PURIFICATION AND CHARACTERIZATION OF HEXOKINASE ISOENZYMES FROM *Rhizopus oryzae*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

DİDEM DEDEOĞLU

IN PARTIAL FULLFILMENT OF REQUIREMENTS FOR
THE DEGREE OF MASTER FOR SCIENCE
IN
BIOTECHNOLOGY

FEBRUARY 2007

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Canan Özgen
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science

Prof. Dr .Fatih Yıldız
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for degree of Master of Science

Dr. Şeyda Açar
Co-supervisor

Prof.Dr. Haluk Hamamcı
Supervisor

Examining Committee Members:

Prof. Dr. Meral Yücel	(METU BIO)	
Prof. Dr. Haluk Hamamcı	(METU FDE)	
Prof. Dr. Hüseyin Avni Öktem	(METU BIO)	
Prof. Dr. Zümrüt Begüm Ögel	(METU FDE)	
Assist. Prof.Dr. Füsun İnci Eyidoğan	(BAŞKENT SCI. EDU.)	

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Didem Dedeoğlu

Signature:

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF HEXOKINASE ISOENZYMES FROM *Rhizopus oryzae*

Dedeoğlu, Didem
MS., Department of Biotechnology
Supervisor: Prof.Dr. Haluk Hamamcı
Co-supervisor: Dr. Şeyda Açar

February 2007, 116 pages

Glycolysis is the central metabolic pathway for living organisms. Its regulation is important for the yield of the end products which are industrially important. These end products, like lactic acid produced by *Rhizopus oryzae*, are industrially important.

Rhizopus oryzae is a filamentous fungus producing lactic acid and ethanol. The lactic acid yield of *R. oryzae* is low (~70 %) compared to that of lactic acid bacteria (>95 %) still it is noteworthy because *R. oryzae* produces only the L (+) form of lactic acid which can be metabolized in the human body. The yield of an industrial process should be high for the feasibility of the production of a particular product. If a way can be found increase the flux through the glycolysis the yield of lactic acid may increase as well.

Keeping this in mind we wanted to focus on the first step of glycolysis, hexokinase of *R. oryzae*. Hexokinase catalyzes the reaction that converts

glucose to glucose-6-phosphate. In this study for the first time the two isoenzymes of hexokinase of *R. oryzae* were purified and characterized by biochemically and kinetically

Hexokinase has two isoenzymes. The purified enzymes (isoenzymes1 & isoenzymes2) obeyed Michealis-Menten Kinetics. The K_m value of purified isoenzyme 1 is 0.16 mM and isoenzyme 2, 0.21 mM at pH 7.70 for glucose. The K_m value of isoenzyme1 for fructose was 28.8 mM. Essentially isoenzyme 2 can not utilize fructose. None of the isoenzymes were inhibited by trehalose-6-phophate.The monomer moleculer weight of isoenzymes were estimated SDS PAGE analysis. There were two different values for molecular weight of isoenzmye 1; 62.9 and 42.5 kDa and two values for isoenzyme 2; 56.2 and 41.6 kDa

Key words*: Rhizopus oryzae*, hexokinase, glucokinase glycolysis, purification.

ÖZ

Rhizopus oryzae'den HEKSOKİNAZ İZOENZİMLERİNİN SAFLAŞTIRILMASI VE KİNETİK KARAKTERİZASYONU

Dedeoğlu, Didem
Yüksek Lisans, Biyoteknoloji Bölümü
Danışman: Prof. Dr. Haluk Hamamcı
Yardımcı danışman: Dr. Şeyda Açar

Şubat 2007, 116 sayfa

Tüm canlılar için glikoliz merkezi bir izyoludur. Glikolizin kontrolü endüstriyel ürünlerin verimi açısından önemlidir. Bu ürünlerden bir tanesi olan laktik asit endüstride önemli bir organizma olan *Rhizopus oryzae* tarafından üretilir

Filamentli bir küf olan *Rhizopus oryzae* laktik asit ve etanol üretir. Bakterilerle karşılaştırıldığında *R. oryzae*'nin laktik asit verimi düşüktür. Fakat sadece L(+) laktik asit formunu ürettiği için önemlidir. Laktik asitin L formu insanlarda metabolize edilebilir. Endüstride, belirli bir ürünün üretiminin uygulanabilir olması için verim yüksek olmalıdır. Eğer glikoliz boyunca akımın arttırılması için bir yol bulunabilirse aynı zamanda laktik asit verimi de artabilir.

Bu çalışmada buna dayanarak glikolizin ilk basamağı olan heksokinaza odaklanmak istedik. Heksokinaz glikozu glikoz-6-fosfat a çeviren reaksiyonu katalizler. Bu çalışmada ilk kez *Rhizopus oryzae*'nin heksokinazının iki izoenzim formunu saflaştırıldı ve kinetik karakterizasyonunu yapıldı.

Heksokinaz iki tane izoenzime sahiptir. Saflaştırılan izoenzimler Michealis-Menten Kinetiğine uyumlu bulunmuştur. pH 7.70 de izoenzyme 1 için glikoza K_m değeri 0.16 mM ve izoenzim 2 için glikoza K_m değeri 0.21

mM olarak tespit edildi. İzoenzim1 için fruktoza K_m değeri 28.8 mM iken izoenzyme 2 nin K_m değeri belirlenememiştir. Her iki izoenzim de trehaloz-6-fosfatla inhibe olmamaktadır. İzoenzimlerin moleküler ağırlıkları SDS PAGE yöntemi ile belirlendi. İzoenzim 1'in moleküler ağırlığı 62.9 ve 45.2 kDa iken izoenzim 2' nin moleküler ağırlığı 56.2 kDa ve 41.6 kDa dır.

Anahtar Kelimeler: *Rhizopus oryzae*, heksokinaz, glikokinaz glikoliz, saflaştırma.

To my family

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Prof.Dr. Haluk Hamamcı for his guidance, supervision and support. My special thanks go to Dr. Şeyda Açar for her guidance, patience, understanding support, advices, criticism and discussions invaluable helps and supervision throughout this study.

I would like to express thank to Prof Dr Meral Yücel for her valuable advices and criticism throughout the study.

I am grateful to thesis committee member Prof. Dr. Meral Yücel, Prof Dr. Hüseyin Avni Öktem, Prof Dr. Zümrüt Begüm Ögel, Assist Prof Dr. Füsun İnci Eyidoğan and Prof Dr. Haluk Hamamcı for their comments and critcism.

Love and thanks go to my laboratory friends A Oğuz Büyükkileci (Oğuz Abi) Aslı Erdoğ (Aslıcan), Ayşem Batur, Aysu Acar, Banu Temur Öztürk, Benek Abat, B. Gökcen Mazi, Ceylan Şahin, Eda Alagöz,Gül Fidan Sarıbay, Lütfiye Kurt for their helps, cooperation friendship and creating fun atmosphere in laboratory. Also I am grateful to Gökhan Duruksu for his friendship in the laboratory.

I would thank to METU Central Laboratory and Ceren Nur Biler for helping column chromatography studies.

My ever friends Burcu Coskuner (kanka), Elif Beşel, Serap Barak and Selda Türkoğlu. Your presence always made me feel warm and happy, thanks your friendship…

I would like to thank to Burak Çorman for helping my graphs.

The last but never the least I want to send my love to my family Harun, Şerife and Mehmet Görkem Dedeoğlu and wish to thank them for their love, support and patience, understanding, encouragement of every steps of this study. I would not been possible to complete this study without their love and support

This study was supported by BAP-2005-07-02-00-04

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE

LIST OF TABLES

TABLE

LIST OF ABBREVIATIONS

A.niger	*Aspergillus niger*
EDTA	Ethylene diamine tetra acetic acid
hxk	Hexokinase gene
Hxk2	Hexokinase2
HXT	Hexose tansporter
K. lactis	*Kluyvermyces lactis*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PFK	Phosphofructokinase
Pgi	Phsophoglucose isomerase gene
PYK	Pyruvate kinase
PMS	Phenazine methosulfate
PVDF	Polyvinylidene difluoride
R. oryzae	*Rhizopus oryzae*
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodyum dodecyl sulphate

CHAPTER 1

INTRODUCTION

Before the existence of atmospheric oxygen, anaerobic glucose metabolism involving glycolysis evolved to produce energy for ancient anaerobic life forms. The glycolytic pathway and its enzymes, which convert glucose to pyruvic acid using the oxidative potential of NAD^+, are among the most ancient molecular metabolic networks (Figure 1.1). In the absence of oxygen, NAD^+ is regenerated from reduced NADH by the conversion of pyruvate to lactic acid. Glycolysis has been highly conserved among the species, even after the emergence of atmospheric oxygen, which provided a means to further oxidize pyruvate by oxidative phosphorylation, resulting in high yield of energy (Kim and Dang 2005).

As it is illustrated in Figure 1.1 glycolysis has ten main steps which convert one molecule of glucose to two molecules of pyruvate with the concomitant generation of two molecules ATP. These ten reactions between glucose and pyruvate can be considered as two distinct phases. The first five reactions constitute energy investment phase in which sugar phosphates are synthesized at the expense of two molecules of ATP and six carbon is split into two molecules of three-carbon sugar phosphates. In the energy investment phase the sugar is metabolically activated by phosphorylation. This process yields a six carbon phosphorylated sugar, fructose-1,6-biphosphate, which undergoes phosphate cleavage to yield two moles of triose: glyceraldeyde-3-phosphate and dihydroxyacetone phosphate.

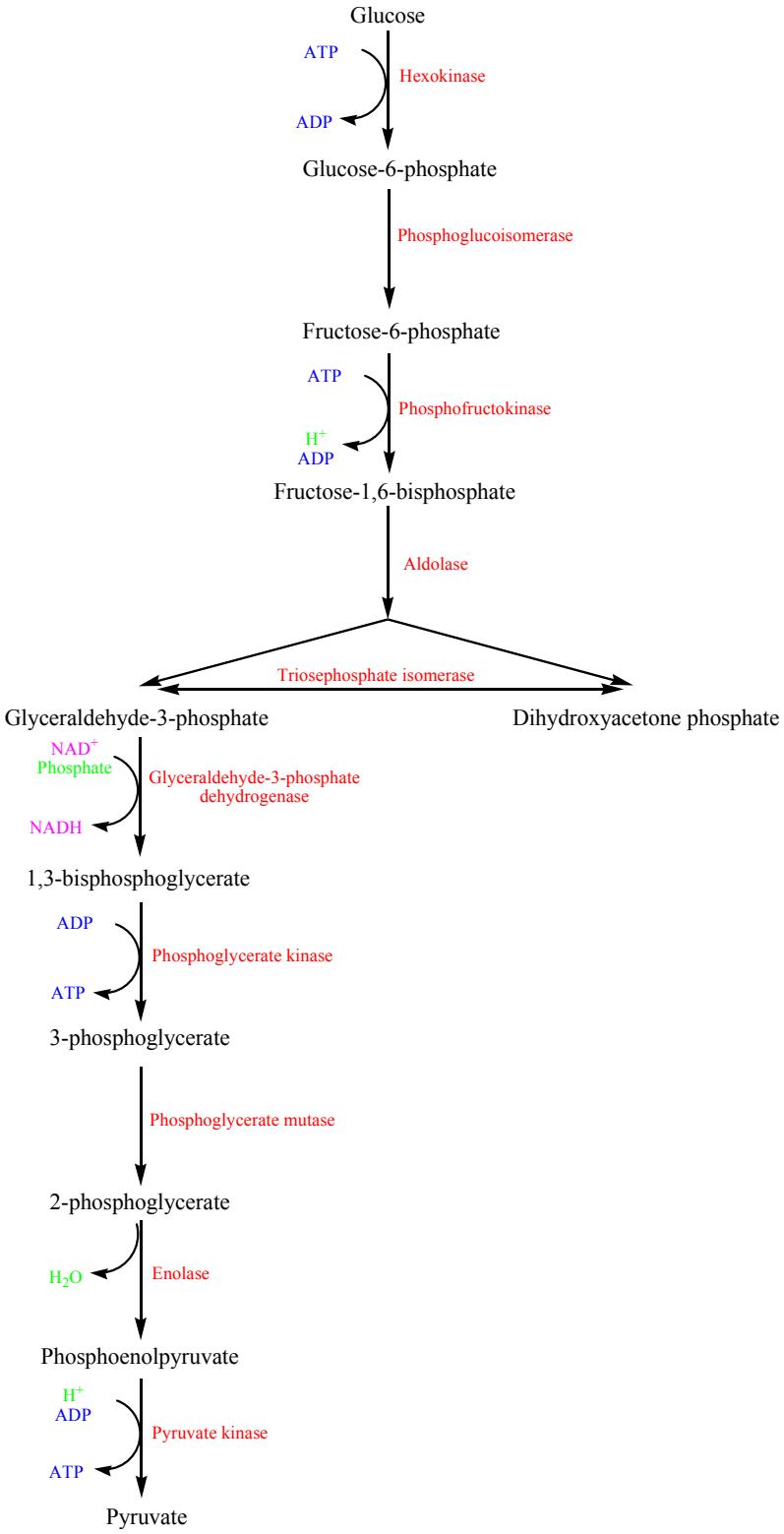


Figure 1.1: Glycolytic pathway

The last five reactions represent an energy generation phase in which the triose phosphates are converted to energy compounds. These compounds transfer four moles of phosphate to ADP leading to four moles of ATP. The net yield per mole glucose metabolized, is two moles of ATP and two moles of pyruvate. In the energy generation phase the triose phosphates undergo further activation to yield compounds containing energy rich phosphate bonds first 1, 3-biphosphoglycerate and phoshoenolpyruvate. During the energy generation phase, each of compounds transfers its high energy phosphate to ADP, yielding ATP. This process is called substrate-level phosphoprylation: The transfer of a phosphoryl group from a super-high-energy compound to ADP yielding ATP. Note that two reducing equivalents are generated as well, in the form of NADH. After the production of the pyruvate the reaction is followed by citric acid cycle in the presence of oxygen. However, in the absence of oxygen pyruvate is converted to lactate, ethanol or any other fermentation product depending on the type and physiology of the organism. For example lactic acid bacteria mainly produce lactic acid as *Saccharomyces cerevisiae* may produce ethanol.

Glycolysis lies at the heart of many important branching pathways (Figure 1.2). These branches involve complex pathways like pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and small branches like glycerol, lactic acid, and ethanol and acetate production (Figure 1.2).

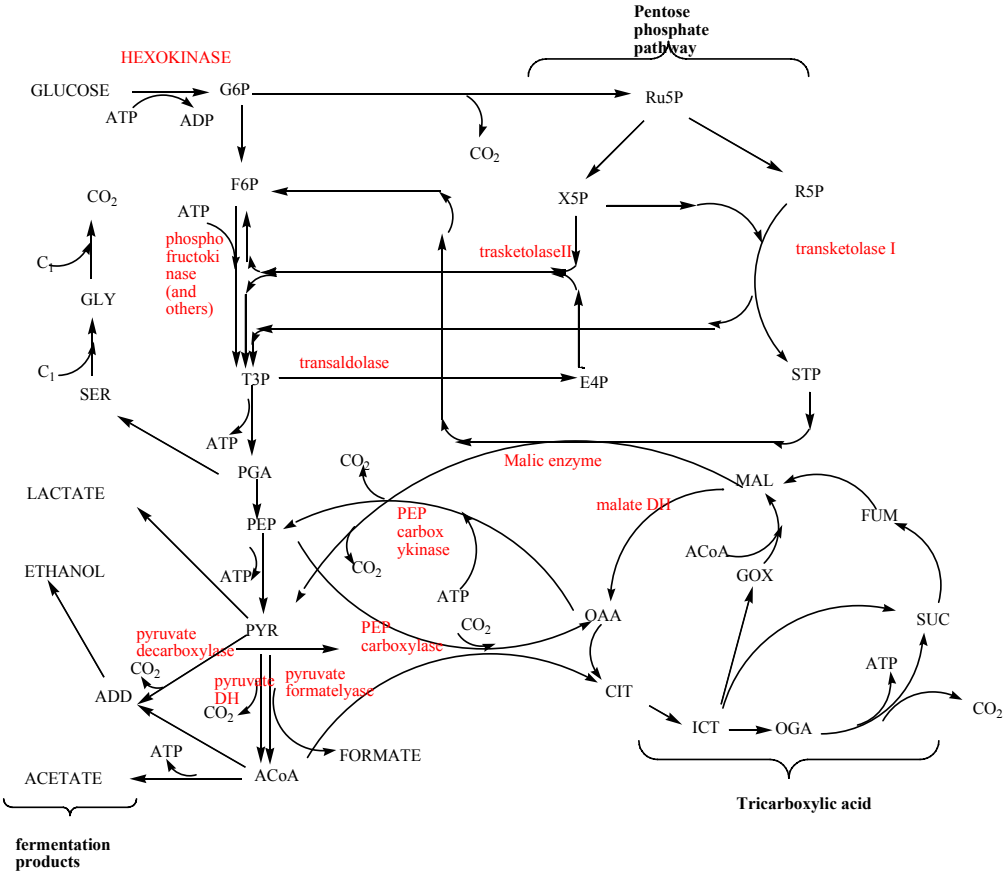


Figure 1.2: Glycolytic pathway and the branching pathways

Glycolysis is a widely studied pathway in plants, fungi, yeasts and other eukaryotes. Most of these studies are focused on increasing the metabolic flux by metabolic engineering which use the analytical methods to determine the regulation of the glycolysis and quantify the flux at different physical conditions. The main focus of the studies that are conducted on glycolytic enzymes, overexpression of the genes that are coding these enzymes, the modeling of the pathway is to find an explanation to the question of "What controls the glycolytic pathway?" (Hauf, Zimmermann et al. 2000)

Saccharomyces cerevisiae, (Baker's yeast) is used as a model organism to understand glycolytic pathway because *S. cerevisiae* is an important industrial organism which is used in brewing and baking industry. Moreover, it

is a genetically amenable organism and there is a considerable amount of mutant collection of this organism e.g. triple hexokinaseless mutant, hexose transporter null mutant, pyruvate decarboxylase (*pdc*) mutant etc. Genetic manipulation and its wide spread use in the industry has made *S. cerevisiae* a widely studied organism. The genome sequence of this organism is available and information about its genes and proteins can be found on web site of National Center for Biotechnology Information (NCBI; www.ncbi.nlm.gov and www.yeastgenome.org).

Organisms, for example *Saccharomyces cerevisiae* can also grow on the non fermentable carbon sources like ethanol, glycerol, and pyruvate etc. Essential cellular components are derived from sugar phosphates; growth on nonsugar carbon sources requires synthesis of these compounds from the nonsugar precursor. This process is called gluconeogenesis. Gluconeogenesis implies a reversal of all, or some of glycolytic reactions depending on the level at which the nonsugar substrate is incorporated to the glycolytic pathway.

Glycolytic pathway and gluconeogenesis share most of their enzymes, so a strict regulatory system is necessary to avoid simultaneous action of both. Moreover cells must prepare themselves for the time when the supply of nutrients is used up. It has to store nutrients in order to survive at periods of starvation (Zimmermann and K.D.Entian 1997).

In order to have an understanding of the regulation of glycolysis various control points, the mechanism involved in these controls and global control mechanism will be further analyzed in the rest of the chapter.

1.1. Regulation of Glycolysis

The organisms have developed several kinds of controls to serve regulation of glycolysis. These are allosteric and covalent action inhibition and regulation of the activities of enzymes by, repression, derepression, and degradation regulating the amount of enzymes. Allosteric activation and

inhibition are dependent upon the concentration of intermediate or specifically synthesized metabolites, while other regulatory processes need specific mechanisms to sense environmental conditions and transduce the information into cellular response. The main allosteric regulations are exerted on the irreversible steps; hexokinase, phosphofructokinase and pyruvate kinase as once called 'key points' in glycolysis. It was thought that the key steps are the main regulating points and in order to prove this some over expression studies were performed. However, overexpression of single genes coding for the enzymes at these key points were not very successful in that there were strong controls on them by other factors (Ruijter, Panneman *et al.* 1996). There was a study done by Schaaff *et al*. 1989 in which eight different enzymes including hexokinase, phosphofructokinase and pyruvate kinase as well as phosphoglucoisomerase, phosphoglyceratekinase, pyruvatedecarboxylase, alcohol dehydrogenase, hexosetranspoter 7 were overexpressed in *S. cerevisiae*. Overproduced specific enzyme activities were increased between 3.7-13.9 fold compared to the wild type level. However no increase in the ethanol production was observed (Schaaff, Heinisch *et al*. 1989). As discussed by authors of this study it might have failed because of an excessive protein burden. If we criticize these studies it can be still proposed that some of the steps are 'more important' than the others and may be the ratio of the enzymes to each other should be taken into account. However, doing an over expression study of eight genes with fine tuning by taking the ratio into account is not easy. Nevertheless, these overexpression studies have enlightened some of the unknowns of glycolysis regulation.

Regulation of the glycolysis at the genetical level is another issue that should be taken into account. In this sense Gcr1p (elements affecting the transcription of glycolytic genes in yeast) was shown as transcription factor in the regulation of the glycolytic genes in *S. cerevisiae* (Baker 1991). In *gcr1* mutant strains the levels of most glycolytic enzymes are between 2 % and 10 % of the wild type (Baker 1991).

