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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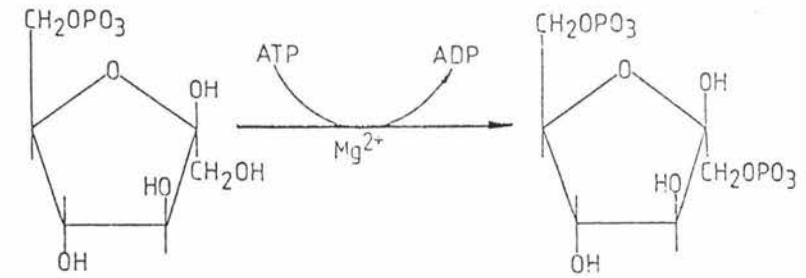
CHAPTER ONE: INTRODUCTION

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).

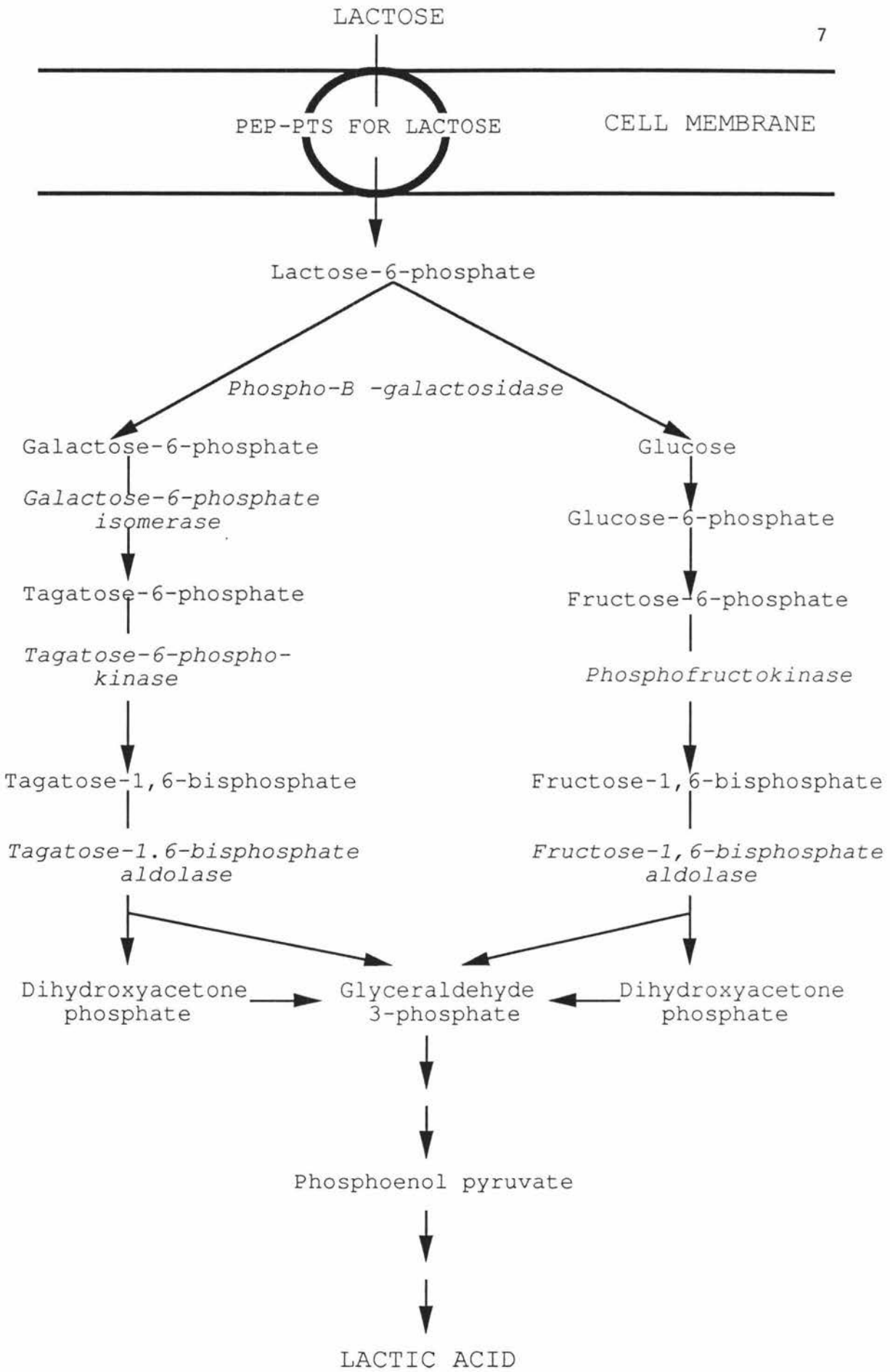
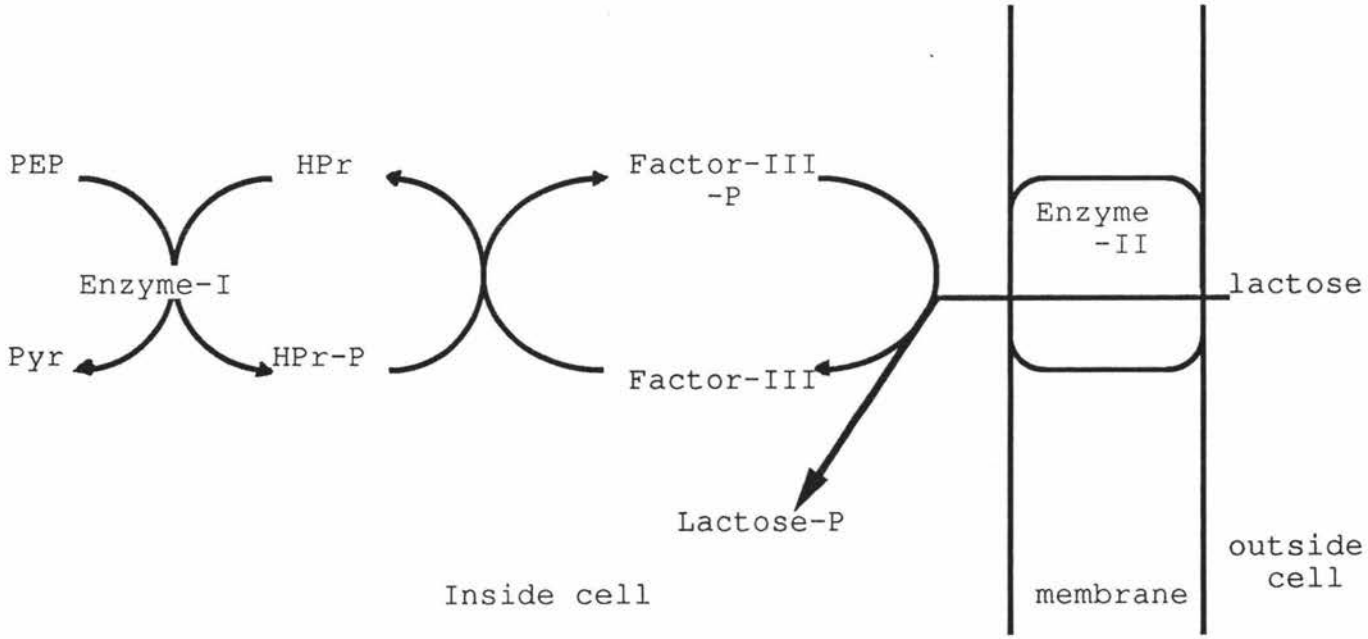


FIGURE 1 LACTOSE METABOLISM IN S. lactis

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

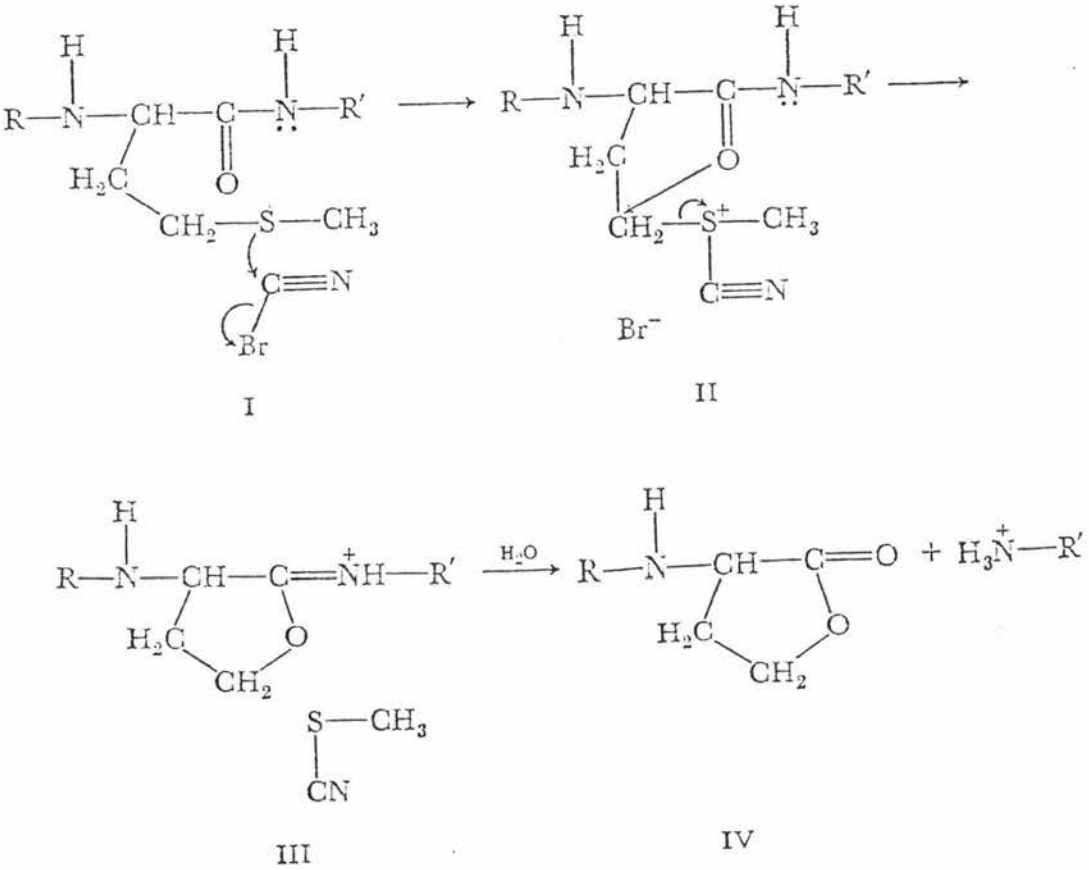


FIGURE 3: CNBr CLEAVAGE OF PROTEIN
I: Protein + CNBr
II: methionine cyanosulfonium derivative
III: homoserine iminolactone
IV: shortened protein + homoserinelactone peptide

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

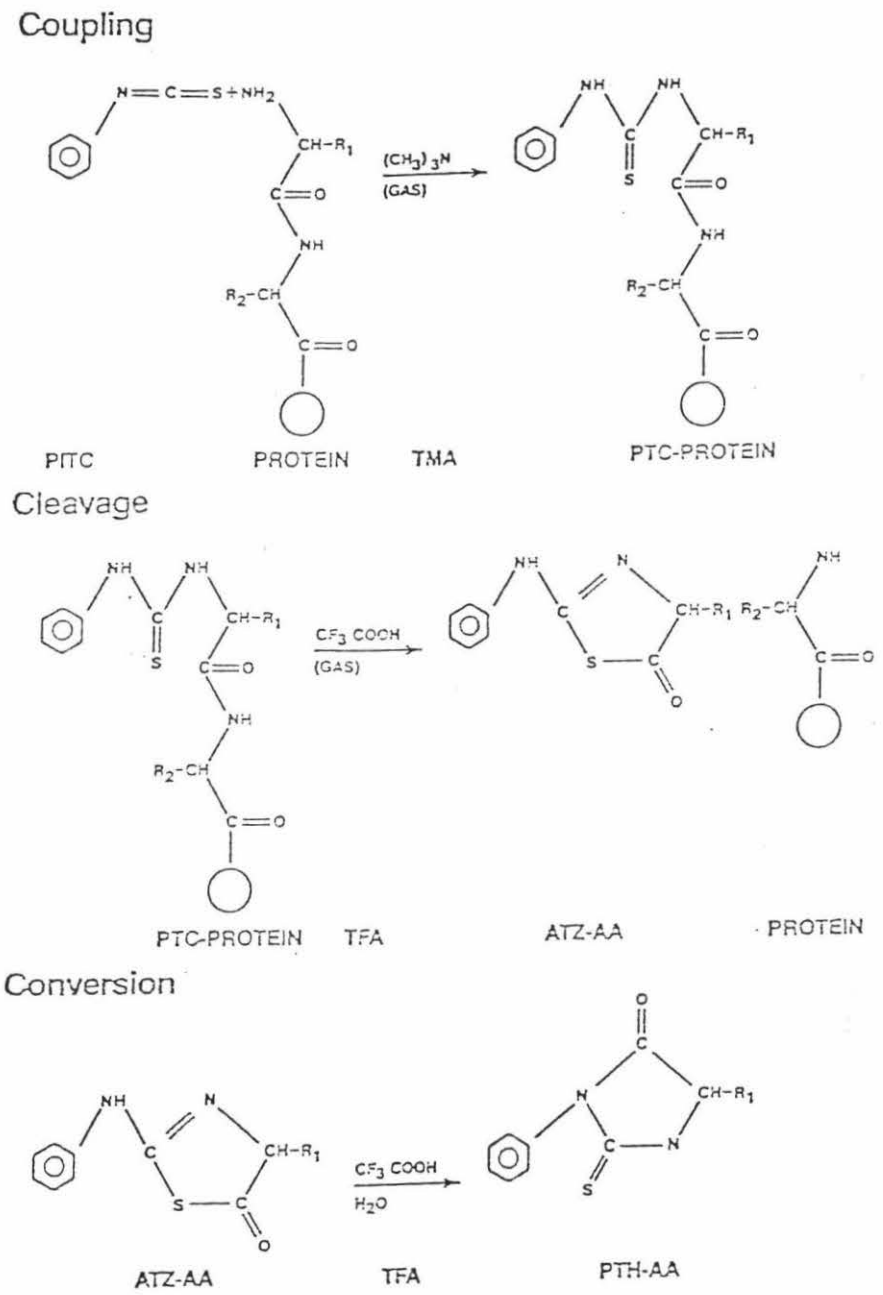


FIGURE 4: EDMAN DEGRADATION MECHANISM
PITC: phenyl isothiocyanate
TMA: trimethylamine
PTC-protein: phenylthiocarbamoyl(PTC)-protein
TFA: trifluoroacetic acid
ATZ-AA: thiazolinone-amino acid
PTH: phenylthiohydantoin

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.

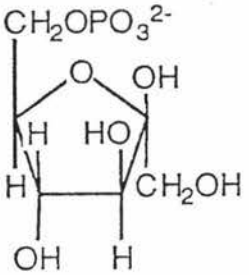
1.4 AIMS

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.

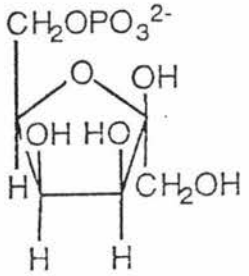
In *S. lactis,* the lactose metabolism includes two parallel and similar pathways, the T6P pathway to metabolize galactose-6-phosphate from the cleavage of lactose and the glycolysis pathway to metabolize glucose from the cleavage of lactose. The end products of the two pathways are the same, dihydroxyacetone 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.

CHAPTER TWO MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes

Fructose-1.6-bisphosphate aldolase (EC 4.1.2.6), trypsin (EC 3.4.4.4) (TPCK treated), chymotrypsin and carboxypeptidase A were purchased from Sigma Chemical Co. St. Louis; glycerol-3-phosphate dehydrogenase was obtained from Boehringer Mannheim GmbH, West Germany.

2.1.2 Materials for bacterial culture

Peptone and yeast extract were from Difco Laboratories; beef extract was from the Oxo Co. Ltd..

S. lactis starter cultures were provided by Dr.G.G.Prichard, Massey University, New Zealand.

2.1.3 Chemicals used for SDS-PAGEs

Bio-Rad Laboratories , Richmond, supplied acrylamide and bis-acrylamide (>99.9%). Glycine (Chromatographically homogeneous) and sodium dodecyl sulphate (SDS) (Specially pure) were obtained from British Drug House (BDH), Poole, England.

2.1.4 Others

Most of the other chemicals used were provided by either Sigma Chemical Company, British Drug House (BDH) Ltd., or May and Baker Ltd., Dagenham, England.

2.2.1 Tris/glycerol buffer

The buffer used for isolating the *S. lactis* PFK contained the following ingredients:

Glycerol	20% (v/v)
Tris/HCl, pH7.5	50mM
$MgCl_2$	5mM
*EDTA	5mM
ßME	10mM

*Present or absent in the buffer

This buffer will be referred to subsequently as Tris/glycerol buffer.

2.2.2 Preparation of Cibacron Blue Sepharose resin

Cibacron Blue Sepharose resin was prepared according to the method introduced by Atkinson et al. in 1981.

800g sucked-dry water-washed Sepharose 4B-CL was suspended in water to give the final volume of 2.8 litres. 12g of triazine dye freshly dissolved in 800ml of water was added to the suspension followed by 400ml of 4M NaCl and 40ml of 10M NaOH. After gentle swirling at 60°C for 16 hours, the resin was washed sequentially with excess 1) water; 2) 1M NaCl in 25% ethanol; 3) water 4) 1M NaCl in 0.2M phosphate, pH7.0; 5) water. Finally the resin was equilibrated with Tris/glycerol buffer. It was then stored in Tris/glycerol buffer at 0-4°C.

2.2.3 Assay systems for measuring PFK activity

During the PFK preparation process, PFK activity was assayed as an indicator for collecting fractions and also as an criterion of PFK yield.

The assay system used in this study was called Glycerol-3-phosphate dehydrogenase assay system. PFK activity was determined by coupling the reaction catalysed by PFK to the oxidation of NADH (Figure 5) and monitored spectrophotometrically at 340nm. Phosphorylation of 1 mole of F6P resulted in oxidation of 1 mole of NADH.

0.5ml reaction mixture in a 1ml microcuvette was needed for each assay which contained:

Tris/HCl, pH8.0	70mM
$MgCl_2$	1.5mM
ßME	5mM
ATP	1.5mM
NADH	0.4mM
BSA	0.01% (w/v)
Glycerol-3-phosphate dehydrogenase (GPD)	1unit/ml
F1,6BP aldolase	0.27unit/ml
F6P	2mM
Sample	10µl

The reaction was started by the addition of the F6P.

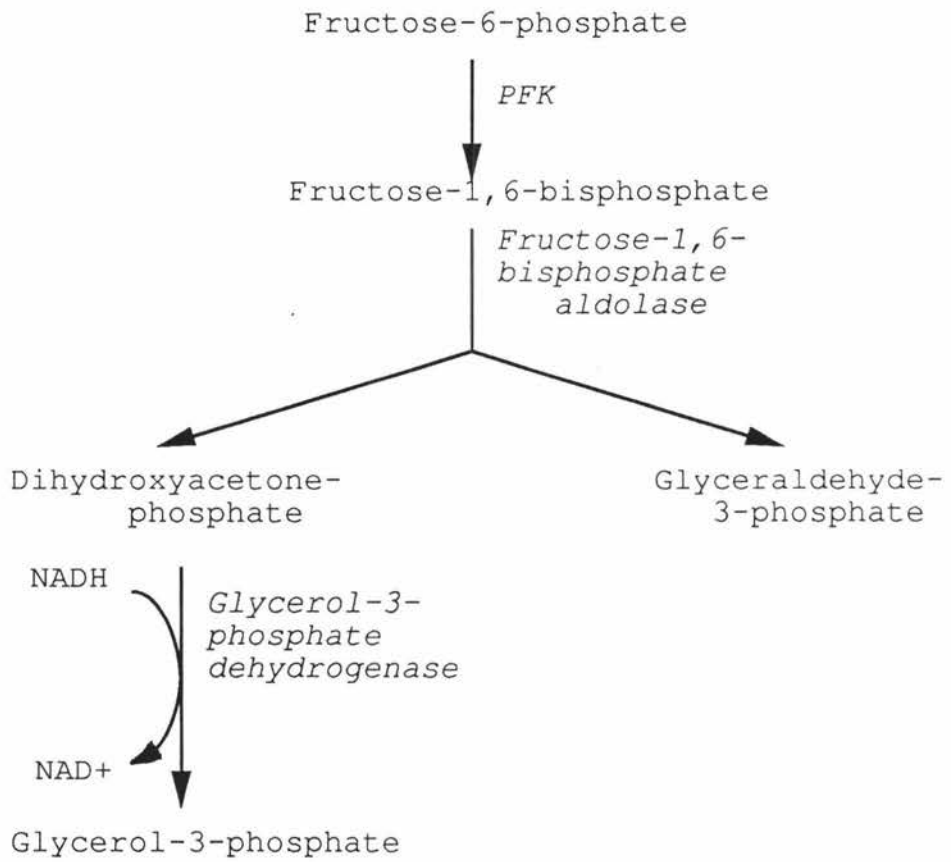


FIGURE 5: GLYCEROL-3-PHOSPHATE DEHYDROGENASE ASSAY SYSTEM

2.2.4 Protein level determination

Bovine serum albumin (BSA) was used as the standard protein for all three protein determination methods mentioned below. Concentration of BSA was determined spectrophotometrically using an extinction coefficient of 6.68 at 280nm for 1% BSA (w/v) solution (Tanford and Robert, 1952).

2.2.4.1 Biuret method

The Biuret method of protein determination as described by Layne (1957) was used to determine the amount of protein in cell free extracts and samples obtained from DEAE cellulose column elution where the protein concentration was high.

2.2.4.2 Modified Lowry method

The small amount of protein in samples from the first Cibacron Blue column elution was determined by a modified Lowry method (Lowry et al., 1951).

2.2.4.3 Coomassie Blue dye binding method

Determination of protein amount in samples from the second Cibacron Blue column elution was carried out using the Coomassie Blue dye binding method (Bradford, 1976).

2.2.5 SDS polyacrylamide gel electrophoresis (SDS PAGE)

10% SDS polyacrylamide slab gels were polymerized in a vertical apparatus with 1cm sealing gel, 10cm

running gel and 2cm stacking gel.

The solutions for running the gels are listed below:

Running and sealing gel

Acrylamide	10% (w/v)
Bis-acrylamide	0.3% (w/v)
Tris/HCl, pH8.8	0.38M
SDS	1.0% (w/v)
TEMED	0.05% (v/v)
Ammonium persulphate	0.05% (w/v)

Stacking gel

Acrylamide	4.5% (w/v)
Bis-acrylamide	0.1% (w/v)
Tris/HCl, pH6.8	0.125M
TEMED	0.05% (v/v)
SDS	1.0% (w/v)
Ammonium persulphate	0.05% (w/v)

Electrode buffer (pH8.3)

Tris	5mM
Glycine	0.3% (w/v)
SDS	1% (w/v)

Sample buffer

Tris/HCl, pH6.8	0.06M
Glycerol	10% (v/v)
ßME	5% (v/v)

Bromophenol blue	0.02% (w/v)

Staining solution

Coomassie Brilliant	
Blue R250	0.125% (w/v)
Methanol	45% (v/v)
Acetic acid	5% (v/v)
Distilled water	50% (v/v)

Destaining solution

Destaining solution has the same contents as staining solution, but no Coomassie Brilliant Blue R250.

Gels were polymerized at room temperature. The bottom of the apparatus was sealed to a depth of 1cm with sealing gel. The running gel was poured to 3cm from the top of the gel plates. To produce an even gel surface, a 0.5cm water layer was overlaid over the runing gel before it polymerized. Stacking gel solution was added when the water layer was poured off and a sample slot comb was inserted immediately into the solution. After polymerization of the stacking gel, the sample slot comb was removed and the slots were washed with electrode buffer. The gel was fixed to a slab gel electrophresis tank whose upper and lower reservoirs were filled with electrode buffer.

