagnosis. The second disorder is *Hereditary fructose* intolerance or *Fructosemia*[32]. It is potentially a lethal disorder resulting from the lack of aldolase B[33], an enzyme needed for the generation of DHAP and glyceraldehyde from F1P. Deficiency in aldolase B results in the accumulation of F1P in the cytoplasm leading to the sequestration of inorganic phosphate resulting in the activation of AMP deaminase that catalyzes the irreversible deamination of AMP to IMP (inosine monophosphate), a precursor of uric acid[34]. Depletion of tissue ATP occurs through massive degradation of uric acid and impairment of regeneration by the oxidative phosphorylation in the mitochondria because of inorganic phosphate depletion[34]. Fructosemia is characterized by severe hypoglycemia and vomiting following fructose intake. Prolonged fructose intake leads to vomiting, poor feeding, jaundice, hepatomegaly, hemorrhage and eventually hepatic failure and death[32].

The third abnormality is Hereditary fructose *-1,6 bisphosphatase deficiency*. It results in severely

impaired hepatic gluconeogenesis and leads to episodes of hypoglycemia, ketosis and lactic acidosis. These episodes are triggered by fasting and febrile infections[35]. This disorder is classified as an error in fructose metabolism. Fructose-1, 6-bisphosphatase, also known as hexose bisphoshatase, is the key enzyme of gluconeogenesis. It catalyzes the splitting of fructose -1,6-bisphosphate to fructose-6-phosphate and Pi. This reaction is irreverssible; the oppoasite conversion can be accomplished by the equally irrevessible phosphofructokinase reaction, which forms fructose-1, 6-bisphosphate from fructose-6-phosphate, using ATP as a phosphoryl donor.

Inactivation of this enzyme prevents the endogenous formation of glucose from the precursors lactate, glycerol, and gluconeogenic amino acids such as alanine[31]. That is why episodes are manifested during fasting since normoglycemia depends on the amount of available liver glycogen.

### 3- Galactokinase Family

Galactokinase is a crucial enzyme needed in the pathway of entry of galactose into glycolysis[32]. Galactokinase phosphorylate galactose to yield

galactose-*1*- phosphate that can be converted into glucose-1-P through action of galactose-1-phosphate uridyltransferase[36]. UDP-galactose generated is converted into UDP-glucose by UDP-galactose-4 epimerase. The UDP portion is exchanged for phosphate generating glucose-1-phosphate that is then converted to G6P by phosphoglucose mutase.

Three inherited disorders of galactose metabolism have delineated. **Classic galactosemia[37] is a** major symptom of two enzyme defects. The first is due to a deficiency in galactose-1-phosphate uridyl transferase. The second form of galactosemia results from a loss of galactokinase[40]. Individuals with deficiency in these enzymes are considered as lactose intolerant. Toxicity in galactokinase deficiency is mainly characterized by cataracts[40].

The third disorder of galactose metabolism results from a deficiency of UDP-galactose-4-epimerase known as epimerase deficient galactosemia. This enzyme catalyzes the reaction of unique pathway in which galactose is converted to glucose[41]. This enzyme is responsible for the inversion of the hydroxyl group ($OH^-$) at the fourth carbon of the hexose chain to form glucose from

from galactose. This enzyme is bifunctional, it catalyzes the interconversion of UDP-glucose with UDP-galactose and UDP-acetylglucosamine with UDP-N-acetylgalactesamine[42]. This enzyme is important for the conversion of UDP-glucose to UDP-galactose in the situation where only glucose is available to the organism and galactose is required as a constituent of complex polysaccharides[44]. Two different forms of this deficiency have been found, one is benign affecting only red and white blood cells while the other affects multiple tissues and manifests symptoms similar to the transferase deficiency[45].

In each of the three forms of galactosemia, diminished enzyme activity produces an accumulation of the substrates proximal to the metabolic block: galactose in galactokinase deficiency; galactose and galactose-1 phosphate in transferase deficiency; and galactose, galactose-1-phosphate and UDP-galactose in epimerase deficiency.

In all of these disorders, there is an alternative metabolic route of galactose metabolism through reduction to galacticol and oxidation to galactonate[39]. Transferase deficiency is associated with the accumulation of galactose-1-phosphate in the tissues in the addition to galacticol and

galactonate accumulation. In epimerase deficiency cellular uridine disphosphate galactose is also elevated[38,39].

In this study, the product of a gene that is expected to code for a putative kinase is identified. This gene belongs to *E.coli K12* strain whose complete genome was sequenced in 1997 by Blattner, F.R., Plunkett, G.III, Bloch, C.A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick ,H.A., Goeden, M.A., Rose, D.G., Mau, B., and Shao, Y. at the university of Wisconsin. The gene was amplified, cloned, and the product was purified and its activity was identified. Gene selection was done taken into consideration the following points: 1) the size of the gene for amplification by PCR, 2) the size of the gene product for purification, 3) the high probability of obtaining a soluble protein, and 4) finding sample assay to determine enzymatic activity of this protein. Putative kinase gene (b1772) found in locus AAC74842 was chosen to be cloned hoping it fits these criteria.

galactomnate accumulation. In epimerase deficiency cellular uridine diphosphate galactose is elevated as well[38,39].

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# II. MATERIALS and METHODS

## A. Media

The media used was Luria Bertani (LB) containing 10g of bactotryptone, 10g NaCl, and 5g of bacto-yeast extract in a final volume equal to 1 liter and adjusted to pH 7.5 using NaOH. The media was sterilized before usage.

## B. Bacterial Strain

The bacteria used for both gene selection and cloning is E. coli DH5α with the following genomic specification: Ø80d lac z ΔM15, rec A1, end A1, gyrA96, thi-1, hsdR17 ($r^-_k$, $m^+_k$), supE44, relA1, deoR, Δ (lac ZyA$^-$ ZA-argF) U169.