1.1.1. The Key Steps in Glycolysis

There are three key steps in glycolysis; catalyzed by hexokinase, phoshofructokinase and pyruvate kinase. All of these steps are irreversible.

1.1.1.1. Hexokinase

Hexokinase is the first step of glycolysis. It catalyses the activation of respective hexoses (glucose, fructose, mannose) by a transfer of a phosphate group provided by ATP to the C6-residue.

$$\text{Glucose} + \text{ATP} \xrightarrow{Mg^{+2}} \text{Glucose -6-phosphate} + \text{ADP}$$

There are many studies in the literature focused on hexokinase step. For example in *S. cerevisiae* (Baker's yeast) three types of hexokinases were elucidated genetically and biochemically (Kaji, Colowick *et al*. 1961; Maitra 1970; Kopetzki and Entian 1982). One is a glucokinase which uses glucose and mannose as substrate and the others are hexokinase A and B (alternatively called hexokinase PI and hexokinase PII) that use glucose mannose and fructose as substrate.

Hexokinase step is an important point of glycolysis because there a few metabolic 'roles' attributed to this enzyme one of which is sugar sensing in lower and higher eukaryotes including plants (Xiao, Sheen *et al*. 2000; Rolland, Winderickx *et al*. 2001; Frommer, Schulze *et al*. 2003) . The characteristics of hexokinase and the role of it in the regulation of glycolysis will be described in next part of this chapter.

1.1.1.2. Phoshofructokinase

Phosphofructokinase is the second irreversible step of the glycolysis. It is an allosteric enzyme that catalyzes the transfer of the γ-phosphoryl group of Mg ATP to the C1 hydroxyl group of fructose 6-phosphate according to the following equation.

$$\text{Fructose-6-phosphate} + \text{ATP} \xrightarrow{Mg^{++}} \text{fructose1,6-bisphosphate} + \text{ADP}$$

Mg^{++}, NH_4 or K^+ are essential for the enzyme activity

Phosphofructokinase (PFK) activity can be regulated by a large number of allosteric effectors, not only in yeast but also in most of the other organism from bacteria to human (Viana, Perez-Martinez *et al.* 2005; Stechmann, Baumgartner *et al*. 2006). In vivo inhibition by ATP and activation by AMP and fructose-2,6-bisphosphate may be the predominant mechanisms. Probably the most important regulation of phosphofructokinase is controlled by fructose-2,6-bisphosphate formed from the fructose-6-phosphate and ATP by 6-phosphofructo-2-kinase. Fructose-2,6-bisphosphate is degraded by fructose-2,6-bisphosphate-2-phosphohdyrolase (F26bPase). At the same time fructose-2,6-bisphosphate inhibits the fructose-1,6-bisphosphatase which catalyses the reverse reaction in gluconeogenesis (Zimmermann and K.D.Entian 1997).

Yeast-6-phosphofructokinase is activated by micromolar concentrations of F26bP and is the most potent activator known. Fructose-2,6-bisphosphate has been proposed to be the predominant effector of glycolysis in *S. cerevisiae* because a tight correlation exists between the production of ethanol and intracellular concentration of F26bP under various experimental conditions. On the other hand, during gluconeogenesis, PFK activity is low due to the low concentration of its substrate. While yeast PF1K is largely regulated at the level of enzyme activity, its gluconeogenetic antagonist

FBpase, additionally is subject to regulation of enzyme. FBpase is strongly inhibited by F26bP and by AMP (Zimmermann and K.D.Entian 1997)

There are two types of genes for encoding the phosphofructokinase *pfk1* and *pfk2*.The *pfk1* codes larger α subunit and *pfk2* encodes the smaller β-subunit (Heinisch, Vogelsang *et al*. 1991). Nadakarni *et al.* implies that the *pfk1* encoded the α-subunits serve a regulatory function in the cytoplasmic form of the enzyme and a catalytic function in the particulate version (Nadkarni, Lobo *et al*. 1982). The sugar phosphate binds exclusively on the β-subunit (Laurent and Yon 1989).

1.1.1.3. Pyruvate Kinase

Pyruvate kinase (PYK) catalyzes the final step in glycolysis, producing the second of two ATP molecules generated in the glycolytic pathway. The enzyme converts phosphoenolpyruvate and ADP to pyruvate and ATP.

$$\text{Phosphoenolpyruvate} + H^+ + \text{ADP} \xrightarrow[K^+]{Mg^{++}} \text{pyruvate} + \text{ATP}$$

This reaction is committed step leading to either anaerobic fermentation or oxidative phosphorylation of pyruvate. In most cells the reaction is essentially irreversible and is one of the major control points of glycolysis. The regulation of PYK is important for controlling the levels of ATP and GTP and glycolytic intermediates in the cell. Pyruvate kinase also serves as a switch between the glycolytic and gluconeogenic pathways in certain tissues (Jurica, Mesecar *et al.* 1998).

Allosteric regulation of enzyme activity is a mechanism for finely tuning biochemical reaction pathways in order to maintain an appropriate balance of intracellular substrate and product concentration. Allosteric processes also allow an enzyme activity to be coordinated with other cellular reactions and signaling pathways. As the metabolic requirements of an

organism fluctuate over time and with cell type, many organisms produce related but differentially regulated form of the same enzyme in different tissues to satisfy disparate metabolic and growth patterns (Jurica, Mesecar *et al.* 1998).

Regulation of the pyruvate kinase is important according to the availability of different carbon sources and oxygen supply. Under the conditions of excess amounts of glucose, the activity of pyruvate kinase should be high enough to provide sufficient ATP and pyruvate. On the other hand under gluconeogenic conditions, inhibition of pyruvate kinase-catalyzed reaction is necessary in order to avoid a futile cycle via pyruvate carboxylase and phosphoenolpyruvate carboxykinase mediated steps(Zimmermann and K.D.Entian 1997). Pyruvate is regulated by fructose-1,6-bisphosphate, ATP, ADP , citric acid, and phosphoenolpyruvate (Barwell, Woodward *et al*. 1971; Barwell and Hess 1972). When the extracellular glucose is high, the intracellular content of phosphoglyceric acids and phosphoenolpyruvate decrease. As extracellular glucose is depleted and glycolytic flux decrease, the level of these intermediates increase, indicating a regulatory change in the activity of pyruvate kinase. Measurement of intracellular content showed that there was a direct relationship between pyruvate kinase and fructose-1,6-bisphosphate. Therefore it has been concluded that the positive control of pyruvate kinase by fructose-1,6 –bisphosphate, the intracellular concentration of which is low during growth on ethanol and high during growth on glucose, provides the switching mechanism between glycolysis and gluconeogenesis. Low amount of fructose-1,6-bisphosphate will block the pyruvate kinase reaction and spare enough amounts of phosphoenolpyruvate to support gluconeogenic flux (Zimmermann and K.D.Entian 1997)

1.1.2. Hexose Transport

Availability of sugars; hexoses, pentoses and disaccharides might also play role in the regulation of glycolysis. Thus transport of these sugars might be regulatory to some extent.

In *S. cerevisiae*, hexoses are transported by facilitated diffusion by more than 20 different hexose transporters (HXT1 to 17 GAL2, SNF3 and RGT2) (Ozcan and Johnston 1999) which display distinct affinities for glucose and are all members of the major facilitator. In a null strain lacking all known hexose transporters, glucose consumption and transport activity were completely abolished (Elbing, Larsson *et al.* 2004). The transporters HXT1 and HXT4 plus HXT6 and HXT7 are most important for the uptake of glucose. In addition HXT5, HXT8 to 11, HXT13 to 17, GAL2 and the maltose transporters AGT1, YDT247w and YJR160c are all the able to transport glucose when ectopically produced, individually support growth in a strain lacking all other transporters (cited in Elbing, Larsson *et al*. 2004).

Hexose transporters are regulated by different regulatory proteins. For example HXT2 and HXT4 are high affinity transporters and are regulated by two independent repression mechanisms. Rgt1 repressor which is a C6 zinc finger DNA binding protein and it binds to the promoters of the *hxt2* and *hxt4* genes and repress their transcription in the absence of glucose. At high concentrations of glucose their expression is repressed by Mig1 repressor, which is responsible for expression of many glucose repressed genes such as *suc2* and *gal2* (cited in Ozcan and Johnston 1999). Only at low concentrations of glucose (~0.05 to 0.4 %) both repressor proteins are inactive, resulting in the expression of *hxt2* and *hxt4*. When *rgt* is deleted *hxt2* and *hxt4* are expressed in the absence of glucose, but has no effect on Mig1-mediated repression of these genes at high glucose concentrations. Conversely deletion of *mig1* causes expression of *hxt2* and *hxt4* become inducible but has no effect on Rgt1-mediated repression in the absence of glucose therefore the

combination of different repression mechanisms result in an 8-10 fold induction of *hxt2* and *hxt4* expression only by low level of glucose.

Expression of *hxt6* only modestly induced by glucose, that is *hxt6* and *hxt7* have higher basal level expression than *hxt1* to *hxt4* (cited in Ozcan and Johnston 1999). Glucose sensor protein Snf3 has a negative effect on the expression of *hxt6*. At the low concentrations of glucose Snf3 has also positive role in expression *hxt6.*

The expression of *hxt1* low affinity transporter is repressed by the Rgt1 in the absence of glucose. Rgt 1 activates transcription t high glucose concentrations.

The expression of *hxt3* gene is induced both low and high glucose concentrations. Because Rgt1 repressor is inactivated by Grr1 and so *hxt3* is activated. It is suggested that the modest increase in *hxt3* expression caused by inactivation of various genes (*mig1 ssn6* and *tup1*) involved in glucose repression so glucose repression play a minor role in regulating *hxt3* expression (cited in Ozcan and Johnston 1999) This modest repression may be mediated by Mig1 since *hxt3* promoter contains several potential binding sites for Mig1 (Ozcan and Johnston 1999).

It was claimed by Ozcan *et al. hxt3* is maximally induced when the extracellular concentration reaches the optimal so it is suggested that *hxt3* is repressed by the initial high levels of glucose (Ozcan and Johnston 1999).

Overexpression of *hxt8-17* (except *hxt12*) individually in an *hxt1-17 gal2* deletion strain in the CEN.PK background restored growth on glucose, indicating that Hxt8-11p and Hxt13-17p are also able to transport glucose (Wieczorke, Krampe et al. 1999), but expression is low under normal growth conditions (Ozcan and Johnston 1999). Recently it has been shown that Hxt5p also has glucose transporter capacity (Diderich, Schuurmans *et al.* 2001). It was shown that in glucose-grown batch cultures, *hxt5* was expressed prior to glucose depletion. Verwaal *et al.* claimed independent of the carbon source used in batch cultures, *hxt5* was expressed after 24 h of growth and during growth on ethanol or glycerol, which indicates that growth on glucose was not

necessary for expression of *hxt5*. Increasing the temperature or osmolarity of the growth medium also induced expression of *hxt5*. In fed-batch cultures, expression of *hxt5* was only observed at low consumption rates, independent of the extracellular glucose concentration. Therefore it was implied that an increase of *hxt5* is accompanied by a decrease of growth rate of cells also their study showed that *hxt5* is regulated by growth rates of cells rather than by extracellular glucose concentrations (Verwaal, Paalman *et al*. 2002).

The galactose permease (Gal2) is more than 60% identical to the Hxt proteins and thus belong to the Hxt protein family *gal2* repressed at high concentration of glucose (mediated byMig1) (Ozcan and Johnston 1999). Hexose transporters are the important in industrial application since they regulate the movement of glucose into the cell.

A recent study which was done on hexose transporter null mutant yeast is a good example of potential effect of hexose transport in the fate of glucose. Elbing *et al.* developed a strain in which *hxt* s have been deleted and which therefore does not take up any glucose and glucose is carried by manipulating hexose uptake system. It was developed a series of strains which had chimeric hexose transportes (HXT1 and HXT7). The strains displayed a range of glycolytic rates resulting in proportional decrease in ethanol production. Using this strain it was shown that for the first time at high glucose levels, the glucose uptake capacity of wild type *S. cerevisiae* did not control the glycolytic flux during the exponential batch growth. However the chimeric HXT transporters controlled the rate of glycolysis to a high degree. Strains whose glucose uptake was mediated by these chimeric transporter undoubtedly provide a powerful tool with which to understand the mechanism underlying switch between the fermentation and respiration in *S. cerevisiae* and will provide new tools for the control of industrial fermentations (Elbing, Larsson *et al*. 2004; Otterstedt, Larsson *et al*. 2004).

1.1.3. Global Regulation

Control of glycolysis can not be attributed to a single regulatory mechanism. Rather all the enzymes of glycolytic pathway contribute to the regulation to an extent as well as there is regulation at genetic level by GCR proteins.

Hauf *et al.* claimed that especially enzymes of the lower part of the glycolysis are regulated at the transcriptional level by glucose induction, suggesting an important role for those enzymes in determination of the glycolytic flux. There were no increase in the glycolytic flux when the overexpression of *tdh3* (glyceraldehde-3-phosphate dehydrogenase), *gpm1* (phsophoglycerate mutase), *pgk1* (phosphoglycerate kinase), *eno2* (Enolase), *pyk1* (pyruvate kinase), *pdc1* (pyruvatedecarboxylase) were performed and also other glycolytic enzymes were overexpressed like phosphfructo-1-kinase genes and activator of this enzyme fructose-2,6-bisphosphate did not show increase in glycolytic rates. These results suggested that glycolytic flux regulation is distributed among all the enzymes (Hauf, Zimmermann *et al.* 2000).

The other important regulatory mechanism at genetical level for glycolysis is regulation of glycolytic genes by Gcr1 (glycolysis regulation-1) and Rap1. These two DNA binding proteins coordinately regulate the expression of the glycolytic genes (cited in Hauf, Zimmermann *et al*. 2000). Rap1 is capable of carrying out many diverse cellular functions, such as activation and repression of transcription depending on the sequence context of binding site, it has been suggested that its function may be determined by interaction of other regulatory proteins (Baker 1991). The most likely role of Rap1 at glycolytic promoters is to facilitate the binding of Gcr1p (cited in Haw, Yarragudi *et al.* 2001).

It was shown that there were physical interactions between Gcr2p and Gcr1p and postulated that Gcr2p is a co-activator in the Gcr1p-Gcr2p complex (cited in Haw, Yarragudi *et al*. 2001). Consistent with this view similar

profiles of reduction of the levels of most glycolytic enzymes were observed in gcr1 and gcr2 mutants (Haw, Yarragudi *et al.* 2001).

1.2. The Enzyme: Hexokinase

Hexokinase (EC. 2.7.1.1) catalyzes the first step of glycolysis in which one phosphoryl group from ATP to glucose is transferred to form glucose-6-phosphate.

Hexokinase is a relatively non specific enzyme contained in all cells that catalyzes the phosphorylation of hexoses such as D-glucose, D-mannose, and D-fructose. Some organisms also contain e.g.: *Saccharomyces cerevisiae* (Maitra 1970) and *Aspergillus niger* glucokinases (Panneman, Ruijter *et al*. 1996), which catalyzes the same reaction. The second substrate for hexokinase, as with other kinases is an Mg^{+2}-ATP complex. In fact uncomplexed ATP is a potent competitive inhibitor of hexokinase (Voet and Voet 1995).

Yeast hexokinase has an ordered Bi Bi mechanism with 10 % probability of random has been postulated as reaction mechanism hexokinase PI and hexokinase PII (cited in Zimmermann and K.D.Entian 1997).

1.2.1. Substrate Specificity

Thomas' s X ray (Bennett and Steitz 1978) structure studies showed that hexokinase and glucose-hexokinase complex different from each other. Comparison of the high resolution structures indicated that one lobe of the molecule is rotated by 12° relative to the other lobe, resulting in movement of as much as 8A° in the polypeptide backbone closing the gap between the lobes into which glucose is bound, the conformational change is produced by binding of glucose (cited in Bennett and Steitz 1978) and essential for the catalysis and thus provide an example of induced-fit. This movement places the ATP in close proximity to the $-C6H_2OH$ group of glucose and excludes

water from active site. If the catalytic and reacting groups were in the proper position for reaction while the enzyme was in the open position, ATP hydrolysis would almost certainly be the dominant reaction. This conclusion is confirmed by the observation that xylose (Figure 1.3) which differs from the glucose by the lack of –$C6H_2OH$ group, greatly enhances the rate of ATP hydrolysis by hexokinase (presumably xylose induces the activating conformational change while water occupies the binding site of the missing hydroxymethyl group). Clearly this substrate-induced conformational change in hexokinase is responsible for enzyme's specificity. In addition to active site polarity is reduced by exclusion of water, thereby expediting the nucleophilic reaction process (Voet and Voet 1995). Also the sugar substrate specificity of yeast glucokinase is broader than that of Aerobacter or hepatic enzymes in that it phosphorylates mannose, 2-deoxyglucose, and glucosamine (Maitra 1970). Figure 1.3 shows the substrates for hexokinase and glucokinase.

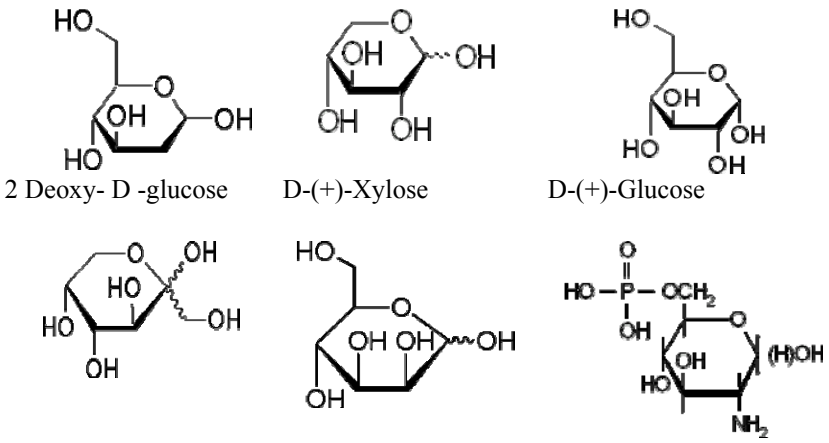


Figure 1.3: Substrates for hexokinases and glucokinases

1.2.2. The Isoenzymes of Hexokinase

Hexokinases from a wide variety of organisms have been cloned and/or characterized. Although the eukaryotic hexokinase and glucokinase genes cloned so far appear to have arisen from a common ancestor, the kinetic and regulatory properties of the corresponding proteins vary considerably. Three isoenzymes with different substrate specifities have been identified in *Saccharomyces cerevisiae* which are hexokinase PI (also referred to hexokinase A) and hexokinase PII (also referred to hexokinase B) and glucokinase. These enzymes differ from one another in their activity with various hexoses. Both hexokinase PI and PII can use range of hexose substrates and can be distinguished from one another by their activity differences in their electrophoretic mobility and serologically distinct. Yeast glucokinase on the other hand shows rather narrow substrate preference, activities highest with glucose and mannose. Both yeast hexokinase PI and PII can exist as dimmers, although both readily dissociate to monomers of 50 kDa by addition of glucose or by raising the pH or the ionic strength. Yeast glucokinase appears to exist only as a monomer and has a slightly higher molecular mass (51 kDa) (Maitra 1970), *Aspergillus niger* has one hexokinase and one glucokinase (Panneman, Ruijter *et al*. 1996; Panneman, Ruijter *et al.* 1998). There are four hexokinase isoenzymes (hexokinase I-IV) in mammalian cells; hexokinase I, II, III have molecular weights of approximately 100 kDa, and sequence data showed that tandem duplication is responsible for the higher molecular weight as compared to yeast hexokinase (Zimmermann and K.D.Entian 1997). The fourth hexokinase IV is known also as glucokinase having a molecular weight of 50 kDa (Zimmermann and K.D.Entian 1997). In contrast to yeast glucokinase liver glucokinase has a very low affinity for glucose and is mainly active in liver to remove exceeding glucose concentrations from the bloodstream. Additionally, mammalian glucokinase also accepts fructose as substrate whereas yeast glucokinase cannot phosphorylate fructose (cited in Albig and Entian 1988).

1.2.2.1: Purification of Hexokinases and Glucokinases of Different Organisms

For purification of hexokinase or glucokinase from different organisms basic chromatographic techniques like anion exchange chromatography (DEAE cellulose Mono Q), gel filtration chromatography (Sephadex and Superose varities) dye affinity chromatography (various dyes immobilized on Sepharose CL-4B), affinity chromatography (glucoseamine immobolised on Sepharose CL-6B) were used ((Panneman, Ruijter *et al.* 1996; Panneman, Ruijter *et al*. 1998; Caceres, Portillo *et al.* 2003); (Karp, Jarviste *et a*l. 2003).

For a short survey of different organisms of hexokinase studies the kinetic characteristic and isoenzyme pattern are given in Table 1.1.

