PRIMARY STRUCTURE STUDY OF PHOSPHOFRUCTOKINASE FROM *Streptococcus lactis*

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ABSTRACT

Phosphofructokinase is an important regulatory enzyme in the glycolytic pathway catalysing the phosphorylation of Fructose-6-phosphate to Fructose-1,6-bisphosphate.

Phosphofructokinase from *Streptococcus lactis* was isolated by chromatographic methods including DEAE Cellulose ion-exchange and Cibacron Blue Sepharose dye-ligand chromatographies.

Phosphofructokinase from *Streptococcus lactis* was digested with trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease and CNBr and the peptides obtained were sequenced and aligned against the sequences of *Escherichia coli* and *Bacillus stearothermophilus* phosphofructokinases.

326 out of 328 amino acid residues of *Streptococcus lactis* phosphofructokinase were obtained in this study.

The comparison of *Streptoccus lactis* phosphofructokinase with *Escherichia coli* and *Bacillus stearothermophilus* phosphofructokinases showed that the sequence similarities among them are above 50%.

Most of the secondary structures are conserved in

Streptococcus lactis phosphofructokinase. The two α-helices at the carboxyl terminal of bacterial phosphofructokinases are longer in *Streptococcus lactis* than in *Escherichia coli* and *Bacillus stearothermophilus*.

The residues involved in binding of Fructose-6-phosphate/Fructose-1,6-bisphosphate are the same in the bacterial phosphofructokinase and the residues involved in binding of ATP/ADP and binding of effectors have high degree of similarity.

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LIST OF ABBREVIATIONS

ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
bis acrylamide	N′N-methylene-bis-acrylamide
ßME	ß-mercaptoethanol
BS	*Bacillus stearothermophilus*
BSA	bovine serum albumin
cAMP	cyclic-AMP
DEAE	diethyl amino ethyl
DNA	deoxyribonucleic acid
EC	*Escherichia coli*
EDTA	ethylenediamine tetra acetic acid
F6P	fructose-6-phosphate
F1,6BP	fructose-1,6-bisphosphate
FPLC	Fast protein liquid chromatography
Hpr	heat stable protein in PEP-PTS
NADH	nicotinamide adenine dinucleotide, reduced form
PEP	phosphoenolpyruvate
PEP-PTS	phosphoenolpyruvate phosphotransferase system
PFK	phosphofructokinase
P	inorganic phosphate
PTH	phenylthiohydantoin
RPLC	Reverse-phase liquid chromatography
SL	*Streptococcus lactis*
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TFA	tetrafluoro acetic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
Tris	tris (hydroxymethyl) aminomethane

AMINO ACID ABBREVIATIONS

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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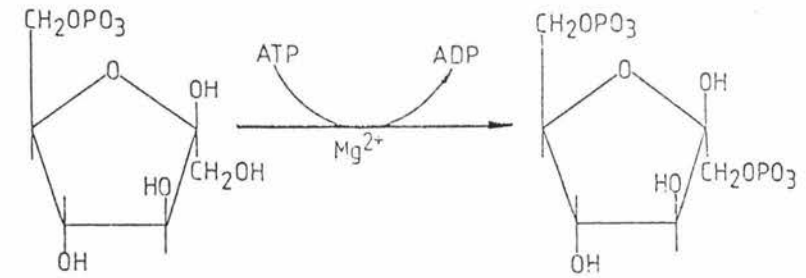
CHAPTER ONE: INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 General Background of Phosphofructokinase

Phosphofructokinase (ATP:D-frutose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) catalyses the transfer of the γ-phosphate group of ATP to the C-1 hydroxyl group of fructose-6-phosphate (F6P) to form fructose-1,6-bisphosphate F1,6BP) and ADP as shown below:



Fructose-6-phosphate

Fructose-1,6- bisphosphate

This reaction is involved in the glycolytic pathway of all organisms. In 1936, the first report of PFK in glycolysis was published (Negelein, 1936). Since then, great effort has been concentrated on the study of PFK from various sources and the knowledge of the function, the structure, the reaction mechanism and the regulatory properties of PFK has been expanded.

The conversion of F6P to F1,6BP is catalysed by PFK while the reverse reaction is catalysed by fructose-1,6-bisphosphate (F1,6BP) phosphatase. This division of the reaction between two enzymes in the metabolic pathway enables the reaction to be easily controlled by different metabolites according to the need of the cell

for energy and for metabolic intermediates provided by the glycolytic pathway.