Protein samples were diluted with sample buffer and heated at 100°C for 5 minutes and cooled. 5-50μl treated samples were loaded.

Electrophoresis was performed at room temperature with currents of 10mA for stacking and 20mA for running until the dye indicator approached the sealing gel.

The gels were then stained in staining solution overnight and then were destained in destaining solution until the protein bands were clearly visible.

2.2.6 Amino acid analysis

Protein or peptide samples were transferred to Pyrex hydrolysis tubes (12mm x 100mm) and dried. Hydrolysis was carried out under vacuum in 200µl 6M HCl containing 1% phenol (w/v) at 110°C for 16 hours. The hydrolysates were dried in vacuum dessicator over NaOH and P_2O_5.

The dried hydrolysates were analysed on a Beckman Model 119BL Amino Acid Analyser with an automatic sample injector.

2.2.7 Amino terminal sequencing

The amino acid sequence information was obtained by the automated Edman method using an Applied Biosystems 470 gas-phase sequencer (Hewick et al., 1981).

About 2nmoles of peptide was dissolved in 30 l distilled, deionized water and loaded onto an ABI Biobrene Plus treated filter. The amino acids sequentially cleaved, were detected as PTH amino acids

which were interpreted by comparison with a standard run of PTH amino acids.

During sequencing, the signal gradually decreased from one cycle to another while the background tended to increase.

The repetitive yield (RY) was calculated for each sequencing experiment as one method to examine the efficiency of the experiment.

For an amino acid which elutes at cycles m and n, the RY is defined as:

$$RY=(Yn/Ym)^{1/(n-m)}$$ where Ym=the peak height at cycle m - height at cycle (m - 1).

Yn=the peak height at cycle n - height at cycle (n - 1).

The total efficiency equals to the average of the RY for each amino acid.

The efficiency of the sequencing was also shown by plotting the yield (log(pmoles)) obtained by comparison of the height of the peak for the amino acid residue with that for a known standard against the cycle number. A typical plot is shown in figure 25.

2.2.8 FAB Mass Spectrometric analysis

Mass spectrometric analyses of peptides was performed by liquid secondary ion mass spectrometry (SIMS) (Seki et al., 1985) in a VG70-250S double-sector mass spectrometer.

The peptide sample was dissolved in glycerol matrix, which had been acidified with HCl, and was bombarded to produce molecular ions as an secondary ion beam by a beam of ceasium ions produced and accelerated by a fast atom gun. Secondary ions were accelerated out of the ion source and were separated in the magnetic sector according to their mass to charge ratio.

The secondary ions were formed actually by protonation. Thus the molecular weight of the peptide equalled to the mass of molecular ion minus one.

The glycerol matrix could be ionised during the process to give molecular ions of different glycerol aggregates of one glycerol molecule difference ($(Glycerol)_1H^+$, $(Glycerol)_2H^+$, $(Glycrol)_3H^+$ etc.). They were detected and provided a calibration of the spectra. In figure 15 of mass spectrometric analysis of T4, there were two of these kinds of aggregates (Mass of 461, $(Glycerol)_5H^+$ and 553, $(Glycerol)_6H^+$). More accurate calibration was obtained from peaks produced by the bombardment of caesium iodide.

2.2.9 Tryptic digestion

Trypsin was dissolved in 0.5% NH_4HCO_3, pH8.0 and added to PFK or CNBr peptide samples dissolved in 0.5% NH_4HCO_3 in a molar ratio of 1/200. The digestion was performed at 37°C for one hour and stopped by lyophilization.

2.2.10 Chymotryptic digestion

PFK and tryptic peptide T7 were digested with chymotrypsin.

About 20mg of PFK dissolved in 0.5% NH_4HCO_3 was denatured by heating at 80°C for 10 minutes before digestion.

Chymotrypsin was dissolved in 0.5% NH_4HCO_3 and added to substrate in a ratio of 1/100, w/w. The reaction was carried out at 37°C for 1.5 hours and stopped by lyophilization.

2.2.11 *S. aureus* V8 protease digestion

PFK or CNBr CN5 was digested with S. aureus V8 protease.

The substrate and enzyme were dissolved in 0.5M NH_4HCO_3 separately and were mixed in an enzyme/substrate ratio of 1/100 (w/w). The reaction was carried out at 37°C for 5 hours and stopped by lyophilization.

2.2.12 CNBr digestion

About 15mg of PFK was dissolved in 1ml of 70%

formic acid and gassed with nitrogen.

CNBr was dissolved in acetonitrile and was added to PFK solution in 200-fold molar excess over total methionyl groups in PFK.

The reaction was carried out in a stoppered flask at room temperature overnight and was diluted with distilled water to reduce formic acid concentration to below 5% (v/v). The sample was then dried by lyophilization.

2.2.13 Separation of CNBr peptides by gel filtration chromatography on the Fast Protein Liquid Chromatography (FPLC) apparatus

Gel filtration chromatography was carried out on a Pharmacia Fast Protein Liquid Chromatography (FPLC) apparatus using a Pharmacia Superose-12 10/30 gel filtration column. The CNBr peptides were separated according to their sizes in 50% formic acid by this method.

2.2.14 Separation of S. aureus V8 protease digested PFK peptides on G-50 Sephadex gel filtration column

The initial separation of the S. aureus V8 protease digested SL PFK peptides was carried out on a G-50 Sephadex gel filtration column.

The peptide sample was dissolved in 2ml of 1% NH_4HCO_3 and loaded onto the column. The separation of

the peptides was achieved according to their sizes in 1% NH_4HCO_3.

2.2.15 Separation of peptides on Reverse Phase Liquid Chromatography (RPLC)

Separations of peptides from the mixtures produced by enzymic digestion of PFK or CNBr peptides from FPLC or S. aureus V8 protease digested peptides from G-50 gel filtration column were carried out on RPLC using a Vydac C18 column of 250mm x 4.6mm diameter connected to a Spectrophysics SP8800 HPLC. The solvent systems used were based on acetonitrile and aqueous TFA or ammonium bicarbonate, acetonitrile and isopropanol (See below).

TFA system

Equilibration buffer (Solution A):

0.1% aqueous TFA

Elution buffer (Solution B):

0.1% TFA in acetonitrile

Ammonium bicarbonate system

Equilibration buffer (Solution A):

0.1M ammonium bicarbonate in
5% aqueous acetonitrile

Elution buffer (Solution B):

0.1M ammonium bicarbonate:acetonitrile:
isopropanol=1:1:1 (v/v/v)

Formic acid system

Equiulibration buffer (A):

0.1% NaCl (w/v) and 0.1% formic acid (v/v) in 2% acetonitrile (v/v) in water

Elution buffer (B):

0.1% NaCl (w/v) and 0.1% formic acid (v/v) in 60% acetonitrile (v/v) in water

The elution of the peptides or proteins was achieved by increasing the hydrophobicity of the mobile phase, that is the gradual increase of the ratio of solution B to solution A.

CHAPTER THREE RESULTS
ISOLATION OF PFK FROM *S. lactis*

3.1 GROWTH OF *S. lactis*

S. lactis starter cultures were provided by Dr.G.G.Prichard, Massey University, and the large scale fermentations were done by the Biochemical Processing Centre, DSIR.

Bacteria were grown in a Fermacell Fermentor (New Brunswick Scientific Company) in a medium containing the following ingredients per litre of distilled water:

Lactose	20g
Peptone	10g
Yeast extract	10g
KH_2PO_4	5g
Beef extract	2g
$MgSO_4$	0.2g
$MnCl_2$	0.05g

Total medium volume was 35 litres.

Cells were harvested at late-log phase by centrifugation (Sorvall RC-2B, GS3 rotor, 10,000rpm ,15minutes, 0-4°C) and were washed twice by resuspension in cold 50mM Tris/HCl buffer, pH7.5. Cells were stored at -20°C. The yield was about 15g cells per litre of medium.

3.2 PFK ISOLATION PROCEDURE

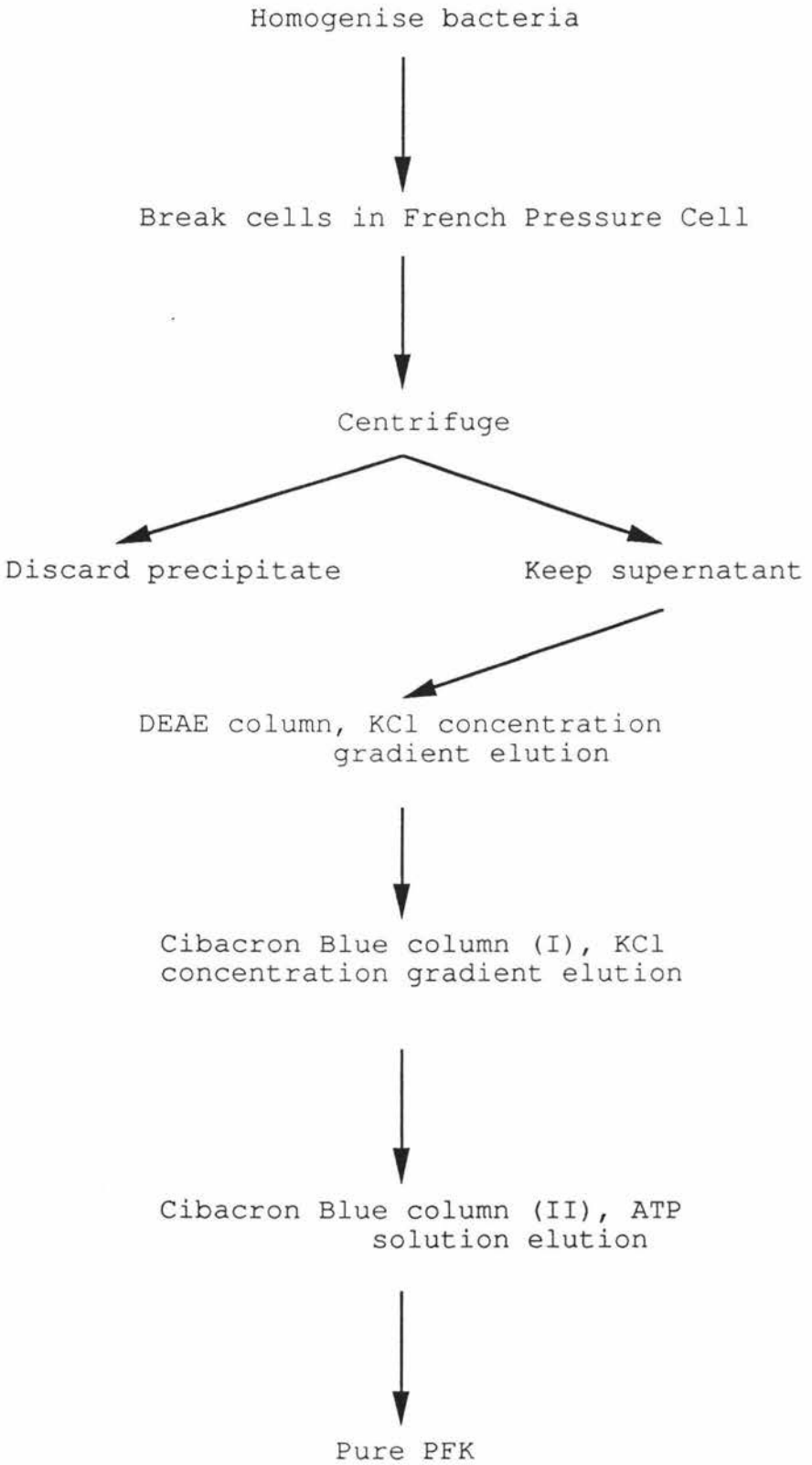
Figure 6 shows the flow chart of the isolation of PFK.

100g frozen bacteria were thawed in 200ml Tris/glycerol buffer. The cells were disrupted in a French Pressure Cell (American Instrument Company Inc. Washington D.C., U.S.A.) at 5500psi. Cell debris and unbroken cells were removed by centrifugation at 15,000rpm for 15 minutes at 0-4°C (Sorvall RC-2B, SS34 rotor).

Cell free extract was diluted with Tris/glycerol buffer until conductivity dropped to below 1.5mMho to ensure that PFK could bind to the DEAE cellulose column. It was then loaded onto DEAE cellulose anion-exchange column at a flow rate of 2ml/min. The column was washed with Tris/glycerol buffer until no more protein eluted (Monitored spectrophotometrically at 280nm). Bound proteins were eluted with a 2000ml linear salt concentration gradient of 0-400mM KCl at a flow rate of 2ml/minute.

Fractions with PFK activity were pooled and loaded directly onto a Cibacron Blue Dye column at a flow rate of 1.2ml/minute. The column was eluted with a 500ml linear salt concentration gradient of 0-2.5M KCl at a flow rate of 1.2ml/minute after being washed with Tris/glycerol buffer to remove the unbound proteins. PFK has higher affinity for the dye column than most of

FIGURE 6: PROCEDURE OF *S. lactis* PFK ISOLATION



the other proteins in the sample from the DEAE cellulose column. At this step, most of the unwanted proteins were removed (See figure 8: The Elution profile of Cibacron Blue column (I).).

Fractions with PFK activity were pooled and dialysed against several changes of Tris/glycerol buffer until the conductivity dropped to below 5mMho so that PFK could bind to a second Cibacron Blue column. The dialysed sample was then loaded onto a second Cibacron Blue column. After being washed with Tris/glycerol buffer, the column was eluted with 20mM ATP solution (pH7.5) at a flow rate of 1.2ml/minute. In this process, not only was the binding of PFK to the column specific, but also the elution of PFK from the column was specific since ATP was one of the substrates for PFK. The PFK obtained at this step was sufficiently pure for sequence study.

Fractions with PFK activity were pooled and concentrated by ultrafiltration (PM30 membrane). The concentrated PFK was dialysed against several changes of Tris/glycerol buffer to remove ATP and stored at -20°C in Tris/glycerol buffer, pH7.5 with the concentration of glycerol increased to 50% (v/v).

3.3 PFK isolation results and discussion

The isolation process was repeated several times and the results were reproducible. The enzyme was

purified 100 to 200 fold with a yield of about 0.7 to 1.0mg PFK per 10g bacteria. The recovery was about 40-50%. In the presence of EDTA in the Tris/glycerol buffer, the activity of PFK was around 160 units per mg protein; in the absence of EDTA in the Tris/glycerol buffer, the activity of PFK was about 80-90 units per mg protein. The EDTA appeared to be essential to inhibit proteases present in the initial cell free extract to protect PFK from inactivation by proteases. The specific activity of PFK was comparable to that of PFK from other origins such as mammalian skeletal muscle (Uyeda, 1979), *C. pasteurianum* (Uyeda and Kurooka, 1970) and *E. coli* (Griffen et al,1967).

The results of a typical PFK preparation from 100g bacteria are summarised in table 1.

Figure 7, figure 8 and figure 9 show the elution profiles from the DEAE cellulose column, the first and the second Cibacron Blue columns. Figure 8 shows clearly that the first Cibacron Blue column removed most of the unwanted proteins from PFK; figure 9 shows that after passing through the second Cibacron Blue column, the PFK sample was reasonably pure.

10% acrylamide SDS gel electrophoresis of samples from different purification steps showed the increase in PFK purity. The final product appeared as a single band (Figure 10).

Table 1: Typical results of the isolation of SL PFK

Samples	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification	Recovery (%)
CFE	300	17.0	5100	0.4	1912	1	100
	205	14.7	3024	1.8	5421	1	100
DEAE	362	2.2	784	2.1	1639	5.6	85.7
	923	1.3	1192	2.6	3049	1.4	55.9
BLUE (I)	179	0.11	19	63.2	1199	168.6	62.7
	244	0.09	21	139.6	2930	77.6	54.0
BLUE(II)	167	0.05	8	81.8	628	218.1	32.8
	198	0.06	12	186.4	2362	103.5	42.7

Abbreviation: CFE=Cell free extract; DEAE=Sample from DEAE column;
BLUE (I)=Sample from Cibacron Blue column (I),
BLUE (II)=Sample from Cibacron Blue column (II).

* Top values were from the isolation in the absence of EDTA in Tris/glycerol buffer.
Bottom values were from the isolation at the presence of EDTA in Tris/glycerol buffer.

FIGURE 7: DEAE CELLULOSE COLUMN ELUTION PROFILE

Column:	DEAE cellulose ion-exchange column
Size:	15cm X 6cm diameter
Temperature:	0-4°C
Equilibration buffer:	20% Tris/glycerol buffer
Elution buffer:	0-400mM KCl, linear gradient
Flow rate:	2ml/minute
Sample:	Cell free extract from *S. lactis*
Keys: ●	Conductivity
■	PFK activity

DEAE—CELLULOSE COLUMN

ELUTION PROFILE

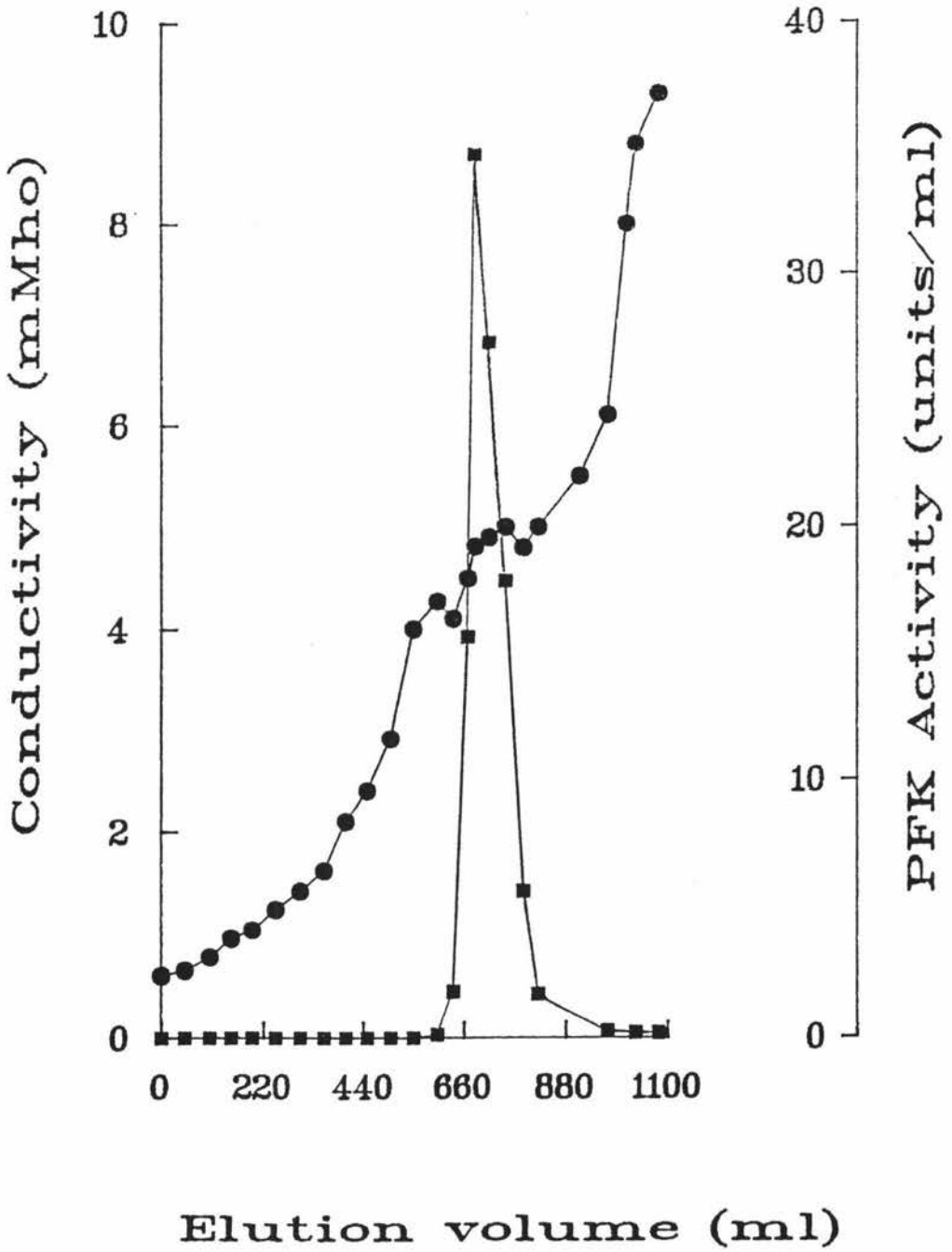


FIGURE 8: CIBACRON BLUE COLUMN (I) ELUTION PROFILE

Column:	Cibacron Blue column
Size:	15cm X 3cm diameter
Temperature:	0-4 °C
Equilibration buffer:	20% Tris/glycerol buffer
Elution buffer:	0-2.5M KCl, linear gradient
Flow rate:	1.2ml/minute
Sample: eluent	Collected DEAE column

Keys:

■ Abs280

× Conductivity

● PFK activity

CIBACRON BLUE COLUMN (I)

ELUTION PROFILE

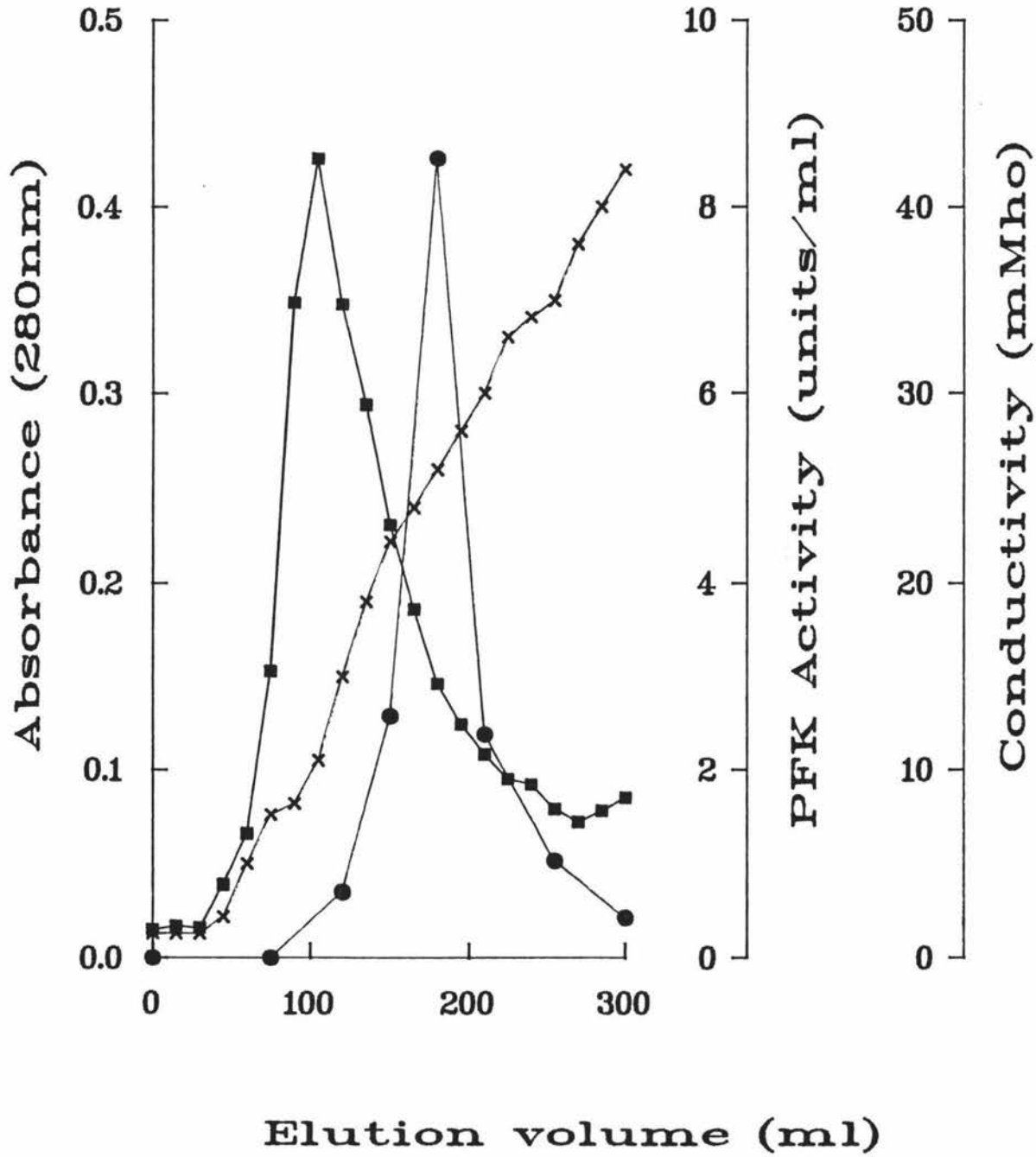


FIGURE 9: CIBACRON BLUE COLUMN (II) ELUTION PROFILE

Column:	Cibacron Blue column
Size:	6cm X 2cm diameter
Temperature:	0-4°C
Equilibration buffer:	20% Tris/glycerol buffer
Elution buffer:	20mM ATP solution
Flow rate:	1.2ml/minute
Sample:	Collected Cibaron Blue column (I) eluent
Keys: ●	Abs595 from the Coomassie Blue protein determination
■	PFK activity