Table 1.1: The characteristic of hexokinase from different organisms

The name of the organism	Isoenzymes	Monomer molecular weight (kDa)	Inhibitor	K_m	References
Aspergillus niger	Hexokinase	54.1 kDa	Trehalose-6-phosphate	0.35 mM glucose 2 mM fructose	(Panneman, Ruijter *et al.* 1998).
	Glucokinase	54.5		0.063 mM glucose 120 mM fructose	
Scwaniomyces occidentalis	Hexokinase	N.D.	N.D	1 mM for glucose 9.3 mM fructose	(Rose 1995)
	Glucokinase	N.D.	N.D	N.D	
Hansenula polymorpha	Hexokinase	49 kDa -79 kDa	Trehalsoe-6-phopsphate and ATP	0.26 mM for glucose, 1.1 mM fructose and 0.32 ATP	(Karp, Jarviste *et al.* 2003)
	Glucokinase	51.6 kDa	N.D.		(Laht, Karp *et al.* 2002)
Kluyveromyces lactis	Hexokinase	53 kDa	N.D.	0.196± 0.017 (monomer) 0.451 ±0.049 (homodimer)	(Bar, Golbik *et al.* 2003)
Tuber borchii vittad	Hexokinases (three distinct forms)	57.64 kDa	N.D.	0.069 D-glucose 0.87 D-fructose 0.28 D-mannose 1.3 D-glucoseamine	(Ceccaroli, Saltarelli *et al.* 2001)

The regulatory properties of the hexose-phosphorylating enzymes vary amongst species. The mammalian hexokinase I-III are mainly controlled by allosteric inhibition by reaction product glucose-6-phosphate. In contrast no physiological control of glucose-6-phosphate is observed for the mammalian hexokinase IV and yeast glucokinase and hexokinase PI (A) and PII (B). Instead *Saccharomyces cerevisiae* hexokinase PII, however, is inhibited by trehalose-6-phosphate (Eastmond and Graham 2003). It has been suggested

that trehalose -6-phosphate plays an important role in the regulation of glycolytic flux in *S. cerevisiae* (Zimmermann and K.D.Entian 1997).

1.2.3. Regulation of Glycolysis by Hexokinase

Hexokinase not only catalyzes the ATP dependent phosphorylation of glucose but also senses glucose levels and phosphorylation status of glucose, transmitting this information to the nucleus through signal transduction pathway. For example in higher organisms such as yeast isoenzymes interact with the other proteins and with cellular membranes. In addition to their cytosolic localization, hexokinase isoforms are found associated with membrane of endoplasmic reticulum and plasma membrane (cited in Frommer, Schulze *et al.* 2003). In plants hexokinases are associated with chloroplast outer envelope, where they might help in glucose export by phosphorylating glucose before it enters the cytosol. Mammalian glucokinase is associated with the actin cytoskeleton but, depending on the physiological conditions it can move to nucleus and alter gene expression (cited in Frommer, Schulze *et al*. 2003). The marked differences in subcellular localization of hexokinase reflect the many activities and suggest that this enzymes behaves as jack of all trade (Frommer, Schulze *et al.* 2003)

Hexokinase also is an indirect sensor of glucose. True sensor would undergo a conformational change in response to glucose that alter the signaling, leading to a change in gene expression. This idea is strongly supported by Moreno *et al* in terms of some isoforms which is direct glucose sensors (Rolland, Winderickx *et al*. 2001; Moreno, Ahuatzi *et al*. 2005).

As it was reviewed in Rolland *et al.* (Rolland, Winderickx *et al*. 2001) different eukaryotic cell use specific mechanisms to sense in the presence of glucose. These mechanisms include glucose phosphorylating enzyme (hexokinase2) glucose carrier homologue (Snf3 and Rgt2), glucose receptor (Gpr1a G protein couple receptor) (Rolland, Winderickx *et al*. 2001).

Hexokinase has important role for the glucose repression besides the glucose sensing. Especially hexokinase PII trigger glucose repression due the presence of a specific regulatory domain in this protein. It is assumed that in the process of glucose binding and nuclear phosphorylation a conformational change of hexokinase protein takes place that triggers repression directly or through interaction with other proteins. This possibility is supported by following data : (i) hexokinase PII mutants with unchanged catalytic activity but defective in glucose repression have been described; (ii) *S. cerevisiae* glucokinase cannot trigger glucose repression even if overexpreesed; (iii) hexokinase PII protein has been found in the nucleus of glucose-grown *S. cerevisiae* cells and nuclear localization was shown to be crucial for glucose repression (iv) expression of heterologous hexokinases in *S. cerevisiae* hexokinase deficient mutant always restores sugar-phosphorylating activity; but glucose repression is restored only in some cases (cited in Kramarenko, Karp *et al*. 2000).

In the glucose repression in *S .cerevisiae* Hxk2 (hexokinase 2) and Mig1 are the major factors. Figure 1.4 explains that the role of the Hxk2 and Mig1 complex to the carbon catabolite repression schematically.

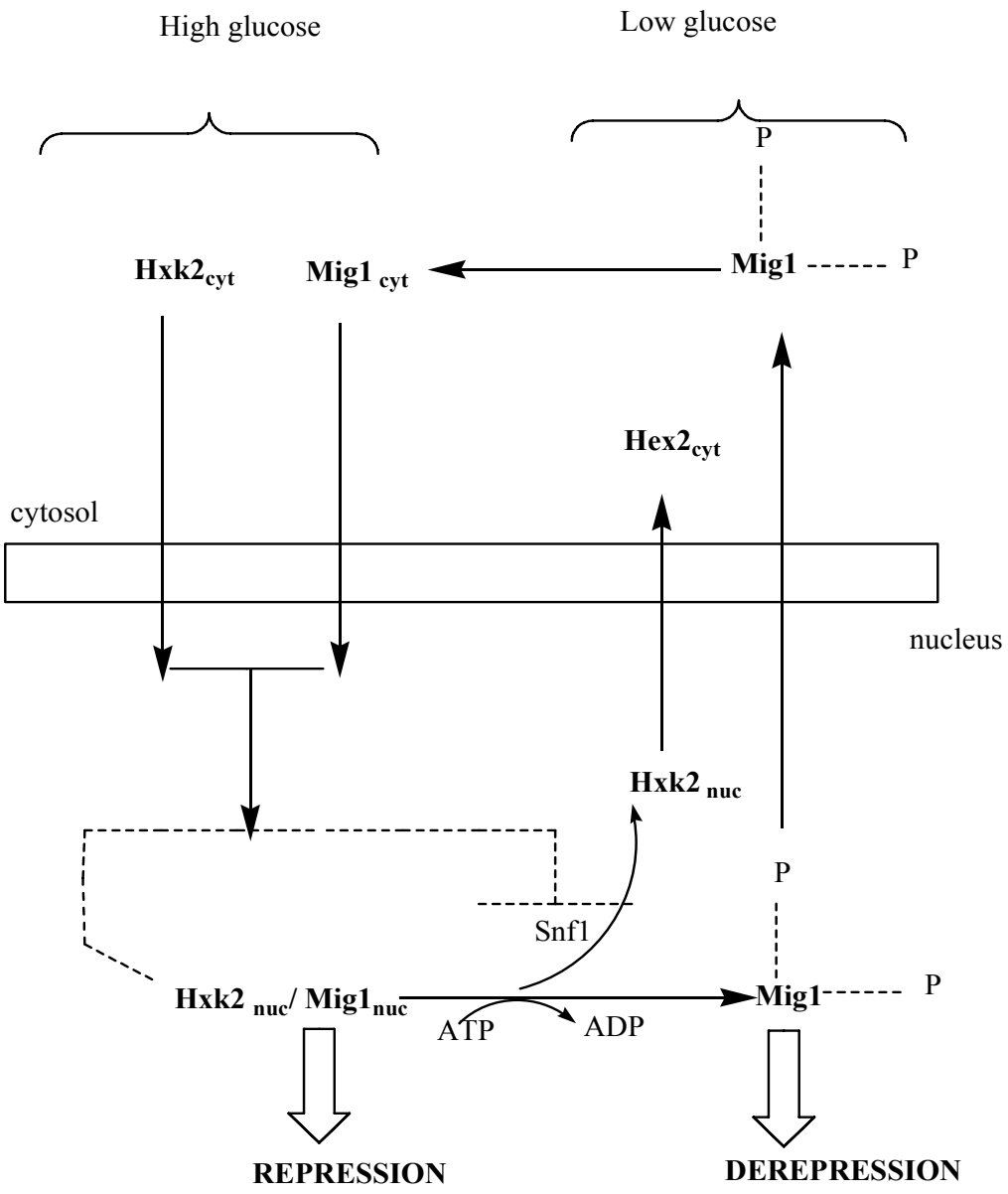


Figure 1.4: Involvement of Mig1 in the subcellular localization of Hxk2 (Moreno, Ahuatzi *et al.* 2005).

As seen in the Figure 1.4 at high glucose concentrations Mig1 is dephosphorylated by the Glc7 phosphatase complex and imported to the nucleus. At the same time Hxk2 enters the nucleus and there it is sequestered

by interacting with Mig1. The amount of Hxk2 present in the nucleus is limited in Mig1 levels of the cell. Once access to the nucleus has been gained the heterodimeric Hxk2-Mig1 complex binds to Mig1 target gene promoters and recruits the general co repressor complex Cyc8-Tup1 to repress the transcription of genes not required for growth in glucose.

Upon glucose depletion, Snf1 protein kinase is activated and phosphorylates Mig1. Phosphorylation induces the Mig1 nuclear export; sequestering the protein in the cytoplasm together with *hxk2* genes needed for growth on other carbon sources different from glucose are thus derepressed and *hxk2* mutation also activates Snf1 complex and therefore Mig1 is phosphorylated and translocated to the cytosol, signaling depression of Mig1 target genes. Thus in the absence of Hxk2, the Snf1 protein kinase complex is active even in the presence of high levels of glucose in the medium. One major function of Hxk2 may be to inhibit Snf1 protein kinase function by blocking Mig1 phosphorylation at the nuclear level (Moreno, Ahuatzi *et al.* 2005).

In yeast another important regulation of glycolysis is the inhibition of Hxk2 by Trehalose-6-phosphate formed from glucose-6-phosphate and uridine-5-diphoshoglucose by trehalose-6-phsophate synthase (TPS). Trehalose-6-phosphate can then be phosphorylated to trehalose by trehalose-6-phophate phosphatase (TPP). Trehalose is a nonreducing disaccharide sugar and it functions as storage carbohydrate and can protect against cellular damage caused by various stress in invertebrates and fungi (Eastmond and Graham 2003).

Some studies which is related to the hexokinase and glucose repression was performed in yeasts other than S. *cerevisiae* for example the yeast *Yarrowia lipolytica* produces extracellular lipase encoded by the *lip2* gene. In the wild type strain CBS6303, the production of lipase is stimulated by long chain fatty acids, whereas in the presence of glucose as the sole carbon source, extracellular lipase activity is detected relatively and only after depletion of this substrate in culture broth (cited in Fickers, Nicaud *et al*. 2005), (cited in

Petit and Gancedo 1999). The unique hexokinase encoded by the *hxk1* gene and its deletion leads to doubling time 15 % longer than the wild type strain on glucose media while no growth could be observed on fructose media. Moreover, expression of *Y. lipolylitica hxk1* gene in a *S. cerevisiae* mutant strain lacking all endogenous phosphorylating activities can restore catabolite repression of genes such as invertase. The involvement of hexokinase Hxk1 in glucose catabolite repression of *lip2* was investigated in a lipase overproducing mutant less sensitive to glucose repression. This mutant has a reduced capacity to phosphorylate hexose compared with the wild type strain, but no differences could be observed between the *hxk* sequences in the two isolates. This suggested that the reduced phosphorylating activity of the mutant strain probably resulted from a modification level of *hx1* gene in this mutant led to a decrease of both *lip2* induction and extracellular lipase activity, suggesting that hexokinase is involved in catabolite repression of *lip2* in *Y. lipolylitica.* (Fickers, Nicaud *et al.* 2005)

In *Hansenula polymorpha* a methylotrophic yeast glucokinase is sufficient in the cell for establishment of glucose repression. In methanol-grown cells enzymes executing methanol oxidation are very highly expressed and constitute majority of the cell protein. If glucose as the preferred carbon source is added to methanol-grown cells, synthesis of methanol specific enzymes has to be stopped rapidly, and enzymatic machinery adjusted to glucose utilization. Glucokinase is the main glucose-phosphorylating enzyme in methanol-grown *H. polymorpha* and it can contribute to the rapid initiation of glucose repression during switch from methylotrophic to glycolytic growth. (Laht, Karp *et al.* 2002).

Hexose phosphorylation was studied in *Aspergillus nidulans* wild type and fructose non-utilising mutant *fr*A1. In the wild type *A. nidulans* has at least one hexokinase and glucokinase, while the *fr*A1mutant lacks hexokinase activity. The *A. nidulans* gene encoding hexokinase was isolated by complementation of the *fr*A1 mutation. The absence of hexokinase activity in *fr*A1 did not interfere with glucose repression of the enzymes involved in

alcohol and L-arabinose. It is suggested that hexokinase does not mediate glucose repression of these enzymes which is involved in alcohol and L-arabinose metabolism (Ruijter, Panneman *et al*. 1996).

There are also contradictory studies like the one on *Candida utilis.* In this study they showed that high hexokinase activity was not related to glucose repression in *Candida utilis* IGC3092. Espinel, *et al.* claimed that in contrast to *S. cerevisiae*, low hexokinase activity did not enough to relieve glucose repression and high hexokinase activity did not trigger repression. (Espinel, GomezToribio *et al.* 1996)

1.3. The organism: *Rhizopus oryzae*

In this study *Rhizopus oryzae* which is a filamentous fungus was used. The fungi are important as primary agents of decay in the cycling of carbon, nitrogen and other nutrients in biosphere in the deterioration of useful materials and products. However they cause serious disease in plants and animals; including human beings, not only by their direct attack and invasion, but also indirectly by the secretion of toxins. Their abilities to synthesize many strange and wonderful compounds significant to us are not limited to deleterious activities, but include the production of many materials important to the food, drug, and chemical industries (Griffin D.H. 1994). For example *Rhizopus oryzae* and *Aspergillus niger* are important industrially organisms. *R. oryzae* produces L(+)-lactic acid and *A. niger* is used in a variety of industrially processes for the production of primary metabolites. Two groups of primary metabolites are readily overproduced and secreted: organic acids and polyols (Panneman, Ruijter *et al*. 1998). Truffles are ectomycorrhizal fungi that form long-living symbiosis with roots of higher plants. They are products of great economic value and are well known for the organoleptic properties of their hypogenous fruitbodies. Ectomycorrizal fungi have received a great deal of attention in recent years and have been used world-wide in reforestation, since they can increase the growth and water nutrient

absorption of host trees and provide protection against root diseases (Agostini, Polidori *et al.* 2001).

1.3.1. Classification of *Rhizopus oryzae*

Rhizopus oryzae has full lineage given as Eukaryote; Fungi /Meteozoa group; Fungi; Zygomycota; Zygomyctes; Mucorales; Mucoraceae; *Rhizopus* (www.ncibi. nlm.nih.gov). It has many synonyms *R .trictic, R thermosus, R. tamari, R. suinus, R. peka, R. hangchow, R. formosaensis, R. formosaensis* var. *chylamydorups, R delemar, R. chinian,R. arrhizus, R. liguefaciens, R. pseudochinesis* (www.ncibi. nlm.nih.gov). The group in which *R. oryzae* belongs to, Zygomycetes, is named after the zygospores which are formed during the sexual cycle of organisms. Zygospore are formed by a process initiated by fusion of two opposing strains of different mating type [called (+) and (-)] (Elliot 1994). Asexual reproduction is characterized by sporangia in which non-motile spores (sporangiospores) are produced (Elliot 1994)] another characteristic of this group is that organisms are coenocytic which means that they lack regular septa formation in their hyphae. This is one of the reasons why they are classified as lower fungi.

1.3.2. *Rhizopus oryzae* in Biotechnology

Rhizopus oryzae is an obligate aerobe and produces optically pure L-(+)-lactic acid. The majority of lactic acid is used in food industry and food related application, which in the U.S., accounts for approximately 85% of the demand. The rest (~15%) of the uses are for non-food industrial applications. As a food acidulant, lactic acid has a mild acidic taste in contrast to other food acids. Lactic acid is nonvolatile, odorless and is classified as GRAS (generally recognized as safe) for use as a general purpose food additive by FDA in the U.S. other regulatory agencies elsewhere. It is a preservative and pickling agent for sauerkraut, olives, and pickled vegetables. It is used as acidulant / flavoring /pH buffering agent or inhibitor of bacterial spoilage in wide variety processed

foods, such as candy, breads, bakery products, soft drinks, soups, sherbets, dairy products, beer, jams, and jellies mayonnaise (Datta, Tsai et al. 1995). In addition to these its use in the form of nonchlorinated solvent, ethyl lactate and biodegradable plastic poly-lactic acid gives it potential to increase in the amount of industrial production. Lactic acid is also produced by lactic acid bacteria mainly lactobacillus species which have at least 95 % yield on the basis of glucose converted into lactic acid. Lactic acid bacteria produce both L(+)-lactic acid and D(-)-lactic acid form. *Rhizopus oryzae* produces only L form of lactic acid. L(+)-lactic acid is preferred form of lactic since D(-)-lactic acid can not be metabolized in the human body. Another advantage over lactic acid bacteria fermentations is that *R. oryzae* can grow in minimal medium which lowers the costs as well as it makes the downstream processing of lactic acid easier. *Rhizopus oryzae* produces mainly lactic acid from glucose with yields of 60-80% and also ethanol, carbon dioxide and minor amounts of malic acid, fumaric acid and citric acid (Maas, Bakker *et al.* 2006).

1.4.Aim of the Study

Glycolysis and its regulation are important for the end product formation. These end products, like lactic acid produced by *R .oryzae*, are industrially important. The yield of an industrial process should be high for the feasibility of the production of a particular product. The lactic acid yield of *R. oryzae* is low (~70 %) compared to lactic acid bacteria (>95 %) (Longacre, Reimers *et al.* 1997; Skory 2004) still it is noteworthy that *R. oryzae* only produces L-(+) form of lactic acid. If one can find a way to increase the flux through the glycolysis the yield of lactic acid may increase as well. Keeping this in mind we wanted to focus on the first step of glycolysis, hexokinase of *R. oryzae*. We have purified and biochemically and kinetically characterized two isoenzymes of *R. oryzae* for the first time in this study.

CHAPTER 2

MATERIALS AND METHODS

2.1. Material

2.1.1. The Microorganism

In the present study *Rhizopus oryzae* ATCC 9363 was used. It was purchased from American Type Culture Collection.

2.1.2 The Chemicals

NADP was purchased from Roche-Boehringer Mannheim (10 128 040 001), glucose-6-phosphate dehydrogenase (Fluka 49271), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M-2128), phenazine methosulfate (PMS; P-9625) was supplied from Sigma. The chemicals used in all experiments were all of analytical grade and commercially available from Sigma, Aldrich, Merck, Roche, Fluka, Riedel de Haen, and Lab M.

2.2. Methods

2.2.1. Purification of Hexokinase from *Rhizopus oryzae*

2.2.1.1. The Growth of *Rhizopus oryzae*

Rhizopus oryzae was sporulated on plates containing potato dextrose agar (PDA) for 4-5 days at 30 °C. For the inoculation of the liquid medium (10^6 spores/ml) spore suspension was used. The spore suspension was prepared in sterile distilled water aseptically and spores were counted on Thoma type hemocytometer to determine the spore concentration (Appendix B). For shake flask cultures salt medium (Appendix A) containing 15 mM $(NH_4)_2SO_4$), 5 mM (K_2HPO_4), 1 mM ($MgSO_4$,) 0.2µM ($ZnSO_4$), 0.8 % $CaCO_3$ and 111 mM D(+)-glucose and 111 mM D(-)fructose were prepared. The media were prepared as 70 ml of liquid medium in each 250 ml Erlenmeyer flask. The medium including minerals and carbon sources were sterilized by autoclaving at 121°C for 15 minutes. The liquid media were inoculated with spores at a final concentration of and/or and were incubated at 30°C and 175 rpm for 18 hours and/or 24 hours in shaker incubator (Infors). The mycelium was harvested by filtration through nylon gauze and washed with extraction buffer 0.1 M sodium phosphate containing 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTE pH 7 and it was dried between layers of filter paper and frozen in liquid nitrogen. The frozen mycelia was either grinded immediately by micro-dismembrator (Sartorius Microdismembrator U) using pre-cooled (in liquid nitrogen) 20 ml Teflon containers of the apparatus or it was stored at -80°C for further use.

2.2.1.2. Extraction of Proteins from *Rhizopus oryzae*

One gram of frozen powdered mycelium was suspended in 10 ml 0.1 M sodium phosphate buffer pH 7.0 containing 5 mM $MgCl_2$ 1 mM DTE, 0.5

mM EDTA and 50 µl protease inhibitor cocktail. The suspension is mixed well using of a glass rod to make a homogenous mixture. It was centrifuged at 10000 rpm (Sigma centrifuge and the rotor code: 12159) for 30 minutes at +4°C. The pellet was discarded and the supernatant was called crude extract. Crude extract was used for further purification studies. All the subsequent purification steps were performed a t +4°C.

2.2.1.3. Protein Determination

Protein content of the crude extract was determined according to Bradford (Bradford 1976) using bovine serum albumin (BSA) (1mg/ml) as standard. The composition of the reagents and preparation of the standard curve is given in Appendix C. (All experiments were carried out in three parallel runs).

2.2.1.4. Gel Filtration and Anion Exchange Column Chromatography

The Fast Performance Liquid chromatography (FPLC) system (Varian Prostar) in Central Laboratory in Middle East Technical University (www.centrallab.metu.edu.tr) was used for all chromatographic separations.