1.1.2 Structural aspects of PFK

PFK has been isolated and studied from various sources, including bacteria, plants, yeast and animals (Mansour, 1972; Ramaiah, 1975; Hofmann, 1976; Goldhammer and Paradies, 1979; Uyeda, 1979). A common feature of the PFKs from different biological origins is their tetrameric structure although their molecular weights are quite different (Rutherfurd, 1988). The PFKs from different bacteria are similar in their primary sequence and their secondary, tertiary and quarternary structures (Shirahara and Evans, 1988). The primary structure of mammalian PFKs has an internal homology and is similar to that of bacterial PFKs, which suggests that they evolved by duplication of an ancestral gene now represented by the bacterial PFK (Poorman et al., 1984).

The subunit molecular weight of mammalian PFKs is about 85,000 (Uyeda, 1979). While the smallest active form of mammalian PFKs is the tetramer structure, the enzymes are capable of aggregating to form the more active high molecular weight multimeric structures (Paetkau and Lardy, 1967; Layzer et al., 1969; Kemp, 1971; Tarui etal., 1972; Massey and Deal, 1973; Dunaway, 1974; Brand and Soling, 1974; Trujillo and Deal, 1977; Reinhart and Lardy, 1980; Foe and Kemp, 1985).

Yeast PFKs have subunit molecular weight of about

100,000 (Kopperschlager et al., 1977). They consist of two non-identical subunits α and ß, differentiated by their antigenic properties (Herrmann et al., 1973). The enzymes can form high molecular weight multimers the same as the mammalian enzymes, but they do not undergo the association and dissociation that occurs in mammalian enzymes.

Plant PFKs have a subunit molecular weight of about 80,000 (Glodhammer and Paradies, 1979). They have similar regulatory properties to the PFKs from animals and bacteria and they may have homology to the enzymes from animals and bacteria.

Bacterial PFKs have a subunit molecular weight of 35,000 (Blangy, 1968; Hengartner and Harris, 1975; Uyeda and Kurooka, 1970). Unlike the eukaryotic PFKs, they do not aggregate to form structures higher than tetramers. The known primary, secondary, tertiary and quarternary structures of *Escherichia coli* (Shirakihara and Evans, 1988) and *Bacillus stearothermophilus* (Evans and Hudson 1979) PFKs are similar. Four subunits, each of which is divided into two domains, are arranged in a way that one contacts with only two of the others. The enzyme has three sites which bind the substrates F6P and ATP and effectors. The F6P binding site sits between two subunits of the tetramer while the allosteric effector binding site sits between another pair of subunits. These ligand bridges between subunits mediate the substrate cooperativity and the allosteric control.

The cooperativity and the allosteric control of the EC PFKs are explained well by the two-state concerted allosteric model described by Monod et al (1965), that is the transition between the conformations of active R-state and the inactive T-state (Evans et al, 1981) and this is based on the conclusions of Blangy et al (1968) that the enzyme's two states have different affinity for F6P but the enzyme does not change the catalytic rate once the substrate binds to the enzyme.

1.1.3 Regulation of PFK activity

Eukaryotic PFKs and prokaryotic PFKs are allosterically controlled and the binding of substrate F6P shows cooperativity. Although enzymes from different sources have their particular ways of regulation, the following features are conserved (Fordyce, 1982):

i. Enzyme activity has a sigmoidal dependence on F6P concentration.
ii. Enzyme activity changes with the energy requirements of the cell, increasing when ATP is required.
iii. Enzyme activity has a feedback inhibition by the intermediates in the carbohydrate metabolic pathways. These intermediates are citrate in mammals and phosphoenol pyruvate (PEP) in bacteria.

The way by which the effectors regulate PFK activity is to alter the affinity of the enzyme for substrate F6P. The inhibitors decrease the affinity and the activators increase the affinity. The eukaryotic PFKs are inhibited by citrate and ATP and activated by phosphate, AMP, and cAMP; while the bacterial PFKs are inhibited by PEP and activated by ADP. The mammalian PFKs are also subjected to hormonal regulation through phosphorylation/dephosphorylation (Brand and Soling, 1975; Hussey et al., 1977). For example, the rabbit muscle PFK is activated by phosphorylation catalysed by a cyclic 3',5'-AMP-independent kinase and inactivated by a phosphatase catalysed dephosphorylation and these two enzymes are subjected to hormonal control. The mammalian PFK can self-associate to an oligomeric form larger than the active tetramer giving it a higher affinity for the substrate F6P and the association/dissociation transition changes with a variety of factors such as the enzyme concentration, the level of metabolic effectors and the pH (Rutherfurd, 1988). The regulation of bacterial PFKs is relatively simple. They are only regulated allosterically and the number of effectors is less than that for eukaryotic PFKs. They are not activated by AMP and not inhibited by either citrate or ATP.

1.2 LACTOSE METABOLISM IN *S. lactis*

S. lactis can utilize lactose in milk to form lactic acid so that milk pH drops and as a result, milk proteins precipitate to start cheese formation and thus it takes an important role in New Zealand cheese production.