CIBACRON BLUE COLUMN (II)

ELUTION PROFILE

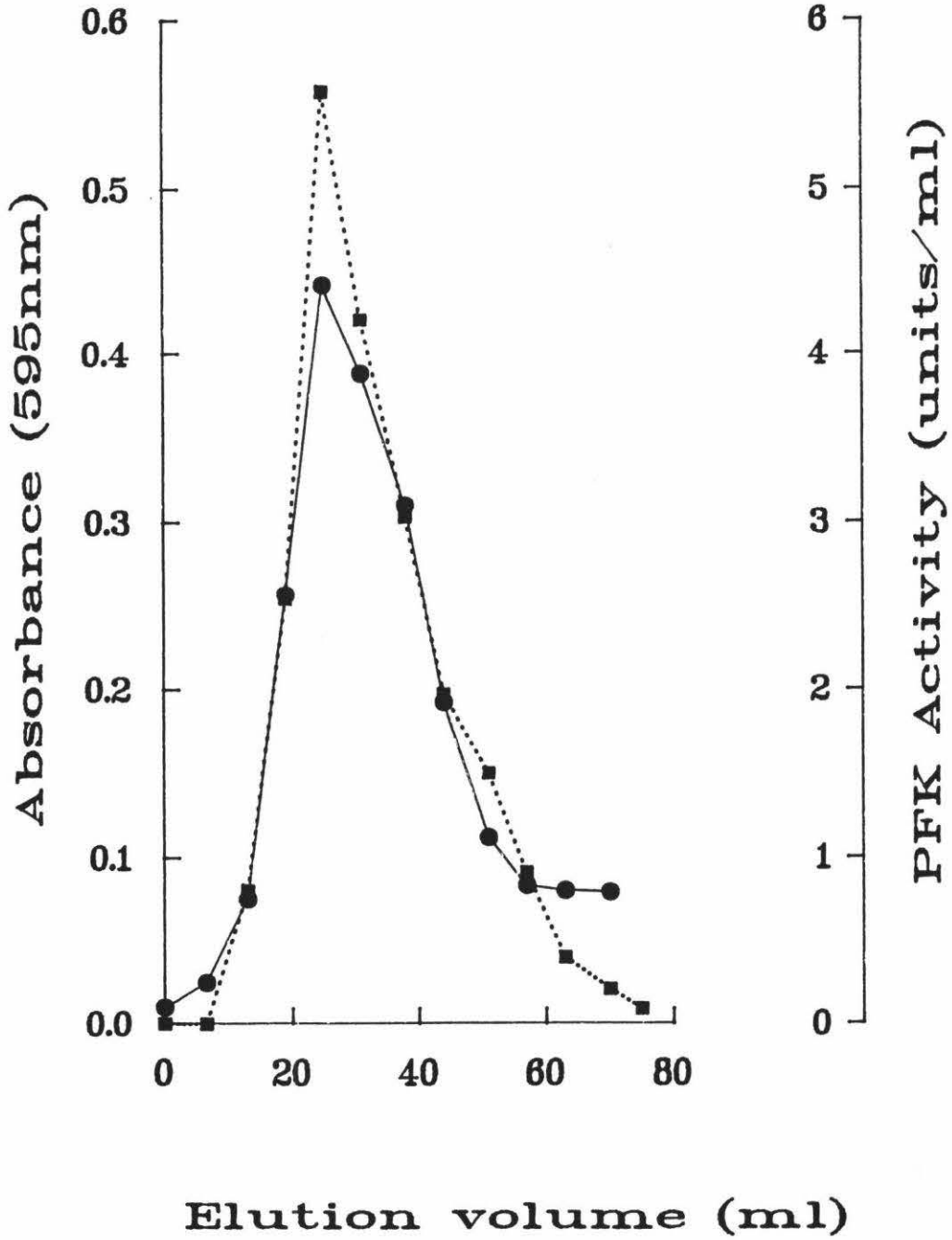
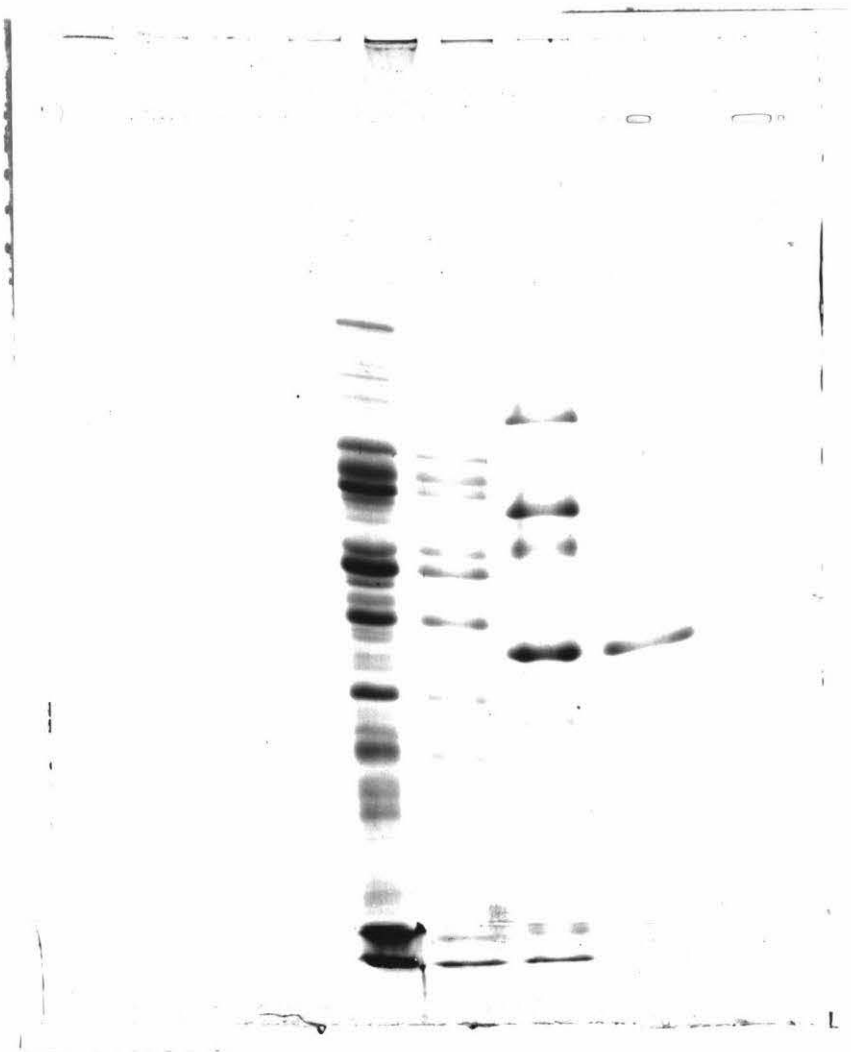


FIGURE 10: SDS PAGE OF PFK SAMPLES



1 2 3 4

1. Cell free extract
2. Sample from DEAE cellulose column
3. Sample from Cibacron Blue column (I)
4. Sample from Cibacron Blue column (II)

CHAPTER FOUR RESULTS
PRIMARY STRUCTURE ANALYSIS OF PFK FROM *S. lactis*

The *B. stearothermophilus* (*BS*) PFK and the *E. coli* (*EC*) PFK have been studied well. They have not only very high similarities in the amino acid sequences, but also similar tertiary and quarternary structures. An initial assumption was made that the *S. lactis* PFK would be similar to *BS* and *EC* PFKs so that the alignment of *SL* PFK sequence could be made on the basis of direct comparison with *BS* and *EC* PFKs.

4.1 AMINO ACID ANALYSIS OF *SL* PFK

The amino acid content of SL PFK was analysed using the method described in 2.2.6.

Four SL PFK samples, each of which was about 2nmoles, were hydrolysed for 16, 24, 38 and 48 hours separately before being analysed on the amino acid analyser.

Figure 11 shows the time-dependency yield of some amino acids during hydrolysis.

Table 2 lists the amino acid contents of SL PFK obtained according to the sequence and by the amino acid analysis. The valine and isoleucine amounts were from the 48-hour hydrolysis result; tyrosine, serine and threonine amounts were the interception values on the

amino acid number axis when the lines representing threonine, serine or tyrosine was extrapolated to zero time and other amino acid amounts were the average values of the four hydrolysis results.

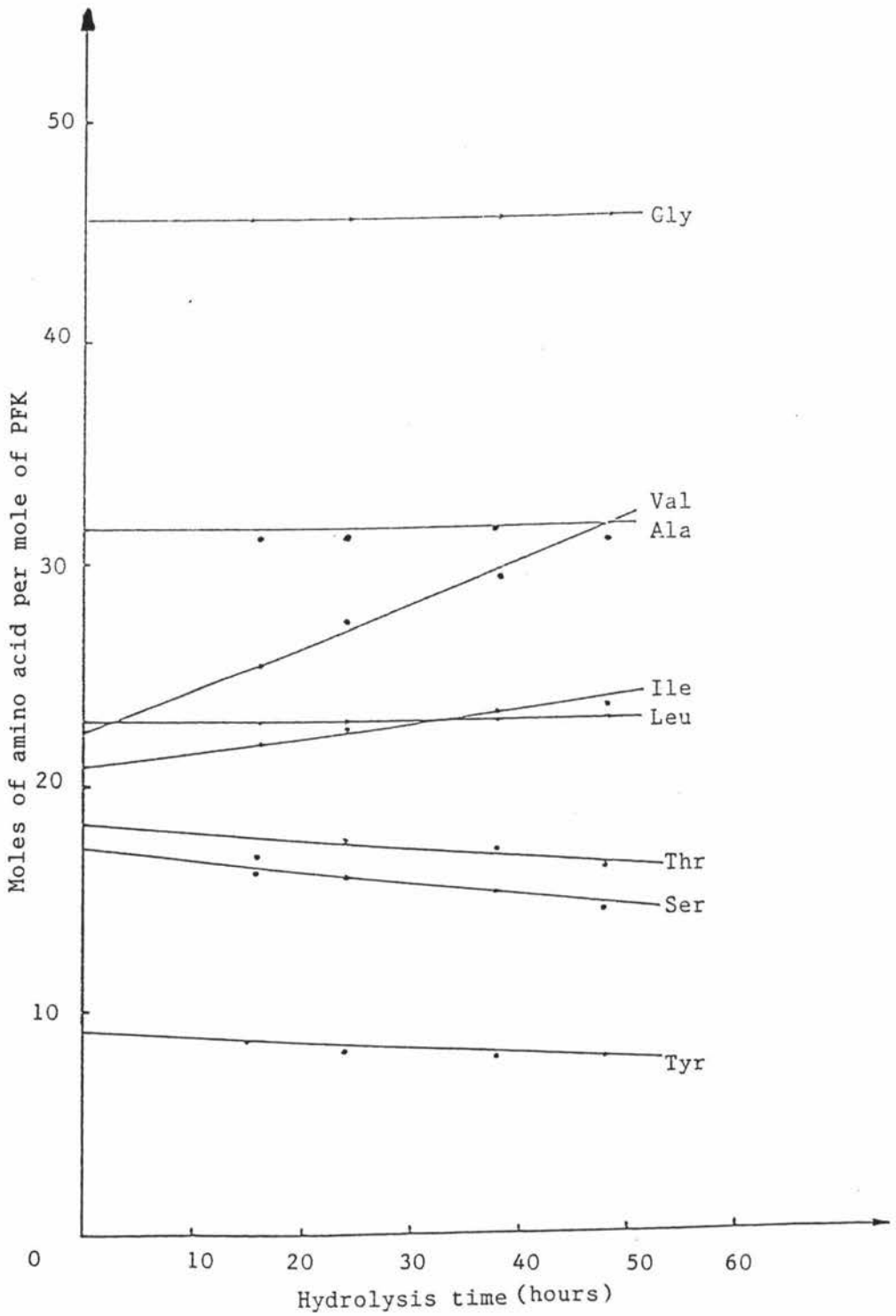


FIGURE 11: TIME-DEPENDENCY OF AMINO ACIDS DURING HYDROLYSIS

Table 2: Amino acid contents in bacterial PFKs

	SL (sequence)	SL (analysis)	BS (sequence)	EC (sequence)
A	34	31.3	26	27
C	0	0	3	6
D+N	35	34.0	28	31
E+Q	28	26.9	29	26
F	12	12.5	7	10
G	48	45.5	46	38
H	8	7.6	12	7
I	27	23.3	30	28
K	15	16.3	18	14
L	25	22.7	22	25
M	7	6.9	5	12
P	9	9.0	7	8
R	18	17.9	19	22
S	17	17.0	12	19
T	18	18.0	19	15
V	30	30.6	26	25
W	0	0	1	1
Y	8	8.9	9	11

* The amino acid analysis results of I and L were low because the hydrolysis time was not long enough.

4.2 AMINO TERMINAL SEQUENCE OF *SL* PFK

About 2nmoles of pure *SL* PFK were sequenced on the Applied Biosystems 470 gas-phase sequencer by the method described in 2.2.7 to get the amino terminal sequence.

28 residues at the amino terminal of *SL* PFK were sequenced which are shown below:

MKRIABLTSGGDAPGMNAAIRAVVRKAI

4.3 TRYPTIC PEPTIDES ANALYSIS

Pure PFK was digested with trypsin by the method described in 2.2.9. Peptides obtained were separated and purified on RPLC and were called T1, T2, T3.....T17, T18 respectively in the order of amino terminal to carboxyl terminal of the *SL* PFK. The amino acid composition and the mass spectrometry analysis result for each peptide are shown in table 3.

4.3.1 Tryptic peptides separation

Tryptic peptides were separated on RPLC using Vydac C18 column with the ammonium bicarbonate buffer system. Figure 12 shows the RPLC elution profile. The numbers in the figure indicate the fractions analysed which contained the tryptic peptides of SL PFK which were studied further. Peak A, B and C in the figure were only partially sequenced and were not studied further because A had the same amino terminal sequence as fraction 8 (T9), B had the same amino terminal sequence as fraction 11 (T5) and C contained peptide which equaled to T2 plus T3.

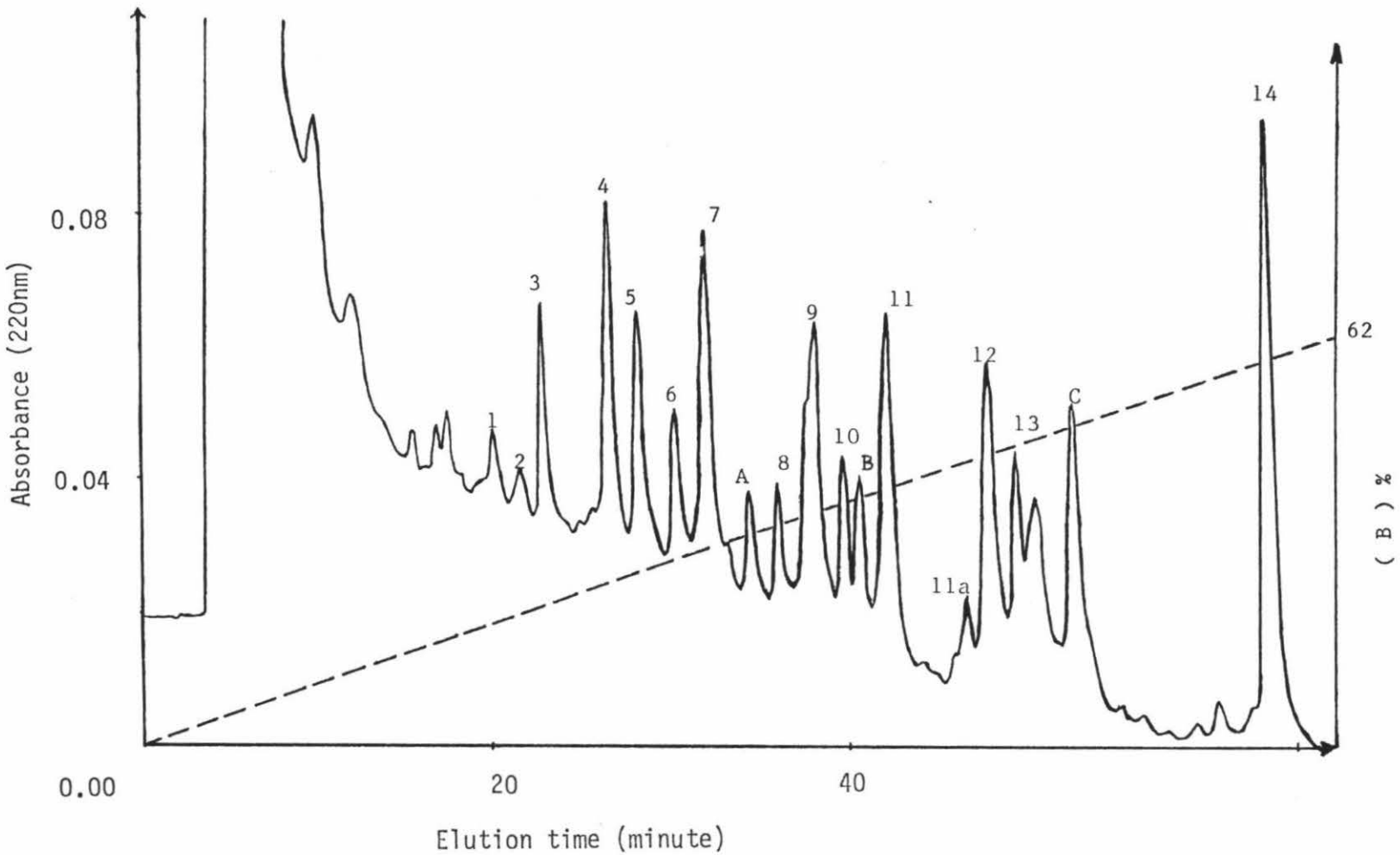
4.3.2 Analysis of fractions collected from the RPLC separation of tryptic peptides

Fractions collected from the RPLC separation of tryptic peptides were passed through the RPLC Vydac C18 column a second time using the TFA system to obtain pure peptides which were subjected to further study.

FIGURE 12: TRYPTIC PEPTIDES MAPPING ON RPLC

Column:	Vydac C18
Temperature:	Room temperature
Equilibration buffer (A):	0.1M Ammonium bicarbonate in 5% Acetonitrile in water
Elution buffer (B):	0.1M Ammonium bicarbonate: Acetonitrile:isopropanol 1:1:1 (V/V/V)
Elution gradient:	Linear gradient, 0% to 60%B in 60 minutes
Flow rate:	0.5ml/minute
Sample:	Tryptic PFK from *S. lactis*

*: The numbers in the figure indicate the fractions analysed which contained the tryptic peptides of *S. latis* PFK and were further studied.


Absorbance (220nm)
0.08
0.04
0.00
20
40
Elution time (minute)
1
2
3
4
5
6
7
A
8
9
10
B
11
11a
12
13
C
14
62
(B) %

4.3.2.1 Results of fraction 1 analysis

Figure 13 shows the purification of fraction 1 on RPLC. Peak B contained T11 whose amino terminal sequence was:

AEGVMTGEEFATK

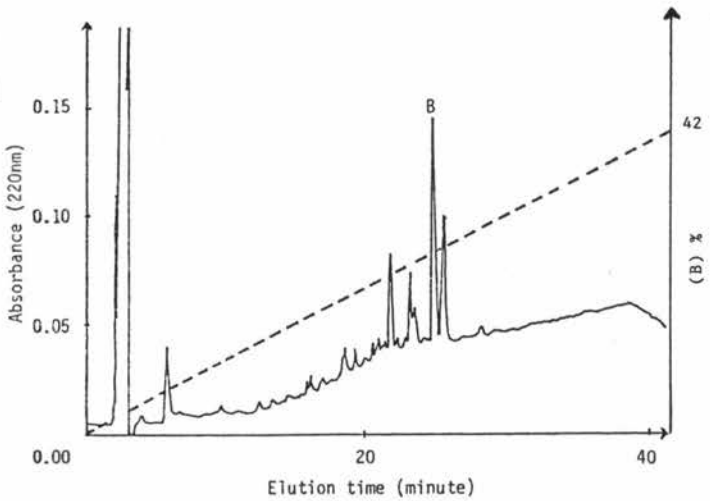


Figure 13. Purification of RPLC fraction 1 of tryptic peptides

4.3.2.2 Results of fraction 2 analysis

Fraction 2 was subjected to sequencing and the following sequence was obtained:

VLASR

4.3.2.3 Results of fraction 3 analysis

Figure 14 shows the purification of fraction 3 on RPLC. Peak A contained T2 whose sequence was:

AISEGIEVY

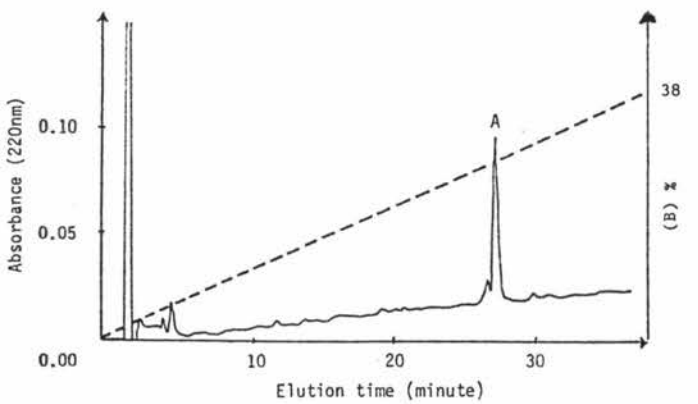


Figure 14. Purification of RPLC fraction 3 of tryptic peptides

4.3.2.4 Results of fraction 4 analysis

Amino terminal sequencing showed that fraction 4

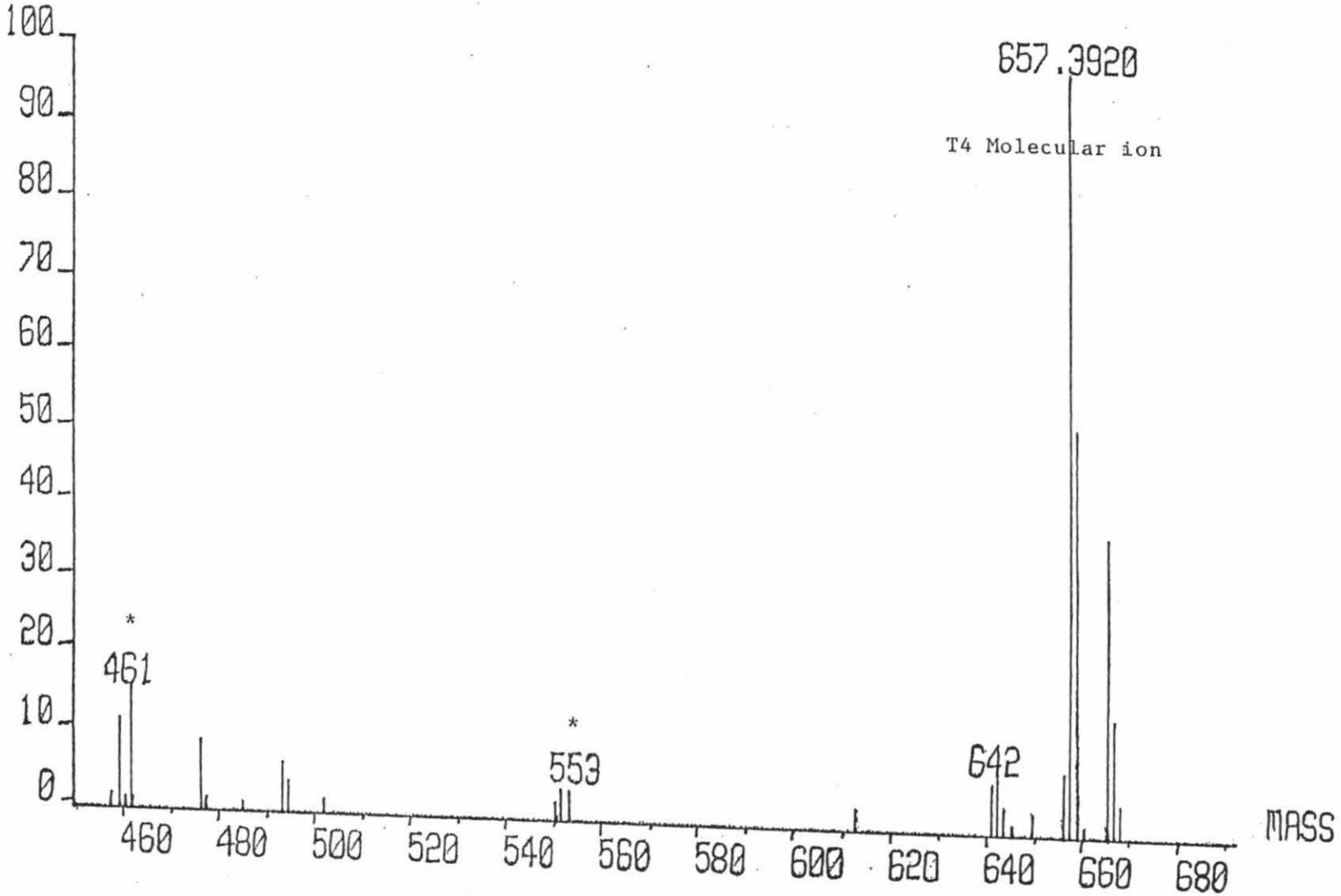


Figure 15. Mass spectrometry analysis of T4

contained T4 whose sequence was **GGTFLY**. Its molecular weight was 656 and the mass spectrometry analysis showed the molecular ion mass of 657. This confirmed the sequence result. Figure 15 was the result of the mass spectrometry analysis of T4.