The first step of purification was gel filtration. The column used was HiLoad^{TM} 16/60 which is a pre-packed column supplied from Amersham Life Sciences. The column material was Superdex 200 with a molecular sieve of 10,000-600,000. The detailed information about to characteristic of column is given in the Appendix K.

The column was equilibrated with column buffer which is 10 mM BisTrisHCl, pH 7 containing 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM DTE and 0.5 mM EDTA 8.5 ml crude extract contained 17.43 mg protein was loaded to the column. The flow rate of the column was 30 ml/hour; fractions of 2.5 ml were collected and assayed for hexokinase activity. The fractions having hexokinase

activity was pooled and the total volume of these fractions was 20 ml (eight fractions).The pool was concentrated by Vivapore 5 concentrator (product no: VP0501) to 4.5 ml and it was stored at -20°C in 20 % of glycerol.

The pooled active fractions of was loaded onto anion exchange column The column was equilibrated with column buffer (10 mM BisTrisHCl buffer pH 7 containing 5 mM $MgCl_2$, 1 mM DTE, 0.5 mM EDTA). The flow rate was 150 ml per hour and each fraction volume was 3.5 ml. The gel filtration pools which were obtained from two separate runs were concentrated and a total of 8.5 ml sample contained 2.88 mg proteins was loaded to anion exchange column. Following the washing of the column with same buffer linear gradient of 0-0.5 M NaCl in a total volume of 400 ml was applied. Fractions having hexokinase activity were pooled separately as isoenzyme 1 and isoenzyme 2. The volume of isoenzyme 1 was 30 ml (9 fractions) and isoenzyme 2 was 26.5 ml (8 fractions). Isoenzyme 1 was concentrated to 10 ml and isenzyme 2 was concentrated to 8 ml by using Vivapore 10/20 (product no: VP2003). The protein content of isoenzyme1 was 0.036 mg/ml and the protein content of isoenzyme 2 was 0.021 mg/ml the enzymes were not pure exactly. Therefore the isoenzyme 1 and isoenzyme 2 was loaded to the DEAE column separately. The volume of isoenzyme 1 loaded was 8.5 ml and the total protein content was 0.30 mg. The volume of isoenzyme 2 loaded was 3.2 ml and the total protein content was 0.067 mg. For isoenzyme 1 fractions of 2.5 ml were collected, the total volume of fractions containing activity 42 ml and these fractions concentrated to 19 ml. The concentrated samples were stored as 20 % glycerol stocks at -20°C. For isoenzyme 2 fractions of 2.5 ml were collected the total volume of fractions containing activity 33 ml and these fractions concentrated to 18 ml

2.2.1.5. Enzyme Assay

Enzyme activity was assayed spectrophotometrically by following the NADPH formation at 340 nm. The cell of spectrophotometer was kept at constant temperature (30°C) by using a circulator water bath.

Absorbance change per minute at 340 nm($\Delta OD_{340)}$ was recorded and used for calculation of enzyme activity The unit of enzyme activity was calculated using extinction coefficient of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) according to the equation given below.

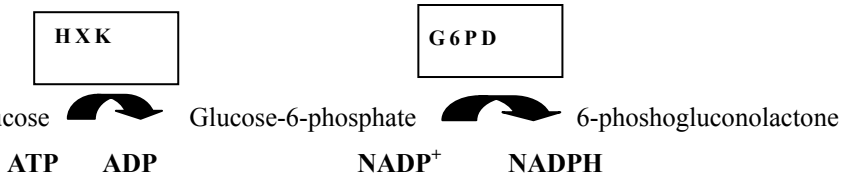
$$Enzyme\ activity\ (U/ml) \equiv \frac{\Delta OD_{340/\min}}{\varepsilon} \times dilution\ \ factor$$

One unit of enzyme activity is described as the amount of enzyme that catalyzes the conversion of 1 µmole of substrate per minute under assayed conditions. The specific activity of the enzyme is given as units per mg of protein**.**

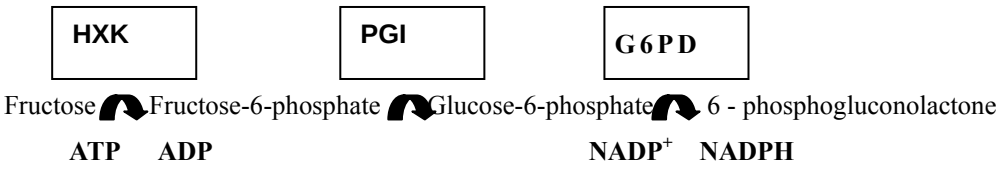
Hexokinase enzyme activity was measured by a coupled assay system taken from (Bergmeyer 1965) with some modifications.

The schematic representation of the coupled assay systems are given below:

The schematic representation for fructose phosphorylating activity



The schematic representation for fructose phosphorylating activity



In the assay glucose-6-phosphate formation was measured by coupling it with a further reaction in which glucose-6-phosphate is converted into 6-phophogluconate in the presence of glucose-6-phosphate dehydrogenase which simultaneously catalyzes the reduction of $NADP^+$ to NADPH. Formation of NADPH is measured spectrophotometrically at 340 nm.

Reaction mixture (1.2 ml) contained 100 mM Tris-HCl (buffer pH 7.70) containing 10 mM $MgCl_2$, 10 mM glucose, 5 mM ATP, 0.5 mM NADP, 1U/ml glucose-6-phosphate dehydrogenase. For fructose phosphorylating activity, 4 U/ml phoshoglucose isomerase and 10 mM fructose (instead of glucose) were used. The volume of hexokinase was 50 µl (1.78 mg/ml protein) in the reaction mixture buffer, distilled water and enzyme (enzyme was incubated for 5 minutes) were incubated at 30 °C before the reaction was started. The reaction was initiated by the addition of $NADP^+$.

The reaction rate was measured as the increase the absorbance of NADPH formed at 340 nm with 10 seconds intervals for 3 minutes in spectrophotometer (Hitachi U1800). The slope of this curve gives the reaction rate. One unit of hexokinase was defined as the amount of enzyme, which catalyzes the formation of 1 µmole glucose-6-phosphate per unit under assayed conditions.

2.3. Characterization of Hexokinase

2.3.1. Electrophoretic Analysis

The degree of purity of the enzyme at each step of purification was determined by SDS-PAGE and Native-PAGE analysis. BioRad Protean II xi Cell vertical slab electrophoresis system and power supply Wealtech Elite 300/300 plus were used.

SDS PAGE gels were prepared as 4 % stacking and 10 % separating (Laemmli 1970). The amount of protein loaded to SDS gel was 5-10 μg. (Appendix D). After electrophoresis SDS PAGE was visualized by silver staining.

Isoenzyme pattern was determined by running the samples obtained from different purification steps on 10 % native gels. The amount of enzyme loaded to each well of native gel was within the range of 0.003 U - 0.005 U (Appendix E). After electrophoresis the native gels were stained by activity staining

2.3.1.1. Silver Staining

The gels were silver stained using the procedure of (Blum, Beier et al. 1987). The reagents and their preparations and details of procedure were given appendix F. The procedure was composed of 6 steps: 1) Fixing 2) Washing by 50% ethanol 3) Pretreatment 4) Impregnate 5) Developing 6) Stop. After completion of procedure gel photos were taken by using Bio-Rad Gel Doc^{Tm}XR 170-8170 and imaging system and the molecular weight determination were done on these photos. (Appendix J)

The relative mobility (R_f) of each protein was determined by dividing its migration from the top of the separating gel to the center of the protein band by the migration distance of the tracking dye from the top of separating gel.

$$R_F = \frac{dis\tan ce\ migrated\ by\ protein}{dis\tan ce\ migrated\ by\ tracking\ dye}$$

The R_f values were plotted against known molecular weights and standard line was drawn and used for calculating the molecular weight of hexokinase

2.3.1.2 Activity Staining

The native gels were stained by using activity staining method (Manchenko 1994). The reagents and procedure were given in the Appendix G. All of the chemicals were dissolver in 100 mM Tris-HCl pH 7.70 .Agarose was prepared as described in the Reference G . Agarose gel and staining solution was mixed immediately pour on the gel surface. Incubate the gel in dark at 30°C until dark blue bans appear on the light background. The principle under laying the activity staining of hexokinase was given in the Appendix G.

2.3.1.3. Kinetic Analysis

For isoenzyme 1 and isoenzyme 2 K_m and V_{max} for both glucose and fructose were determined. For isoenzyme 1 glucose concentrations used were between 0.08 and 10 mM. For isoenzyme 2 glucose concentrations were between 0.25 mM and 10mM. While fructose concentrations used for isoenzyme 1 were between 2 mM and 50 mM for isoenzyme 2 fructose concentrations 10 mM and 150 mM were used. The velocity (v) versus substrate concentration (*S*) curves for hexokinase were determined using a coupled assay given above. The temperature and pH was kept constant at 30°C and 7.70 respectively. All measurements were done in triplicate, and the mean values were used for the final evaluation.

Data were analyzed by fitting them into Michealis-Menten Kinetics. The K_m values of the isoenzymes were calculated from the 1/ν versus 1/S (Lineweaver-Burk Plot).

The effect of trehalose-6-phosphate on the activity of isoenzyme 1 and isoenzyme 2 were investigated. The concentration of trehalose-6-phosphate was 1 mM and 4 mM. Commercial yeast hexokinase 7.5 U/ml was used to check the trehalose-6-phosphate inhibition. One way Anova method was used to evaluate the effect of trehalose-6-phosphate on hexokinase.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of Isoenzyme Pattern

In order to appreciate the significance of hexokinase in the glycolytic pathway various studies have been performed so far by using different organism. The studies showed that in some organism's hexokinase possesses isoenzymes. For example there are two hexokinases and one glucokinase in *Saccharomyces cerevisiae* (Zimmermann and K.D.Entian 1997) and a hexokinase and a glucokinase in *Aspergillus niger* (Panneman, Ruijter *et al.* 1998;Panneman, Ruijter *et al*.1996). Furthermore biochemical and genetical characterization of hexokinases were done from various organisms from ranging bacteria to mammalian cells (Magnusson, .Shelton. 1989; Imriskova, Langley *et al*. 2001). Considering the possibility of existence of hexokinase isoenzymes in *Rhizopus oryzae* number of experiments were performed.

Rhizopus oryzae was grown in two different media containing glucose or fructose as carbon sources. Crude extract was prepared as mentioned in materials and methods (section 2.2.1.). In order to prove the existence of hexokinase the native PAGE and activity staining studies were made for crude extracts. Crude extracts obtained from media containing 2 % of glucose crude G and crude extracts obtained from media containing 2 % of fructose crude F was loaded to the native gel and activity staining was used for staining the gel as mentioned in materials methods (section 2.3.1.2). Either glucose or fructose was used in the activity staining. The result of this study is shown in Figure 3.1.

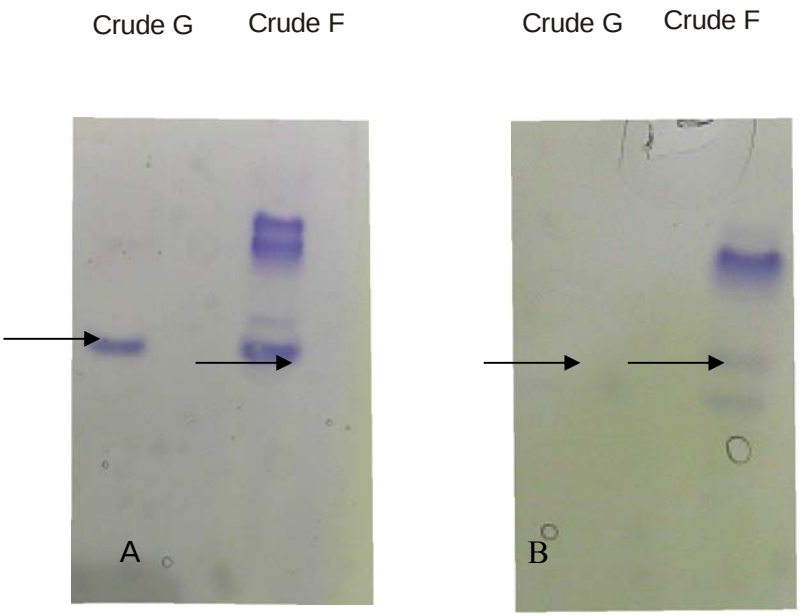


Figure 3.1: The Native PAG (10 %) and activity staining of crude extractCrude G: Extracts obtained from *R .oryzae* samples grown on 2 % glucose. Crude F: Extracts obtained from *R. oryzae* grown on 2 % fructose. In gel A glucose was used as substrate. In gel B fructose was used as substrate.

In Figure 3.1, the gel marked as 'A' was activity stained using glucose ad the gel marked as 'B' was activity stained using fructose as substrate. The arrows on the figure show the place of the band which is activity stained by using glucose but not stained by using fructose. This showed us that there must be at least two different enzymes one of which can not utilize or utilize fructose but very slowly. The activity stained banding pattern is different crude G and crude F which might have caused by loading different amounts of total protein into the wells. Another cause might be that of the isoenzymes might be present at different ratios in crude G and crude F.

In order to prove the presence of hexokinase isoenzymes of *R. oryzae* further experiments were performed. The two isoenzymes were purified and

separated from each other in a three steps purification protocol. The details of these experiments are given in the next part of this chapter.

3.2. The Effect of the Using the Different Carbon Sources

Rhizopus oryzae can utilize different carbon sources like glucose, fructose and mannose. In order to see whether isoenzyme pattern differs on different carbon sources we performed activity staining of crude extracts obtained from biomass grown on either 2 % glucose or 2 % fructose or 2 % mannose. The crude extracts were run on native gel activity stained by using glucose as substrate for activity staining. The result of this experiment is given in Figure 3.2.

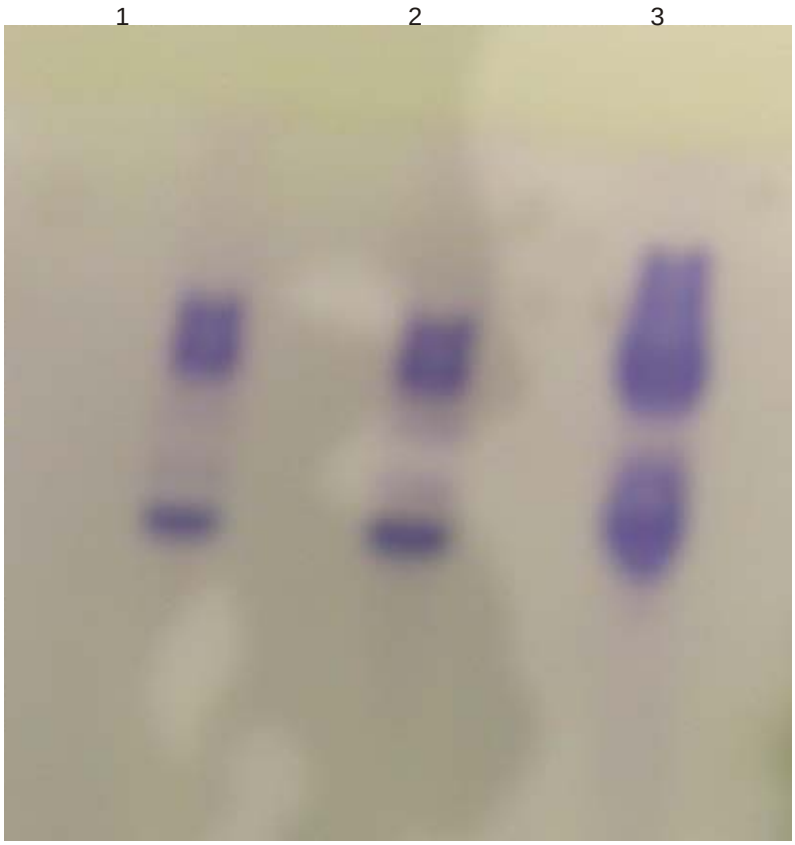


Figure 3.2: The Native PAG (10 %) and activity staining of crude extractsobtained from mannose (Lane 1), glucose (Lane 2) and fructose (Lane 3) grown biomass. The gel is a 10 % native polyacyrylamide gel. Glucose was used as substrate for activity staining.

Figure 3.2 displays the hexokinase isoenzyme pattern of *Rhizopus oryzae* grown on different carbon sources. This result showed that hexokinase isoenzymes are present in all three conditions regardless of carbon source used.

3.3. Purification of Hexokinase Isoenzymes from *Rhizopus oryzae*

The biomass obtained from glucose or fructose grown mycelia were used in purification studies. Two steps FPLC purification protocol was used; gel filtration and anion exchange. Gel filtration steps were repeated fifteen times and anion exchange experiment was replicated ten times. The results of these chromatographic separations were proved to be reproducible as we find exactly the same results in each run. In the first step of purification; gel filtration chromatography, hexokinase activity was eluted as a single peak. A typical gel filtration chromatography elution is shown in Figure 3.3.

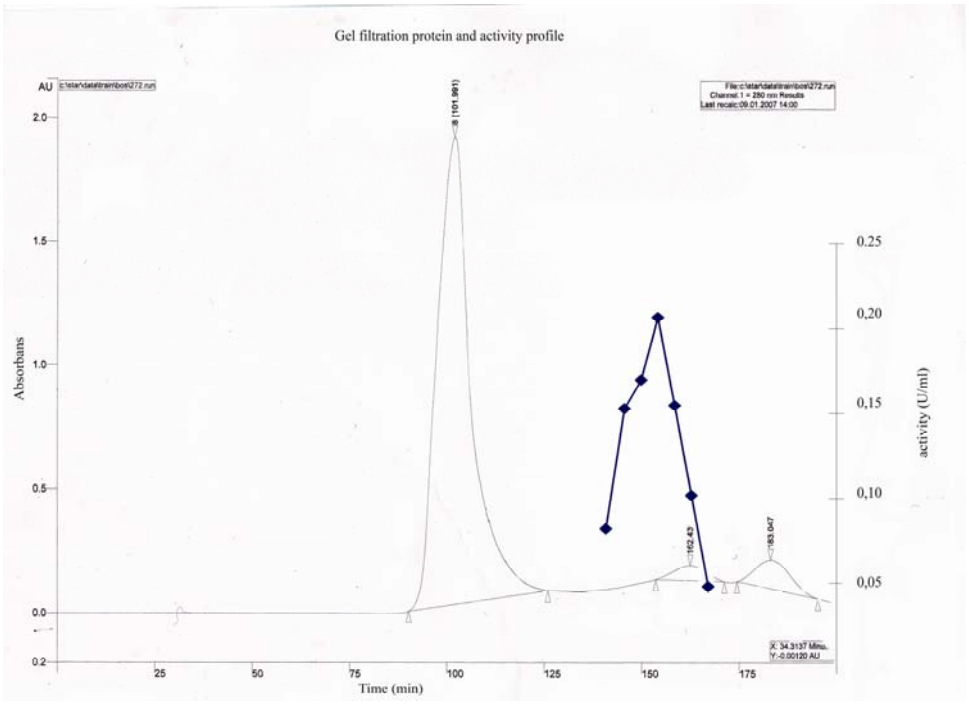


Figure 3.3: The elution profile of hexokinases from *Rhizopus oryzae* using the gel filtration column chromatography 16/60 Superdex 200 prep grade (M_r 10000-600000) "—" protein profile (OD at 280 nm) "◊" Activity (U/ml).The column was washed 10 mM Bis-Tris buffer pH 7.0 fractions of 2.5 ml were collected. Flow rate of the column was 0.5 ml/min.

The single activity peak from gel filtration column meant that there was no separation of the isoenzymes. The fractions, containing the hexokinase activity were collected, concentrated and loaded to anion exchange chromatography. The two isoenzymes were separated from each other in anion exchange chromatography. In Figure 3.4 the elution profile from anion exchange column is shown.

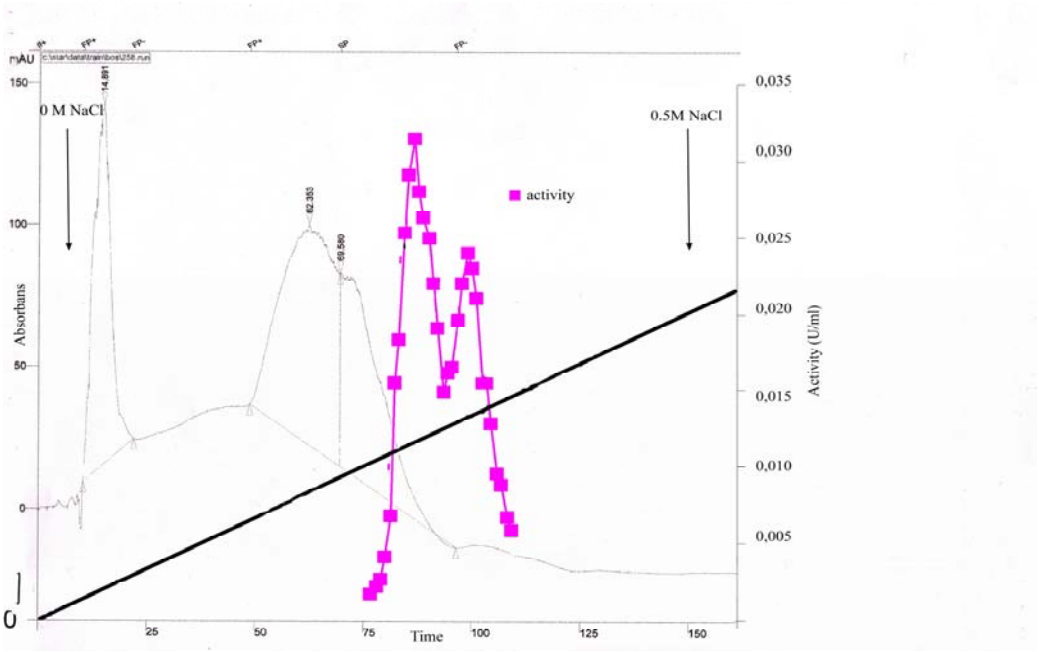


Figure 3.4: The elution profile of hexokinases from *Rhizopus oryzae* using the anion exchange column HiPrep16/10 Q XL matrix 6% highly cross-linked spherical agarose charged group $N^{+}(CH_3)_3$ (OD at 280 nm) "□" Activity (U/ml) "▬"The linear of NaCl gradient ↓ start and end concentration of NaCl. The column was washed with 10 mM Bis-Tris Buffer pH 7.0 and fractions of 3.5 ml were collected. The column was developed with a linear NaCl gradient (0- 0.5 M) fractions of 3.5 ml were collected. Flow rate of the column was 2.5 ml/min.