## C. DNA Extraction

A DNA extraction Kit (Amersham Pharmacia Biotech) was used to extract DNA from bacterial cells. DH5α cells were grown with aeration in LB medium for 12 hrs. An aliquot of 1 ml was pelleted at 12000 r.p.m. for 5s at $4^0$C in a micro centrifuge (Mikro 22R). The cell pellet was resuspended in 600µl of cell lysis

solution containing tris [hydroxymethyl] amino methane, ethylene diamine tetra acetic acid [EDTA] and Sodium dodecyl sulfate. The suspension was incubated at 80$^{0}$C for 30 min to lyse the cells and cooled to room temperature. Three µl of RNase A solution were added to the cell lysate, then the suspension was mixed by inversion many times before incubation at 37$^{0}$C for another 30 min. After cooling to room temperature, 200µl of protein precipitation solution containing ammonium acetate were added to the cell lysate with vortexing at high speed for 20 s followed by centrifugation at 12000 rpm for 3 min at 4$^{0}$C, the proteins precipitate and form a white pellet. The DNA in the supernatant was pipetted carefully and removed into a 1.5 ml tube containing 600µl 100% isopropanol. The sample is mixed by inverting many times before centrifugation at 12000 rpm for 1 min, and the supernatant was drained. The pellet was washed with 600µl of 70% ethanol followed by centrifugation. The supernatant was discarded and the DNA left to air dry for 5 min. For hydration, 100µl of DNA hydration solution containing tris EDTA was added to the DNA pellet. The DNA was allowed to rehydrate overnight at room temperature then stored at 4°C

## D. Cloning Vectors

1- Vector used

The vector pUC18 (Amersham Pharmacia biotech), a derivative of pBR322, was used. Its size is 2.7 Kb plasmid. This vector posses a polylinker region that contains a variety of restriction sites inserted into *lacZ* gene and a gene for antibiotic resistance $Amp^R$ **(fig.5)**.

2-Vector digestion

pUC18 was double digested with Hind III and EcoRI, starting with Hind III. An aliquot of 10µl of pUC18 vector (50µg/ml) was pipetted into a 1.5ml tube with 5µl of HindIII buffer (10X), 32µl of autoclaved water and 3µl of HindIII enzyme (10U/µl) in a total volume of 50µl. The mixture was incubated at 37°C for 1 hr to allow digestion. Before digestion with the second restriction enzyme, EcoRI; an aliquot of 8µl of the digested vector were removed and tested for complete digestion and used as a control in other experiments. To the remainder 42µl, 5µl of EcoRI buffer (10x), 3µl of EcoRI enzyme (10U/ul) and 50µl of autoclaved water were added and the mixture was incubated for1.5 hrs at 37°C. The digested vector was