The lactose metabolic pathways in S. lactis are shown in figure 1. Lactose is transferred into the cell by a specific phosphoenol pyruvate phosphotransferase system (PEP-PTS) (Figure 2). Four kinds of protein are involved in this system, in which enzyme II and factor III are specific for lactose while enzyme I and a small heat stable protein (HPr) are nonspecific. Lactose is phosphorylated at carbon 6 position when it is transferred into cells. The lactose-6-phosphate is then cleaved into glucose and galactose-6-phosphate (Gal-6-P) through the reaction catalysed by phospho-ß-galactosidase. Glucose is metabolized to triose-phosphates through the glycolytic pathway while Gal-6-P produces the same triose-phosphates through the tagatose-6-phosphate pathway (T6P pathway). The triose-phosphates are then converted to lactic acid through the same pathway.

The glycolytic pathway and the T6P pathway are similar processes. Tagatose-6-phosphokinase (T6PK) and tagatose-1,6-bisphosphate aldolase (T1,6BP aldolase) are equivalent to PFK and fructose-1,6-bisphosphate aldolase (F1,6BP aldolase).

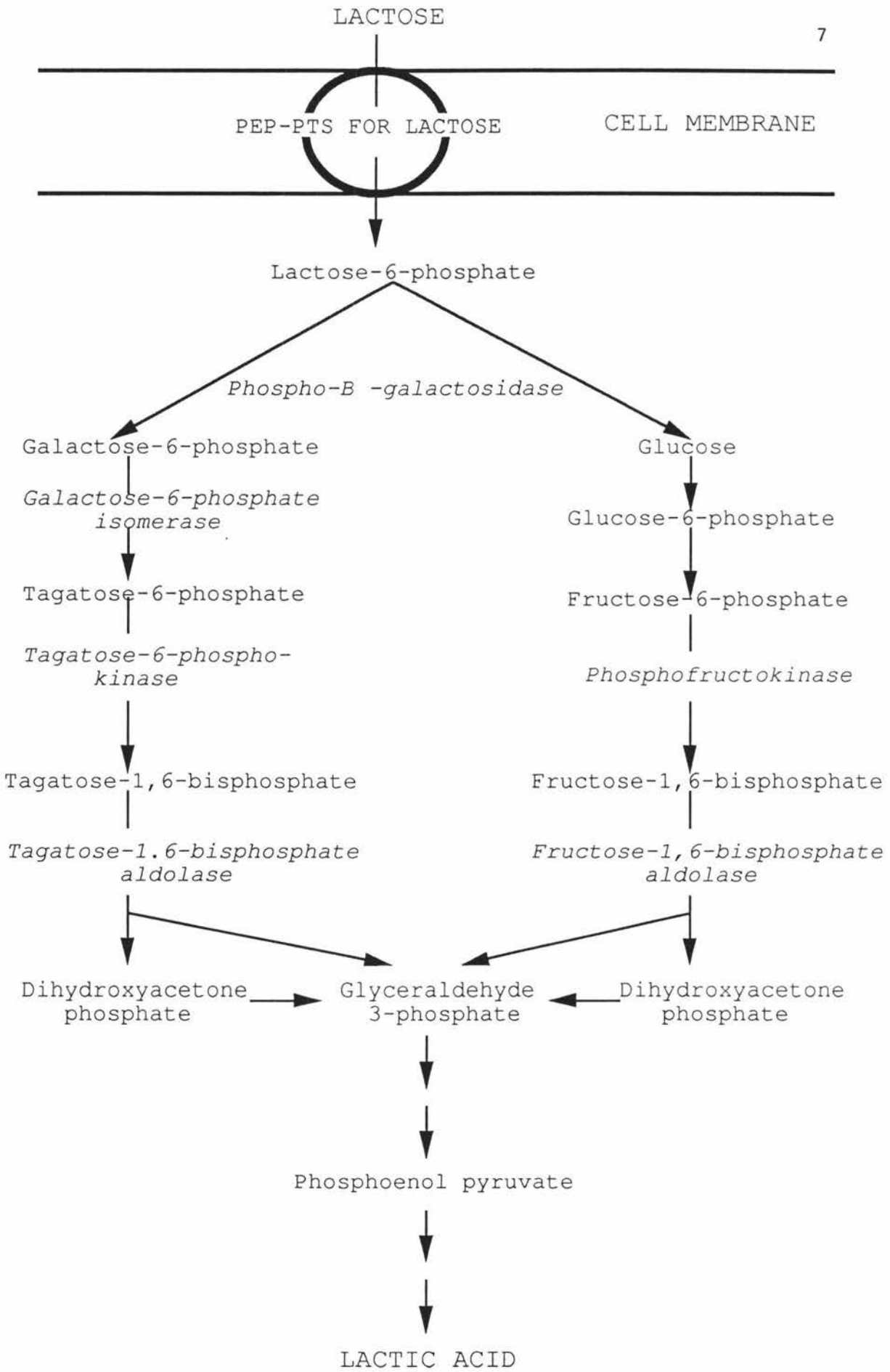
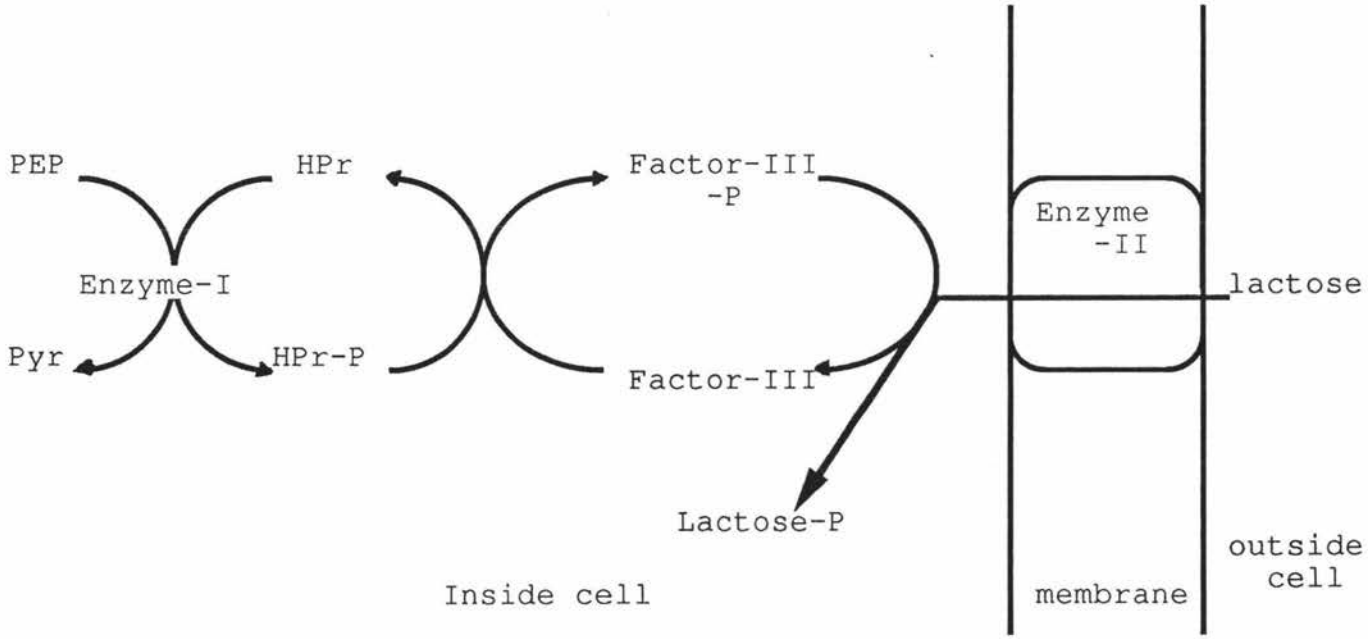