Gradient elution from 0 to 0.5 NaCl was used to elute the hexokinase activity from anion exchange column. The protein concentration of each fraction was given as OD measurements at 280. Enzyme activities of fractions were measured by coupled enzyme assay. There were two intersecting activity peaks, Peak 1 and Peak 2 the numbers in the name of the peaks is given depending the order of elution from the column For convenience we will refer them as "isoenzyme 1" and "isoenzyme 2" respectively in the rest of chapter."

Fractions were collected separately as isoenzyme 1 and isoeznyme 2 concentrated and stored at -20 °C in 20 % of glycerol.

Isoenzyme 1 and isoenzyme 2 from the first anion exchange column were further characterized by determining K_m and V_{max} values for glucose. The kinetic data showed that these different pools are not separated exactly. The velocity versus substrate concentration graph was linear which might be caused by presence of two isoenzymes in the samples. Thus, in the need to further separate the isoenzymes from each other the isoenzymes were loaded onto the same anion exchange column once more. The elution profile of isoenzyme 1 and isoenzyme 2 are shown in Figure 3.5 and Figure 3.6

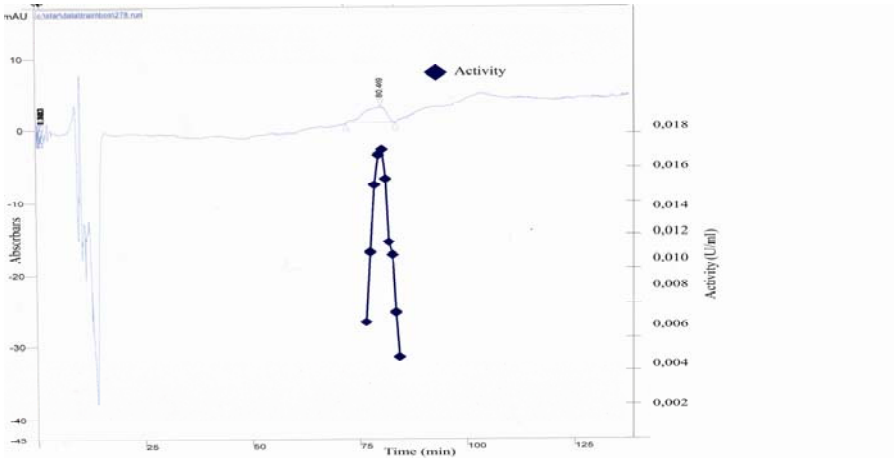


Figure 3.5: The elution profile of hexokinase isoenzyme 1 from *Rhizopus oryzae* using the anion exchange column HiPrep16/10 Q XL matrix 6% highly cross-linked spherical agarose charged group $N^{+}(CH_3)_3$ (OD at 280 nm) "□" Activity (U/ml) "▬"The linear of NaCl gradient ↓ start and end concentration of NaCl the column was washed with 10 mM Bis-Tris Buffer pH 7 and fractions of 3.5 ml were collected. The column was developed with a linear NaCl gradient (0- 0.5 M) fractions of 3.5 ml were collected. Flow rate of the column was 2.5 ml/min.

As can be seen from figure 3.5 the isoenzyme 1 was eluted as a single peak from the column the elution profile was very well in accordance with the first anion exchange column as the activity was eluted at the same elution volume.

The fractions containing activity were collected, concentrated and stored at -20°C in 20 % of glycerol for further characterization studies.

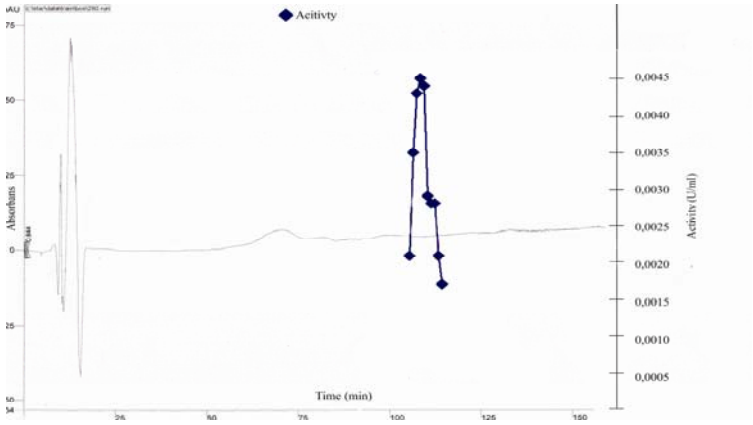


Figure 3.6: The elution profile of hexokinase isoenzyme 2 from *Rhizopus oryzae* using the anion exchange columnHiPrep16/10 Q XL matrix 6% highly cross-linked spherical agarose charged group $N^{+}(CH_3)_3$ (OD at 280 nm) "□" Activity (U/ml) "▬"The linear of NaCl gradient ↓ start and end concentration of NaCl the column was washed with 10 mM Bis-Tris Buffer pH 7 and fractions of 3.5 ml were collected. The column was developed with a linear NaCl gradient (0- 0.5 M) fractions of 3.5 ml were collected. Flow rate of the column was 2.5 ml/min.

Represented in Figure 3.6 isoenzyme 2 was eluted as a single peak as well. The activity peak eluted at the same elution volume where isoenzyme 2 was eluted in the first anion exchange column. For further kinetic studies fractions were collected concentrated and stored at -20°C in 20 % of glycerol.

The overall purification of hexokinase isoenzymes of *R. oryzae* from glucose grown and fructose grown mycelia are given in Table 3.1 and Table 3.2 respectively.

Table 3.1: Purification table of hexokinase isoenzymes from *Rhizopus oryzae* grown on 2% glucose.

Purification step		Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
Crude extract		8.5	1.40	2.05	11.92	17.43	0.68	100	1
Gel filtration chromatography		8	0.61	0.36	4.88	2.88	1.69	40.9	2.48
First Anion exchange chromatography	Peak 1	8.5	0.186	0.036	1.58	0.30	5.26	13.2	7.74
	Peak 2	3.2	0.166	0.021	0.53	0.067	7.91	4.4	11.6
Second Anion exchange chromatography	Peak 1	18	0.033	0.0017	0.59	0.031	19.2	4.94	28.2
	Peak 2	19	0.015	N.D	0.29	N.D	N.D.	2.43	N.D.

N.D. Not Determined

Hexokinase isoenzymes were purified from *Rhizopus oryzae* grown on 2 % glucose up to 28.2 fold with 4.94 % yield in three steps composed of gel filtration, column chromatography and two successive anion exchange columns. The yield even after the first step is low which is only 41 %. The sudden decrease in the yield might be because the enzyme is eluted as a very dilute solution. This can be easily seen as the total protein in the gel filtration pool is one sixth of the total protein in the crude extract. The decrease in the yield continues in the other steps of purification. However, it can be said that even if hexokinase isoenzymes were very dilute solutions at the last step we could measure the enzyme activities. The pool obtained for isoenzyme 2 after second anion exchange chromatography was very dilute that we could not determine the protein the concentration by any means (OD measurement,

Bradford's dye binding method). The purification fold achieved for isoenzyme 1 was 28.2 fold and for isoenzyme 2 it was more than 11.6 fold.

Table 3.2: Purification table of hexokinase from *Rhizopus oryzae* grown on 2 % fructose.

Purification step		Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification fold
Crude extract		8.65	1.12	1.76	9.64	15.22	0.63	100	1
Gel filtration chromatography		3.25	0.86	0.72	2.78	2.34	1.18	28.8	1.87
First anion exchange chromatography	Peak 1	2.25	0.24	0.031	0.54	0.07	7.74	5.64	12.2
	Peak 2	5	0.047	0.0087	0.24	0.044	5.4	2.43	8.5
Second anion exchange chromatography	Peak 1	7	0.018	N.D.	0.13	N.D.	N.D.	1.3	N.D.
	Peak 2	4.4	0.020	N.D	0.088	N.D.	N.D.	0.91	N.D.

N.D. Not determined

Comparison of Table 3.1 and Table 3.2 shows us that the hexokinase isoenzyme pattern of *R. oryzae* is the same in glucose and fructose grown mycelia. The yields throughout the purification decreased as seen in Table 3.2 and were the case in Table 3.1. The enzyme was eluted as a very dilute solution from second anion exchange column that the protein concentration could not be measured by any means. Therefore the purification fold achieved

at the last step could not be determined. But the kinetic analysis showed that two isoenzymes were completely separated from each other.

3.4. Characterization of Hexokinase Isoenzymes

3.4.1. Molecular Weight Determination

The monomer molecular weights of isoenzymes were determined by SDS PAGE analysis. The SDS PAG analysis of samples from the first anion exchange column has shown that the isonzyme 1 and isoenzyme 2 were not pure and contaminating bands were present. At the end of second anion exchange enzymes were separated from each other and they were almost pure. However, the protein concentration was too low and the bands was not observed after silver staining even if a maximum volume of sample was loaded on the denaturing gel (the results were not shown). In the end the molecular weight of the isoenzymes could not be determined in the samples from first and second anion exchange chromatography. Therefore, to determine the molecular weight of isoenzymes a unique method was used. The activity stained bands from native gel were cut and loaded onto denaturing gel. This way the bands cut from the native gel was ensured to be free from contaminating proteins.

The samples from gel filtration and first anion exchange column having hexokinase activity were loaded to the native gel and activity staining was performed using glucose as substrate. Figure 3.7 shows the picture of the activity stained native gel.

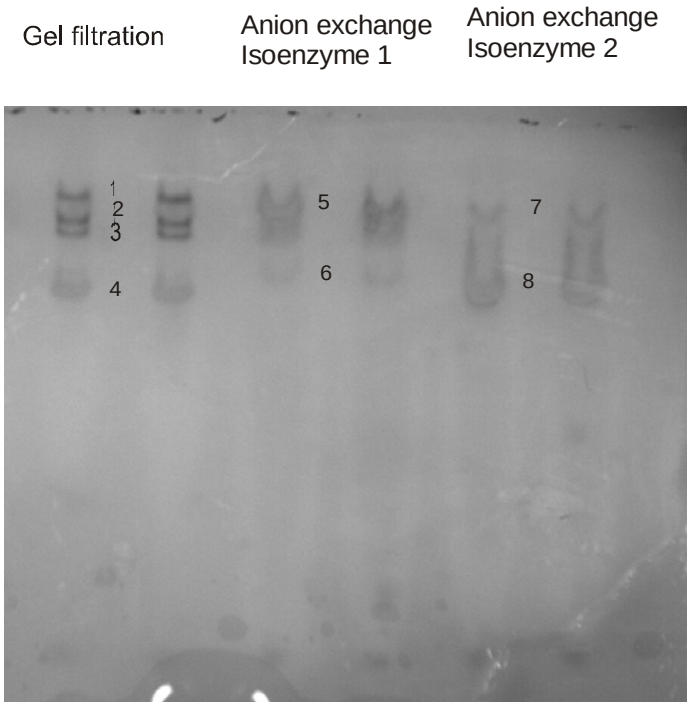


Figure 3.7: The Native PAG (10 %) of the gel filtration and first anion exchange fractions 1, 2, 3, 4 isoenzymes of hexokinase obtained from gel filtration. 5, 6 isoenzyme 1 of hexokinase from first anion exchange. 7, 8 isoenzyme 2 of hexokinase from first anion exchange. Equal amounts of proteins were loaded to each well. The gel visualized by activity staining using glucose as substrate.

As seen in Figure 3.7 four different bands were observed in the gel filtrations fractions (Lanes 1 and 2). These bands were numbered from 1 to 4 and were cut from the gel as thin slices. Cut bands were loaded to denaturing gel to determine monomer molecular weight and silver staining was performed. Figure 3.8 shows the results of this experiment.

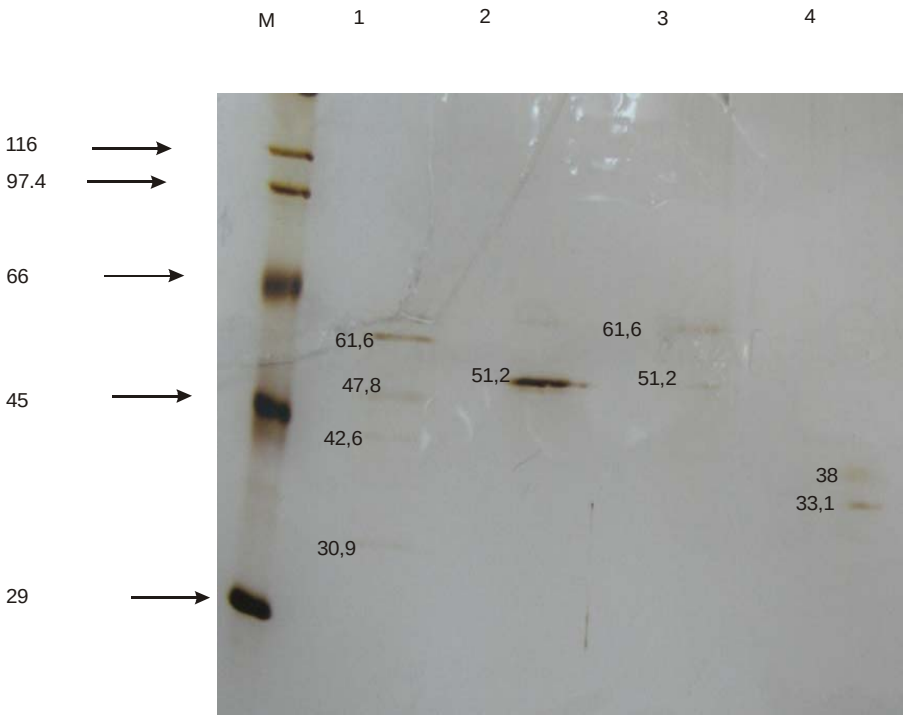


Figure 3.8: The SDS PAG (10 %) of the Native PAGE bands from gel filtration fractionsThe molecular weight of standards. 1, 2, 3, 4 the bands from the native gel. The gel was stained by silver staining.

A more clear picture of the molecular weight of the isoenzymes were obtained when the bands cut from the sample of first anion exchange column, numbered as 5-8, were loaded to the denaturing gel (Figure 3.9).

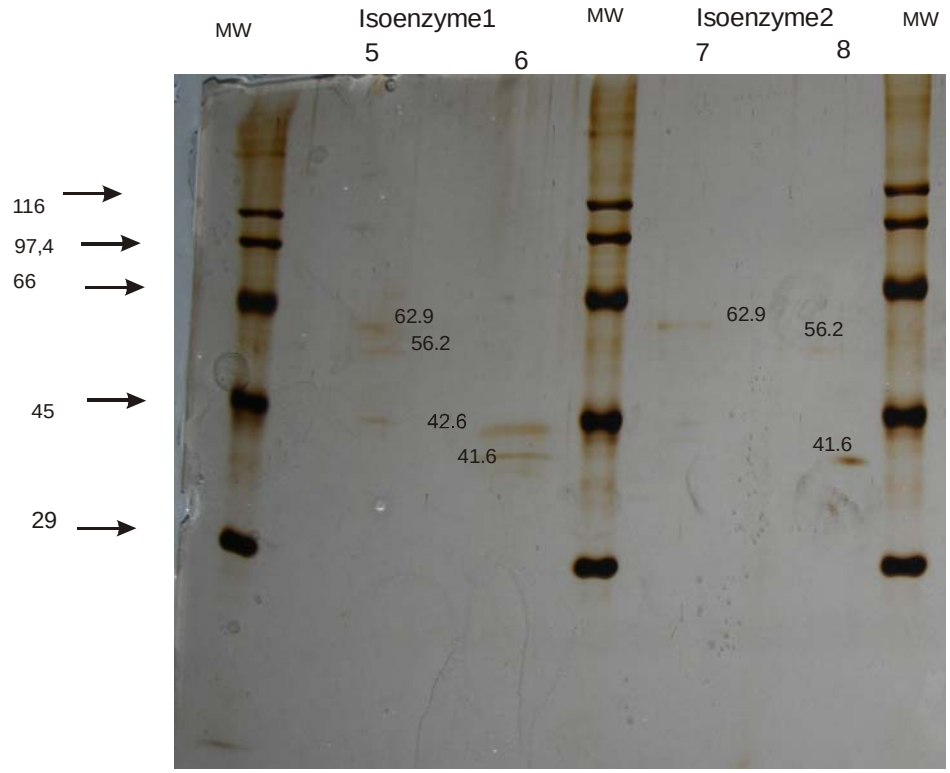


Figure 3.9: The SDS PAG (10 %) of the Native PAGE bands from anion exchange fractions. MW molecular weight marker. 5, 6 isoenzyme 1 after first anion exchange. 7,8 isoenzyme 2 after first anion exchange

Monomer molecular weight of the purified hexokinase isoenzymes were calculated from R_f vs log molecular weight graph (Appendix J). There were two different values for molecular weight of isoenzmye1; 62.9 and 42.5 kDa and two values for isoenzyme2; 56.2 and 41.6 kDa. The bands of isoenzyme 2 were also present in the lanes containing samples from isoenzyme 1. This might be because the bands are not clearly separated in the samples from first anion exchange chromatography leading to a mixture of both isoenzymes. The calculated monomer molecular weight of these isoenzymes was similar to the hexokinase from other organism [for example *Aspergillus niger* of hexokinase is 54.1 kDa (Panneman, Ruijter *et al.*1998)

and *Kluyvermyces lactis* is 53 kDa (Bar, Golbik *et al.* 2003). The data displays the convenience to probable hexokinase gene of *Rhizopus oryzae* from *R. oryzae* genome database http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html). The molecular weight of the probable hexokinase enzymes of *Rhizopus oryzae* were found to be between 44.7 and 53.6 kDa.

3.4.2. Determination of Pure Isoenzyme Pattern

Isoenzymes from the first and second anion exchange were loaded to native gel and activity staining was performed by using glucose as substrate. Figure 3.8 shows the result of this experiment. This gel was used to transfer of the protein to the PVDF membrane by using the electroblotting technique as described in the Materials and Methods section (2.3.1.3).

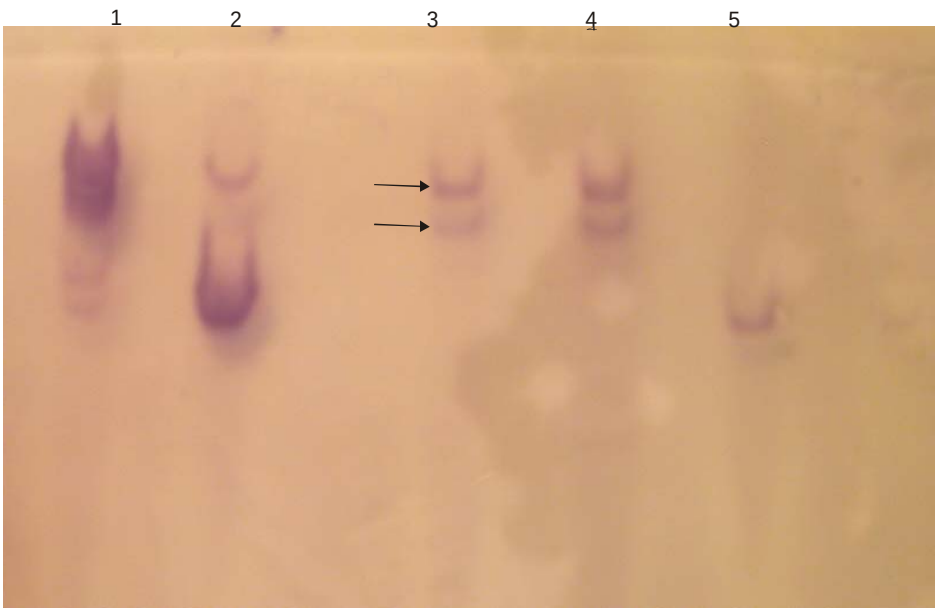


Figure 3.10: The Native PAG (10 %), activity staining of isoenzyme 1 and isoenzmye 2 from *Rhizopus oryzae* grown on 2% glucose as carbon source. Lane 1: isoenzyme 1 from first anion exchange. Lane 2 isoenzyme 2 after first anion exchange. Lane 3 and lane 4 the isoenzyme1 after second anion exchange column and lane 5 the isoenzyme2 after second anion exchange. → isoform of the isoenzyme1. Maximum 100 µl of each sample was loaded.