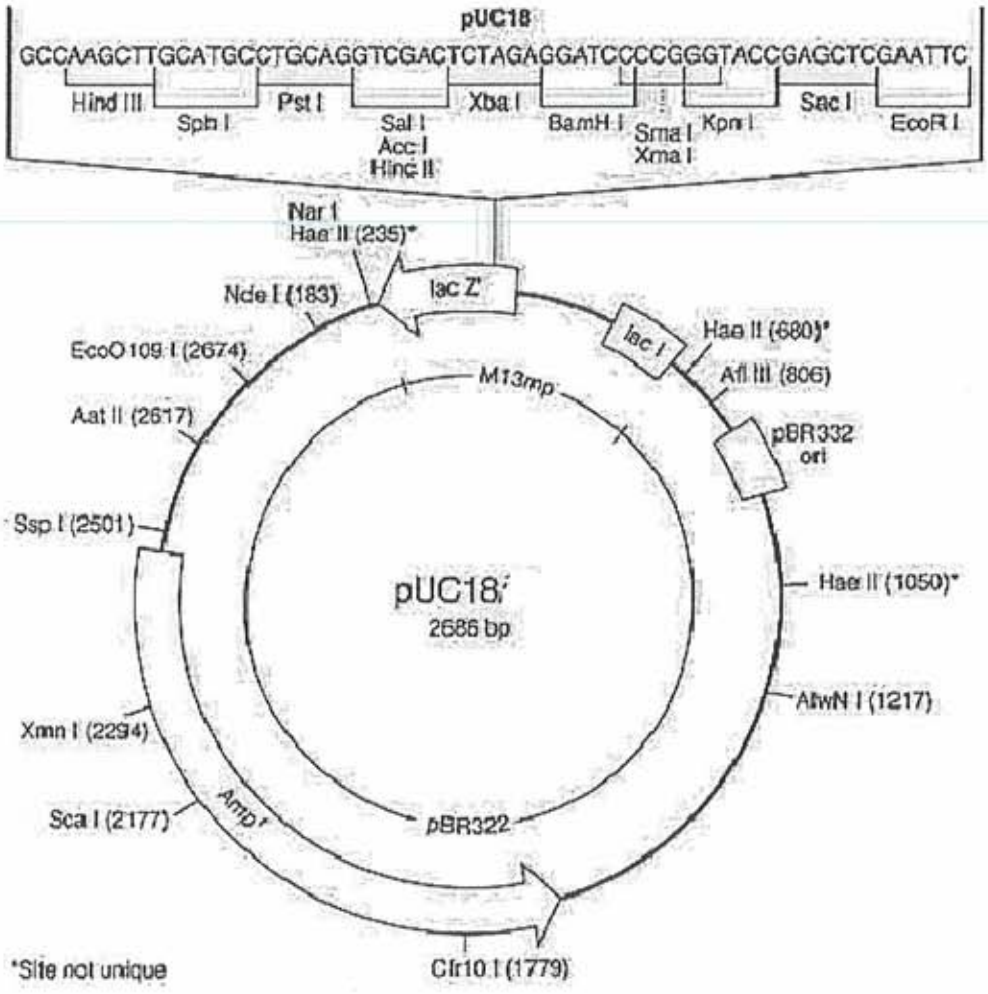



**Fig.** 5 pUC 18 cloning site sequence

purified with phenol/chloroform extraction. An equal volume of phenol i.e. 100µl was added to the digested mixture followed by centrifugation and removal of the supernatant. Equal volume of chloroform was added to the supernatant followed by centrifuqation. Chloroform addition was repeated two times. To precipitate the DNA, 10µl of 3M sodium acetate pH 5.5 were added to the supernatant followed by the addition of 300u1 of 95% ethanol. The mixture was incubated for 1.5 hrs at -80$^0$C. Following incubation, the sample was centrifuged for 30min, at 4$^0$C then the supernatant was carefully pipetted and an additional 100µl of 70% ethanol was added to wash the DNA. The sample was centrifuged for another 30min, and the pelleted DNA was allowed to air dry. The digested DNA was resuspended in 20µl TE buffer and left overnight at 4$^0$C before storage at -20$^0$C.

## E. Use of Polymerase Chain Reaction (PCR) to Amplify the Putative Kinase (b1772) Gene

To amplify the putative kinase qene (b1772) from DHSα genome, PCR was used. One µl of Taq DNA polymerase at 5units/µl was added to reaction mixture

containing the following:

1- 5µl of each deoxynucleotide (C,G,A,and T) in a concentration equal to 25mM for each d'NTP

2- 5µl of 1µM for each primer

3- 5µl of the reaction buffer (10x) containing 750mM tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$, 0.1% Tween 20

4- 3µl of $MgCl_2$ in a final concentration equal to 1.5mM

5- 4µl of Purified genomic DNA (0.36µg/ml)

6- 22µl of autoclaved water

A PCR thermocycler was programmed to perform the following steps:

1- Initial denaturation of DNA at 95°C for 5 min

2- 30 cycles of denaturation at 95°C for 1 min annealing at 51°C for 1 min, and polymerization at 72°C for one min

3- Additional 5 min at 72°C were allowed to complete polymerization.

4- The PCR product was allowed to rest at 8°C for 15 min.

Annealing temperature was chosen as 15°C lower than the melting temperature of the annealed primers. Melting temperature of the primers was calculated according to the following formula:

$$Tm = 4(C+C)+2(A+T)$$

The PCR product was purified using GFX PCR DNA and gel band purification kit(Amersham Pharmacia biotech) according to manufacturer's recommendation.

## E. Ligation of PCR Product and the Digested Plasmid

The amplified gene was digested with the same restriction enzymes used for vector digestion, starting with Hind III. Three µl of HindIII enzyme (10U/µl) were mixed with 60µl purified PCR product (200ng/µl) and 7µl of enzyme buffer. The mixture was incubated for 3 hours at 37°C. After incubation, 4µl of the second enzyme, EcoRI, was added in addition to 5µl enzyme buffer and 1 µl of water. The mixture was incubated for 3 hours at 37°C then stored at -20°C. The digested PCR product was purified using GFX PCR kit.

To ligate the vector to the amplified gene the following mixture was prepared: one µl of digested vector, 2.5µl of digested and purified amplified gene, 1µl of T4 DNA ligase (1U/µl) purchased from Amersham Pharmacia, 1µl enzyme buffer (10x), and

4.5µl water. The mixture was incubated at 4°C for 16 hours and stored at -20°C or used directly for transformation.

## G. Transformation of E.coli Cells with Plasmids

### 1- Preparation of competent cells

Competent Cells were prepared using the Rubidium chloride method. One ml of overnight culture was inoculated into 25ml LB medium to a density of 0.4 - 0.5 OD at λ= 600. An aliquot of 6ml was pelleted at 5000 xg for 5 min at 4°C in Eppendorf centifuge 5403. After discarding the supernatant, the cell pellet was resuspended in 3 ml of chilled buffer containing 100mM MOPS pH 7.0, and 10mM RbCl then repelleted at 5000xg for 5min at 4°C. The pellet was the resuspended in 2.7 ml of a chilled buffer containing 100mM MOPS pH 6.5, 10mM RbCl and 0.3 ml of chilled 500mM $CaCl_2$ was added before incubation for 30min on ice. The Catreated cells were repelleted, and resuspended in 1.5 ml of Ca-containing MOPS pH 6.5 buffer. Finally, 50µl of DMSO were added, to give a final concentration of 33.3%

and the cells were kept at $4^0$C for a period of one week.

2- Transfomation

An aliquot of 100µl of the competent cells were dispensed into 1.5 ml sterile thin-walled tube to which a maximum volume of 10µl of plasmid DNA were added. The cells were incubated on ice for 30 min followed by swirling in 44°c water bath for 3 sec and rechilled.

The transformed cells were spread on LB plates containing ampicilline (50µg/ml), 25µl X-gal (20mg/ml), and 25µl IPTG (100mg/ml) then incubated at $37^0$C overnight.

## H. Induction of Gene Product

An inoculum of 300µl of cells harboring the plasmid with the gene insert was grown with aeration at 37°C overnight. One ml of overnight culture was sub-inoculated into 30 ml LB/Amp and grown with aeration to an O.D of 0.4 - 0.5. Isopropyl thio galactosidase (IPTG) was added to a final concentration of 5mM. An aliquot of one ml of cells was taken every hour to check for protein induction

to determine the proper time for induction.