FIGURE 1 LACTOSE METABOLISM IN S. lactis

FIGURE 2: PHOSPHOENOL PYRUVATE PHOSPHOTRANSFERASE SYSTEM (PEP-PTS)



The PEP-PTS for lactose and the T6P pathway are induced when S. lactis is grown on lactose or galactose. The genes for enzyme II, factor III of the PEP-PTS; galactose-6-phosphate isomerase, T6PK, T1,6BP aldolase of T6P pathway and phospho-galactosidase are on a plasmid (Crow et al, 1983).

1.3 DETERMINATION OF THE AMINO ACID SEQUENCE OF A PROTEIN

The determination of the amino acid sequence of a pure protein involves several steps.

First, the protein has to be fragmented to get peptides of a suitable size for sequencing because the number of residues which can be sequenced by the current sequencing methods is limited due to the loss of material or due to the lag reaction during sequencing.

There are enzymic and nonenzymic methods for protein fragmentation. Partial acid hydrolysis is one of the oldest methods used. It is nonspecific and the peptides produced are unpredictable and small. This method is suitable for small proteins being sequenced manually. Because of the random digestion, some overlapping fragments can occur and this is useful for alignment of the total sequence. But this method produces many di and tri peptides and this can give ambiguous results for large proteins. The studies of the high specificity of the proteolytic enzyme trypsin by Bergmann et al.(1939) led to the application of proteolytic enzymes in the specific cleavage of proteins. Trypsin is the most commonly used proteolytic enzyme and it cleaves the peptide chain specifically on the carboxyl side of lysine and arginine. The cleavage can be restricted with the blocking of the ε-amino groups of lysine by carbamylation or acetylation.