As illustrated in Figure 3.10 the second anion exchange chromatography proved to be successful to separate the isoenzymes from each other. In the samples from first anion exchange chromatography there are multiple activity stained bands with some of them bolder than the others, i.e., the higher bands are sharper in lane 1 and lower bands are sharper in lane 2. However, in the samples form second anion exchange chromatography (lanes 3-5) there are fewer bands and isoenzyme 1 and isoenzyme 2 have different banding patterns. The two bands of isoenzyme 1 might be because there are

different isoforms of the enzyme. These isoforms may be the charge isomers or molecular weight isoforms or phosphorylated/unphosphorylated forms of the same enzyme since the gel is a nondenaturing (native) gel. Isoenzyme 2 (lane 5) seem to have only one form represented as one band. However, when the banding patterns from lane 1 and lane 2 are compared to the one in lane 5 it seems like one of the isoforms at the lower part might be missing in the picture. The absence of this isoform in lane 5 can be explained by the very low protein concentration of the sample from second anion exchange chromatography or one of the forms might have been lost during the purification steps.

3.4.3 Protein Sequencing

In order to prove the existence of hexokinase isoenzymes amino acid sequence can be used since different isoenzymes have different amino acid sequence. Therefore purified enzymes from DEAE column were loaded on native PAG, activity staining was done to determine the place of hexokinase isoenzymes. Then proteins were transferred into PVDF membrane by using electroblotting technique (Appendix H). The PVDF membrane has high mechanic strength, chemical stability, and enhanced binding capacity. The use of the PVDF membrane is the best way for N-terminal sequencing because the protein degradation was prevented during transportation. PVDF membrane was stained by Coomassie staining (Appendix I). In order to check the efficiency of transfer also gel was stained by Coomassie staining. If transfer is achieved no bands should be visible on the gel.

PVDF membrane was sent to Astbury Building, School of Biochemistry and Molecular Biology, University of Leeds, UK to determine the sequence of protein by using the N-terminal sequencing method. However the protein concentration on the PVDF membrane was too low for sequencing causing the sequence analysis to be unsuccessful. The sequencing to be

successful the bands on the membrane must be sharp and pure which can be achieved by using an SDS PAG blot.

3.4.4. Kinetic Analyses of Hexokinase

3.4.4.1.Determination of the K_m and V_{max} for the Isoenzyme 1 and Isoenzyme 2

Kinetic analyses were done for both isoenzyme1 and isoenzyme2. The ν versus S graphs showed that these isoenzymes followed Michealis-Menten Kinetics (Figure 3.11, Figure 3.13, and Figure 3.15). The K_m of isoenzyme 1 and isoenzyme 2 for glucose were found as 0.16 mM and 0.21 mM respectively. The K_m of isoenzyme1 for fructose was found as 28.8 mM. Isoenzyme2 could not utilize fructose as substrate even at 150 mM fructose concentration.

Depending on the K_m values of isoenzymes for glucose and fructose we can conclude that isoenzyme 1 is a hexokinase and isoenzyme 2 is a glucokinase. *Rhizopus oryzae* hexokinase is different from yeast hexokinases in that K_m of yeast hexokinases for fructose are lower than that of the K_m of hexokinase of *Rhizopus oryzae*. Isoenzyme 2; glucokinase of *Rhizopus oryzae* is a true glucokinase like the bacterial glucokinase(Cardenas, Cornish-Bowden *et al*. 1998) or glucokinase of *Hansenula polymorpha* (Laht, Karp *et al*. 2002) in that it can not phosphorylate glucose even at very high glucose concentrations. The other behaves like a glucokinase but it can phosphorylate the fructose even if it has low affinity for fructose. Further verification has to be achieved by sequence analysis of the purified enzymes.

The kinetic analysis of hexokinase isoenzyme 1 showed that the velocity curve of the enzyme follow the normal Michealis-Menten Kinetics. (ν) versus substrate concentration ([S]) curve was drawn at glucose concentration between 0.08 mM and 10 mM (Figure 3.11).

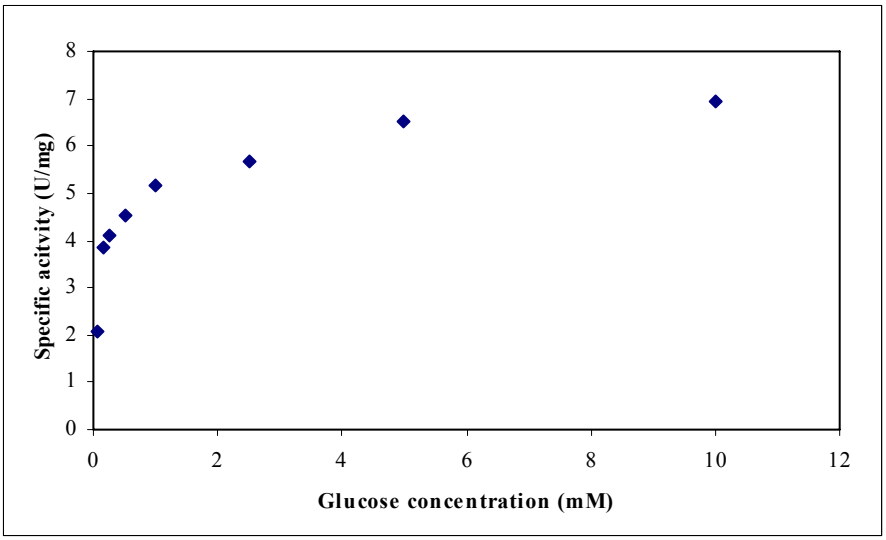


Figure 3.11: Glucose concentrations versus hexokinase activity for isoenzyme1 Kinetics of purified hexokinase for glucose (0.08-10mM). The reactions measured at 30 ˚C pH 7.70 in a mixture containing 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM NADP 1 U/ml glucose- 6-phosphate dehydrogenase the concentration of the enzyme in the reaction was approximately 0.012 mg/ml.

The kinetic data at different glucose concentrations were analyzed by means of the Lineweaver-Burk plot (Figure 3.12). V_{max} of the isoenzyme 1 was 6.53 U/mg and K_m for glucose 0.16 mM glucose.

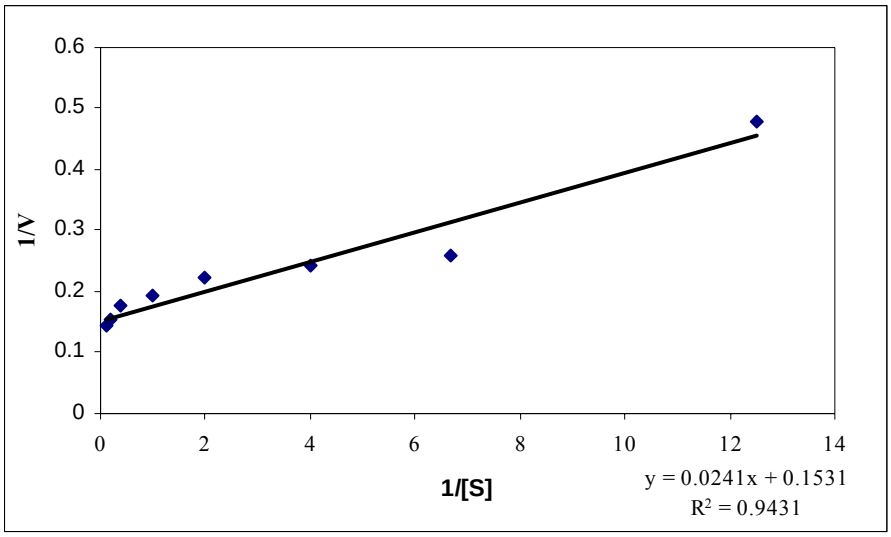


Figure 3.12. Lineweaver-Burk plot of the purified hexokinase of *R. oryzae* isoenzyme1

The kinetic analysis of hexokinase isoenzyme2 showed that the velocity curve of the enzyme follow the normal Michealis-Menten Kinetics. (ν) versus substrate concentration ([S]) curve was drawn at glucose concentration between 0.25 mM and 10 mM (Figure 3.13).

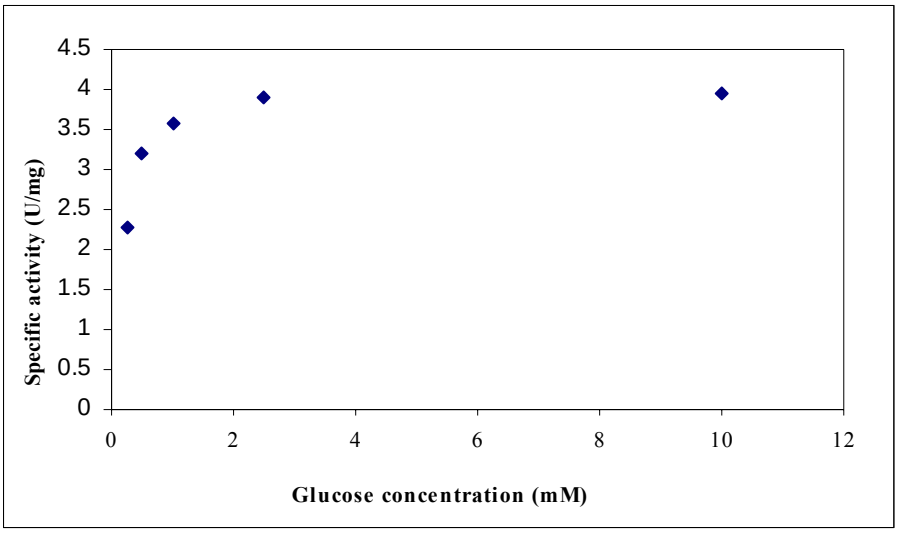


Figure 3.13: Glucose concentrations versus hexokinase activity for isoenzyme2Kinetics of purified hexokinase for glucose (0.25-10mM). The reactions measured at 30 ˚C pH 7.70 in a mixture containing 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM NADP 1 U/ml glucose-6-phosphate dehydrogenase the concentration of the enzyme in the reaction was approximately 0.014 mg/ml.

The kinetic data at different glucose concentrations were analyzed by means of the Lineweaver-Burk plot (Figure 3.14). V_{max} of the isoenzyme 2 was 4.24 U/mg and Km for glucose 0.21 mM glucose.

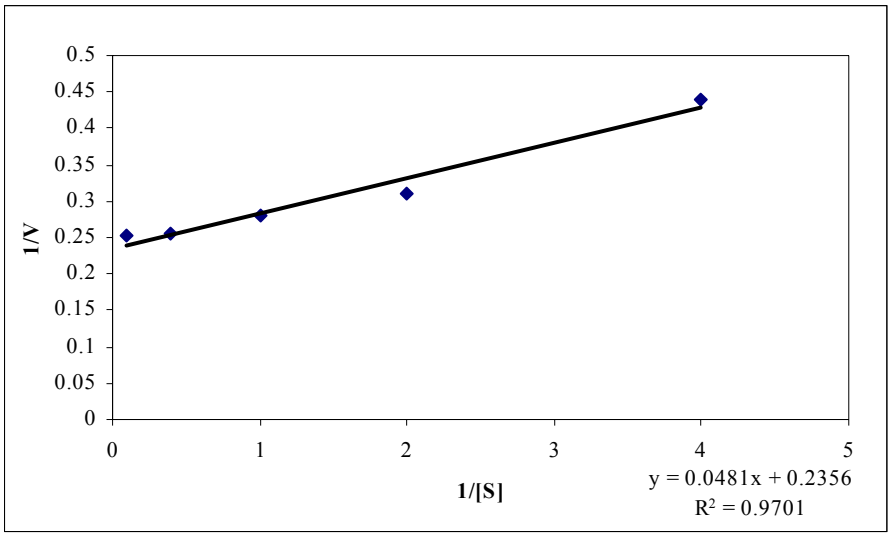


Figure 3.14: Lineweaver-Burk plot of the purified hexokinase of *R. oryzae* isoenzyme2

The kinetic analysis of hexokinase isoenzyme 1 showed that the velocity curve of the enzyme follow the normal Michealis-Menten kinetics. (v) versus substrate concentration ([S]) curve was drawn at fructose concentration between 2 mM and 50 mM (Figure 3.15).

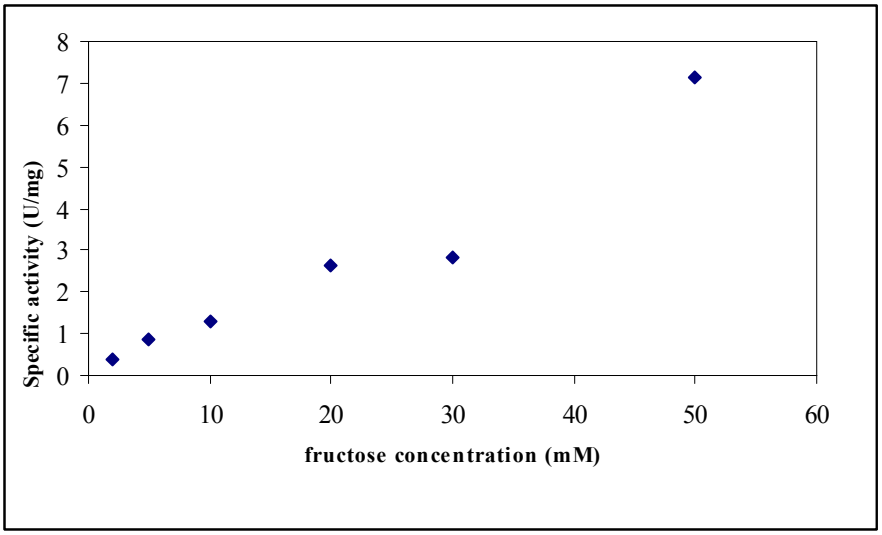


Figure 3.15: Fructose concentrations versus hexokinase activity for isoenzyme 1. Kinetics of purified hexokinase for glucose (2 -50 mM) the reactions measured at 30 ˚C pH 7.70 in a mixture containing 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM NADP 1 U/ml glucose-6-phosphate dehydrogenase 4 U/ml phosphor glucose isomerase the concentration of the enzyme in the reaction was approximately 0.012 mg/ml.

The kinetic data at different glucose concentrations were analyzed by means of the Lineweaver-Burk plot (Figure 3.16). V_{max} of the isoenzyme was 5.95 U/mg and Km for glucose 28.8 mM fructose.

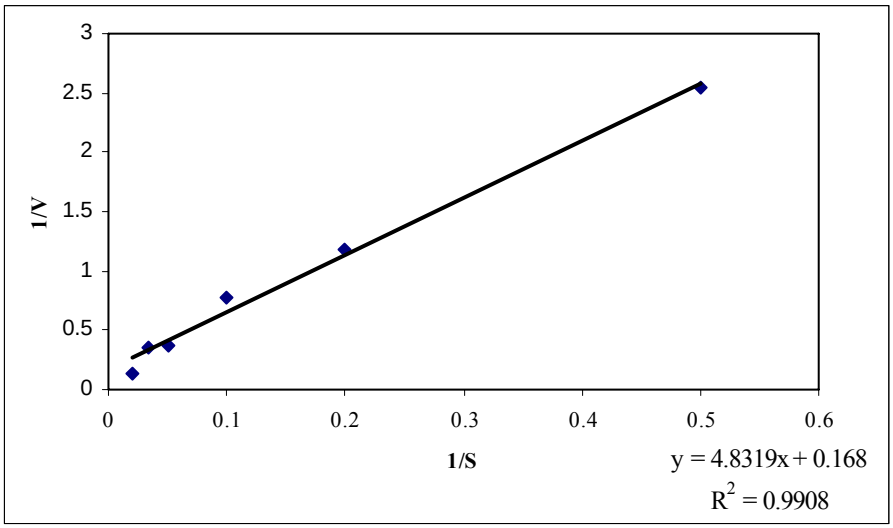


Figure 3.16: Lineweaver-Burk plot of the purified hexokinase of *R. oryzae* isoenzyme 1

3.4.4.2. The Inhibition of Hexokinase by Trehalose-6-phosphate

Trehalose-6- phosphate is the potent inhibitor of *Saccharomyces cerevisiae* hexokinase and *Aspergillus niger* hexokinase while glucokinase from both organism are not be affected by this intermediate metabolite. It can be said that inhibition by trehalose 6-phosphate may be an evidence for the presence of glucokinase. The inhibition of *R. oryzae* isoenzyme 1 and isoenzyme 2 were investigated by measuring the activity in the presence and absence of trehalose-6-phosphate. Neither isoenzyme 1 nor isoenzyme 2 can be inhibited by trehalose-6-phosphate. In order to show the inhibition effect of trehalose-6-phosphate commercial yeast hexokinase was used as a positive control. The activity measurement result displayed that this commercial hexokinase is inhibited by trehalose-6-phosphate (Table 3.3.).

Table3.3 The Effect of the Trehalose-6-phosphate on Isoenzyme 1 and Isoenzyme 2

The effect of traholse-6-phosphate	Isoenzyme 1 Activity (U/ml)	Isoenzmye 2 Activity (U/ml)	Commercial yeast hexokinase Activtiy (U/ml)
No Trehalose-6-phosphate	0.027	0.021	11.92
Trehalose-6-phosphate (1mM)	0.025	0.020	8.08

Table 3.3 displays the activities of isoenzyme1 and isoenzyme2 and commercial hexokinase in the presence of trehalose-6-phosphate.

When trehalose-6-phosphate was not used the activity of isoenyzme 1 was 0.027 U/ml. While in the presence of trehalose-6-phosphate (1mM) the activity of isoenzyme 1 was 0.025 U/ml. According to statistical analysis addition of trehalose-6-phosphate was not effective on hexokinase isonezyme1 of *Rhizopus oryzae* ($p=0.28>0.05$) (Appendix L). The activities of isoenzyme 2 in the absence and presence of trehalose-6-phosphate (1mM) were 0.021 U/ml and 0.020 U/ml respectively. According to statistical analysis addition of trehalose-6-phosphate was not effective on hexokinase isonezyme 2 of *Rhizopus oryzae* ($p= 0.415>0.05$) (Appendix L).

The inhibition of yeast hexokinase was investigated. The results showed that yeast hexokinase was inhibited by trehalose-6-phosphate. The activity of hexokinase was 11.92 U/ml when trehalose-6-phosphate was not used. However when the trehalose-6-phosphate 1(mM) was used the activity was 8.08 U/ml. Statistical analysis showed that addition of trehalose-6-phosphate was effective on commercial hexokinase of yeast ($p=0.027<0.05$) (Appendix L). Therefore it was seen that yeast hexokinase was inhibited by trehalose-6-phosphate.

It was concluded that *Rhizopus oryzae* hexokinase isoenzymes are not inhibited by trehalose-6-phosphate like yeast glucokinase (Blazquez, Lagunas *et al.* 1993). The two yeast hexokinases are inhibited by trehalose-6-phosphate (Blazquez, Lagunas *et al*. 1993; Eastmond and Graham 2003).

CHAPTER 4

CONCLUSIONS

Glycolysis and its regulation are important for the end product formation like lactic acid and ethanol. *Rhizopus oryzae* is industrially important organism and it produces L form of lactic acid. However the yield of lactic acid is low when compared that of lactic acid bacteria (Longacre, Reimers *et al*. 1997; Skory 2004). If a way can be found to increase the glylcolytic pathway yield of lactic acid may be raised. Hexokinase is the first and also irreversible step. It has role on the regulation of glycolysis. Therefore in this study the isoenzymes of hexokinase of *Rhizopus oryzae* have been purified and characterized. This study can be a step for further investigation of glycolysis

Rhizopus oryzae was grown on two different media as carbon source; one was containing glucose the other was including fructose. Crude extracts from these mediums were loaded to native gel and activity staining was performed. Glucose and fructose were used as substrate for staining. At the end of the activity staining different isoenzymes pattern was observed (Figure 3.1). This study showed that *R. oryzae* hexokinase had isoenzymes.

In order to purify these isoenzymes FPLC system was used. Isoenzymes was separated from each other exactly after three steps including gel filtration, first anion exchange and second anion exchange. After gel filtration isoenzymes came as single peak (Figure 3.3). Therefore active fractions were loaded to anion exchange column and isoenzymes came as double peaks. However kinetic studies showed that the isoenzymes could not be separated from each other exactly. Therefore isoenzyme1 and isoenzyme2 were loaded to same column. Isoenzyme1 and isoenzyme2 could be separated from each other exactly (Figure 3.5 and 3.6).

At the end of the tree purification steps the activity of isoenzymes was lost significantly and also the protein concentration was too low to determine by using Bradford method (Table 3.1 and 3.2).

In order to determine the monomer molecular weight of these isoenzymes SDS PAG and silver staining were performed. Since the protein concentration of isoenzymes was too low the monomer molecular weight of pure isoenzymes could not be determined by using silver staining. Therefore the unique method was used for estimation of monomer molecular weight of isoenzymes. Firstly fractions from gel filtration and fractions from anion exchange were loaded to native gel and activity staining were performed (glucose as substrate) (Figure 3.7) Bands obtained this gel was loaded to SDS PAGE and silver staining was performed. There were two different values for molecular weight of isoenzyme 1; 62.9 and 42.5 kDa and two values for isoenzyme2; 56.2 and 41.6 kDa. The bands of isoenzyme 2 were also present in the lanes containing samples from isoenzyme 1. This might be because the bands are not clearly separated in the samples from first anion exchange chromatography leading to a mixture of both isoenzymes (Figure3.8 and Figure 3.9).