## I. Electrophoretic Methods

### 1-Agarose Gel Electrophoresis

High melting Agarose (Fluka) was used to separate and visualize PCR products, purified plasmids, and products of restriction enzyme digestions. To prepare 1% agarose horizontal slab gels, agarose was dissolved in Tris acetate EDTA (TAE) buffer at a pH 8.5, and 10g/ml of ethidium bromide solution was added for staining. A volume of 150 ml of agarose was prepared to load into gel bed size 14cm x 14 cm (C.B.S. Scientific Co.). A 10 well comb was inserted into the gel and left for polymerization. DNA samples were suspended in a loading buffer for electophoresis. The loading buffer consisted of 30% glycerol, 30mM EDTA, 0.03% Xylene cyanol, and 0.03% bromophynol blue. The electrophoresis was carried out at 90-100 volts. The DNA bands were visualized on a UV transilluminator (Fotodyne).

### 2- Protein SDS-polyacrylamide Gel Electrophoresis

The SDS-PAGE was used to analyze bacterial

induced proteins. The VWR Scientific products electrophoresis cell was used. Estimation of the molecular weight of protein bands was done using BenchMark Prestained Protein Ladder as a control (Life Technologies).

a- Preparation of Acrylamide

38.9 g of acrylamide and 1.1 g of bis-acrylamide were dissolved in autoclaved water to a final volume of 100 ml. The mixture was filtered and stored in a dark bottle at $4^0$C.

b- Upper and lower gel buffers

The upper gel buffer consisted of 0.4% SDS in 0.5M Tris-HCl, pH 6.8.

The lower gel buffer consisted of 0.4% SDS in 1.5M Tris-HCl, pH 8.8.

c- Gel Running buffer

The gel running buffer consisted of 0.1% SDS in 25mM Tris-base, 192 mM glycine, pH~8.3.

d- Preparation of 12 % resolving gel

To prepare 12% gel, 7.5 ml of 40% acrylamide solution, 11 ml of water, 6.25 ml lower gel buffer,

125µl of ammonium persulfate (10%), and 12.5µl of TEMED were mixed then poured into 16cm x 14 cm electrophoresis cell and overlayed with 25 % lower gel buffer. The gel was left to polymerize at room temperature for 20 min.

e- preparation of stacking gel

After removal of the overlay buffer and washing twice with water, the stacking gel was added. The stacking gel consisted of 0.8 ml acrylamide solution, 6.6 ml water, 2.5 ml of upper gel buffer, and 100 µl 10% APS and 10 µl TEMED. A 20- well comb was inserted into the stacking gel and left for polymerization at room temperature. Electrophoresis was carried out at 25mA. After electrophoresis, the gel was stained for 30 min with 0.05% coomasie blue dissolved in 50% methanol and 10% acetic acid and destained in 10% methanol and 5% acetic acid.

## J. Protein Purification

1- Buffers and solutions

Solubilization buffer

The solubilization buffer consisted of 50mM Tris-HCl pH 8, 2mM EDTA and 0.1M NaCl, 20 µl/ml

DNase I and 2mM phenyl methyl sulfonyl fluoride (PMSF).

Washing buffer

Washing buffer consists of 10 mM Tris-HCl pH 8, 1 mM EDTA and 10 mM NaCl.

2- Purification Steps

Two liters of E.coli DH5α harboring the recombinant plasmid were grown to 0.5 $O.D_{600}$. at 37°C. A final concentration of 5mM IPTG was added to induce protein production for 3 hours. The cells were then pelleted by centrifugation at 12000 rpm for 15 min, washed with washing buffer and lysed with solubilization buffer. The cell suspension was then sonicated with pulses of 30s and increasing amplitude as follows:

| Amplitude | Time | Frequency |
|---|---|---|
| 30 | 30s of sonication resting on ice for 30s | 3X |
| 40 | 30s of sonication resting on ice for 30s | 3X |
| 50 | 30s of sonication resting on ice for 30s | 3X |
| 60 | 30s of sonication resting on ice for 30s | 3X |

The cell lysate was then pelleted for 40 min at 6000xg in a Sorvall (SS34) centrifuge. The supernatant was placed in an ice-bath and 10.6g of ammonium sulfate were added gradually for every 100 ml, thus bringing the ammonium sulfate saturation up to 20%. The precipitate was removed with centrifugation for 10 min at 10,000 r.p.m. The supernatant was further saturated with ammonium sulfate up to 40% and 55 % by the addition of 11.3g and 8.9g respectively for every 100ml of solution. The precipitated protein was collected by centrifugation for 10 min at 10,000 r.p.m. The precipitated protein collected were dissolved in

50mM Tris-HCl and 100mM KCL in a volume equal to 10% of the original volume. The resulting solutions were placed in a cellophane dialysis bag (Medicell. International and Ltd.) and dialyzed against 2L of 50mM Tris-Cl PH 8 and 100 mM KCl for 5 hours. Dialysis was repeated overnight in a fresh buffer solution. After complete dialysis, the dialyzed solution was centrifuged for 10 min at 10,000 r.p.m. Thus removing any inactive precipitate. The sample was loaded onto a DEAE -Sepharose (dimension 2cm x 30cm) column preequilibrated with the same buffer. The bound proteins were then eluted with increasing concentration of KCl (50-450mM) gradient made in 50mM Tris-HCl pH 8 buffer. Fractions of 1 ml were collected and tested by SDS-PAGE. Fractions containing the suspected protein were pooled, dialyzed against 50mM tris-HCl and 100 mM KCl then loaded onto Sephadex G- 200 column 1cm x 30cm) and eluted with 100 mM KCl made in 50 mM Tris- HCl pH 8. Protein fractions of 1 ml were collected and tested.