Trifluoroacetylation and maleylation may be used to block the ε- amino groups of lysine reversibly (Kasper, 1975). Aminoethylation of the cysteine residues with ethyleneimine was introduced to form another site for tryptic cleavage (Raftery and Cole, 1966). There are also some other proteolytic enzymes which can be used to fragment proteins or to further digest the large peptides obtained by other procedures. Proteins may be cleaved by chymotrypsin at the carboxyl side of tyrosine, phenylalanine, tryptophan or methionine. Leucine is cleaved more slowly. Thermolysin cleaves proteins and peptides on the amino side of isoleucine, leucine, valine, phenylalanine, alanine, methionine and tyrosine. If proline is at the carboxyl side of the thermolysin-sensitive residue , the cleavage will not be successful. *Staphylococcus aureus* V8 protease, a microbial enzyme, can hydrolyse proteins or peptides on the carboxyl side of glutamic acid specifically (Houmard and Drapeau, 1972). Pepsin can hydrolyse peptide bonds formed by either amino or carboxyl groups of phenylalanine, glutamic acid, cysteine and cystine. Among the non-enzymic methods of specific cleavage of proteins and peptides, CNBr is the most specific and the most useful reagent. CNBr selectively cleaves the peptide bond on the carboxyl side of the methionine residues (Figure 3). The initial reaction is the formation of a methionine cyanosulphonium derivative(II), which is converted into the transient homoserine iminolactone(III). Then the reaction spontaneously forms a new amino terminal peptide and a

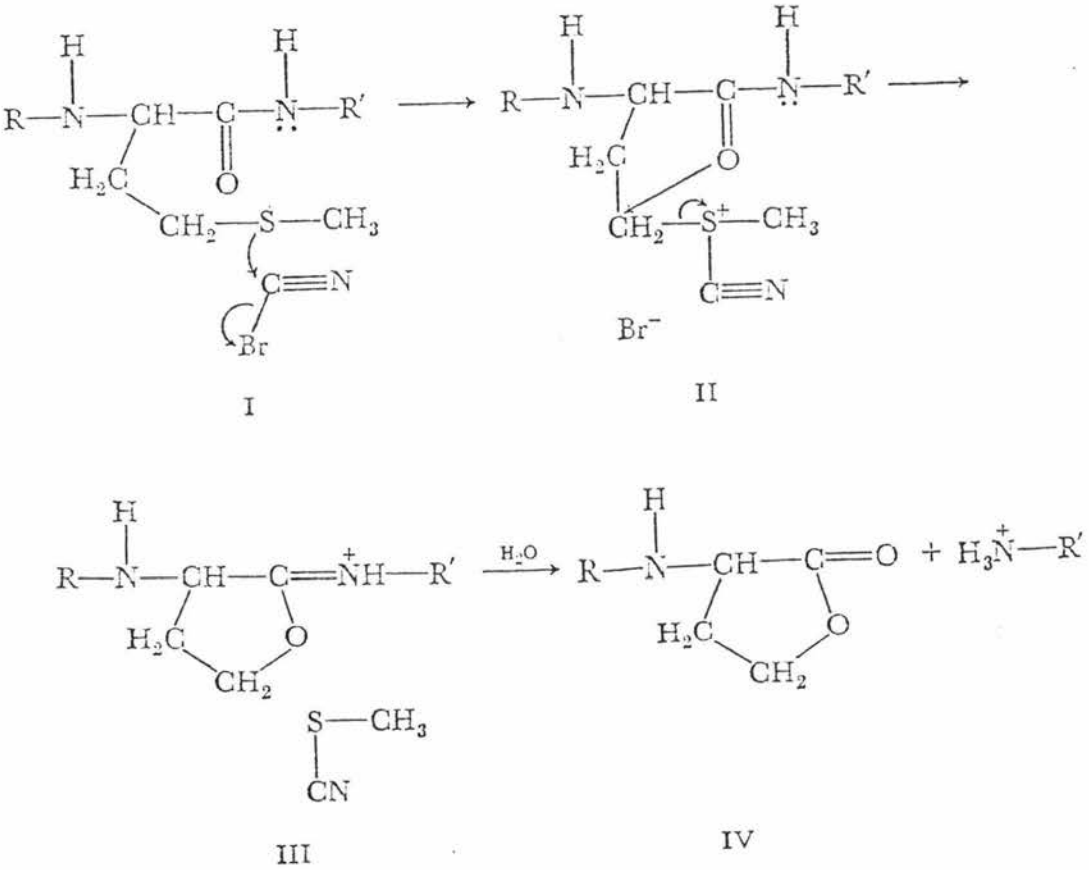


FIGURE 3: CNBr CLEAVAGE OF PROTEIN
I: Protein + CNBr
II: methionine cyanosulfonium derivative
III: homoserine iminolactone
IV: shortened protein + homoserinelactone peptide