4.3.2.5 Results of fraction 5 analysis

Figure 16 shows the purification of fraction 5 on RPLC. Peak A contained T17 whose sequence was:

AGLELYR.

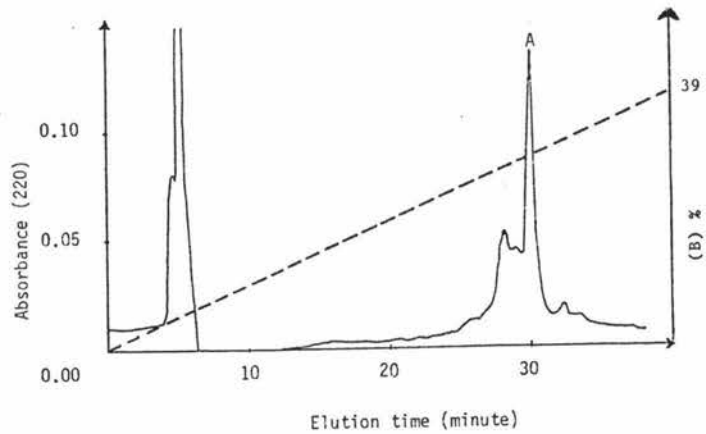


Figure 16. Purification of RPLC fraction 5 of tryptic peptides

4.3.2.6 Results of fraction 6 analysis

Figure 17 shows the purification of fraction 6 on RPLC. Peak A contained T15 whose sequence was:

DGIGGVAVGIR

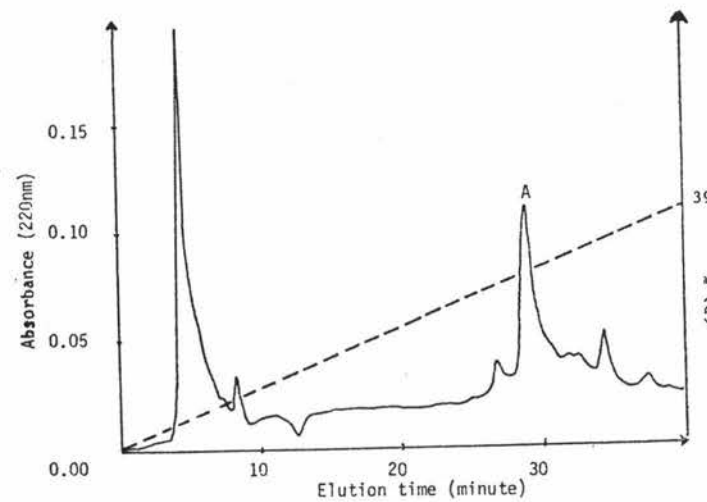


Figure 17. Purification of RPLC fractio 6 of tryptic peptides

4.3.2.7 Results of fraction 7 analysis

Amino terminal sequencing showed that this

fraction contained T14 and T16. T14 gave the sequence of **AVELLR**, and T16 was sequenced up to 12 residues: **NEELVESPILGT**. Figure 18a shows the plot of log(pmoles) against the cycle number (2.2.7) for T14 and T16. It is clear on the plot that the drop in the amount of T14 with sequencing cycle is more rapid than that of T16 and because small peptide are easily washed out during extraction, the amounts of the last two residues of T14 dropped seriously. Thus the sequence of T16 could be separated from T14.

T14 and T16 were loaded onto RPLC for further separation. But T14 was lost in this process. Figure 18 shows the RPLC profile in which peak A contained T16: **NEELVESPILGTAEEGALF**.

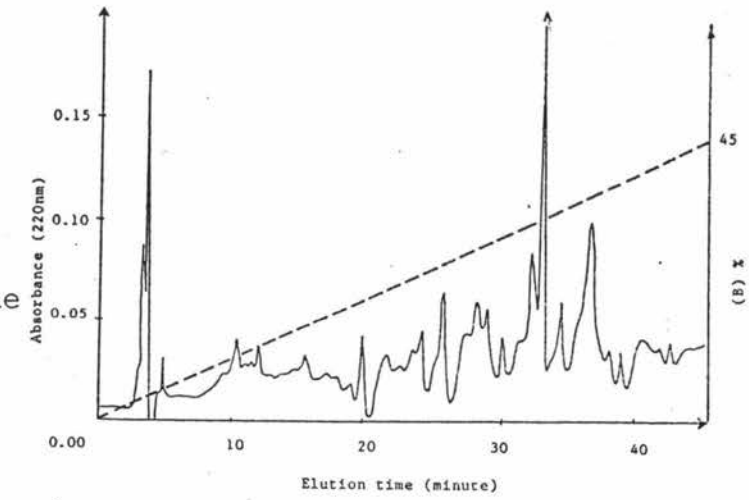


Figure 18. Purification of RPLC fraction 7 of tryptic peptides

4.3.2.8 Results of fraction 8 analysis

Figure 19 shows the purification of fraction 8 on RPLC. Peak B contained T9 whose sequence was: **NAGDIALNAGIAAGADDISIPELEFK**

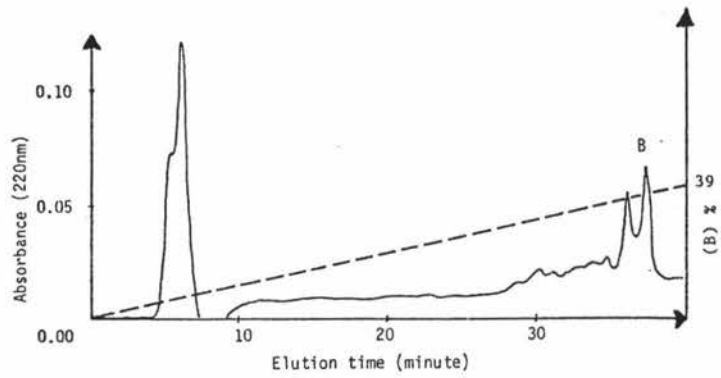


Figure 19. Purification of RPLC fraction 8 of tryptic peptides

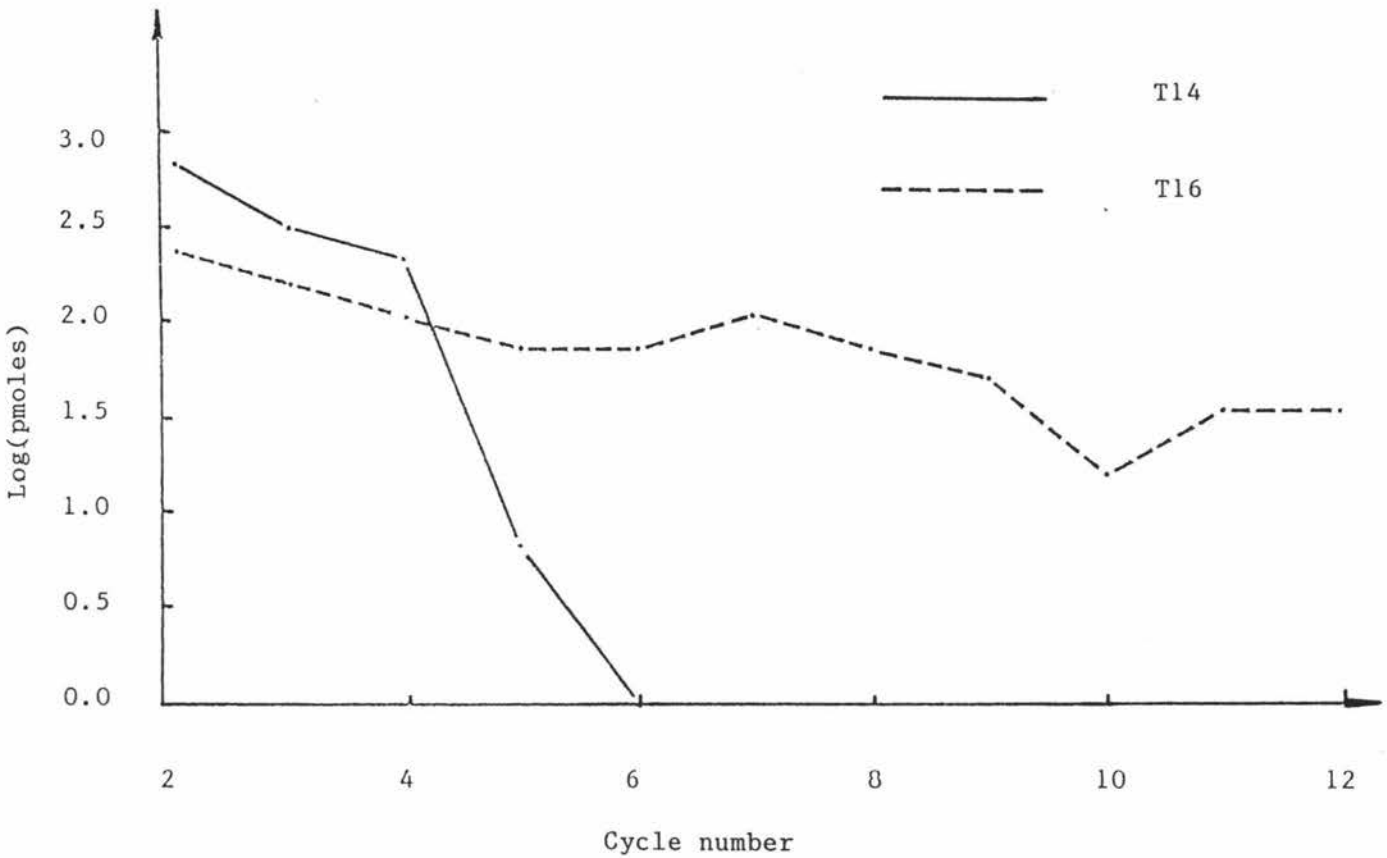


Figure 18a. Plot of log(pmoles) against cycle number for T14 and T16

4.3.2.9 Results of fraction 9 analysis

Fraction 9 contained T8, T10 and T12, which were separated on RPLC (Figure 20).

Peak A contained T10 which was **FENVVNNINK;** peak B contained T12 which was **VSVLGHIQR;** peak C contained T8 which was **TFVLEVMGR.**

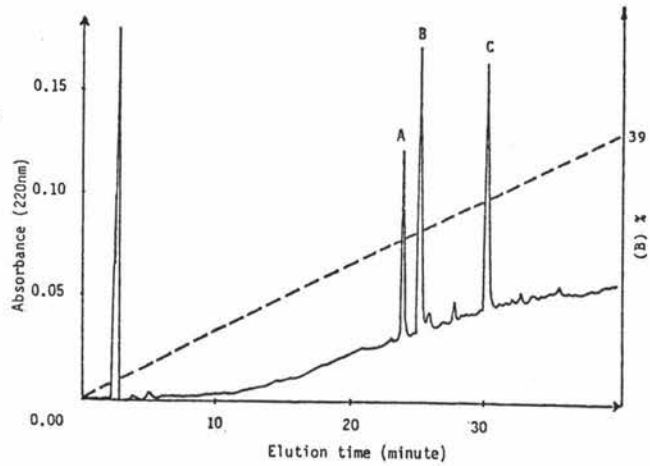


Figure 20. Purification of RPLC frcation 9 of tryptic peptides

4.3.2.10 Results of fraction 10 analysis

Figure 21 shows the purification of fraction 10 on RPLC. Two peptides were obtained.

Peak A contained T1 whose sequence was

IAVLTSGGDAPGMNAAIR

Peak B contained T5 whose sequence was

YPEFAQVEGQLAGIEQLK

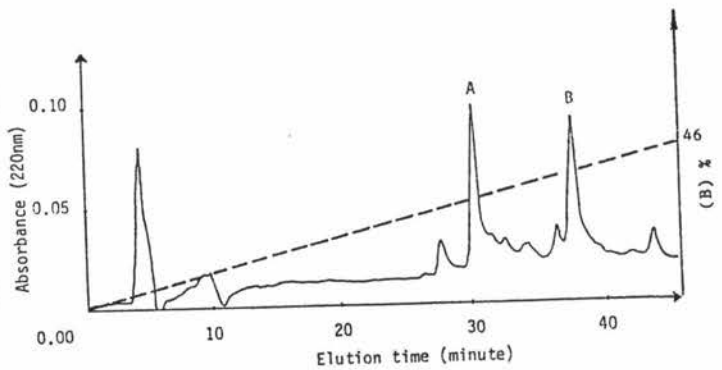


Figure 21. Purification of RPLC fraction 10 of tryptic peptides

4.3.2.11 Results of fraction 11 analysis

Figure 22 shows the purification of fraction 11 on RPLC.

Peak A contained T5 which was

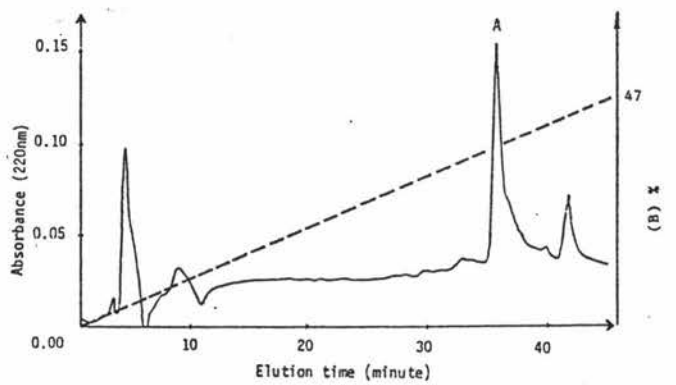


Figure 22. Purification of RPLC fraction 11 of tryptic peptiges

YPEFAQVEGQLAGIEQLK.

4.3.2.12 Results of fraction 12 analysis

Figure 23 shows the purification of fraction 12 on RPLC.

Peak A and B were the same containing T6 which was

FGIEGVVVIGGDGSYHGAMR.

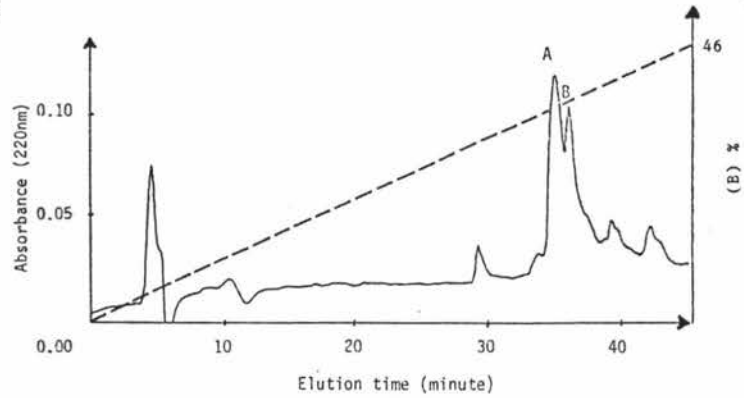


Figure 23. Purification of RPLC fraction 12 of tryptic peptides

4.3.2.13 Results of fraction 13 analysis

Figure 24 shows the purification of fraction 13 on RPLC.

Peak A contained T3 which was

GINHGYAGMVAGDIGFPLTSASVGDK.

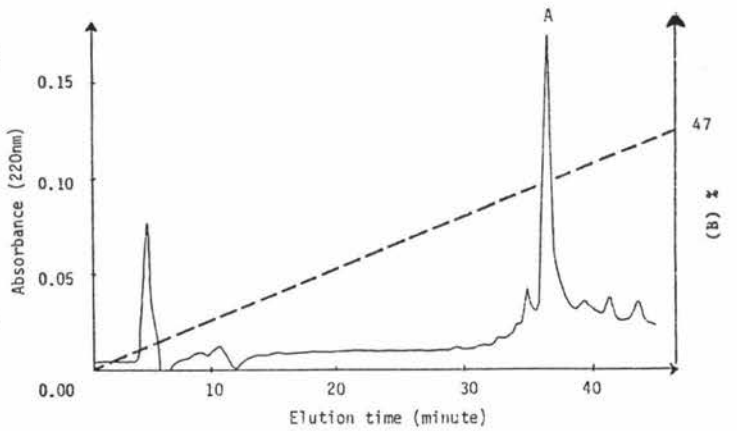


Figure 24. Purification of RPLC fraction 13 of tryptic peptides

4.3.2.14 Results of fraction 14 analysis

Fraction 14 contained T7. Amino terminal sequence on the gas phase sequencer gave the following sequence **LTEHGFPAVGLPGTIDNDIVGTDFTIGFDTAVSTVVD.** The total repetitive yield was 88.7% (2.2.7). Figure 25 is the curve of log(pmoles) against the cycle number (2.2.7).

T7 was further digested with chymotrypsin by the

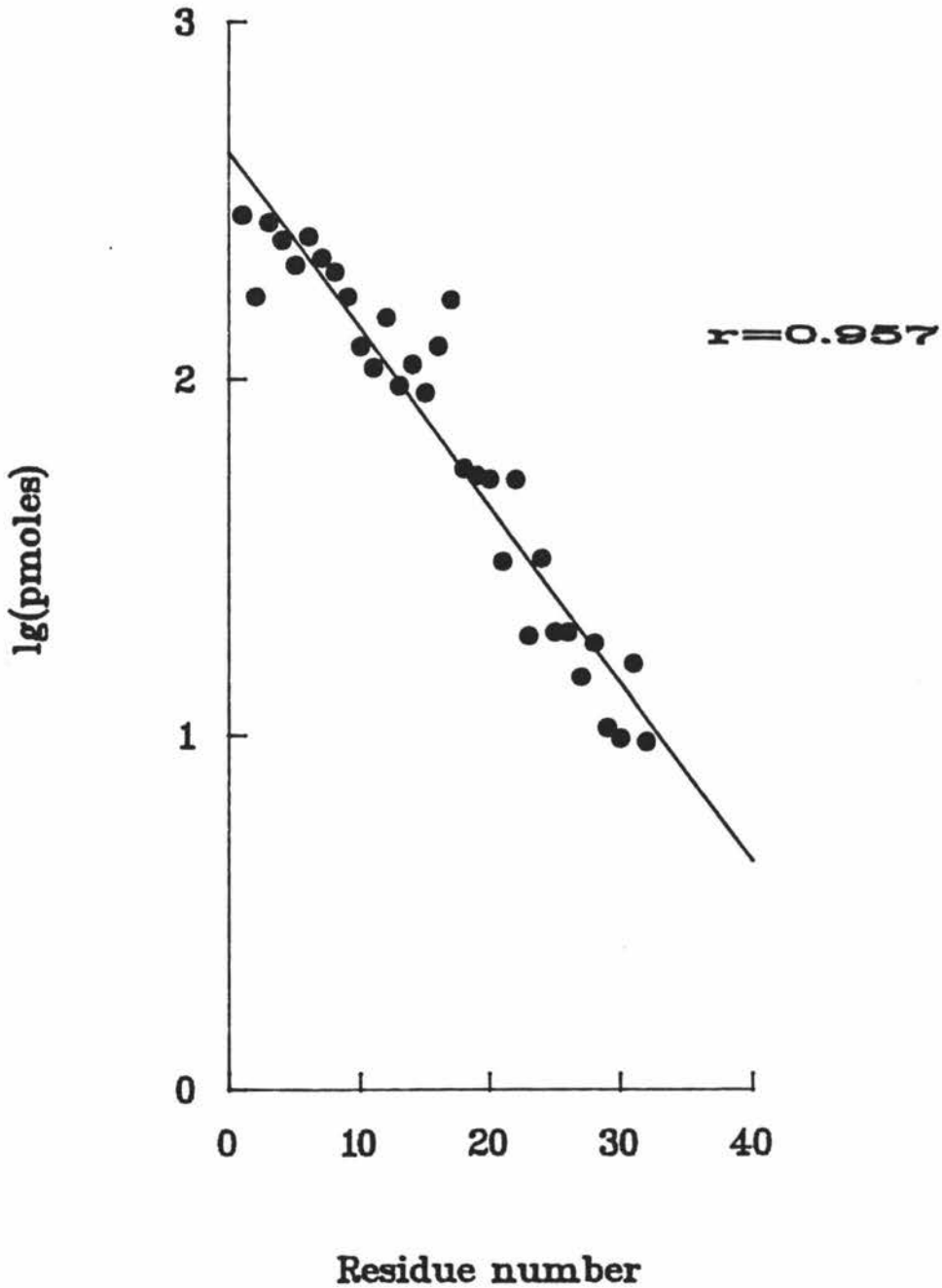


FIGURE 25: PLOT OF Log(pmoles) AGAINST CYCLE NUMBER OF T7 ANALYSIS ON SEQUENCER

method described in 2.2.10. Two subpeptides T7-1 and T7-2 were separated on RPLC using Vydac C18 column. The separation result is shown in figure 26. T7-1 was at the amino terminal while T7-2 was at the carboxyl terminal of T7.

Peak A contained T7-1 which was **LTEHGFPAVGLPGTIDNDIVGTDF**; peak B contained T7-2 which was **TIGFDTAVSTVVDALDK**.