## K. Hexokinase Assay

The hexokinase assay test catalyzes the following reaction:

$$ATP + Hexose \longrightarrow ADP + Hexose\text{-}6\text{-}P + H^{+}$$

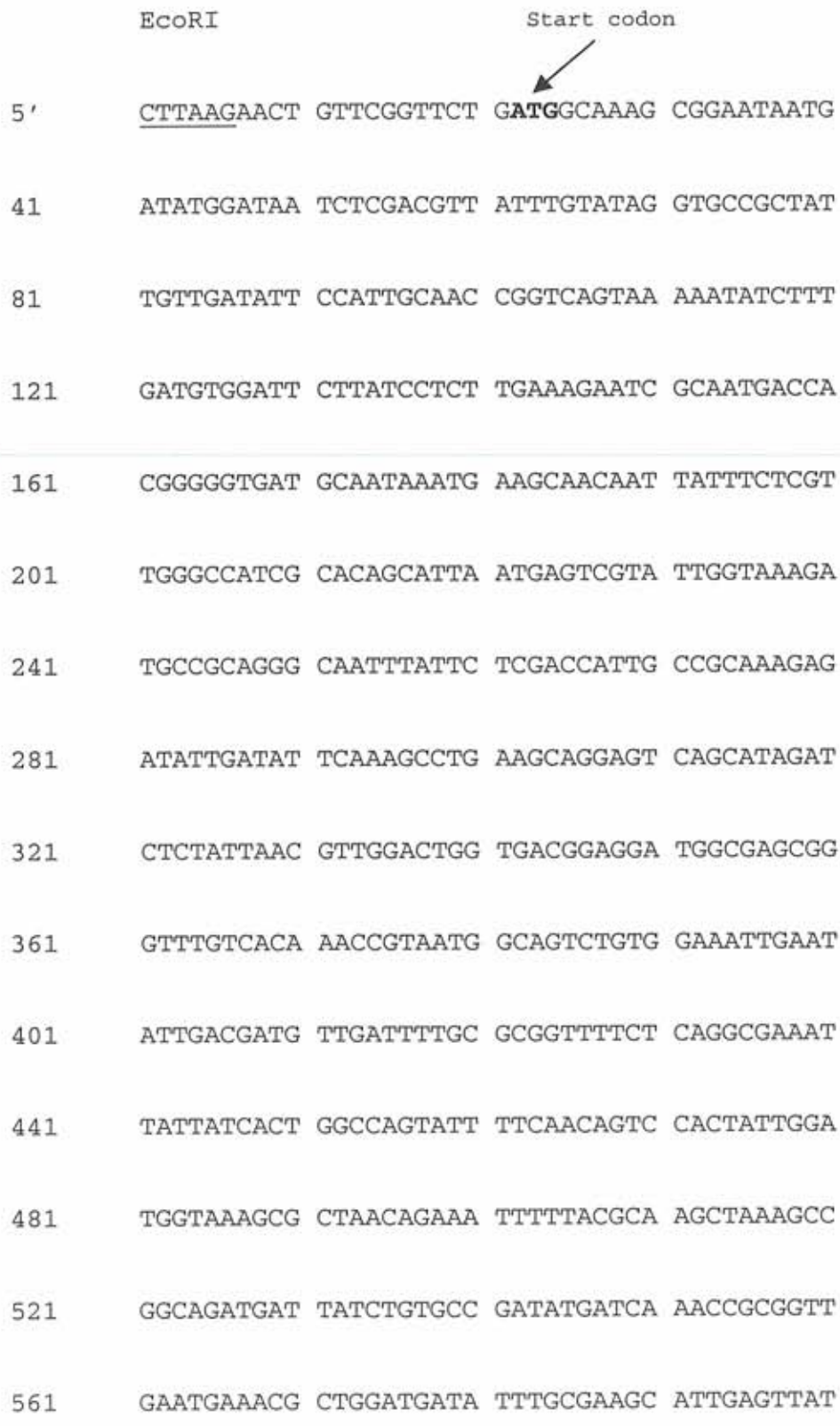
The assay test is adapted from Wajzer method. Its basis is that for each mole of phosphate transferred from ATP to a hexose an acid equivalent is liberated. The rate of acid production is proportional to the hexokinase concentrateion. An acid-base indicator was used to test the acid produced in the hexokinase reaction. A stock solution was made by mixing 7ml of 0.006% cresol red, 1.6 % $MgCl_2$ $.6H_2O$, and 1.5ml of 0.1M ATP neutralized with 0.1M NaOH until indicator became reddish Purple. Three ml of 0.1 M Tris-Cl PH 9 were then added and the mixture was diluted with water to 30 ml. This stock solution was prepared fresh daily as needed. To perform the test 0.1 ml of the partially purified enzyme or total supernatant was added to a cuvette containing 2.5 ml of stock solution with 0.4 ml of 0.2 M sugar. The decrease of optical density was measured at 560 mμ. Qualitative determination of enzyme activity was done by visualization of color change from purple to yellow

using the spot test. A decrease in optical density gives a quantitative measure of the acid production i.e. sugar phosphorylation.

# III. RESULTS

*The results described here attempts to show that the activity of the product of b1772 gene in E.coli is a sugar kinase and this kinase showed to be a hexokinase with high specificity to glucose.*

The Putative kinase gene (b1772) is found in locus AAC74842. It was chosen to be cloned since it fits the criteria mentioned in the introduction. Follows is the nucleotide sequence of selected gene:


EcoRI
Start codon
5'
CTTAAGAACT GTTCGGTTCT GATGGCAAAG CGGAATAATG
41
ATATGGATAA TCTCGACGTT ATTTGTATAG GTGCCGCTAT
81
TGTTGATATT CCATTGCAAC CGGTCAGTAA AAATATCTTT
121
GATGTGGATT CTTATCCTCT TGAAAGAATC GCAATGACCA
161
CGGGGGTGAT GCAATAAATG AAGCAACAAT TATTTCTCGT
201
TGGGCCATCG CACAGCATTA ATGAGTCGTA TTGGTAAAGA
241
TGCCGCAGGG CAATTTATTC TCGACCATTG CCGCAAAGAG
281
ATATTGATAT TCAAAGCCTG AAGCAGGAGT CAGCATAGAT
321
CTCTATTAAC GTTGGACTGG TGACGGAGGA TGGCGAGCGG
361
GTTTGTCACA AACCGTAATG GCAGTCTGTG GAAATTGAAT
401
ATTGACGATG TTGATTTTGC GCGGTTTTCT CAGGCGAAAT
441
TATTATCACT GGCCAGTATT TTCAACAGTC CACTATTGGA
481
TGGTAAAGCG CTAACAGAAA TTTTTACGCA AGCTAAAGCC
521
GGCAGATGAT TATCTGTGCC GATATGATCA AACCGCGGTT
561
GAATGAAACG CTGGATGATA TTTGCGAAGC ATTGAGTTAT

601 GTCGATTATC TGTTTCCTAA TTTTGCCGAG GCAAAATTAC

641 TCACCGGGAA AGAGACACTG GATGAAATTG CTGACTGCTT

701 TCTTGCGTGC GGCGTAAAAA CGGTGGTGAT TAAAACGGGT

741 AAAGACGGCT GCTTTATCAA GCGTGGTGAC ATGACGATGA

801 AGGTGCCGGC GGTCGCAGGA ATAACCGCCA TCGACACCAT

841 TGGCGCGGGC GATAACTTTG CTTCAGGTTT TATTGCGGCA

881 CTGTTAGAAG GCAAAAATCT GCGTGAATGC GCACGCTTTG

921 CCAATGCAAC GGCGGCTATC TCGGTTCTAA GCGTCGGTGC

961 CACCACCGGC GTAAAAAACA GAAAGCTGGT CGAACAATTG

1001 TGGAAGAATA CGAAGGA**TAA** TGAAGGCTTC GAA Hind III 3'

stop codon

**Fig 6** The complete nucleotide sequence of b1772 gene. The sequences written in bold indicate the start codon and stop codon. The underlined nucleotides are the restriction sited of EcoRI and Hind III.

This nucleotide sequence codes for the following protein:

```
  1 makrnndmdn ldvicigaai vdiplqpvsk nifdvdsypl
 41 eriamttggd aineatiisr lghrtalmsr igkdaagqfi
 81 ldhcrkenid iqslkqdvsi dtsinvglvt edgertfvtn
121 rngslwklni ddvdfarfsq akllslasif nsplldgkal
161 teiftqakar qmiicadmik prlnetlddi cealsyvdyl
201 fpnfaeakll tgketldeia dcflacgvkt vviktgkdgc
241 fikrgdmtmk vpavagitai dtigagdnfa sgfiaalleg
281 knlrecarfa nataaisvls vgattgvknr klveqlleey
301 eg
```

The estimated molecular weight for this protein is 34.96 Kd. When compared to protein data bank, a high degree and identity and similarity was obtained with the following gene products:

1- YDJH ECOLI:hypothetical sugar kinase

2- RBSK HAEIN:ribokinase

3- KDGK BACSU:2- dehydro-3-deoxyglyconokinase

Sense and antisense primers were designed to amplify this gene. These primers were flanked at 5′ end with additional sequence for cloning purposes.

The sequence of the primer of the sense strand:

5'AAG A↓AT TCA ACT GTT CGG TTC TGA TGG C3'.

The underlined part includes the sequence for EcoRI site.

Primer of the antisense strand:

5'AGA↓ AGC TTG CCT TCA TTA TCC TTC GTA T3'.

The underlined part includes the restriction site of HindIII.

To amplify this gene, PCR protocol described under materials and methods was used. In preliminary experiments, a set of experiments was done in order to optimize the conditions under which maximum amount of specific PCR product was obtained.

In these experiments, increasing amount of DNA with constant primer concentrations or increasing amount of primers with constant amount of DNA were used. **Fig.7** shows agarose gel electrophoresis for purified PCR product along with 1 Kb DNA ladder. As shown, the size of the PCR product obtained is around 1 Kb running along the 1 Kb fragment of the control DNA ladder.

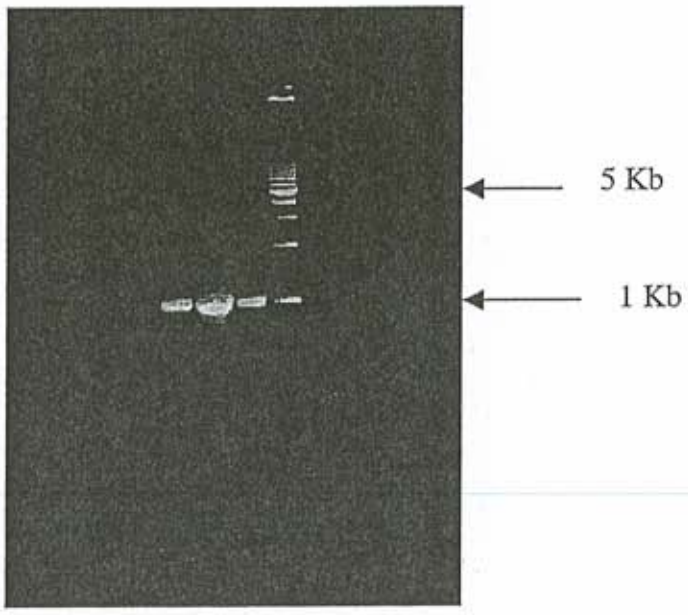



**Fig.7:** Agarose gel electrophoresis of purified PCR product of gene b1772.1% agarose showing the mobility of th PCR product ofdifferent reaction utilizing different amount od DNA. In lane 1, 3µl of DNA were used, in lane 2, 4µl of DNA were used, in lane 3, 2µl of DNA is used. Lane C is the Kb DNA ladder, used as a control marker. The brightest fragment represent 5 Kb DNA, the size of the fragment that runs along with the purified PCR product is 1 Kb.