The kinetic studies showed that isoenzymes obeyed Michealis-Menten Kinetics (Figure 3.11, Figure 3.13 and Figure 3.15). When glucose was substrate the K_m value of isoenzyme 1 and isoenzmye 2 were estimated as 0.16 mM and 0.21 mM respectively. When the fructose was substrate the K_m value was calculated as 28.8 mM for isoenzyme 1. Although 150 mM fructose was used as substrate the K_m value of isoenzyme 2 could not be calculated. Therefore it was concluded that isoenzyme 2 can not phosphorylate fructose. Since the isoenzyme 2 could not be inhibited by trehalose-6-phosphate it can be said that isoenzyme 2 is true glucokinase. It can be implied that the isoenzyme 1 was hexokinase because it can phosphorylate fructose even if the K_m value is very high. *Rhizopus oryzae* hexokinase shows different behaviors from the yeast and A. *niger* hexokinase toward inhibition of trehalose-6-phosphate.While *A. niger* and yeast hexokinases can be inhibited by this intermediate metabolite (Panneman, Ruijter *et al*. 1998) (Eastmond and Graham 2003). *R. oryzae* hexokinase is not inhibited by trehalose-6-phosphate.

The other important characteristic of these isoenzymes was observed after the native gel electrophoreses. After first and second anion exchange isoenzymes were loaded to the native gel and activity staining was performed (glucose as substrate) (Figure 3.10). This study showed that isoenyzme 1 has two isoforms and also the isoenzyme 2 may two isoforms. However these isoforms could not be observed after second anion exchange column they were lost at one of the step of purification.

In order to support the existence of isoenzymes and the amino acid sequence of isoenzyme were investigated. Pure protein was loaded to native gel and after activity staining; bands on the gel were transferred to PVDF membrane. The amino acid sequence was not determined because the band was not sharp and pure. If pure protein is loaded to SDS PAG the sharp bands can be obtained and N terminal sequence can be achieved. This sequence will be helpful further studies for example overexpression of these isoenzymes. After overexpression of these isoenzymes the more detailed further studies related to feature of isoenzymes may be performed and the role of *R. oryzae* hexokinase on glycolytic pathway may be understood.

REFERENCES

Albig, W. and K. D. Entian (1988). "Structure of Yeast Glucokinase, a Strongly Diverged Specific Aldo-Hexose-Phosphorylating Isoenzyme." Gene **73**(1): 141-152.

Baker, H. V. (1991). "Gcr1 of *Saccharomyces-Cerevisiae* Encodes a DNA-Binding Protein Whose Binding Is Abolished by Mutations in the Cttcc Sequence Motif." Proceedings of the National Academy of Sciences of the United States of America **88**(21): 9443-9447.

Bar, D. Golbik, R., Hubner, G., Lilie, H., Muller, E. C., Naumann, M., Otto, A., Reuter, R., Breunig, K. D., Kriegel, T. M.al. (2003). "The unique hexokinase of *Kluyveromyces lactis* - Molecular and functional characterization and evaluation of a role in glucose signaling." Journal of Biological Chemistry **278**(41): 39280-39286.

Barwell, C. J. and B. Hess (1972). "Application of Kinetics of Yeast Pyruvate-Kinase in-Vitro to Calculation of Glycolytic Flux in Anaerobic Yeast-Cell." Hoppe-Seylers Zeitschrift Fur Physiologische Chemie **353**(7): 1178-1184

Barwell, C. J., Woodward, B., Brunt, R. V. (1971). "Regulation of Pyruvate Kinase by Fructose 1,6-Diphosphate in *Saccharomyces-Cerevisiae."* European Journal of Biochemistry **18**(1): 59-64

Bennett, W. S. and T. A. Steitz (1978). "Glucose-Induced Conformational Change in Yeast Hexokinase." Proceedings of the National Academy of Sciences of the United States of America **75**(10): 4848-4852.

Bergmeyer, H. U., 1965, (1965). Methods of Enzymatic Analysis, Verlag Chmeie GmbH Academic Press, USA.

Blazquez, M. A., Lagunas, R., Gancedo, C., Gancedo, J. M. (1993). "Trehalose-6-Phosphate, a New Regulator of Yeast Glycolysis That Inhibits Hexokinases." Febs Letters **329**(1-2): 51-54.

Blum, H., Beier, H., Gross, H. J. (1987). "Improved Silver Staining of Plant-Proteins,Rna and DNA in Polyacrylamide Gels." Electrophoresis **8**(2): 93-99.

Bradford, M. M. (1976). "Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding." Analytical Biochemistry **72**(1-2): 248-254.

Caceres, A. J., Portillo, R., Acosta H. (2003). "Molecular and biochemical characterization of hexokinase from *Trypanosoma cruzi*." Molecular and Biochemical Parasitology **126**: 251-262.

Cardenas, M. L., Cornish-Bowden, A., Ureta, T. (1998). "Evolution and regulatory role of the hexokinase." Biochimica Et Biophysica Acta-Molecular Cell Research **1401**(3): 242-264.

Ceccaroli, P., Saltarelli, R., Cesari P. (2001). "Effects of different carbohydrate sources on the growth of *Tuber borchii* Vittad. mycellium stains in pure culture." Molecular and cellular Biochemistry **218**: 65-70.

Datta, R., Tsai, S. P., Bonsignore, P., Moon, S. H., Frank, J. R (1995). "Technological and Economic-Potential of Poly(Lactic Acid) and Lactic-Acid Derivatives." Fems Microbiology Reviews **16**(2-3): 221-231.

Diderich, J., A.Schuurmans, J. M.Van Gaalen, M. C.Kruckeberg, A. L. Van Dam, K. (2001). "Functional analysis of the hexose transporter homologue HXT5 in *Saccharomyces cerevisiae*." Yeast **18**(16): 1515-1524.

Eastmond, P. J. and I. A. Graham (2003). "Trehalose metabolism: a regulatory role for trehalose-6-phosphate?" Current Opinion in Plant Biology **6**(3): 231-235.

Elbing, K., Larsson, C., Bill, R. M., Albers, E., Snoep, J. L., Boles, E., Hohmann, S., Gustafsson, L. (2004). "Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*." Applied and Environmental Microbiology **70**(9): 5323-5330.

Elliot, C. G. (1994). Zygomycetes in Reproduction in fungi: genetical and physiological aspects. London, pp. 129-145, Chapman and Hall.

Espinel, A. E., GomezToribio, V., Peinado, J. M. (1996). "The inactivation of hexokinase activity does not prevent glucose repression in *Candida utilis*." Fems Microbiology Letters **135**(2-3): 327-332.

Fickers, P., Nicaud, J. M., Destain, J., Thonart, P.2005. "Involvement of hexokinase HXK1 in glucose catabolite repression of LIP2 encoding extracellular lipase in the yeast *Yarrowia lipolytica*." Current Microbiology **50**(3): 133-137.

Frommer, W. B., W. X. Schulze, et al. (2003). "Plant science - Hexokinase, jack-of-all-trades." Science **300**(5617): 261-263

Griffin D.H. (1994). Fungal Physiology. New York, John Wiley &Soul Publication.

Hauf, J., Zimmermann, F. K., Muller, S. (2000). "Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*." Enzyme and Microbial Technology **26**(9-10): 688-698.

Haw, R., Yarragudi, A. D., Uemura, H. (2001). "Isolation of GCR1, a major transcription factor of glycolytic genes in *Saccharomyces cerevisiae*, from *Kluyveromyces lactis*." Yeast **18**(8): 729-735.

Heinisch, J., Vogelsang, K., Hollenberg, C. P (1991). "Transcriptional Control of Yeast Phosphofructokinase Gene-Expression." Febs Letters **289**(1): 77-82.

Imriskova, I., Langley, E., Arreguin-Espinosa, R., Aguilar, G., Pardo, J. P., Sanchez, S. (2001) "Rapid purification and biochemical characterization of glucose kinase from *Streptomyces peucetius var. caesius.*" Archives of Biochemistry and Biophysics **394**(2):137-144

Jurica, M. S., Mesecar, A., Heath, P. J., Shi, W. X., Nowak, T., Stoddard, B. L. (1998). "The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate." Structure **6**(2): 195-210.

Kaji, A., Colowick, S. P., Trayser, K. A.,. (1961). "Multiple Forms of Yeast Hexokinase." Annals of the New York Academy of Sciences **94**(3): 798-811.

Karp, H., Jarviste, A., Kriegel, T. M., Alamae, T.. (2003). "Cloning and biochemical characterization of hexokinase from the methylotrophic yeast *Hansenula polymorpha.*" Current Genetics **44**(5): 268-276.

Kim, J. W. and C. V. Dang (2005). "Multifaceted roles of glycolytic enzymes." Trends in Biochemical Sciences **30**(3): 142-150.

Kopetzki, E. and K. D. Entian (1982). "Purification of Yeast Hexokinase Isoenzymes Using Affinity-Chromatography and Chromatofocusing." Analytical Biochemistry **121**(1): 181-185.

Kramarenko, T., Karp, H., Jarviste, A., Alamae, T. (2000). "Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants." Folia Microbiologica **45**(6): 521-529.

Laemmli, U. K. (1970). "Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4." Nature **227**(5259): 680-685.

Laht, S., Karp, H., Kotka, P.,.Jarviste, A., Alamae, T."Cloning and characterization of glucokinase from a methylotrophic yeas*t Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*." Gene **296**(1-2): 195-203.

Laurent, M. and J. M. Yon (1989). "Yeast phosphofructokinase," in: allosteric enzymes. Florida, CRC Press Inc.

Longacre, A., Reimers, J., M.Gannon, J. E., Wright, B. E. (1997). "Flux analysis of glucose metabolism in *Rhizopus oryzae* for the purpose of increasing lactate yields." Fungal Genetics and Biology **21**(1): 30-39.

Maas, R. H. W., Bakker, R. R., Eggink, G., Weusthuis, R. A. (2006). "Lactic acid production from xylose by the fungus *Rhizopus oryza*e." Applied Microbiology and Biotechnology **72**(5): 861-868.

Maitra, P. K. (1970). "A Glucokinase from *Saccharomyces-Cerevisiae*." Journal of Biological Chemistry **245**(9): 2423-2431.

Magnusson, M. A.and Shelton K.D. 1989 "An alternate promoter in the glucokinase genes is active in pancreatic beta cell". Journal of Biological Chemistry **264:** 15936-15942

Manchenko, G. P. (1994). Handbook of Detection Enzymes on Elcetrophoretic Gels, CRC Press.

Moreno, F., Ahuatzi, D.,Riera, A., Palomino, C., A.Herrero, P. (2005). "Glucose sensing through the Hxk2-dependent signalling pathway." Biochemical Society Transactions **33**: 265-268.

Nadkarni, M., Lobo, Z., Maitra, P. K. (1982). "Particulate Phosphofructokinase of Yeast - Physiological-Studies." Febs Letters **147**(2): 251-255.

Otterstedt, K., Larsson, C., Bill, R. M., Stahlberg, A., Boles, E., Hohmann, S., Gustafsson, L. (2004). "Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*." Embo Reports **5**(5): 532-537.

Ozcan, S. and Johnston M. (1999). "Function and regulation of yeast hexose transporters." Microbiology and Molecular Biology Reviews **63**(3): 554-569.

Panneman, H., Ruijter, G. J. G., VandenBroeck, H. C., Visser, J. (1998). "Cloning and biochemical characterisation of *Aspergillus niger* hexokinase - The enzyme is strongly inhibited by physiological concentrations of trehalose 6-phosphate." European Journal of Biochemistry **258**(1): 223-232.

Panneman, H., Ruijter, G. J. G., VandenBroeck, H. C., Driever, E. T. M., Visser, J. "Cloning and biochemical characterisation of an *Aspergillus niger* glucokinase - Evidence for the presence of separate glucokinase and hexokinase enzymes." European Journal of Biochemistry **240**(3): 518-525.

Petit, T. and. Gancedo C. (1999). "Molecular cloning and characterization of the gene *HXK1* encoding the hexokinase from *Yarrowia lipolytica.*" Yeast **15**(15): 1573-1584.

Rolland, F., Winderickx, J., Thevelein, J. M. (2001). "Glucose-sensing mechanisms in eukaryotic cells." Trends in Biochemical Sciences **26**(5): 310-317.

Rose, M. (1995). "Molecular and Biochemical-Characterization of the Hexokinase from the Starch-Utilizing Yeast *Schwanniomyces-Occidentalis.*" Current Genetics **27**(4): 330-338.

Ruijter, G. J. G., Panneman, H., VandenBroeck, H. C., Bennett, J. M., Visser, J. (1996). "Characterisation of the *Aspergillus nidulans* frA1 mutant: Hexose phosphorylation and apparent lack of involvement of hexokinase in glucose repression." Fems Microbiology Letters **139**(2-3): 223-228.

Schaaff, I., Heinisch, J., Zimmermann, F. K. (1989). "Overproduction of Glycolytic-Enzymes in Yeast." Yeast **5**(4): 285-290.

Skory, C. D. (2004). "Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity." Applied Microbiology and Biotechnology **64**(2): 237-242.

Stechmann, A., Baumgartner, M., Silberman, J. D., Roger, A. J. (2006). "The glycolytic pathway of *Trimastix pyriformis* is an evolutionary mosaic." Bmc Evolutionary Biology **6**.

Verwaal, R., Paalman, J. W. G., Hogenkamp, A., Verkleij, A. J., Verrips, C. T., Boonstra, J. (2002). "HXT5 expression is determined by growth rates in *Saccharomyces cerevisiae*." Yeast **19**(12): 1029-1038.

Viana, R., Perez-Martinez, G., Deutscher, J., Monedero, V. (2005). "The glycolytic genes pfk and pyk from *Lactobacillus casei* are induced by sugars transported by the phosphoenolpyruvate: sugar phosphotransferase system and repressed by CcpA." Archives of Microbiology **183**(6): 385-393.

Voet, D. and J. Voet (1995). Biochemistry. New York, Willey Sons.
Wieczorke, R.Krampe, S.Weierstall, T.Freidel, K.Hollenberg, C. P.
Boles, E. (1999). "Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*." Febs Letters **464**(3): 123-128.

Xiao, W.Y., Sheen, J., Jang, J. C.. (2000). "The role of hexokinase in plant sugar signal transduction and growth and development." Plant Molecular Biology **44**(4): 451-461.

Zimmermann, F. K. and . K.D.Entian (1997). Yeast Sugar Metabolism:, Technomic Publishing Co. Inc. USA.

APPENDICES

APPENDIX A

GROWTH MEDIUM FOR *Rhizopus oryzae*

Table A1: Defined medium composition

Chemicals	**gram/100 ml**	**Final concentration**
Calcium carbonate ($CaCO_3$)	0.43	43 mM
Ammonium sulfate ($(NH_4)_2SO_4$)	0.2	15 mM
Potassium phosphate (KH_2PO_4)	0.065	5 mM
Magnesium sulfate ($MgSO_4.7H_2O$)	0.025	1 mM
Zinc sulfate ($ZnSO_4.7H_2O$)	0.005	0.2 µM
Glucose or Fructose*	2	111 mM

If you are preparing a solid medium use Potato Dextrose Agar (PDA) which is readily available from different suppliers (e.g.: Merck or Lab M).

Sterilize the solid and liquid medium by autoclaving at 121°C for 15 minutes.

*Fructose contains little amount of glucose (contains less than 0.05 % glucose by enzymatic assay) which is enough for hexokinase to show activity. In order to remove the traces of glucose in 1M fructose solution, it was treated by adding glucose oxidase to the solution at a final concentration of 50 units/ml and incubation at 35°C for four hours. At the end of four hours glucose oxidase was inactivated by incubating the fructose solution at 95°C for 5 minutes

APPENDIX B

PREPARATION OF SPORE SUSPENSION AND SPORE COUNT FROM *Rhizopus oryzae*

Materials:

Sterile screw capped bottles and pipette tips.

Protocol:

1.*Rhizopus oryzae* sporulated on plates containing PDA agar for 4-5 days, at 30°C is used for spore suspension preparation

2.Lift the mycelium which is spread over the surface of the agar and which carries spores by using sterile tweezers.

3.Put the mycelium into a sterile bottle containing 30-40 ml sterile distilled water

4.Agitate by shaking and vortexing this spore suspension

5.Take out as much of the mycelium from the bottle. Alternatively you can filter through a sterile glass funnel which contains glass wool loosely inserted (to allow the passage of spores but not the mycelium) in its hole.

6.Transfer the suspension into a sterile centrifuge tube and centrifuge in a Sigma Centrifuge at 10095 xg for 10 minutes at room temperature

7.Discard the supernatant aseptically

8.Add the sterile distilled water to the pellet shake till the pellet is dissolved in distilled sterile water and transfer it to a screw capped bottle

9.Take 1 ml spore suspension to a 1.5 ml Eppendorf tube to count the spores.

10. Prepare a 10 fold dilution of this suspension to make the count. Make sure the suspension is even when you are doing the dilution.

11. Take 10 µl dilute sample drip on the haemocytometer (Thoma counting chamber). Care should be taken not to overfill or underfill the chamber

12. Put the cover slip starting at a slightly angled position so that no air bubbles are trapped

13. Focus on the etched side of the Thoma counting chamber using the 40X objective and 10X ocular

14. A scheme of etched side of Thoma counting chamber is given in the scheme below. Count the spores found in the each (total 25) large square (with surface area of 1/25 mm^2)

15. Calculate the average of spore number for large square by dividing the total number of spores with the number of large squares counted

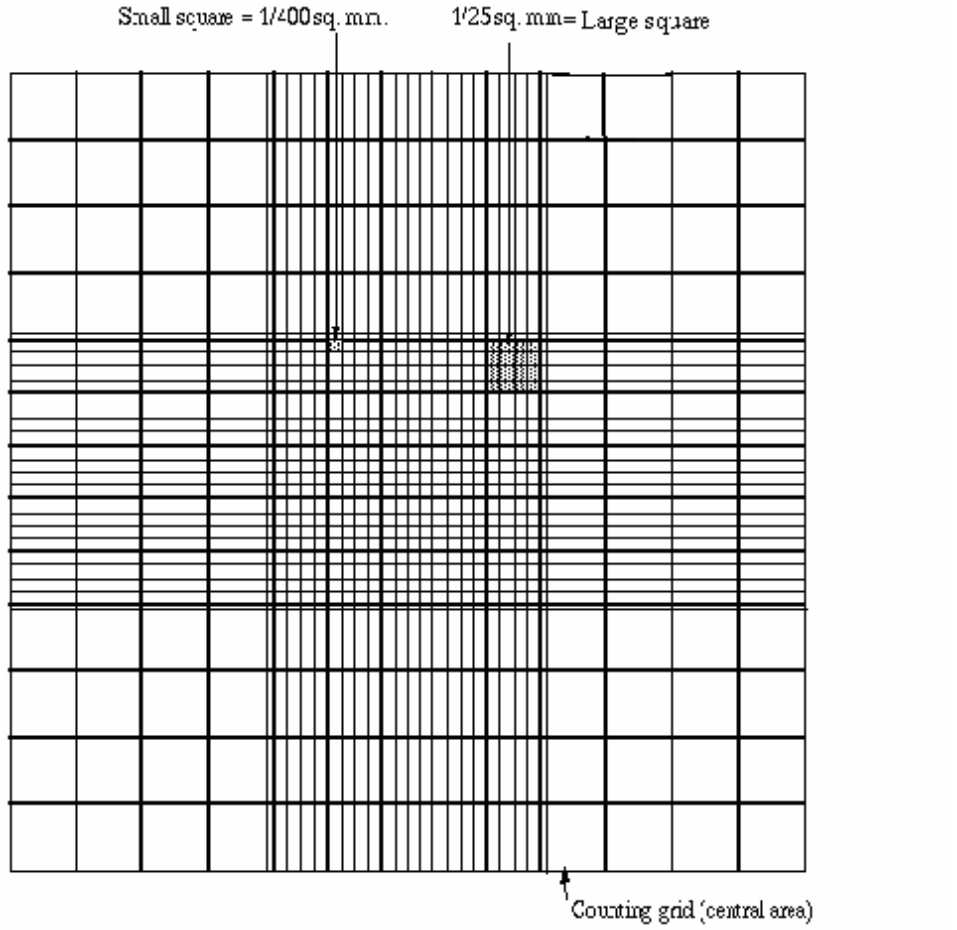


Figure A1: The scheme showing The view of etched site of Thoma counting chamber.

Note: The depth of the counting chamber is 0.1 mm and the minimal area as shown with A 0.0025 mm^2. There are 25 square with 0.00025 mm^3 volume.

APPENDIX C

BRADFROD'S DYE BINDING ASSAY

Stock solution

100 mg Coomassie Brilliant Blue G

100 ml 95 % Ethanol

50 ml 85 % Phosphoric acid

After the dye has completely dissolved in ethanol, add phosphoric acid. Dilute the solution to 1 L by adding distilled water. Filter through normal filter paper inserted in a glass funnel. This solution should be kept in a dark bottle away from light. It can be stored at room temperature (tightly closed) for two weeks.