homoserine lactone peptide fragment. Also some other selective cleavage methods were investigated by a number of researchers. 2-(2-nitrophenylsulphenyl)-3-methylindole (BNPS-Skatole) (Ozol and Gerad, 1977; Mahoney and Hermodson, 1979) were found to be relatively good reagents for selective cleavage at tryptophan. After cyanylation, peptides or proteins can be cleaved at cysteine residues (Degani and Patchornik, 1971). Hydroxylamine can be used to cleave the asparagine-glycine bonds (Bornstein, 1970). In weak acid conditions, such as 10% acetic acid or 70% formic acid, Asparagine-proline bonds can be cleaved (Piszkiewicz, London and Smith, 1970). Because these methods have serious side reactions and are less efficient than CNBr, they are not used as commonly as CNBr.

Since the three dimensional structures of native proteins limit the exposure of the sensitive peptide bonds to the proteolytic enzymes or reagents, the proteins have to be denatured to ensure complete reaction. The methods include heating and trichloroacetic acid precipitation. If the proteins are stabilized by disulphide bonds, they have to be cleaved before unfolding. The methods include reduction followed by S-carboxymethylation of the reduced thiol groups (Crestfield, Moore and Stein, 1963) and performic acid oxidation.

The methods used for separation of the peptides obtained from digestions are paper electrophoresis, gel

electrophoresis, gel filtration chromatography, and ion-exchange chromatography. The recent introduction of the Fast Protein Liquid Chromatography (FPLC) and the High Performance Liquid Chromatography with different kinds of columns has improved the separation power greatly enabling small amounts of peptides to be analysed. The Reverse Phase Liquid Chromatography (RPLC) has become the dominant tool for isolation of the peptides (Shively, 1986). It has hydrophobic organic substances such as open-chain hydrocarbon or aromatic hydrocarbon, covalently linked to the surface of silica beads to bind the hydrophobic peptides. The solvent systems used to elute the peptides consist of an aqueous buffer system containing a water-miscible organic solvent called the organic modifier which can be easily changed to suit the properties of the peptides being separated. Acetonitrile, propanol and methanol are the most popular organic modifiers.

After the peptides have been purified, they can be subjected to sequence analysis. In the history of protein sequencing, many methods have been used, but the Edman degradation developed by P. Edman in 1950 is still widely used although the detailed procedures have been modified. The reactions of the Edman degradation include addition, cleavage and conversion (Figure 4). They are carried out by sequentially adding the reagents and removing the excess reagents and byproducts. The thiazolinone is separated from the shortened peptide before converting it to the more stable