Both the purified PCR product as well as the pUC 18 vector were digested with two different restriction enzymes EcoRI and HindIII to ensure directional cloning. **Fig.8** shows the purified PCR product and the control 1 Kb DNA ladder. The pieces of DNA were ligated using T4 DNA ligase and transformed into competent DH5α cells. A set of controls was used to ensure the activity of the T4 DNA ligase as well as the restriction enzymes utilized. Some of these controls included:

1- pUC 18 digested with HindIII with and without ligase.

2- pUC 18 digested with HindIII and EcoRI with and without ligase.

The pUC 18 vector encodes the alpha fragment of the *E.coli* beta-galactosidase gene. Transformation of the bacterial cells with this vector allows the production of functional beta-galactosidase by (alpha-complementation) since the host strain of *E.coli* produces the enzymatically inactive omega fragment only. Colonies harboring the plasmid with an insert will inactivate the *lacz* gene since the multiple cloning sites fall within this gene.

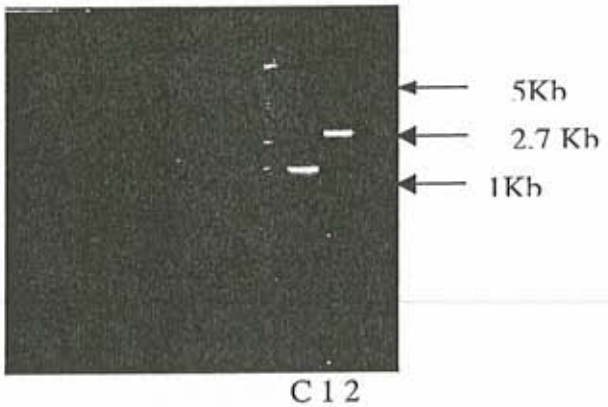



**Fig. 8** Agarose gel electrophoresis of the purified digested vector and amplified gene b1772. The gel shows the mobility of the purified linearized vector as compared to the PCR to be cloned. Lane 1 shows Hind III-EcoRI gene b1772, lane 2 shows HindIII-EcoRI pUC 18, lane C 1 Kb DNA Ladder.

Identification was done through the growth of bacteria on place containing the chromogenic substrate for beta-galactosidase, 5- bromo - 4 - chloro - 3 - indolyl -β-D galactoside (x-gal), and an inducer of *lacz* promoter, IPTG. Colonies containing vectors without inserts release the chromophore from the x-gal and are colored blue. Colonies harboring plasmids with insert were colorless.

After transformation, 8 colonies that were suspected to carry the insert were isolated. The transformed plasmid was purified and tested to check for the presence of the inserted PCR product. Four out of eight plasmids contained the insert.

**Fig.9** shows the electrophoretic mobility of 2 purified plasmids along with pUC 18 vector utilized. The size of the plasmid harboring the insert is expected to be larger than the plamid with ni insert. As shown in figure 9, the insert was released with the restriction enzymes to confirm its presence.

These four positive clones were then induced with IPTG to examine the production of the "putative kinase". Induction was done on these four different clones separately for 1,2,3 and 4 hours to select

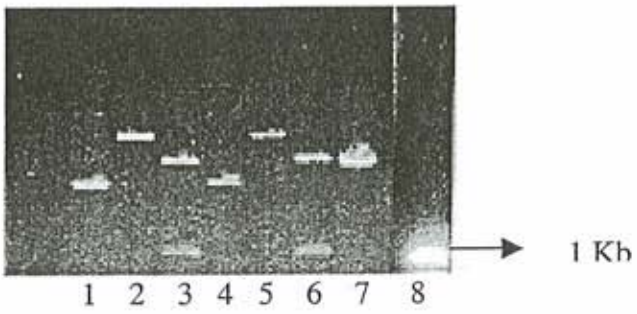



**Fig.9** Electrophoretic mobility of purified positive clones of gene b 1772. 1% agarose showing the mobility of digested and undigested positive clones. Lane 1 shows positive clone number3, lane 2 linearized clone 3 with Hind III, lane 3 EcoR-HindIII digested clone 3, lane 4 undigested clone 6, lane 5 Hind III digested clone 6, lane 6 Hind III-EcoRI clone 6, lane 7 EcoRI-Hind IIIpUC18, lane 8 HindIII-EcoRI PCR product of gene b 1772.

the proper time for the highest amount of protein produced. After induction, the cells were lysed and the proteins produced were examined by SDS-PAGE.

All four positive clones showed similar overproduction of the putative kinase with its expected size. To determine if the putative kinase is a soluble protein, a new induction experiment was performed on the four positive clones. The cells were lysed as indicated under "materials and methods" and both the supernatants and the pellets were tested by SDS-PAGE. The enzyme was found in both the supernatant and in the pellet as well. Qualitative determination of the enzyme activity using hexokinase assay was done using the supernatant obtained from the four positive clones. Two out of the four clones were inactive (clones 6 and 3) while the other two clones showed activity (clones 5 and 8).

To test for the enzyme activity in a purer form, the enzyme was partially purified from clone 5 in three different steps. The enzyme precipitated mainly with 40% ammonium sulfate. The redisolved and equilibrated pellet was further purified on DEAE-cellulose. The protein eluted from this anion exchanger column at a salt concentration of 250mM KCl. Fractions that showed the presence of putative

kinase along with positive hexokinase activity were pooled and further purified on gel filtration column (sephadex G200). Fractions containing the suspected sugar kinase were pooled and tested using the hexokinase test with other sugars. The presence of gp 1772 after induction, in ammonium sulfate precipitation and in fractions eluted from Sephadex G200 column was confirmed in SDS-PAGE (**Fig 10**).