Standard curve preparation:

Prepare stock solution BSA standard 1 mg/ml:

BSA (Sigma A2153 96 %)

Weigh 104.17 mg and dissolve in 0.85 % NaCl and 0.1 % sodium azide this solution can be stored at +2 to +8 °C

Table A2: Standard Curve Preparation table of Bradford

Tube #	BSA standard (µl)	Distilled water (µl)	Bradford's Reagent (ml)	Protein concentration (mg /ml)
1		500	5	0
2	5	495	5	0.01
3	10	490	5	0.02
4	15	485	5	0.03
5	20	480	5	0.04
6	25	475	5	0.05

Bradford's reagent must be at room temperature before addition. After addition of Bradford's reagent vortex and mix well. Let the tubes stand at room temperature for at least 10 minutes. Read the absorbance at 595 nm within one hour.

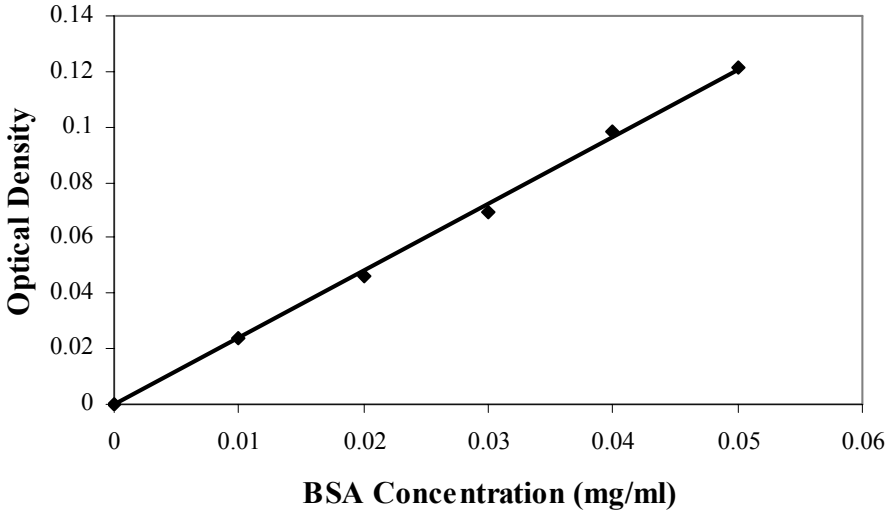


Figure A.2 Standard Curve for Bradford's dye binding assay

APPENDIX D

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Apparatus:

BioRad Protean II xi Cell vertical slab electrophoresis and .Power supply. Wealtech Elite 300/300 plus

Reagents:

A) 30 % Acrylamide/bis acrylamide solution

Acrylamide is a nerve toxin. Therefore, precautions should be taken by wearing gloves and mask during preparation of this solution.

- Weigh 58.4 g acrylamide in a 500 ml beaker
- Add 1.6 g NN-bis-methylene-acrylamide
- Pour 100 ml dH_2O into the beaker, cover the beaker with aluminum foil due to light sensitivity, and mix on a magnetic stirrer
- When it is completely dissolved, complete the volume to 200 ml with dH_2O
- Store the solution in dark bottle at 4ºC.
- Do not use the solutions older than 30 days and before discarding any remaining solution let it polymerize by adding 10 % APS and TEMED

B) 10% SDS Solution

- Dissolve 10 g of SDS (Sodium Dodecyl Sulfate) in 100 ml dH_2O
- Gently stir to prevent foaming of SDS

- This solution can be stored at room temperature for long term

C) 1.5 M Tris-HCl, pH 8.8 buffer (for separating gel)

- Weigh 54.45 g Tris base and dissolve in 150 ml dH_2O by stirring on a magnetic stirrer
- Adjust the pH to 8.8 with concentrated HCl while continuously stirring
- Complete the volume to 300 ml with dH_2O and store at 4ºC
- Discard the remaining solution after 30 days

D) 0.5 M Tris-HCl, pH 6.8 Buffer (for stacking gel)

- Weigh 6 g Tris base and dissolve in 60 ml dH_2O
- Adjust the pH to 6.8 with concentrated HCl while mixing on a magnetic stirrer
- Complete the volume to 150 ml with dH_2O and store at 4ºC
- Discard the remaining solution after 30 days

E) 10% Ammonium persulfate (APS)

- Dissolve 100 mg APS in 1ml dH_2O in an Eppendorf tube by vortexing.
- Prepare freshly on the day of usage

F) Sample Buffer

Mix together
- 1 ml of 0.5 M Tris-HCl, pH 6.8 buffer
- 0.8 ml glycerol
- 1.6 ml of 10% SDS solution
- 0.4 ml β-mercaptoethanol
- 0.4 ml of 0.05% (w/v) bromophenol blue (in water)
- 3.8 ml dH_2O*

* In order to make a concentrated sample buffer (for use with diluted samples) you can exclude 3.8 ml distilled water.

G) Running Buffer

- Weigh 9 g Tris base and 43.2 g glycine and dissolve in 600 ml dH_2O
- Complete to volume to 1000 ml and transfer the solution into the electrophoresis tank.
- Dilute the solution to 3 ml with dH_2O.
- Add 3 g SDS and stir on a magnetic stirrer.
- Place the tank in cold room and stir continuously at a slow pace.

Procedure:

A) Preliminary Preparation

- Clean the surface of the glasses with pure ethanol
- Align the spacers at the two edges of the big glass and place the small glass on it. Adjust the bottom edges very carefully in order not to have leakage.
- Install the clamps and fasten screws.
- Place the glass sandwich on the base and check that the bottoms of the glasses are properly sealed by pouring dH_2O inside the sandwich.
- Dry the glasses with filter paper.

B) Preparation of SDS-PAGE Gel Solution

Separating Gel (for two 10 % Slab Gel of 16X18 cm):
Add the followings into an Erlenmeyer flask and shake gently:
- 33.5 ml of 30 % acrylamide/bis solution
- 40 ml of distilled water
- 25 ml of 1.5 M Tris-HCl, pH 8.8 buffer

- 1.0 ml 10% SDS solution
- 500 µl of 10% APS
- 50 µl of TEMED

Add APS and TEMED when you are ready to pour the gel inside the glass sandwich.

Immediately collect the gel solution into a glass pipette using a pump and discharge it into the space between the glasses very gently not to cause formation of bubbles. Fill the space up to 5 cm below the upper edge of the small glass.

With a Pasteur pipette, pour distilled water onto the gel in order to avoid the contact of the gels with air that prevents polymerization of the gel.

Allow 1 hour for complete polymerization.

Stacking Gel (for one 4% gel):

Add the followings into an Erlenmeyer flask and shake gently:

- 1.3 ml of 30 % acrylamide/bis solution
- 6.1 ml of distilled water
- 2.5 ml of 0.5 M Tris-HCl, pH 6.8 buffer
- 100 µl 10% SDS solution
- 50 µl of 10% APS
- 10 µl of TEMED

Take out the vast of the water by use of a filter paper. But do not over dry which will make the gel surface to run dry. Immediately pour the stacking gel solution between the glasses and place the comb inside the glass sandwich carefully not to cause any air bubbles. Allow to stand for at least 30 minutes for polymerization.

After polymerization is completed, take off the comb and wash the wells with loading buffer in order to remove the remaining of gel that is not polymerized.

C: Sample preparation and loading the gel

Samples and molecular weight markers are loaded into the wells by use of loading tips (Art Gel Molecular Bioproducts). The tip should be inserted to about 1-2 mm from the well bottom before delivery. The samples are prepared by placing them into boiling water in the presence of SDS and β-mercaptoethanol, the aim of this step is to denature the protein chains. Samples are mixed with the sample buffer with ¼ ratio (e.g.:75 µl sample and 25 µl sample buffer) Total volume loaded was 100µl

Load the standard molecular weight markers of 2.5 µl, each sample into one well. Install the slab gel sandwiches to the cooling core. Fill the upper chamber of the core with loading buffer without disturbing the loaded samples.

Gently place the cooling core into the electrophoresis tank. Make sure that there are no bubbles trapped on the lower side of the glass sandwich. If bubbles are present, remove them with a glass rode, since these bubbles can act as an insulator.

Conduct the separation at 4°C by operating at 25 mA/gel constant current in the region of stacking gel and then at 35 mA per gel for the separating gel. End the run when the dye front is approximately 2 cm from the lower side of the gel.

If you are going to do a Silver staining, when the separation is over, carefully extrude the gels and immerse them in fixing solution containing 50 % methanol, 12 % acetic acid and 0.5 ml of 37 % formaldehyde/L. perform shaking for 5 minutes on a platform shaker and keep the gels in fixing solution for at least 1 hour.

Stain the gels with silver staining procedure of Blum *et al.* (1987) (Appendix F).

Molecular weight markers used for SDS-PAGE are given in Table A.3.

Table A3: Molecular weight marker proteins used in SDS-PAGE

Proteins	***Approx. MW (kDa)***
Myosin, Rabbit Muscle	205.000
B Galactosidase, *E. coli*	116.000
Phosphorylase b, Rabbit	97.400
Albumin, Bovine	66.000
Albumin, Egg	45.000
Carbonic anhydrase Bovine Erytrocytes	29.000

APPENDIX E

NATIVE GEL ELECTROPHORESIS

All steps are the same as in the SDS-PAG except for there is no SDS solution and β-mercaptoethanol in the sample buffer, loading buffer and gel solution of Native-PAGE, distilled water is added instead.

APPENDIX F

SILVER STAINING OF THE POLYACRYLAMIDE GEL

The apparatus:

Shaker platform and covered plastic boxes

The solutions:

Fixer:

Mix 150 ml methanol + 36 ml acetic acid +150 µl of 37 % formaldehyde and complete to volume 300 ml with d H_2O.

A) 50 % Ethanol: Prepare 1 L of 50 % solution ethanol.

B) Pretreatment solution (sodium thiosulfate solution)

Dissolve 0.08 g sodiumthiosulfate ($Na_2S_2O_3$. 5 H_2O) in 400 ml d H_2O mix by a glass rod and take 8 ml for further use in developing solution preparation.

C) Silver nitrate solution:

Dissolve 0.8 g silver nitrate in 400 ml d H_20 and add 300 µl 37 % formaldehyde.

D) Developing solution:

In an Erlenmeyer flask measure 9 g potassium carbonate and 8 ml previously kept pretreatment solution and 300 µl 37 % formaldehyde. Complete the volume to 400 ml with d H_20

E) Stop solution:

In an Erlenmeyer flask mix 200 ml methanol and 48 ml acetic acid and complete to 400 ml with d H_2O

Procedure:

The procedure given in the table below is followed. All the steps are performed by a constant shaking on a platform shaker.

Table A4: Steps of Silver Staining

	Step	Solution	Time	Comments
1	Fixing	Fixer	>1 hour	The gel can be kept overnight in this solution
2	Washing	50 % Ethanol	3X20 min	
3	Pretreatment	Pretreatment solution	1 min	Time should be exact
4	Rinse	dH_2O	3X20 sec	Time should be exact
5	Impregnate	Silver nitrate solution	20 min	
6	Rinse	dH_2O	2X20 sec	Time should be exact
7	Developing	Developing solution	5 min*	
8	Wash	dH_2O	2X2 min	
9	Stop	Stop solution	> 10 min	

* Time is adjusted by one's self according to the color development. To decrease the fast reactions distilled water can be added into the developing solution.

APPENDIX G

ACTIVITY STAINING OF HEXOKINASE

Preparation of the native gel for activity staining:

1. Prepare a 10 % native gel as described in Appendix

2. Pre-electrophoreses the empty gel either at 10 mA/gel overnight or 30 mA/gel for 4 hours at 4 °C.

3. Load the samples into the wells so that that each contains at 0.0030-0.0060 U of activity. Dilute samples can be concentrated using Vivapore 2 (product no : VP0201 and even this is not enough to concentrate, load at most 100 µl of sample regardless of the total activity that is loaded.

4. Activity staining of the gel:

Table A5: The chemicals of activity staining

Chemicals	mg/25 ml (Tris HCl pH 7.7)
D(+) glucose	50 mg
ATP	30 mg
NADP	15 mg
MTT	5 mg
PMS	1 mg
$MgCl_2.6\ H_2O$	10 mg
Glucose-6 dehydrogenase	0.4 u/ml

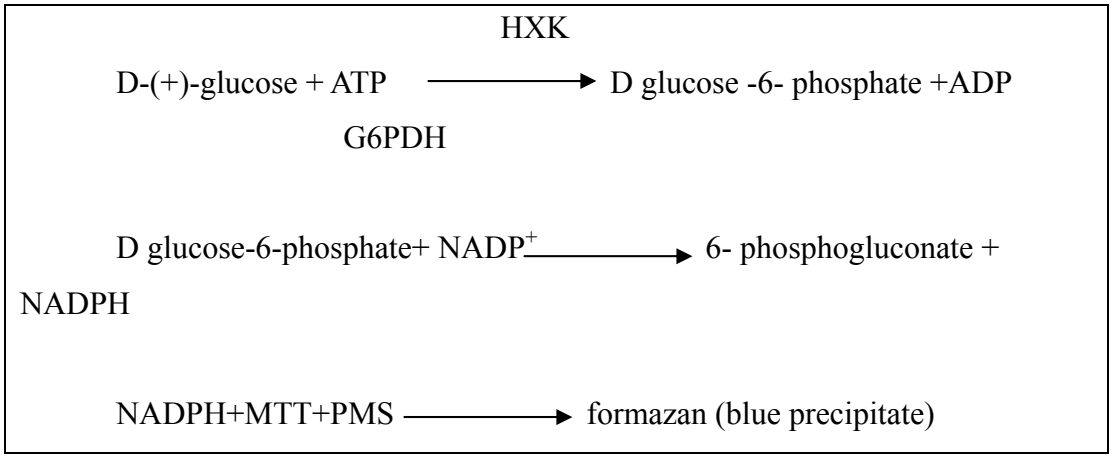
1. Dissolve all of the chemicals in 25 ml of 0.1 M pH 7.8 Tris- HCl.

2. Prepare 2 % agarose in distilled water (25 ml) and keep it at 65 °C.

3. Lay the native gel slab into a plastic box layered with a glass plate (glass plate will help to handle the fragile gel easily)

4. Mix agarose gel and staining solution and immediately pour on the gel surface. Incubate the gel in dark at 30°C until dark blue bands appear on light background. For fructose phosphorylating activity 1 U/ml phosphoglucose isomerase should be added to the staining solution. When fructose phosphorylating activity is measured fructose treated by glucose oxidase is used as substrate.

The principle underlying the activity staining of hexokinase is given in the scheme below.



HXK: Hexokinase

G6PDH: Glucose -6-phosphate dehydrogenase

MTT: 3-[4,5 Dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide

PMS: Phenazine Methosulfate (N-methyldibenzopyrazine methyl sulfate salt)

APPENDIX H

ELECTROBLOTTING

Reagents:

100% HPLC grade methanol

10 mM CAPS (3-(cyclohexylamino)- 1-propane sulphonic acid) buffer pH 11

Procedure:

1. Cut the blotting paper and the gel the same as the size of the membrane (BIO-RAD Sequi-Blot^{Tm} PVDF Membrane for protein Sequencing (0.2µm) (7x8,4 cm) Catalog no162-0186)

2. Overlay 3 sheets of pre-wetted with electroblotting buffer: 10 mM CAPS pH 11 and 10 % methanol) filter papers onto the anode area of the elctroblotting apparatus (Serva Blue Flash M)

3. Pre-wet the membrane with 100 % HPLC grade methanol for 5 minutes and soak thoroughly in electroblotting buffer.

4. Place the pre-wetted membrane onto three layers of filter paper

5. Equilibrate the gel with electroblotting buffer

6. Place the gel on top of the membrane

7. Lay a stack of filter paper soaked in the electroblotting buffer on top of the gel and complete the gel sandwich.

8. Be sure that the gel sandwich is touching to the cathode surface of the electroblotting apparatus.

9. The membrane and the filter papers are cut into the size of the gel to make sure that the current passes through the gel and the membrane. Remove the

air bubbles by rolling a glass tube gently on each layer in order to assure there is no interruption of the current

10. Place the cathode and close the cassette.

11. Connect the power supply and apply a current at 88.4 MA for one hour The gel should be electroblotted at 1-1.5 mA/cm^2 of blotting membrane (*e.g.* 50 mA for a mini-gel) for 60-90 min

12. Stain the membrane by Coomassie staining and destain in 10 % methanol (stain the gel with Coomassie staining and destain to make sure that the protein bands are transferred to the membrane.)

13. When you visualize the bands in destaining wash the membrane with distilled water.

14. Dry the blot thoroughly between two filter papers and store at -20°C

APPENDIX I

COOMASSIE STAINING

Reagents:

0.1 % Coomassie Blue R
12 % Glacial acetic acid
50 % Methanol

Procedure:

Dye is dissolved in methanol and glacial acetic acid is added. The solution is completed to final volume of 1 L with dH_2O & filtered through a course filter paper before use.

Destaining solution:

30% methanol
7% glacial acetic acid

Destainig solution is prepared complete final volume to the 500 ml and gel is washed with this solution. When color of destaining solution turn the blue this solution is poured then fresh solution is added to gel. This process is repeated till the background is clear.

APPENDIX J

THE GRAPH OF LOG MW AND R_F VALUE TO DETERMINE MOLECULAR WEIGHT

The molecular weight of pure protein was calculated according to Log MW versus R_f value.

Take the logarithm of Marker's molecular weight.

Draw the graph Log MW versus R_f value (described in material methods chapter). Calculate the molecular weight of unknown sample from the equation of the line. The graph of the molecular weight determination for hexokinase isoenzymes from *Rhizopus oryzae* grown on 2 % glucose

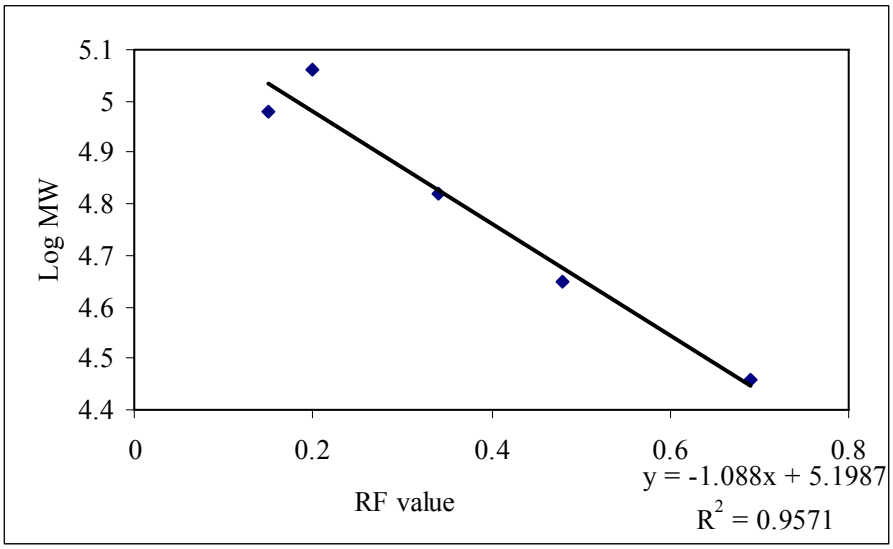


Figure A3: Log molecular weight and R_f value

The graph of the molecular weight determination for hexokinase isoenzymes from *Rhizopus oryzae*

APPENDIX K

GEL FILTRATION COLUMN AND ANION EXCHANGE COLUMN

The volume of the gel filtration column was 120-124 ml and the column material was Superdex 200. Superdex prep grade (pg) is produced by covalent binding of dextran to highly cross-linked agarose. The separation properties of these composite media are determined by the dextran component. The media combines high mechanical strength with high hydrophocity allowing high flow rates and minimal non-specific interactions.

The bed volume of anion exchange column was 20 ml the bed height was 100 mm and i.d. 16 mm HiPrep16/10 Q XL matrix 6% highly cross-linked spherical agarose charged group $N^{+}(CH_3)_3$

APPENDIX L

STATISCAL ANALYSIS OF TREHALOSE-6-PHOSPHATE INHIBITION

ISOENZYME 1

Activity (U/ml)	Trehalose-6-P conc.(mM)
0.0296	0
0.0266	0
0.0249	0
0.0247	1
0.0248	1

One-way ANOVA: Activity (U/ml) versus Trehalose-6-P conc.(mM)

Analysis of Variance for Activity

Source	DF	SS	MS	F	**P**
T-6-P	1	0.0000063	0.0000063	1.66	**0.288**
Error	3	0.0000113	0.0000038		
Total	4	0.0000			

ISOENZYME 2

Activity (U/ml)_1	Trehalose-6-P conc.(mM)_1
0.0220	0
0.2000	0
0.0210	1
0.0192	1

One-way ANOVA: Activity (U/ml) versus Trehalose-6-P conc.(mM)

Analysis of Variance for Activity

Source	DF	SS	MS	F	**P**
T-6-P	1	0.00826	0.00826	1.04	**0.415**
Error	2	0.01584	0.00792		
Total	3	0.02411			

Commercial HXK

Activity (U/ml)_1_1	Trehalose-6-P conc.(mM)
11.92	0
11.15	0
8.08	1
8.77	1

One-way ANOVA: Activity (U/ml) versus Trehalose-6-P conc.(mM)

Analysis of Variance for Activity

Source	DF	SS	MS	F	**P**
Trehalose	1	9.672	9.672	36.19	**0.027**
Error	2	0.534	0.267		
Total	3	10.207			