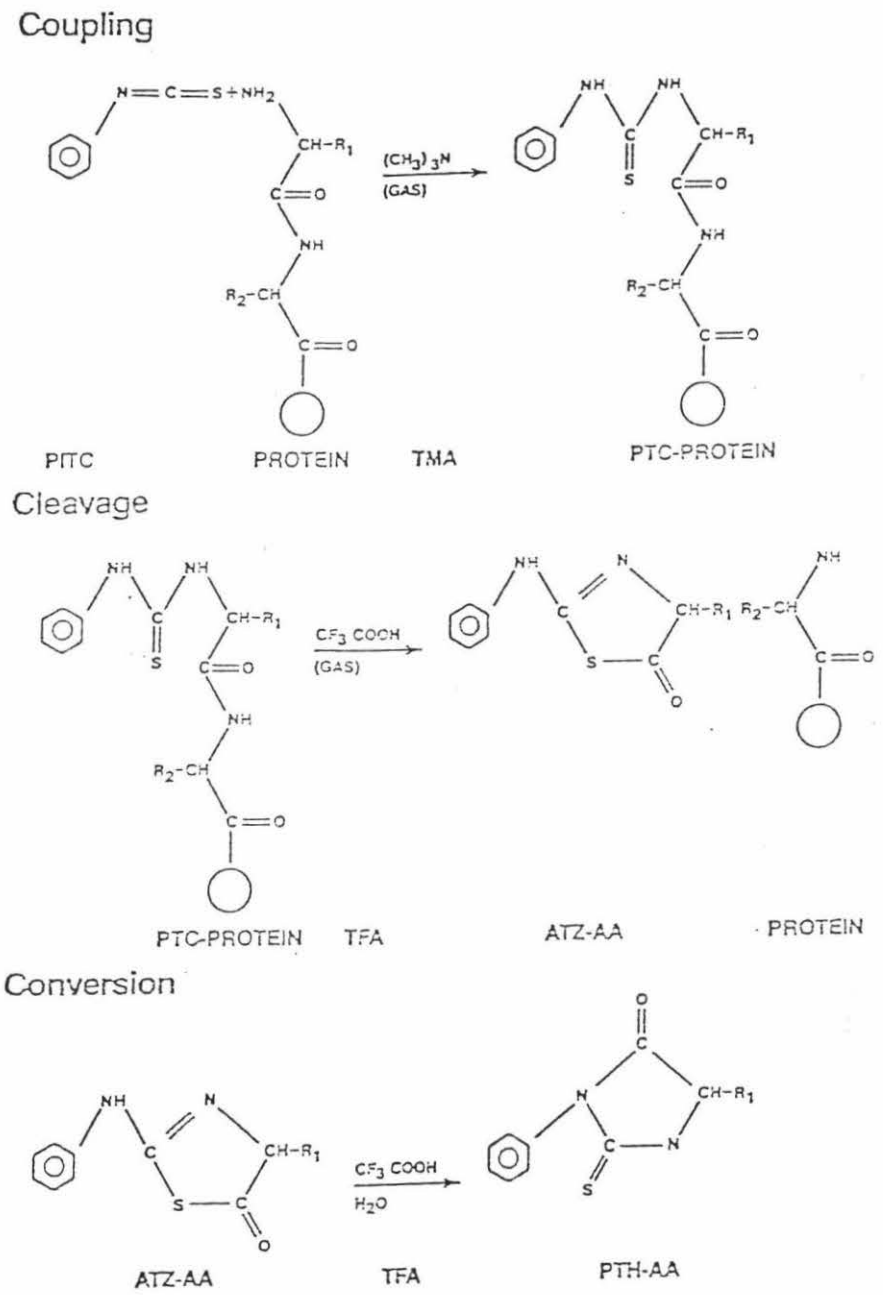


FIGURE 4: EDMAN DEGRADATION MECHANISM
PITC: phenyl isothiocyanate
TMA: trimethylamine
PTC-protein: phenylthiocarbamoyl(PTC)-protein
TFA: trifluoroacetic acid
ATZ-AA: thiazolinone-amino acid
PTH: phenylthiohydantoin

phenylthiohydantoin (PTH) which can then be analysed by chromatographic methods. The newly exposed amino terminal amino acid of the peptide can react again with the Edman reagents and the cycle is repeated. Problems that may occur with this procedure include the loss of peptides during extraction and the desulphurization of the coupled amino terminal residue which prevents the cleavage of this residue.

The Edman-degradation mechanism was first used in protein sequencing as a manual method and the major disadvantages of the early procedures were the difficulties in separating and characterising the PTH derivatives and the replacement of the sulphur atom in the coupled amino terminal with oxygen when air was present during the first extraction step where the excess reagents and byproducts were removed. In the 1960s, Hartley (1969) introduced the dansylation method which enabled the amino terminal derivatives to be easily detected by thin-layer chromatography and improved the sensitivity of sequencing due to the fluorescent nature of the dansyl derivatives. Also this method eliminated the first extraction step and so the desulphurization was reduced. The average number of residues which can be sequenced by manual methods is about fifteen and this limitation restricts the use of manual methods on large proteins.

In 1967, the first automatic sequenator was described by Edman and Begg (1967). The coupling and

cleavage reactions took place in a cup which was spinning to make the reaction mixture form a thin liquid layer on the wall of the cup so that the extraction of the excess reagents and the byproducts was efficient. Some peptides were also extracted during extracting step, but the introduction of polybrene, a polyquaternary amine with positive charges, reduced the loss of the peptides by keeping the peptides in the cup without being extracted by organic solvent through forming hydrogen bonds or providing a stable polar environment.

Laursen (1971) introduced a solid phase sequenator where the peptides were covalently attached to a solid support so that the peptides would not be lost during the extraction step. Peptides could be attached to the resin with their carboxyl terminal residues by different methods. They could be coupled with their α-carboxyl groups of the carboxyl terminal residues, with the amino groups of the carboxyl terminal lysine residues, or with the homoserine carboxyl terminus. But these reactions are often not efficient resulting in the loss of the peptides during the coupling procedure.

In early 1980s, the gas phase sequenator (Hewick et al., 1983) was invented which increased the efficiency and the sensitivity of the automated sequencing procedure. The important feature of this sequenator is that the reagents, which can dissolve the peptides sitting in the glass fibre disc, are supplied in gas

phase to prevent the loss of the peptides through extraction. The automatic sequencing procedures prevent the desulphurization by gassing the reagents and the solvent with argon gas.

In contrast to the amino terminal sequencing, the carboxyl terminal sequencing is not as satisfactory. Some carboxyl terminal exopeptidases are used. Carboxypeptidase A, which hydrolyses most of the amino acids at carboxyl terminus at various rates except for carboxyl terminal of proline and charged amino acids; and carboxyl peptidase B, which cleaves the carboxyl terminal of lysine and arginine, are the most widely used enzymes. These two enzymes are often combined together in practical use. In addition, carboxypeptidase C which can cleave all the amino acids from carboxyl terminal at a good rate except glycine is now commercially available. Carboxypeptidase Y which can cleave most amino acids except aspartic acid, histidine, arginine, lysine and glycine is the most recent exopeptidase to be used extensively for the carboxyl terminal sequence analysis because of its good specificity and strong action on substrates.