Eight different sugars were used for testing: Glucose, galactose, fructose, rhamnose, xylose, maltose, glycerol, and mannose. The activity was detected photometrically and as a spot test in the presence of the acid-base indicator cresol red.

**Table 4** summarizes the results of the spot test upon the addition of the enzyme to the different sugars.

The spot test indicated no change in color with xylose, rahmnose and maltose; however, glucose, fructose, galactose, had shown gradual change to yellow. The hexokinase spot test was also performed on the total supernatant of uninduced and induced cells to further confirm that the activity seen in partially purfied protein is indeed due to the overproduced protein and not due to one of the protein impurities.

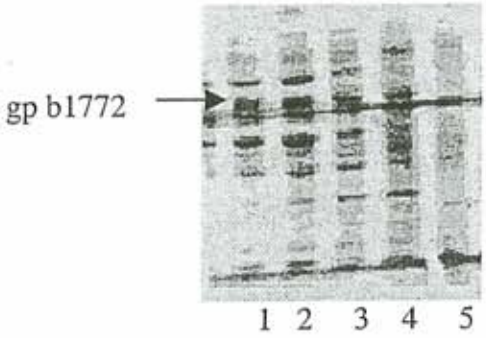



**Fig. 10** Purification of *E.coli* gp 1772. 12.5% SDS-PAGE of protein extract obtained from:1- uninduced cells,2- induced cells, 3- 55% ammonium sulfate precipitation,4- 40% ammonium sulfate precipitation, 5- sephadex G200 fraction that showed hexokinase activity.

**Table 4** Activity of the sugar kinase with different sugars using the hexokinase test.

| Sugar | Result |
|---|---|
| Glucose | + |
| Galactose | + |
| Xylose | - |
| Rahmnose | - |
| Fructose | + |
| Maltose | - |
| Mannose | - |
| Glycerol | - |

In the induced cells, all three sugars showed differentially (fastest reaction with glucose) hexokinase activity while the uninduced cells showed no activity with the sugars with the exception of galactose. These results shows that this putative kinase is indeed a sugar kinase and this sugar kinase is most probably a hexokinase with high affinity to glucose.

# IV. DISCUSSION

The results described here for the activity of the product of a putative kinase gene isolated from *E.coli* DH5α indicate that this product is a sugar kinase. Examination of this gene showed that it shared a high degree of similarity with other genes reflecting the high degree of conservation of this enzyme from *E.coli* with other species. Computer assisted analysis of the primary sequence of this gene product showed also the high degree of similarity and identity with other sugar kinases from other species (i.e. yeast hexokinase, *Bacillus subtilis* 2-dehydro-3-deoxyglucokinase and *Haemophilus influenzae* ribokinase) in addition to other *E.coli* hypothetical sugar kinases and fructokinases (even though there is a difference in their molecular weight, the molecular weight of gp b1772 is 34.96 Kd, yeast hexokinase is 100 Kd and that of *H.influenzae* is 32.2 Kd).

Assaying the gene product after its partial purification with the hexokinase test confirmed the function of this enzyme as a sugar kinase.

In our attempts to clone the gene, four positive clones (clones 3,5,6 and 8) were obtained. The presence of gp b1772 was confirmed by SDS-PAGE in the four clones but the activity of phosphorylation was observed in two only. The inactivity of the two other clones can be attributed most probably to a mutation that had affected the active site of the enzyme produced, or to mutation/s that has/have affected the overall conformation of this enzyme inhibiting it from binding to one of its substrate. These mutations might have risen from the Taq polymerase used during PCR and could not have been alleviated since as compared to other polymerases, Taq has lower percentage of fidelity (even though Taq polymerase is more proccessive in comparison to other polymerases). Anyway, the inactivity of these two clones served as negative controls for the active ones.

During purification of the enzyme from one of the active clones (i.e. clone 5) we noticed that the enzyme was not 100% soluble. It was divided between the supernatant of the cell extract and the pellet. The presence of the protein in an insoluble form could be due to different reasons. First it is possible that upon induction, the high amount of protein produced led to

the formation of inclusion bodies; another reason is that the enzyme itself may adopt two different states a soluble and insoluble one according to specific modification (e.g. ADP-ribosylation, phosphorylation, dephosphorylation and several other types of modifications) based on physiological needs.

The soluble fraction of *E.coli* gp b1772 that was purified behaved similarly to yeast hexokinase and eluted at a salt concentration of 250 mM KCl from DEAE-Cellulose, implying that the two enzymes share similar chemical properties.

In assaying the enzyme activity of the partially purified protein obtained from G200 column we found that the enzyme was able to phosphorylate different hexoses among them glucose, fructose and galactose. Other hexoses like mannose and rhamnose were not phosphorylted; this might be due to the spatial orientation of one (or more) hydroxyl (OH) group of the sugar itself that does not fit the active site of the enzyme. Thus, the substrate-induced cleft closing needed for hexokinase activity was inhibited.

In the hexokinase assay, the total supernatant from induced and uninduced cells were used. The uninduced cells were serving as a negative control to ensure that the hexose activity seen was due indeed to the overproduced protein. These tests were done using glucose, galactose and fructose.

The spot tests had shown that this enzyme is phosphorylating glucose, fructose and galactose; however, we found that only in the presence of galactose both the induced and uninduced cells gave positive results. This might be due to the production of galactokinase that is produced constitutively by the cells in very high quantities that triggered the phosphorylation of galactose even in the absence of hexokinase produced by the cloned gene.

Finally, as a conclusion, we may deduce that the product of this putative kinase gene, b1772, is a hexokinase with higher specificity or preference to glucose than other hexoses.

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