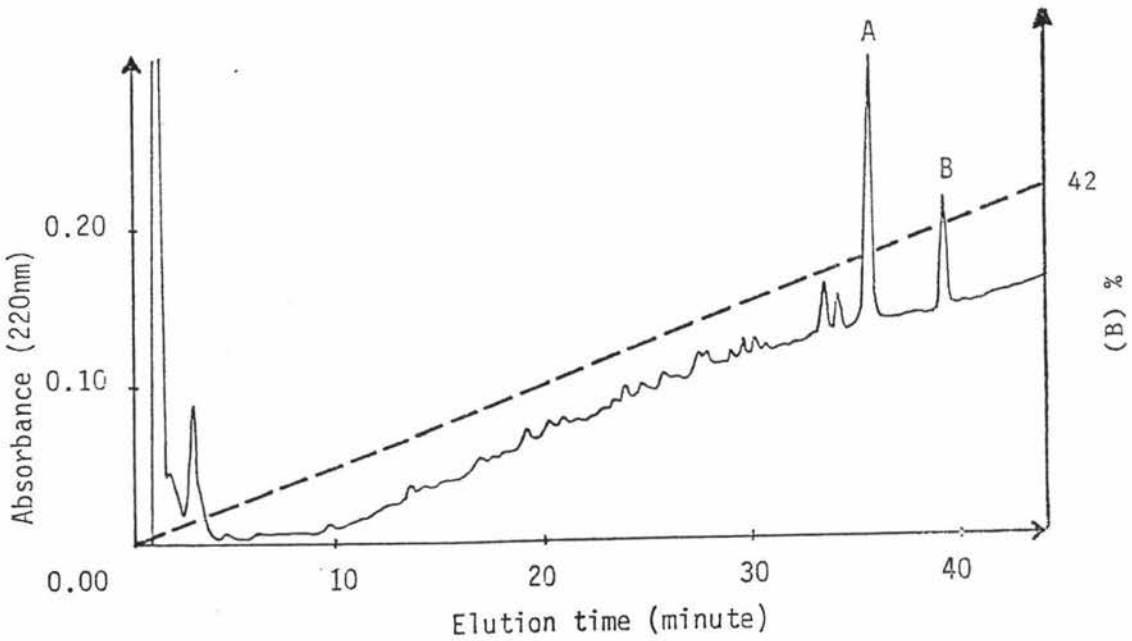


Figure 26. Separation of T7-1 and T7-2 on RPLC

4.3.2.15 Results of fraction 11a analysis

Amino terminal sequencing showed that fraction 11a contained T18 which was **LNSALNNLNL**.

4.3.3 Amino acid composition and mass spectrometric analysis results of the tryptic peptides

Amino acid compositions of tryptic peptides were analysed by the method described in 2.2.6 and in some cases, the results were confirmed by mass spectrometry analysis (2.2.8). Table 3 shows the results.

Table 3: Tryptic peptides characterization.

Peptide	Molecular weight	Molecular ion	Amino acid composition															
			A	D+N	E+Q	F	G	H	I	K	L	M	P	R	S	T	V	Y
T1	1712	1713	4.4	2.3	—	—	3.4	—	2.1	—	1.2	1.0	1.0	1.0	1.3	1.1	1.0	—
			4	2+0			3		2		1	1	1	1	1	1	1	
T2	979	980	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			1		2+0		1		1						1		1	1
T3	2475		2.5	2.7	—	1.2	4.2	1.7	1.7	1.3	1.1	1.0	0.8	—	1.7	1.6	2.2	0.6
			3	2+1		1	5	1	2	1	1	1	1		2	1	2	1
T4	656	657	—	—	—	0.8	1.8	—	—	—	0.9	—	—	—	—	0.8	—	1.0
						1	2				1					1		1
T5	1851		2.2	—	4.7	1.1	2.3	—	1.3	1.3	2.2	—	2.1	—	—	—	1.0	1.0
			2		3+3	1	2		1	1	2		1				1	1
T6	2019	2022	1.4	1.4	1.2	1.4	6.2	1.0	1.9	—	—	0.9	—	1.4	—	—	1.8	0.8
			1	1+0	1+0	1	6	1	2			1		1			3	1
T7-1	2483	2484	1.2	4.2	1.3	1.9	4.0	1.0	1.4	—	2.1	—	2.1	—	—	2.8	1.9	—
			1	3+1	1+0	2	4	1	2		2		2			3	2	
T7-2	1750	1752	2.0	2.7	—	1.1	1.2	—	0.8	1.0	1.0	—	—	—	1.1	2.5	2.5	—
			2	3+0		1	1		1	1	1				1	3	3	
T8	1036	1037	—	—	0.9	1.2	1.4	—	—	—	—	0.9	—	1.3	—	1.2	1.8	—
					1+0	1	1					1		1		1	1	
T9	2583		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
			6	3+2	2+0	1	3		4	1	2		1		1			

T10	1189			3.8	1.2	1.1			1.0	1.3							1.3	
				0+4	1+0	1			1	1							2	
T11	1368	1369																
			2		3+0	1	2			1		1				2	1	
T12	951		2.3		1.1		1.5	0.7	0.7		1.4			0.7	1.2		2.3	
			2		0+1		1	1	1		1			1	1		2	
T13	544																	
			1								1			1	1		1	
T14	699																	
			1		1+0						2			1			1	
T15	1012	1014	1.1	1.0			3.9		2.0					1.0			1.9	
			1	1+0			4		2					1			2	
T16	2302																	
			2	0+1	5+0	1	2		1		3		1		1	1	1	
T17	820	821	0.9		1.2		0.9				1.9			1.0				1.0
			1		1+0		1				2			1				1
T18	1227																	
			1	0+4								4					1	

* The top values were obtained from amino acid analysis and the bottom values were from the sequence. Some peptides have no amino acid analysis result because not enough material was avalaible.

4.3.4 Alignment of SL PFK tryptic peptides against the sequences of BS and EC PFKs

Figure 27 shows the alignment of SL PFK sequence against that of BS and EC PFKs.

FIGURE 27: ALIGNMENT OF SL PFK TRYPTIC PEPTIDES AGAINST THE SEQUENCES OF BS AND EC PFKs

```
     1                                                  50
BS   MKRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYHGYAGLIAGNIK
EC   MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMV
SL       IAVLTSGGDAPGMNAAIR     AISEGIEVYGINHGYAGMVAGDIF

     51                                                 100
     KLEVGDVGDIIHRGGTILYTARCPEFKTEEGQKKGIEQLKKHGIEGLVVI
     QLDRYSVSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVI
     PLTSASVGDK   GGTFLY    YPEFAQVEGQLAGIEQLK FGIEGVVVI

     101                                                150
     GGDGSYQGAKKLTEHGFPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAI
     GGDGSYMGAMRLTEMGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAI
     GGDGSYHGAMRLTEHGFPAVGLPGTIDNDIVGTDFTIGFDTAVSTVVDAL

     151                                                200
     DKIRDTATSHERTYVIEVMGRHAGDIALWSGLAGGAETILIPEADYDMND
     DRLRDTSSSHQRISVVEVMGRYCGDLTLAAAIAGGCEFVVVPEVEFSRED
     DK           TFVVEVMGRNAGDIALNAGIAAGADDISIPELEFKFEN

     201                                                 250
     VIARLKRGHERGKKHSIIIVAEGVGSGVDFGRQIQEATGF ETRVTVLGHV
     LVNEIKAGIAKGKKHAIVAITEHMCDVDELAHFIEKETGR ETRATVLGHI
     VVNNINK              VAEGVMTGEEFATK            VSVLGHI

     251                                                300
     QRGGSPTAFDRVLSARLGARAVELLLEGKGGRCVGIQNNQLVDHDIAEAL
     QRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNEQLVHHDIIDAI
     QR          VLASR    AVELLRDGIGGVAVGIRNEELVESPILGTA

     301
     ANKHTIDQRMYALSKELSI
     ENMKRPFKGDWLDCAKKLY
     EEGALF                AGLELYRLNSALNNLNL
```

4.4 CNBr PEPTIDES ANALYSIS

About 15mg of pure PFK was dissolved in 70% formic acid and digested with CNBr by the method described in 2.2.12. The peptides produced were named sequentially CN1, CN2, CN3, CN4, and CN5 from amino terminal to carboxyl terminal of the PFK.

4.4.1 CNBr peptides mapping

CNBr peptides were separated on FPLC using a Superose-12 gel filtration column. Figure 28 shows the FPLC elution profile.

4.4.2 Amino terminal analysis of the CNBr peptides

Table 4 shows the amino terminal sequences of the CNBr peptides in each fraction collected from FPLC.

Table 4: Amino terminal sequences of CNBr peptides

Fractions	Peptides	Amino terminal sequences
1	CN5	GRNAGDIALNAGIAAGAD
*2	CN3 CN4 CN6	VAGDIFPLTS RLTEHGFPAV GARAVELLRD
3	CN2	NAAIRAVVRKAISEGIEVYGIN
4	CN1	KRIAVLTSGG

* Since it was assumed that SL PFK was similar to BS and EC PFKs in amino acid sequence, by comparison, three peptides could be picked out.

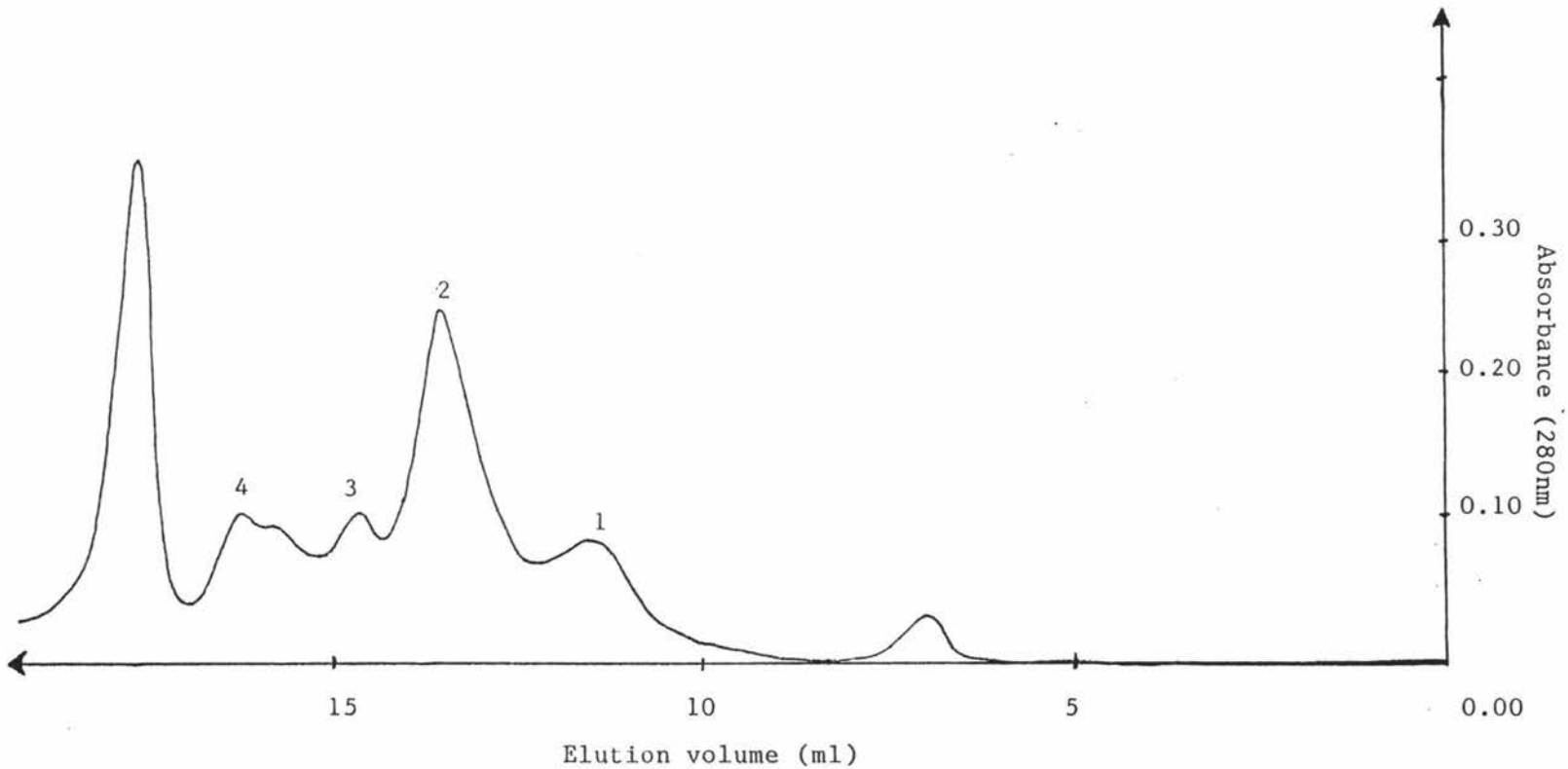
4.4.3 Analysis of fraction 1

Fraction 1 was purified by passing it through a FPLC Superose-12 gel filtration column a second time to

FIGURE 28: CNBr PEPTIDES MAPPING ON FPLC

Column:	Superose-12 gel filtration column
Temperature:	Room temperature
Buffer:	50% Formic acid
Flow rate:	0.4ml/minute
Sample:	CNBr peptides of PFK from *S. lactis*

*: The numbers in the figure indicate the fractions analysed which contained the CNBr peptides of *S. lactis* PFK.


4
3
2
1
0.30
0.20
0.10
0.00
Absorbance (280nm)
15
10
5
Elution volume (ml)

obtain pure CN5.

About 30nmoles of the pure CN5 was digested with *S. aureus* V8 protease using the method described in 2.2.11. The subpeptides were named CN5-V1, CN5-V2, CN5-V3 in the order of amino terminal to carboxyl terminal of CN5 and were separated on RPLC using Vydac C18 column. The buffer system used was the TFA system with the linear gradient of 0 to 60%B in 60 minutes. The flow rate was 0.5ml/min and the elution was monitored at 220nm. Table 5 shows the characterisation of the subpeptides.

Table 5: Subpeptides of CN5 digested with S. aureus V8 protease

Subpeptides	B% when eluted	Amino terminal sequences
CN5-V1	26.8	FKFE
CN5-V2	30.5	NVVNNINKGYE
CN5-V3	32.9	GYKGDLRVSVLGHIQ

About 30nmoles of the pure CN5 was digested with trypsin using the method described in 2.2.9. The subpeptides produced were named CN5-T1, CN5-T2, CN5-T3 and CN5-4 in the order of amino terminal to carboxyl terminal of CN5 and were separated on RPLC using a Vydac C18 column. The solvent system and the elution condition were the same as that used for separating the subpeptides of CN5 digested by *S. aureus* V8 protease. Table 6 shows the features of the subpeptides.

Table 6: Subpeptides of CN5 digested with trypsin

Subpeptides	B% when Eluted	Amino terminal sequences
CN5-T1	38.4	NAGDIALNAGIAAG
CN5-T2	15.5	GYEK
CN5-T3	16.5	EAGYK
CN5-T4	28.7	VSVLGHIQR

The alignment of these subpeptides against the relevant sequence of *B. stearothermophilus* and *E. coli* PFKs is shown below:

```
          170                                                  212
EC        GRYCGDLTLAAAIAGGCEFVVVPEVEFSREDLVNEIKAGIAKG
BS        GRHAGDIALWSGLAGGAETILIPEADYDMNDVIARLKRGHERG
   |CN-N GRNAGDIALNAGIAAGADD
SL |CN-T   NAGDIALNAGIAAG                              GYEK
   |CN-V                                  FKFENVVNNINKGYE

          213                                                  255
EC        KKHAIVAITEHMCDVDELAHFIEKETGRETRATVLGHIQRGGS
BS        KKHSIIIVAEGVGSGVDFGRQIQEATGFETRVTVLGHVQRGGS
   |CN-N
SL |CN-T                                EAGYK    VSVLGHIQR
   |CN-V                                  GYKGDLRVSVLGHIQ

          256      267
EC        PVPYDRILASRM
BS        PTAFDRVLASRM
```

* CN-N: Amino terminal sequence of CN5
CN-T: Subpeptides from tryptic digestion of CN5
CN-V: Subpeptides from V8 digestion of CN5

4.4.4 Analysis of fraction 2

4.4.4.1 Separation of CN3,CN4 and CN6

CN3,CN4 and CN6 in fraction 2 were separated on RPLC using a Vydac C4 column. The equilibration buffer (A) was 0.1% TFA in water (v/v) and the elution buffer (B) was 0.1%TFA in isopropanol. Linear gradient was 0-80%B in 80 minutes and the flow rate was 0.5ml/min.. The elution was monitored at 220nm. Figure 29 shows the elution profile.

Peak A contained CN3 whose amino terminal sequence was **VAGDIFPLTSASVGDKIGRGGT**. Peak B contained CN6 whose amino terminal sequence was **GARAVELLRDGIGGVA**. Peak C contained CN4 whose amino terminal sequence was **RLTEH**.

4.4.4.2 Treatment of CN3

CN3 was digested with trypsin by the method described in 2.2.9. Subpeptides obtained were named CN3-T1, CN3-T2, CN3-T3 and CN3-T4 and were separated using the same method as that for separating CN5 subpeptides. Table 7 shows the features of the CN3 subpeptides.

Table 7: Subpeptides of CN3

Subpeptides	B% when Eluted	Amino terminal sequences
CN3-T1	16.7	TSASVGDK
CN3-T2	33.0	GGTFLY
CN3-T3	29.6	YPEFAQVEGQLAGIEQLK
CN3-T4	37.6	FGIEGVVVIGGDGSYGAM

The alignment of these subpeptides against the

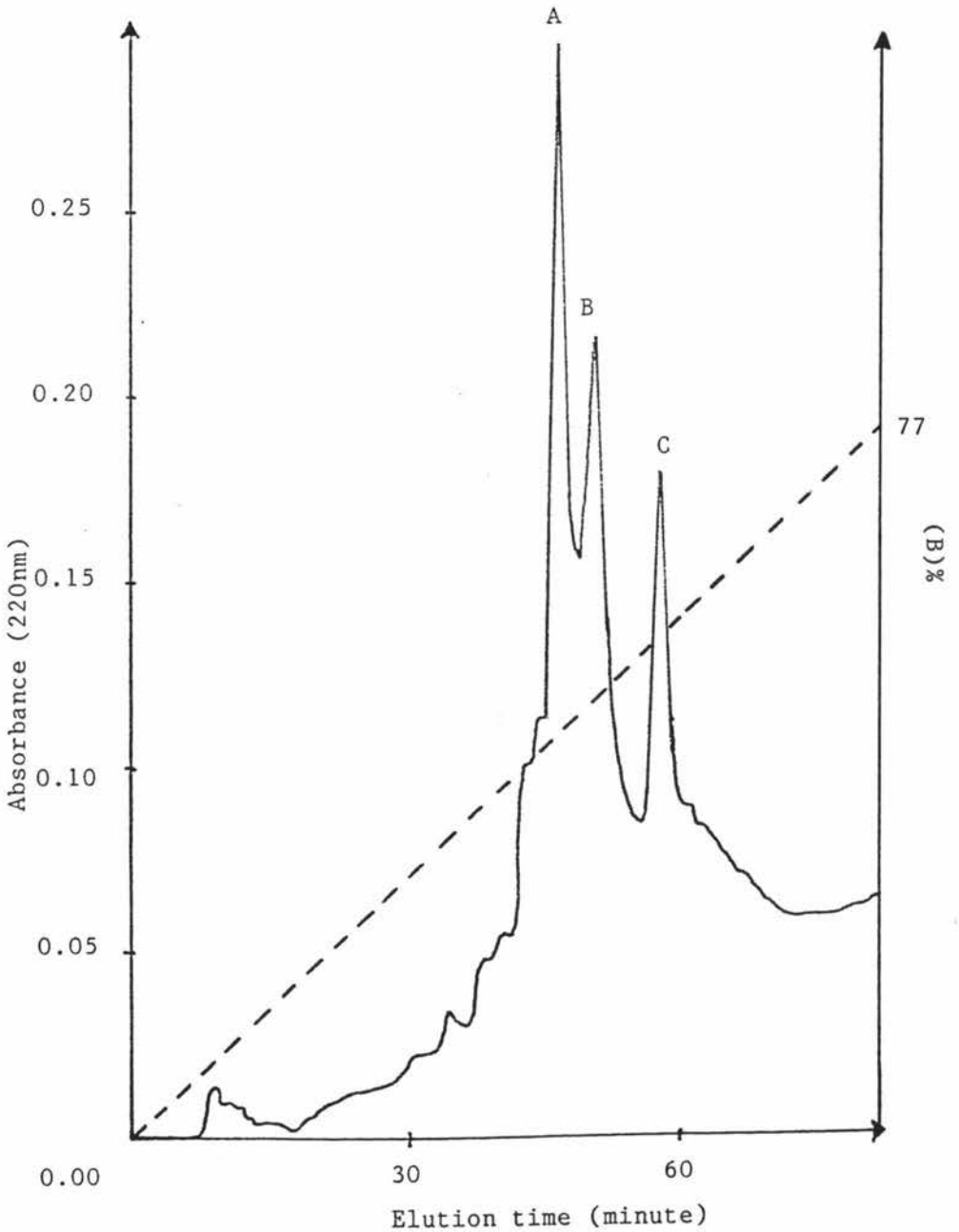


Figure 29. Separation of CN3, CN4 and CN6 on RPLC

relevant sequence of *B. Stearothermophilus* and *E. coli* PFKs is shown below:

```
          45                                                    87
EC        YEDRMVQLDRYSVSDMINRGGTFLGSARFPEFRDENIRAVAIE
BS        IAGNIKKLEVGDVGDIIHRGGTILYTARCPEFLTEEGQKKGIE
   |CN3N  VAGDIFPLTSASVGDKIGRGG
SL |CN3T           TSASVGDK   GGTFLY YPEFAQVEGQLAGIE

          88                    110
EC        NLKKRGIDALVVIGGDGSYMGAM
BS        QLKKHGIEGLVVIGGDGSYQGAK
   |CN3N
SL |CN3T  QLK FGIEGVVVIGGDGSYHGAM
```

* CN3N: Amino terminal sequence of CN3
CN3T: Subpeptides of CN3 digested by trypsin

4.4.4.3 Treatment of CN4

CN4 was digested with trypsin using the method described in 2.2.9 to give two subpeptides named CN4-T1 and CN4-T2 which were separated using the same method as that for separating the subpeptides of CN5. Table shows the features of CN4 subpeptides.

Table 8: Subpeptides of CN4

Subpeptides	B% when Eluted out	Amino terminal sequences
CN4-T1	13.8	TSSHNR
CN4-T2	44.8	TIGFDTAVSTVVDALDK

The alignment of these two subpeptides against the equivalent sequence of *B. Stearothermophilus* and *E. coli* PFKs is shown below:

```
          111                                                   157
EC        RLTEMGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAIDRLRDTS
BS        KLTEHGFPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAIDKIRDTA
SL       *RLTEHGFPAVG                TIGFDTAVSTVVDALDK   TS

          158      169
EC        SSHQRISVVEVM
BS        TSHERTYVIEVM
SL        SSHNR
```

* The amino terminal sequence of CN4

4.4.4.4 Treatment of CN6

CN6 was digested with trypsin by the method described in 2.2.9. The Subpeptides produced were named CN6-T1, CN6-T2, CN6-T3 and CN6-T4 and were separated using the same method as that for separating the CN5 subpeptides. The features of the subpeptides are shown in table 9.

Table 9: Subpeptides of CN6

Subpeptides	B% when Eluted out	Amino terminal sequences
CN6-T1	31.6	AVELLR
CN6-T2	31.6	DGIGGVAVGIR
CN6-T3	41.6	NEELVVESPILGTAEEGALF
CN6-T4	25.6	SLTTEGGIK

The alignment of these subpeptides against the relevant *B.stearothermophilus* and *E. coli* PFKs sequences is shown below:

```
     268                                                  314
EC   GAYAIDLLLAGYGGRCVGIQNEQLVHHDIIDAIENMKRPFKGDWLDC
BS   GARAVELLLEGGKGRCVGIQNNQLVDHDIAEALANKHTIDQRMYALS
     |*GARAVELLRDGIGGVA
SL   |    AVELLRDGIGGVAVGIRNEELVESPILGTAEEGALFSLTTEGGI

     315
EC   AKKLY
BS   KELSI
SL   K
```

* Amino terminal sequence of CN6

4.4.5 Analysis of fraction 3

Fraction 3 was purified by passing it through the Superose-12 gel filtration column on FPLC a second time.