Entering 1980s, mass spectrometry (MS) has appeared as an important tool for the determination of the primary structure of proteins. This technique has become possible in protein study because of the introduction of the Fast Atom Bombardment Mass Spectrometry (FABMS) by Barber (1981) in which a peptide

is dissolved in a solvent such as HCl treated glycerol and is irradiated with a beam of atoms (Ar or Xe) to form secondary ions. This method has removed the previous limitation of MS of vapourizing the substrates. The molecular ions can then be separated precisely by their mass to charge ratio in the magnetic sector of the instrument. Since the FABMS can give the molecular weight of the protonated peptides precisely, it can not only confirm the peptide sequences obtained from Edman-degradation, but also give an indication of the modification of the peptides which cannot be shown by Edman-degradation. Thus FABMS is a good complement to Edman-degradation. Also FABMS can give the sequence information from the masses of different peptide species formed by fragmentation by bombardment.

In 1980s, the development of the DNA sequencing techniques provides an indirect method for amino acid sequence determination because each amino acid has a specific codon and the specific arrangement of these codons in DNA indicates the amino acid sequence. Although this method is quicker than that acting directly on protein, the protein sequencing is still used widely and cannot be replaced by DNA sequencing. There are some reasons for this phenomenon. First, from DNA to protein, several steps are involved. To form mRNA, DNA information has to be processed including splicing. Thus the mRNA sequence is not the simple copy of DNA sequence. After translation, the proteins need to be modified such as cutting of some terminal

sequences and chemical modification (Phosphorylation, methylation and glycosylation etc.). Unfortunately, the DNA sequence cannot tell these changes. Secondly, to clone the DNA sequence, probes are needed which are generally obtained by reverse genetics from a pieces of protein sequence. Thus it is important and necessary for the protein sequencing techniques to exist.

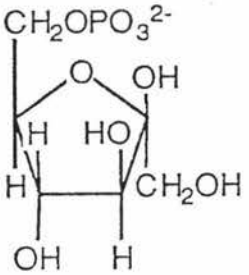
1.4 AIMS

PFK plays an important role in regulating the glycolytic rate so that a suitable energy and metabolic intermediate supply can be obtained. Thus it is necessary to study its catalytic mechanism and regulatory properties. Since these properties are related to enzyme structure which is coded in the protein sequence, the study of the primary structure of PFK is especially important in understanding its properties.

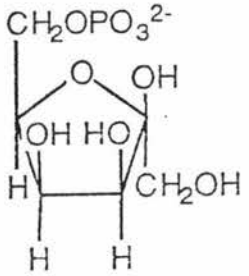
In *S. lactis,* the lactose metabolism includes two parallel and similar pathways, the T6P pathway to metabolize galactose-6-phosphate from the cleavage of lactose and the glycolysis pathway to metabolize glucose from the cleavage of lactose. The end products of the two pathways are the same, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. these two pathways coordinate with each other so that neither glucose nor galactose-6-phosphate accumulate intracellularly. There must be some regulatory mechanisms existing inside cells which adjust the two pathways to operate at equal rates. The study and comparison of the enzymes involved in the two pathways should provide a basis for the understanding of the regulatory mechanisms.

Interestingly, the PFK and T6PK (Tagatose-6 phosphokinase) in the two pathways catalyse similar phosphorylation reactions and their substrates are isomers whose only difference is the position of the

hydroxyl group at carbon 4.



Fructose-6-phosphate



Tagatose-6-phosphate

Thus a study of PFK and T6PK, comparing their primary and tertiary structures and their catalysing mechanism will help us to understand their catalytic mechanisms and also their specificities.

In this study, the PFK was isolated from *S. lactis* and digested with trypsin, chymotrypsin, *S. aureus* V8 protease and CNBr. The peptides obtained were separated by Superose-12 gel filtration on Fast protein liquid chromatography (FPLC), Reverse-phase liquid chromatography (RPLC) and G-50 Sephadex gel filtration chromatography. The amino terminal sequences of the peptides were deduced using a gas-phase sequencer and then confirmed by amino acid analysis and FABMS in some cases.

Chapter three describes the isolation of PFK from *S. lactis*. The amino acid sequences of the PFK peptides, which were obtained from CNBr, trypsin ,chymotrypsin and S. aureus V8 protease digestions are shown in chapter four. Chapter five illustrates the comparison of the PFK primary structures from *E. coli*, *S. lactis* and *B. stearothermophilus*, especially the amino acid sequences around the active site and the regulatory site.