Pure CN2 was obtained and it was sequenced completely. CN2 is aligned against the equivalent *B. stearothermophilus* and *E. coli* PFKs sequences:

```
     17                              44
EC   NAAIRGVVRSALTEGLEVMGIYDGYLGL
BS   NAAIRSVVRKAIYHGVEVYGVYHGYAGL
SL   NAAIRAVVRKAISEGIEVYGINHGYAG
```

4.4.6 Alignment of SL PFK CNBr peptides against EC and BS PFKs

Figure 30 is the alignment of SL PFK CNBr peptides against the sequences of BS and EC PFKs.

FIGURE 30: ALIGNMENT OF SL PFK CNBr PEPTIDES AGAINST BS AND EC PFKs

```
      1                                                  50
BS    MKRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYHGYAGLIAGNIK
EC  MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMV
SL    MKRIAVLTSGG     NAAIRAVVRKAISEGIEVYGINHGYAGMVAGDIF

      51                                                 100
      KLEVGDVGDIIHRGGTILYTARCPEFKTEEGQKKGIEQLKKHGIEGLVVI
      QLDRYSVSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVI
      PLTSASVGDKIGRGGTFLY   YPEFAQVEGQLAGIEQLK FGIEGVVVI

      101                                                150
      GGDGSYQGAKKLTEHGFPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAI
      GGDGSYMGAMRLTEMGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAI
      GGDGSYHGA RLTEHGFPAV                TIGFDTAVSTVVDAL

      151                                                200
      DKIRDTATSHERTYVIEVMGRHAGDIALWSGLAGGAETILIPEADYDMND
      DRLRDTSSSHQRISVVEVMGRYCGDLTLAAAIAGGCEFVVVPEVEFSRED
      DK   TSSSHNR       GRNAGDIALNAGIAAGADD       FKFEN

      201                                                 250
      VIARLKRGHERGKKHSIIIVAEGVGSGVDFGRQIQEATGF ETRVTVLGHV
      LVNEIKAGIAKGKKHAIVAITEHMCDVDELAHFIEKETGR ETRATVLGHI
      VVNNINKGYEK                          KEAGYKGDLRVSVLGHI

      251                                                300
      QRGGSPTAFDRVLSARLGARAVELLLEGKGGRCVGIQNNQLVDHDIAEAL
      QRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNEQLVHHDIIDAI
      QR                GARAVELLRDGIGGVAVGIRNEELVESPILGTA

      301
      A NKHTIDQRMYALSKELSI
      ENMKRPFKGDWLDCAKKLY
      E EGALFSLTTEGGIK
```

4.5 CHYMOTRYPTIC DIGESTION

About *20mg S. lactis* PFK was subjected to chymotryptic digestion by the method described in 2.2.10. The peptides obtained were called in turn from amino terminal to carboxyl terminal as Ch1, Ch2····· Ch16. Ch11-1 and Ch11-2, Ch16-1 and Ch16-2 were two pairs of similar peptides due to different cleavage sites. These peptides were separated on RPLC. Figure 31 shows the chymotryptic peptide map obtained by RPLC.

Each fraction from the RPLC of the chymotryptic peptides was further purified on RPLC using the TFA system (2.2.14). The amino acid composition and sequence of each pure peptide were studied. Table 10 shows the elution features during RPLC mapping, the amino acid compositions and the amino terminal sequences of the chymotryptic peptides.

The alignment of chymotryptic peptides from *S. lactis* PFK against the sequences of *B.stearothermophilus* and *E. coli* PFKs is shown in figure 31**a**.

FIGURE 31: CHYMOTRYPTIC PEPTIDES MAPPING ON RPLC

Column:	Vydac C18
Temperature:	Room temperature
Equilibration buffer (A):	0.1M ammonium bicarbonate in 5% acetonitrile in water
Elution buffer (B):	0.1M ammonium bicarbonate: isopropanol:acetonitrile = 1:1:1 (v/v/v)
Elution gradient:	0 to 60% of B in 60 minutes
Flow rate:	0.5ml/minute
Sample:	Chymotryptic peptides from *S. lactis*

* The numbers indicate the fractions collected and analysed which contained chymotryptic peptides of SL PFK.

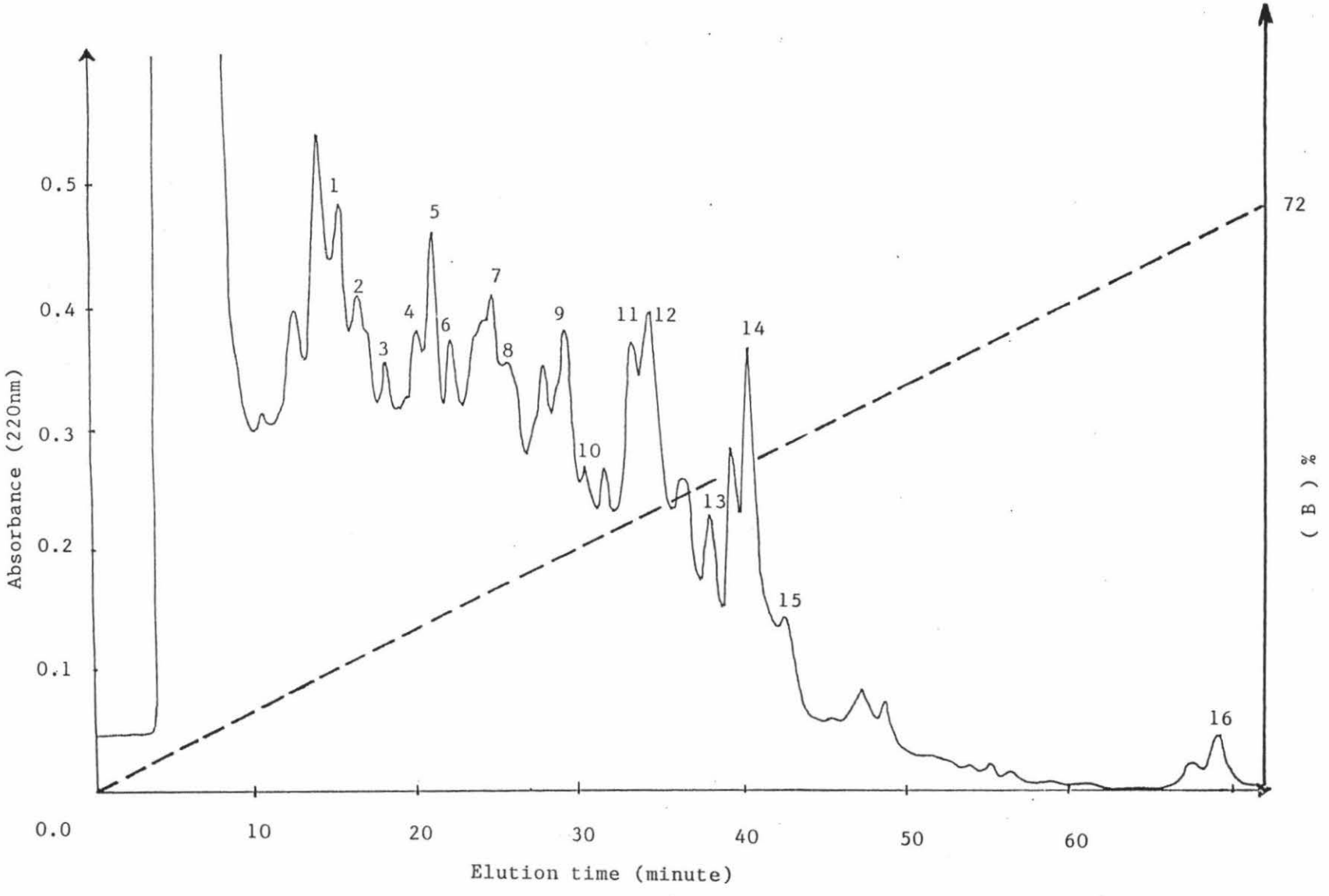

Absorbance (220nm)
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
72
(B) %
10
20
30
40
50
60
Elution time (minute)

Table 10: Characterisation of chymotryptic peptides

Fraction	Peptide	B% when Eluted	Amino terminal sequences	Amino acid composition															
				A	D+N	E+Q	F	G	H	I	K	L	M	P	R	S	T	V	Y
5	Ch1	21.5	KAISEGIEVY	1.3		2.0		1.2		2.0	0.9					1.0		0.9	0.9
				1		2+0		1		2	1					1		1	1
1	Ch2	15.8	GINHGY		1.3			2.1	1.0	1.4									1.0
					0+1			2	1	1									1
8	Ch3	26.0	TSVSGDKIGRGTF	1.5	1.0		1.1	3.6		0.8	1.2				1.2	1.4	1.8	1.1	
				1	1+0		1	4		1	1				1	2	2	1	
2	Ch4	16.8	LY																
												1							1
13	Ch5	34.4	SARYPEFAQVEGQL AGIEQL	3.0		5.8	1.1	2.1		0.9		2.2		1.1	1.0	1.0		1.0	0.9
				3		3+3	1	2		1		2		1	1	1		1	1
9	Ch6	29.7	GIEGVVVIGGDGSY		1.6	1.5		4.6		1.8						1.3		1.9	1.0
					1+0	1+0		5		2						1		3	1
14	Ch7	40.9	LTEHGFPAVGLPGTI DNDIVGTDF	1.4	4.2	1.1	2.1	4.2	0.9	1.7		2.2		2.1			2.4	1.7	
				1	3+1	1+0	2	4	1	2		2		2			3	2	
16	Ch8	69.1	TIGFDTAVSTVVDAL DKIRDTSSS	3.3	4.3		1.2	1.9		1.5	1.8	1.2			0	4.2	3.6	2.0	
				2	4+0		1	1		2	1	1			1	4	4	3	
3	Ch9	18.5	GRNAGDIALN																
				2	1+2			2		1		1			1				

7	Ch10	25.0	AGIAAGADDISIPELEF	3.9 4	2.6 2+0	2.3 2+0	1.3 1	2.3 2		2.8 3	1.0 1	0.2 1		1.0 1		0.2 1			
5	Ch11-1	21.5	ENVVNNINKGY		0+4	1+0		1		1	1							2	1
4	Ch11-2	20.4	KFENBVNN		0+3	1+0	1				1							2	
15	Ch12	42.9	EKGKNHHIIIVAEGVM	1	0+1	2+0		2	2	3	2		1					2	
3	Ch13	18.5	ATKLKEAGY	2		1+0		1			2	1					1		1
10	Ch14	30.7	KGDLRVSVL		1+0			1			1	2			1	1		2	
13	Ch15	38.2	LRDGIGGVAVGIRNEELVESPILGTAEEGALF	3.1 3	2.3 1+1	5.0 5+0	1.1 1	5.8 6		2.7 3		4.1 1		1.1 1	1.9 2	1.1 1	1.0 1	2.9 3	
6	Ch16-1	22.6	SLTTEGGIKVNNPH		1.8 0+2	0.9 1+0		2.2 2	0.9 1	0.9 1	1.1 1	1.3 1		1.0 1		1 1	2 2	1 1	
12	Ch16-2	35.0	SLTTEGGIKVNNPHKAGLELY	1	0+2	2+0		3	1	1	2	3		1		1	2	1	1

* The top amino acid content values were from the amino acid analysis and the bottom values were from the sequence. Some peptides have no amino acid analysis result because not enough material was available for amino acid analysis.

FIGURE 31a: ALIGNMENT OF CHYMOTRYPTIC PEPTIDES OF SL PFK AGAINST THE SEQUENCES OF BS AND EC PFKs

```
      1                                                  50
BS    MKRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYHGYAGLIAGNIK
EC  MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMV
SL                                KAISEGIEVYGINHGY

      51                                                 100
      KLEVGDVGDIIHRGGTILYTARCPEFKTEEGQKKGIEQLKKHGIEGLVVI
      QLDRYSVSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVI
        TSASVGDKIGRGGTFLYSARYPEFAQVEGQLAGIEQL   GIEGVVVI

      101                                                150
      GGDGSYQGAKKLTEHGFPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAI
      GGDGSYMGAMRLTEMGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAI
      GGDGSY    RLTEHGFPAVGLPGTIDNDIVGTDFTIGFDTAVSTVVDAL

      151                                                200
      DKIRDTATSHERTYVIEVMGRHAGDIALWSGLAGGAETILIPEADYDMND
      DRLRDTSSSHQRISVVEVMGRYCGDLTLAAAIAGGCEFVVVPEVEFSRED
      DKIRDTSSS           GRNAGDIALNAGIAAGADDISIPELEFKFEN

      201                                                 250
      VIARLKRGHERGKKHSIIIVAEGVGSGVDFGRQIQEATGF ETRVTVLGHV
      LVNEIKAGIAKGKKHAIVAITEHMCDVDELAHFIEKETGR ETRATVLGHI
      VVNNINKGYEKGKNHHIIIVAEGVM     ATKLKEAGYKGDLRVSVL

      251                                                300
      QRGGSPTAFDRVLSARLGARAVELLLEGKGGRCVGIQNNQLVDHDIAEAL
      QRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNEQLVHHDIIDAI
                              LRDGIGGVAVGIRNEELVESPILGTA

      301
      ANKHTIDQRMYALSKELSI
      ENMKRPFKGDWLDCAKKLY
      EEGALFSLTTEGGIKVNNPHKAGLELY
```

4.6 *S. aureus* V8 PROTEASE DIGESTION

About 20mg of SL PFK were digested with *S. aureus* V8 protease (2.2.11). The initial separation of the peptides was carried out on a G-50 Sephadex gel filtration column (2.2.15) and the process was monitored spectrophotometrically at 280nm (Figure 32).

Fractions shadowed in figure 32 were combined and freeze-dried and were treated according to the following procedure:

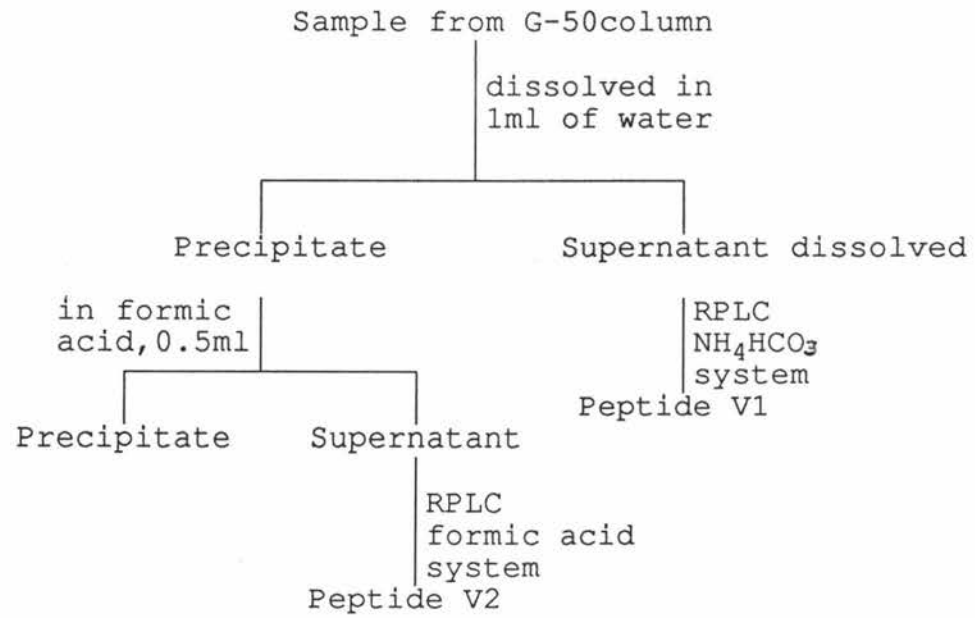


Figure 33 shows the RPLC elution profile from which V1 was obtained. Figure 34 shows the RPLC elution profile from which V2 was obtained.

V1 is **LYRLNSALNNLNL** and V2 is **AGYKGDLRVSVLGHIQRGGSPTA*DRVLASRMG.**

FIGURE 32: SEPARATION OF S. aureus V8 PROTEASE DIGESTED SL PFK ON G-50 COLUMN

Column:	G-50 Sephadex Gel Filtration Column
Size:	113cm X 1.5cm diameter
Temperature:	Room temperature
Buffer:	1% ammonium bicarbonate
Flow rate:	0.5ml/minute
Sample:	S. aureus V8 protease digested SL PFK

* Shadowed area indicates the fractions collected for further study.

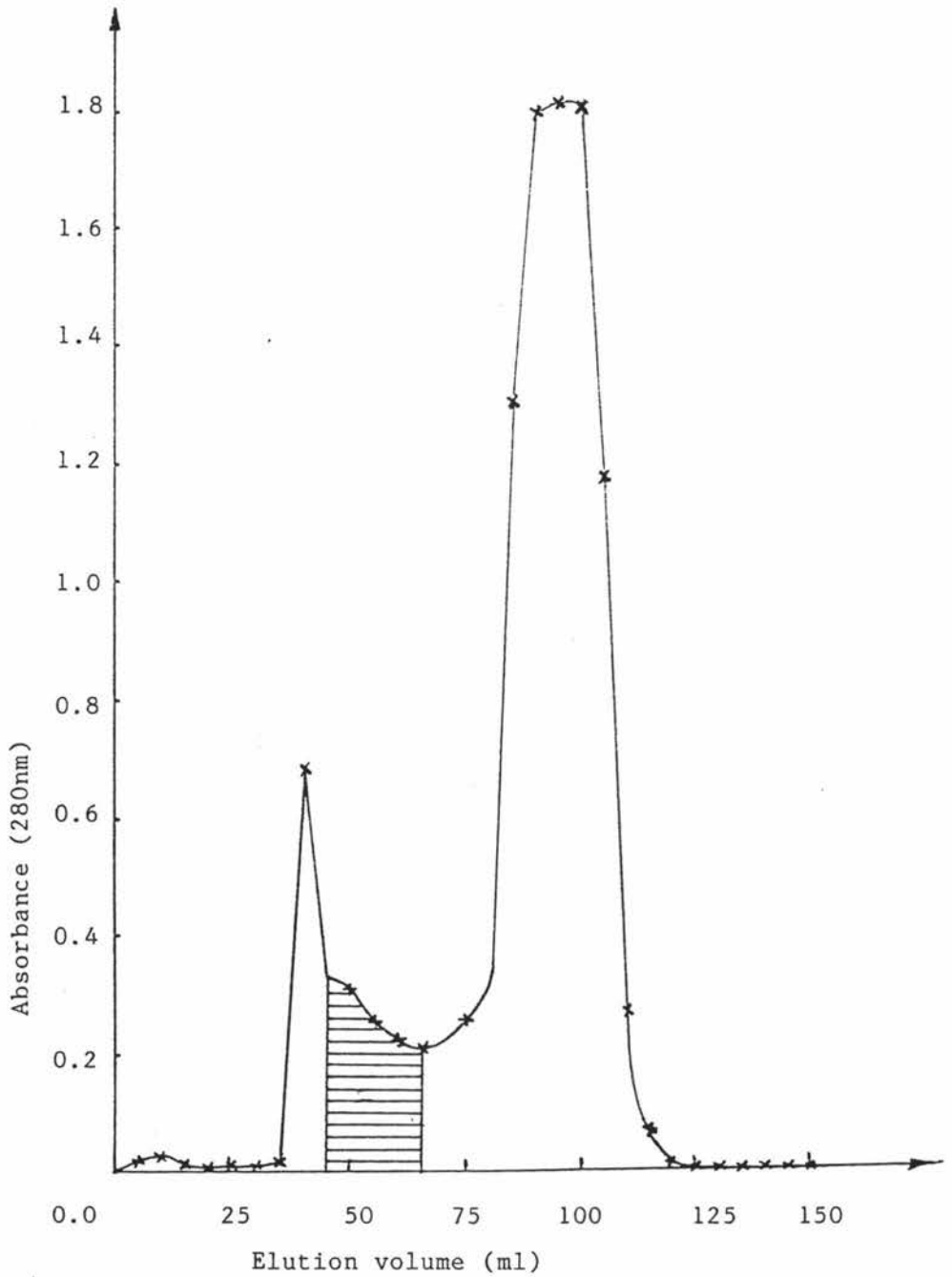


FIGURE 32: SEPARATION OF S. aureus V8 protease DIGESTED SL PFK ON G-50 GEL FILTRATION COLUMN

(Shadowed fractions were collected)

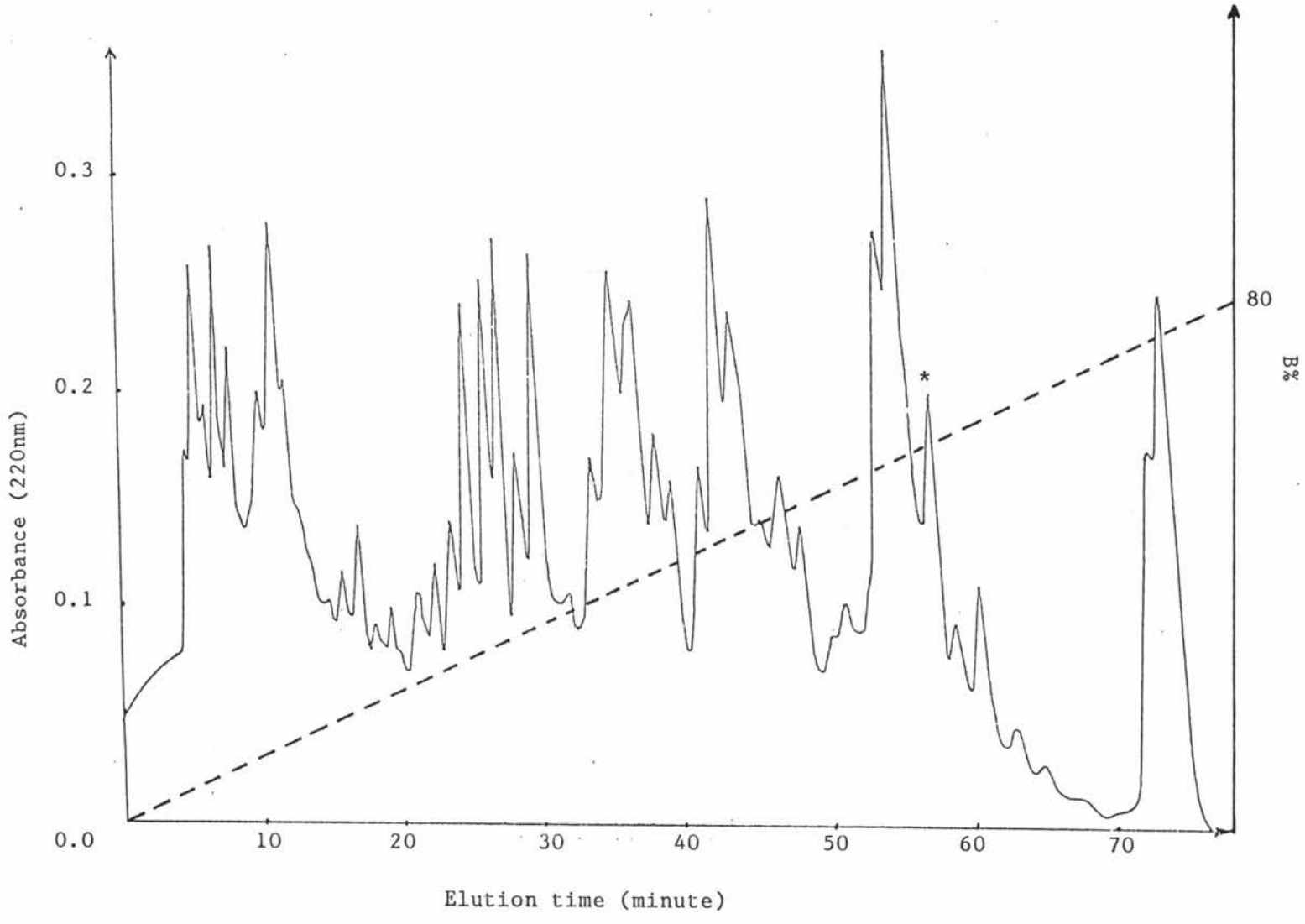


FIGURE 33: RPLC SEPARATION OF WATER-SOLUBLE MATERIAL FROM G-50 ELUTION

* The fraction where V1 was found.

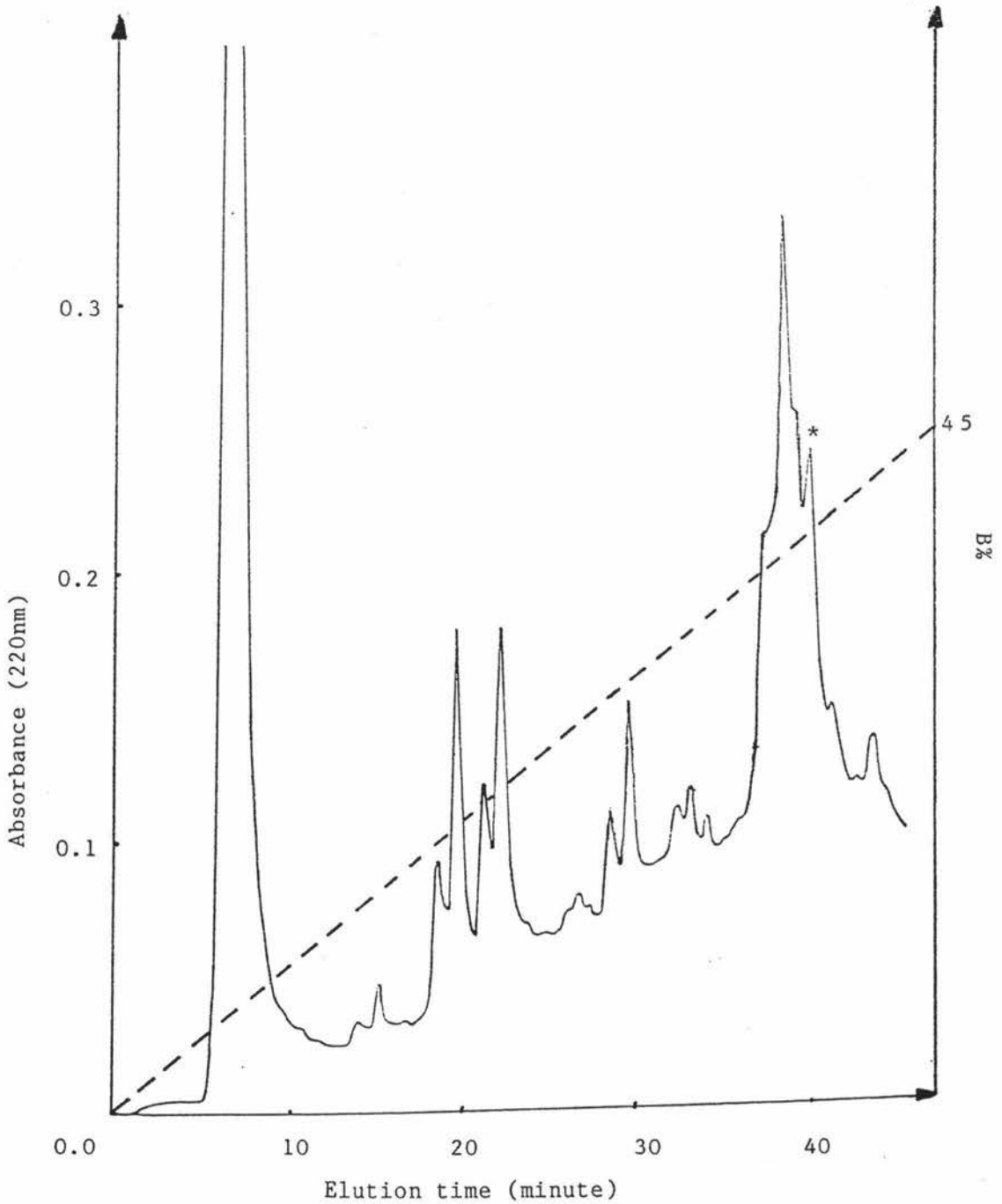


FIGURE 34: RPLC SEPARATION OF FORMIC ACID-SOLUBLE MATERIAL FROM G-50 ELUTION
* Fraction collected where V2 was found.

CHAPTER FIVE DISCUSSION

5.1 SEQUENCE STUDY OF *S. lactis* PFK

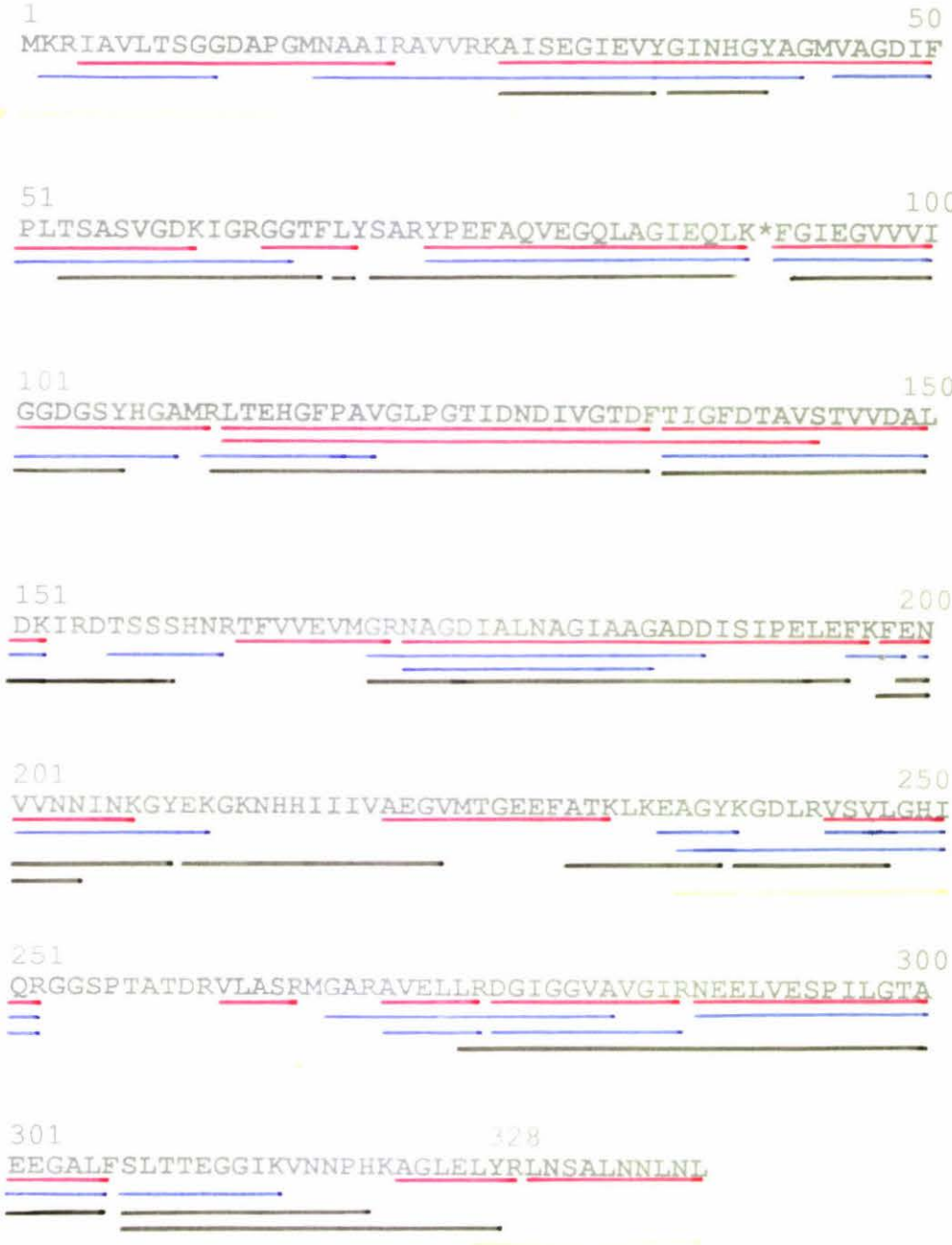
5.1.1 General discussion

The sequence study of *S. lactis* PFK was carried out by digesting the pure enzyme with trypsin, chymotrypsin, CNBr and *S.aureus* V8 protease separately and analysing the sequence of peptides obtained in each digestion followed by the alignment of the peptides against the sequences of PFKs from *B. stearothermophilus* and *E. coli*. The confirmation of the structure of the peptides was made by amino acid analysis and mass spectrometry. 336 out of 338 residues of SL PFK were determined.

Figure 35 shows the sequence of *S. lactis* PFK. The lines underneath in different colours indicate the peptides (or subpeptides) obtained in tryptic, chymotryptic, *S. aureus* V8 protease or CNBr digestion of *S. lactis* PFK.

Residues 91 and 260 in the *S. lactis* PFK sequence were not sequenced in this study. Residue 91 is probably a lysine since the tryptic peptide T5 starts at 92 indicating that it is likely to be adjacint to a basic residue and the double basic sequence KK for residues 90 and 91 occurs also in BS and EC PFKs.

FIGURE 35: AMINO ACID SEQUENCE OF PFK FROM S. lactis



Peptide overlaps
Red: tryptic peptides
Blue: CNBr peptides
Black: chymotryptic peptides
Green: S. aureus V8 digested PFK
*: amino terminal sequence of PFK

The fragmentations gave good overlaps among peptides and confirmed the sequence alignment with each other. No overlaps were found between residue 90 and 92, 162 and 163, 307 and 308. Residues 163 to 169, 226 to 230 were found only in the tryptic peptides. Residues 160 to 162, 269 to 270 were found only in the CNBr peptides. Residues 70 to 72, 153 to 155, 212 to 220 were found only in the chymotryptic peptides. Residues 253 to 261 were found only in V8 protease digestion.

No cysteine was found in *S. lactis* PFK from either the sequence study or the amino acid analysis.

The first 28 residues from the amino terminus of *S. lactis* PFK was obtained by directly sequencing the enzyme. Like *B. stearothermophilus* and *E. coli* PFKs, the *S. lactis* PFK has a methionine residue at the amino terminus. The amino terminal sequence is:

MKRIAVLTSGGDAPGMNAAIRAVVRKAI

5.1.2 Tryptic digestion (3.4)

After tryptic digestion, 18 tryptic peptides from *S. lactis* PFK were obtained and studied. Some small peptides, such as dipeptides ^{212}**GK**^{213} and ^{234}**LK**^{235}; tripeptides ^{61}**IGR**^{63} and ^{70}**TSR**^{72}; tetrapeptides ^{208}**GYEK**^{211}, ^{225}**VVRK**^{229} and ^{267}**MGAR**^{270}; and pentapeptide ^{235}**EAGYK**^{239}, were not isolated because they passed through RPLC without retention. It was considered that

these small sequences would be found by overlaps from other enzymic digestions.

Although trypsin was treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) to inhibit chymotryptic activity, some chymotrypsin activity still remained as some cleavages occurred at chymotrypsin-sensitive residues. For example, T2 which is **AISEGIEVY** and T4 which is **GGTFLY** ended with tyrosine; T16 which is **NEELVESPILGTAEEGALF** ended with phenylalanine.

One interesting cleavage site was at the carboxyl terminal of Val220. This bond is not cleaved by chymotrypsin and, since the chymotryptic peptide Ch12 which contained Val220 was isolated by the same procedures as the tryptic peptides, the cleavage was not likely to be caused by the isolation conditions.

5.1.3 CNBr digestion (3.5)

CNBr digestion is a very specific method for cleaving the protein at the carboxyl terminal of methionine. This method generally has high efficiency, but some methionyl bonds may be poorly cleaved, particularly the linkages of Met-Thr and Met-Ser (Findlay and Geisow, 1989). Between residue 225 and 226, there was a Met-Thr linkage which was not cleaved by CNBr in this study.

S. lactis PFK has 7 methionines. Since one of

them is the amino terminus and another is linked to threonine, only six peptides were found after digestion.

5.1.4 Chymotryptic digestion (3.6)

Chymotrypsin has a relatively broad specificity. It hydrolyses peptide bonds on the carboxyl terminal of tyrosine, phenylalanine, tryptophan, leucine and methionine. Cleavages of other peptide bonds have also been reported. In the case of chymotryptic digestion of *S. lactis* PFK, the cleavages at histidine, serine, and arginine were found. Ch16-1 ended with histidine and Ch8 appeared to end with serine. Ch1 which followed a cleavage at Arg25 could be the result of contamination by trypsin.

No cleavage was found at Phe139 and the hydrolysis at Phe198 was not complete since Ch11-2 whose amino terminal sequence was **KFENVVNNI** was obtained in the chymotryptic S. lactis PFK sample. This is because Phe139 is linked to Asp140 and Phe198 is linked to Glu199. It is hard to cleave at the chymotrypsin sensitive sites if their carboxyl terminals are linked to negatively charged amino acid residues.

5.2 STRUCTURAL COMPARISON OF PFKs FROM DIFFERENT SOURCES

5.2.1 Sequence similarities among PFKs from *B. stearothermophilus, E. coli* and *S. lactis*

The alignment of the sequences of PFKs from *B. Stearothermophilus, E. coli* and *S. lactis* is shown in figure 36. The best alignment is obtained when the extra isoleucine in *E. coli* PFK is inserted after the amino terminal methionine, and the extra glycine in *S. lactis* PFK is inserted after residue Lys40.

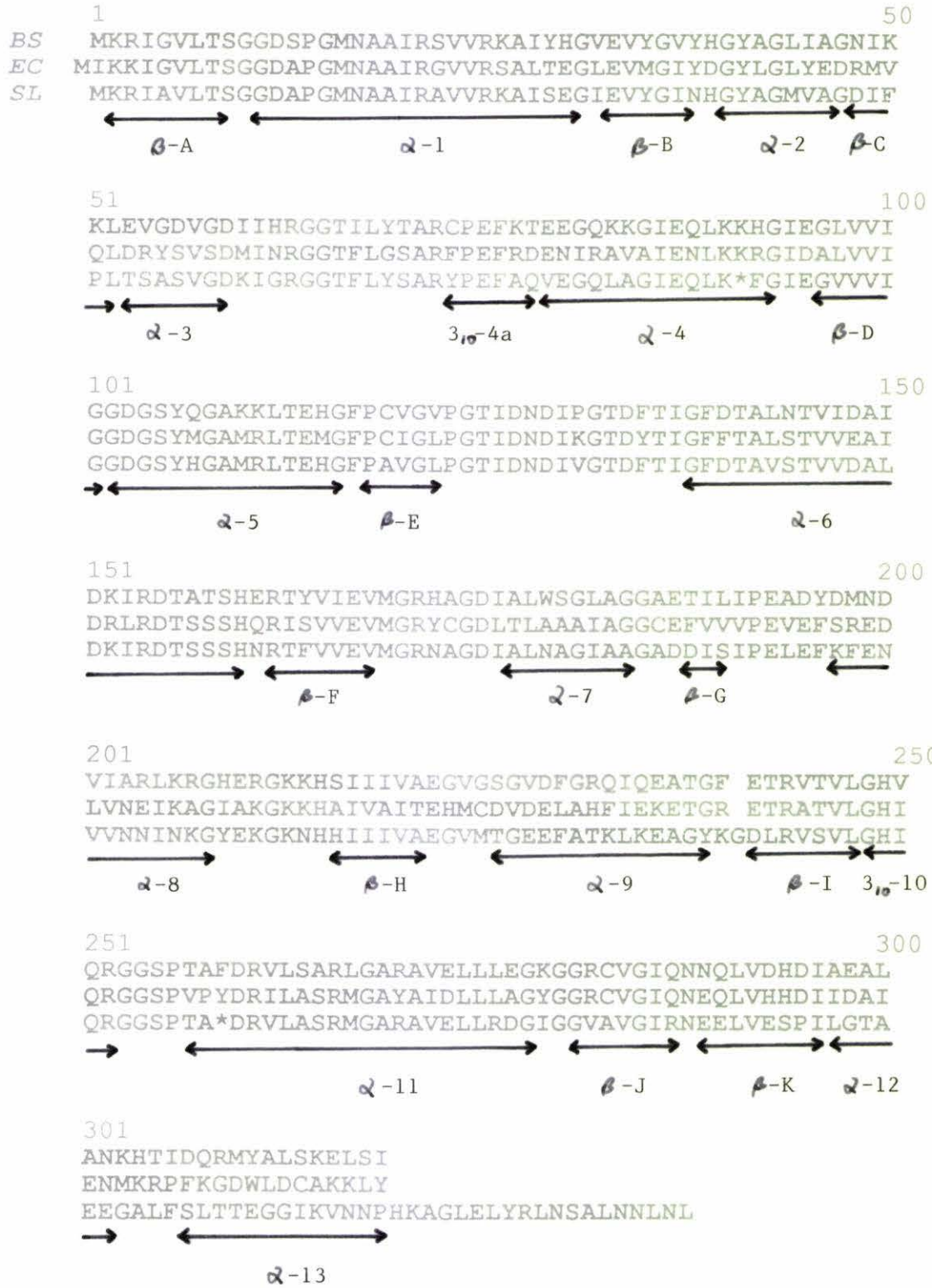
The similarities between PFKs from *B. stearothermophilus, E. coli* and *S. lactis* are shown in table 11. The high degree of similarity suggest a slow rate of evolution for PFKs similar to that found for the other enzymes in glycolytic pathway (Fothergill-Gilmore, 1986).

Table 11: Similarities among bacterial PFKs

PFK / Similarity PFK	*SL*	*EC*
BS	57.3%	53.6%
EC	51.5%	-

The most commonly occurring amino acid changes (Table 12 and table 13) between *BS* and *SL* PFKs, *EC* and *SL* PFKs were consistent with the conclusion drawn by Doolittle (1979) of the most frequently observed amino acid interchanges except the C/A interchange between *EC* PFK and *SL* PFK.

FIGURE 36: COMPARISON OF THE SEQUENCES OF PFKs FROM *S.lactis*, *B.stearothermophilus* and *E.coli*



Secondary structure elements of bacterial PFK based on the study of EC PFK.

Table 12: The most commonly occurring amino acid interchanges between BS PFK and SL PFK

Interchanges	Number	% among total changes	Base changes
V/I	7	7.3	1
S/A	6	6.3	1
D/E	5	5.2	1
T/S	4	4.2	1
G/A	3	3.1	1
K/R	3	3.1	1
L/V	3	3.1	1
A/L	3	3.1	1
Total	34	35.4	-

Table 13: The most commonly occurring amino acid interchanges between EC PFK and SL PFK

Interchanges	Number	% among total changes	Base changes
I/L	7	6.2	1
G/A	6	5.3	1
I/V	6	5.3	1
D/E	5	4.4	1
L/V	4	3.5	1
C/A	4	3.5	2
T/A	3	2.3	1
Total	35	31.0	-

All these interchanges except the C/A interchange are due to single base changes in codons.

The conservative amino acid changes of proteins include the amino acid interchanges among the following sets of amino acids: (G,A,V,L,I,F,P,M,C), (S,T,Y,W,N,Q), (K,R,H), and (D,E).

The substitutions which would cause changes in the secondary structure-packing interactions are less common and are called strictly conservative changes which occur

within the following sets of amino acids: (V,L,I), (G,A), (T,N,Q), (C,S) and (D,E) (Rath,1987).

Table 14 and table 15 show the numbers and the percentages of the conservative changes and the strictly conservative changes between *SL* and *BS* PFKs, *SL* and *EC* PFKs.

Table 14: Conservative and strictly conservative interchanges between SL and BS PFKs

Changes	Number	%
Conservative changes	43	45.3
Restrictly conservative changes	21	22.1

Table 15: Conservative and strictly conservative interchanges between SL and EC PFKs

Changes	Number	%
Conservative changes	50	44.2
Restrictly conservative changes	28	24.8

5.2.2 Secondary structure similarities of PFKs from *S. lactis*, *E. coli* and *B. stearothermophilus*

The secondary, tertiary and quarternary structures of PFKs from *B. stearothermophilus* and *E. coli* have been analysed by X-ray crystallography (Evans and Hudson, 1979; Shirakihara and Evans, 1988). Both enzymes have similar secondary structures including 13 α-helices (Labelled 1 to 13 in figure 37) and 11 ß-

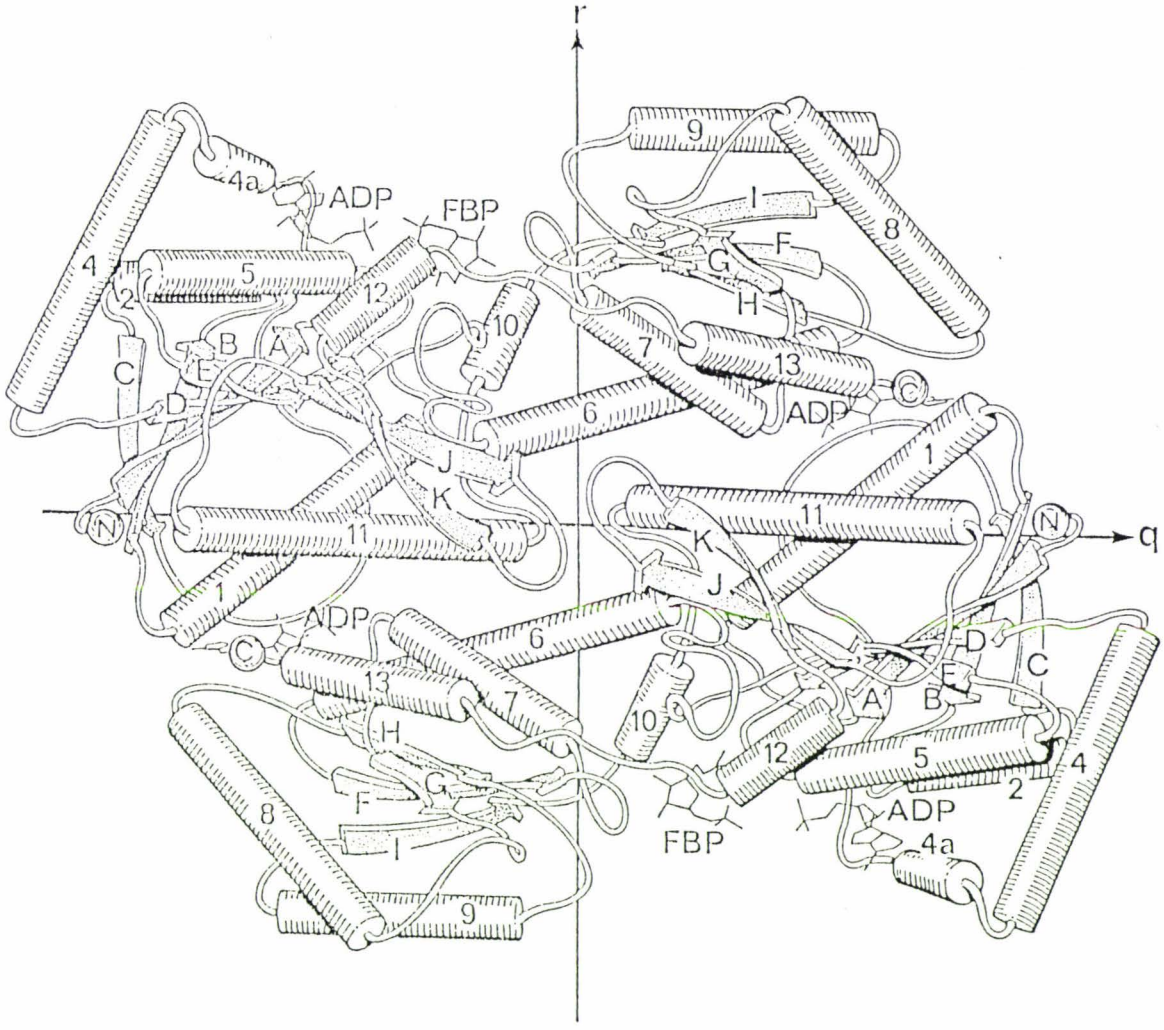


Figure 37. Schematic view of two subunits of EC PFK tetramer
(From Shirakihara & Evans, 1988)

Table 16: Secondary structure elements of bacterial PFKs

Element	Residue numbers	Comments
Helices		
1	15-30	3_{10} end
2	40-47	3_{10} beginning and end
(3)	53-58	Helical angles, but not H-bonds
4a	73-78	All 3_{10}
4	78-93	3_{10} end
5	102-116	
6	138-160	
7	177-185	
8	197-212	3_{10} beginning and end
9	226-239	
10	248-252	All 3_{10}
11	257-278	3_{10} end
12	295-302	
13	308-319	
Sheet strands		Sheet
A	2-9	1
B	33-38	1
C	48-52	1
D	96-101	1
E	118-122	1
F	162-168	2
G	188-190	2
H	216-221	2
I	241-247	2
J	281-287	1
K	290-295	1

* This information is from Shirakihara and Evans, 1988

pleated sheets (Labelled A to K in figure 37). The tertiary and quarternary structures of BS and EC PFKs are also similar. By comparing the sequence of SL PFK with that of BS and EC PFKs and by predicting the secondary structures of SL PFK according to the Chou-Fasman method (Chou and Fasman, 1974), it is apparent that the secondary structures of SL PFK are quite similar to that of BS and EC PFKs. Table 16 lists the residues which form the main secondary structure elements. Also in figure 36, the secondary structure elements are labelled with lines.

In most of the secondary elements, the amino acid residues are similar in all three bacterial PFKs. Although some residues are different in the different PFKs, they have similar abilities in forming α-helices, ß-pleated sheets or turns and thus the secondary structures are probably not disrupted in any of these PFKs.

Large amino acid changes occur at α-8, α-9, α-12 and α-13. In the case of α-8 and α-9, the changes in primary sequence do not prevent the possibility of α-helix formation (Table 17 and Table 18).

Table 17: Comparison of the residues forming α-8 in EC, BS and SL PFKs

SL	K	F	E	N	V	V	N	N	I	N	K	G	Y	E	K	G
	ha	ia	ha	ha	ia	ia	ia	ia	ia	ia	ha	ba	ba	ha	ha	ba
BS	D	M	N	D	V	I	A	R	L	K	R	G	H	E	R	G
	ia	ha	ia	ia	ia	ia	ha	ia	ha	ha	ia	ba	ha	ha	ia	ba
EC	S	R	E	D	L	V	N	E	I	K	A	G	I	A	K	G
	ba	ia	ha	ia	ha	ia	ia	ha	ia	ha	ha	ba	ia	ha	ha	ba

Table 18: Comparison of the residues forming α-9 in EC, BS and SL PFKs

SL	T	G	E	E	F	A	T	K	L	K	E	A	G	Y
	ba	ba	ha	ha	ia	ha	ba	ha	ha	ha	ha	ha	ba	ba
BS	S	G	V	D	F	G	R	Q	I	Q	E	A	T	G
	ba	ba	ia	ia	ia	ba	ia	ha	ha	ha	ha	ha	ba	ba
EC	D	V	D	E	L	A	H	F	I	E	K	E	T	G
	ia	ia	ia	ha	ha	ha	ha	ia	ia	ha	ha	ha	ba	ba

* ha=Helix-favouring amino acid ia=Helix-indifferent amino acid ba=Helix-breaking amino acid

The carboxyl terminal sequences of SL, EC and BS PFKs have no similarities and the carboxyl terminal of SL PFK is longer than that of BS and EC PFKs. It is possible that α-12 of SL PFK is longer than that of BS and EC PFKs. The following shows the α-helix preferences of each amino acid at the carboxyl terminal of SL PFK.

I L G T A E E G A L F S L T T E G G I
ia ha ba ba ha ha ha ba ha ha ia ba ha ba ba ha ba ba ia

K V N N P H K A G L E L Y R L N S A L
ha ia ia ia ba ha ha ha ba ha ha ha ba ia ha ia ba ha ha

N N L N L
ia ia ha ia ha

The lengthening of the carboxyl terminal in SL PFK can be accommodated by increasing the helical portion of α-12 by one turn (Residues GALF single line labelled). The segment SLTT (Double line labelled) would act as a helix-breaking sequence. The remaining extra residues are helix-forming and could extend the helix α-13.

5.2.3 Tertiary and quaternary structure of bacterial PFKs

According to the structural study on BS and EC PFKs, the secondary structural elements of each subunit aggregate to form large and small domains, both of which have a central ß-pleated sheet core sandwhiched between two layers of α-helices. These are shown in figure 37.

The active PFK is a homotetramer with four subunits arranged in a way that one subunit only contacts two of the others. The F6P binding site sits between a pair of subunits while the effector binding site sits between the other pair of subunits (See figure 38, figure 39 ,and figure 40). Thus the ligand bridges between subunits formed when F6P or effector binds to the enzyme may respond to the F6P binding cooperativity and the allosteric regulation property of bacterial PFKs.

Figure 41 highlights α-8, α-9, α-12 and α-13 of PFK where the enzymes of different origins have low similarities in amino acid sequence. These helices are at the outside loops of the enzyme and are far away from the active site and none of them is involved at the interface of subunits. Thus the amino acid changes at these helices should not affect the association of the subunits and the function of the enzyme.

An important point that must be recognised is that the helices α-8 and the carboxyl terminus of the enzyme are close to the effector binding site and the amino

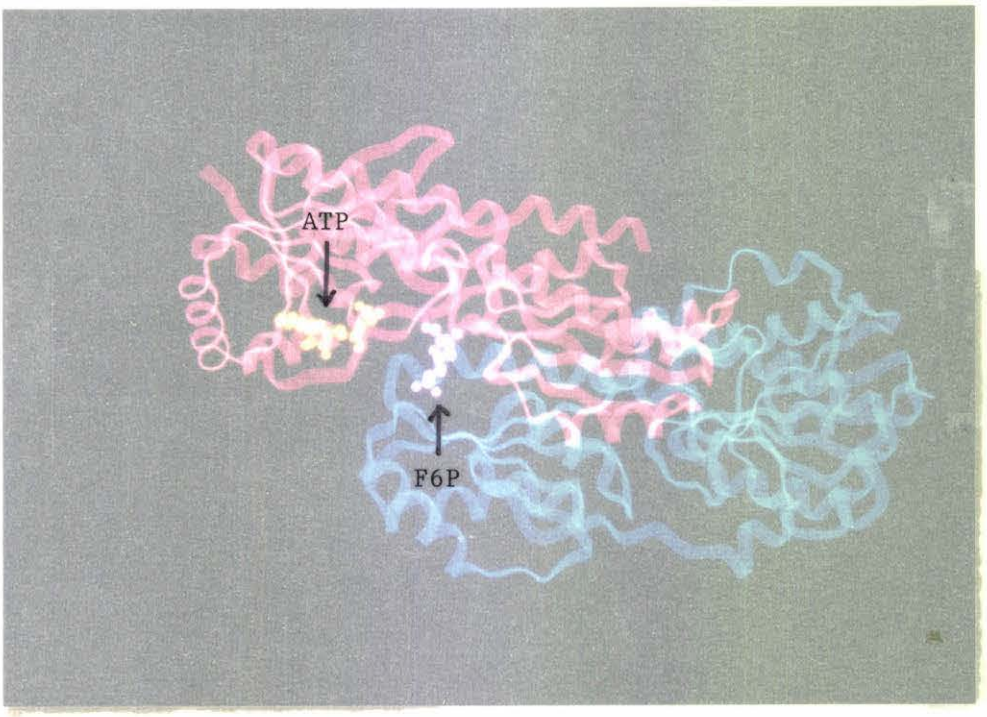


FIGURE 38: Computer-graphic view of two subunits of EC PFK tetramer from the computer program FRODO showing the binding of the substrates

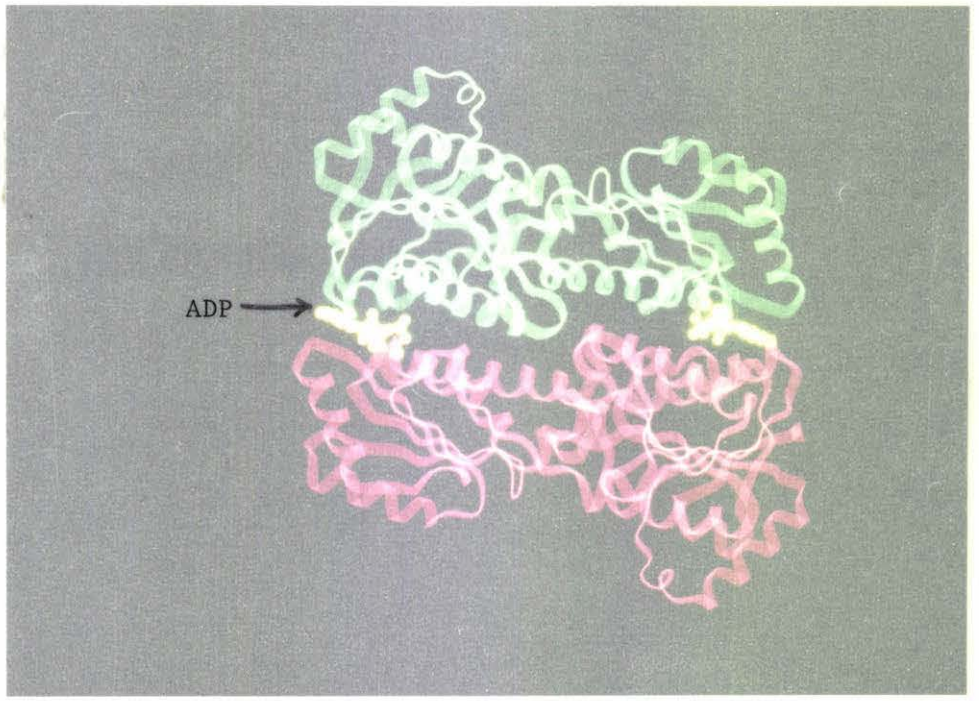


FIGURE 39: Computer-graphic view of two subunits of EC PFK tetramer from the computer program FRODO showing the binding of effector

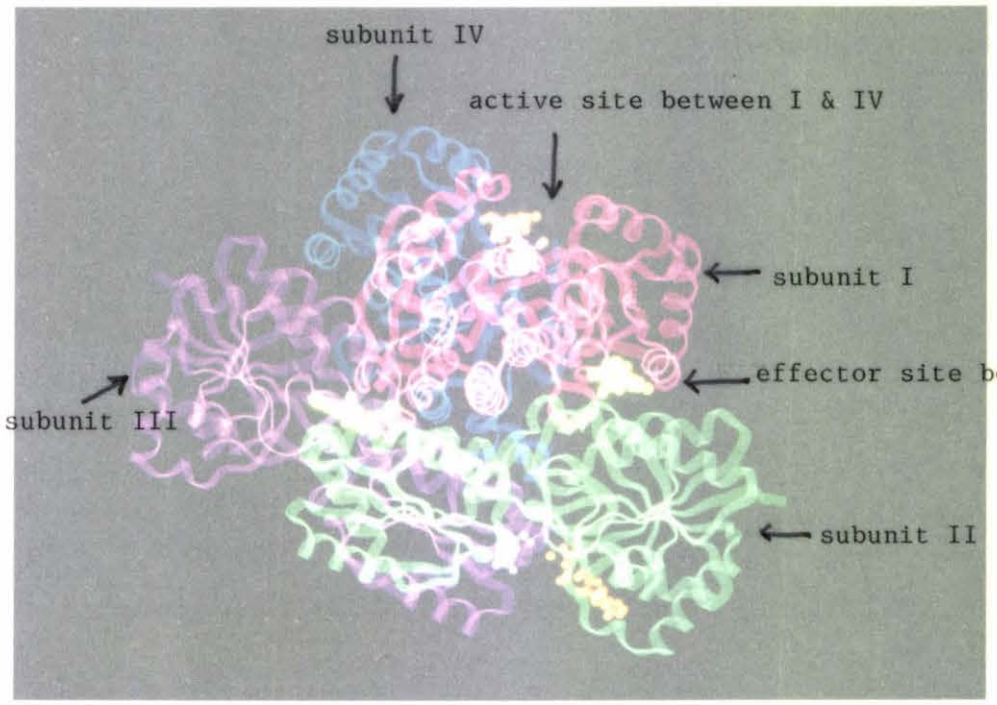


FIGURE 40: Computer-graphic view of EC PFK tetramer from the computer program FRODO

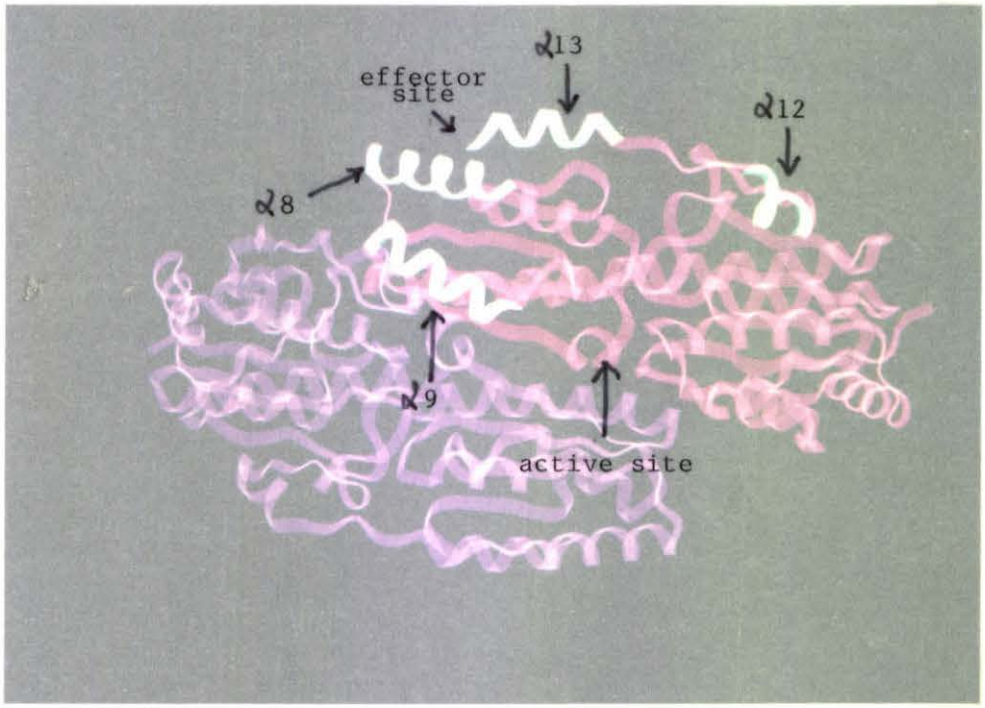


FIGURE 41: Computer-graphic view of two subunits of EC PFK tetramer from the computer program FRODO showing the α-helices which are different in amino acid sequence in PFKs from EC, BS and SL

acid alterations in these places could affect the allosteric control characteristics of the enzyme.

It is known that PEP does not affect the co-operativity of F6P to SL PFK; in contrast, PEP disturbs the cooperativity of EC PFK (Fordyce, 1982) and the cooperativity of BS PFK depends on the binding of PEP (Schirmer and Evans, 1990). These different kinetic properties must result from amino acid changes in the different enzymes.

Schirmer and Evans (1990) in their crystallographic study of the allosteric movement in PFK showed that the structure of PFK changed between the active T-state and inactive R-state. These changes produced significant movements of α-8 and α-9 but these changes in position do not affect the subunit interaction or the positions of the residues involved in ligand binding (Schirmer and Evans, 1990). Thus α-8 and α-9 may only have minor role in changing the allosteric feature of the enzyme and their large diversity in amino acid sequence could be due to evolution which can be tolerated by the enzyme without changing the function.

5.2.4 Comparison of the active site of bacterial PFKs

The active site is where the substrates F6P and ATP bind to the enzyme and where the phosphorylation of F6P takes place to form F1,6BP and ADP. It has F6P/F1,6BP binding site and ATP/ADP binding site.

5.2.4.1 F6P/F1,6BP binding site

The residues involved in F6P/F1,6BP binding in PFKs from EC and BS are shown in table 19 and the relevant residues in SL PFK are listed for comparison. Residues involved in F6P/F1,6BP binding are identical in all these bacterial PFKs.

Site-directed mutagenesis on EC PFK (Hellinga and Evans, 1987) showed that the change of Asp127 to Ser reduced the enzyme activity greatly while the change of Arg171 to Ser only reduced the enzyme activity slightly. Thus Asp127 must take a key role in enhancing the reaction rate, while Arg171 only has a minor effect on enzyme function. It is said that Asp127 probably acts as a base in the reaction mechanism. Because of its important role, Asp127 is conserved not only in bacterial PFKs, but also in the amino terminal half of mammalian PFKs which carries out the catalytical function. The equivalent position of Asp127 in the carboxyl terminal half of mammalian PFKs from rabbit muscle, sheep heart and sheep liver is replaced by a serine residue. This is consistent with the function change of the carboxyl terminal half of mammalian PFK which has no catalytic ability but whose original F6P binding site could become a regulatory site to bind the allosteric activator fructose bisphosphate rather than the substrate F6P.

In BS PFK, the change of the enzyme structure, from R-state to T-state, results in the movement of Arg162, which hydrogen bonds to the negatively charged

Table 19: Site A: F1,6BP/F6P binding

Residue number	*EC	*BS	SL	Comments
125	Thr	Thr	Thr	H bond to 1-phosphate
127	Asp	Asp	Asp	Catalytic residue, H bond to $O_{(3)}$
129	Asp	Asp	Asp	Binds water attached to Mg^{2+}
162#	Arg	Arg	Arg	Binds 6-phosphate
169	Met	Met	Met	Hydrophobic contact to $O_{(2)}$, $O_{(3)}$
170	Gly	Gly	Gly	Main-chain H bond to $O_{(3)}$
171	Arg	Arg	Arg	Near 1-phosphate
222	Glu	Glu	Glu	H bond to $O_{(4)}$
243#	Arg	Arg	Arg	Binds 6-phosphate
249	His	His	His	Binds 6-phosphate
252	Arg	Arg	Arg	H bond to 6-phosphate and $O_{(2)}$

* From Shirkihara and Evans, 1988
# Residues from the subunit different from that contributing the unlabelled amino acids.

phosphate group of F6P, away from F6P and its position is replaced by negatively charged Glu161. This change could contribute to the reduction of the affinity of PFK to F6P. In EC PFK, Glu161 is replaced by Gln161; and in SL PFK, it is replaced by Asn161. Glu and Asn are neutral, and they contribute less to the reduction of the enzyme affinity to F6P than Glu.

5.2.4.2 ATP/ADP binding site

The residues involved in ATP/ADP binding in PFKs from EC and BS are shown in table 20. The relevant residues in SL PFK are listed for comparison. These residues have high similarities among three bacterial PFKs. Residue 73 which forms main-chain amide and carbonyl hydrogen bonds to hydroxyl groups of ribose is Phe in EC PFK, Cys in BS PFK and Tyr in SL PFK. The side chain changes do not affect its function. Residue 77 which is thought to have hydrophobic contact to adenine is Arg in EC PFK, Lys in BS PFK. The corresponding residue in SL PFK is alanine. But this residue may be too small to have an identical function (See the structures below).

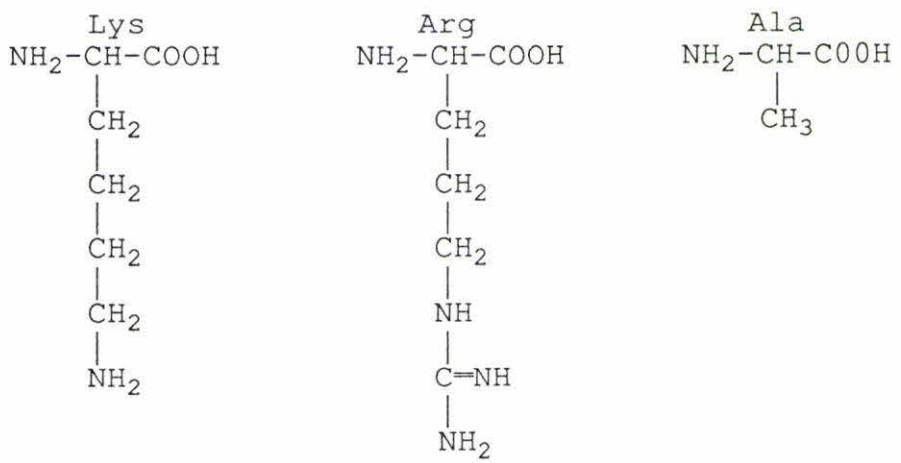


Table 20: Site B: ADP binding

Residue number	*EC	*BS	SL	Comments
11	Gly	Gly	Gly	Main-chain amide H bond to ß-P
41	Tyr	Tyr	Tyr	Contact to ribose
72	Arg	Arg	Arg	Bridges ADP -P and Frul, 6P phosphates P1 and P6
73	Phe	Cys	Tyr	Main-chain amide and carbonyl H bond to ribose OH
77	Arg	Lys	Ala	Hydrophobic contact to adenine
103	Asp	Asp	Asp	Binds to Mg^{2+}
104	Gly	Gly	Gly	Main-chain NH H bond to ß-P, no room for side-chain
105	Ser	Ser	Ser	H bonds to ß-P from OH and NH
107	Met	Gln	His	Hydrophobic contact to adenine
108	Gly	Gly	Gly	Contacts adenine

* From Shirkihara and Evans, 1988

Residue 107 which is thought to have hydrophobic contact to adenine is Met in EC PFK and Gln in BS PFK. The corresponding residue in SL PFK is His which has similar hydrophobicity to Met and Gln and thus may have the same function as them.

5.2.5 Comparison of the effector binding site of bacterial PFK

PFK is a regulatory enzyme which has an effector binding site sitting between a pair of subunits of the PFK tetramer. The residues involved in the binding of effectors in BC and BS PFKs are shown in table 21. The relevant residues in SL PFK are listed for comparison. Most of the residues are conserved in the three bacterial PFKs.

The site-directed mutagenesis showed that the mutation of Glu187 to Ala in EC PFK leads to the change of PEP from an inhibitor to an activator (Lau and Fersht, 1987), which indicates the importance of Glu187 at the effector site; and the mutation of Tyr55 to Gly in EC PFK only results in slight changes in enzyme response to effectors, suggesting a minimal involvement of Tyr55 in binding event (Lau, et al., 1987). Figure 42 is a simplified binding scheme at the effector site of EC PFK in which the important role of Asp187 and the minor role of Tyr55 can been seen clearly.

In SL PFK, residue 187 is Asp instead of Glu. But this change should not affect the important role of this

Table 21: Site C: effector binding

Residue number	#EC	#BS	SL	Comments
21*	Arg	Arg	Arg	H bond to ß-P and to Mg^{2+} -water
25*	Arg	Arg	Arg	H bond to ß-phosphate
54*	Arg	Val	Ser	H bond to -P and C terminus
55*	Tyr	Gly	Ala	Stacks against adenine ring
58*	Ser	Gly	Gly	H bond to -P and Arg25
59*	Asp	Asp	Asp	Main-chain amide H bond to ß-P, side-chain H-bond to ribose $O_{(3)}$
154	Arg	Arg	Arg	Two H bonds to ß-P
185	Gly	Gly	Gly	Main-chain carbonyl bonds to Mg^{2+}
187	Glu	Glu	Asp	Carboxyl binds to Mg^{2+}
211	Lys	Arg	Lys	Alternative to Arg54
213	Lys	Lys	Lys	H bond to C terminus and Glu187
214	Lys	Lys	Asn	Main-chain amide H bond to ribose $O_{(4)}$ (side-chain disordered)
215	His	His	His	Bond to Mg^{2+} via water
C terminus	Try	Ile	Leu	H bond to Arg54 in *E.coli*

From Shirkihara and Evans, 1988
* Residues from the subunit different from that contributing the unlabelled residues.

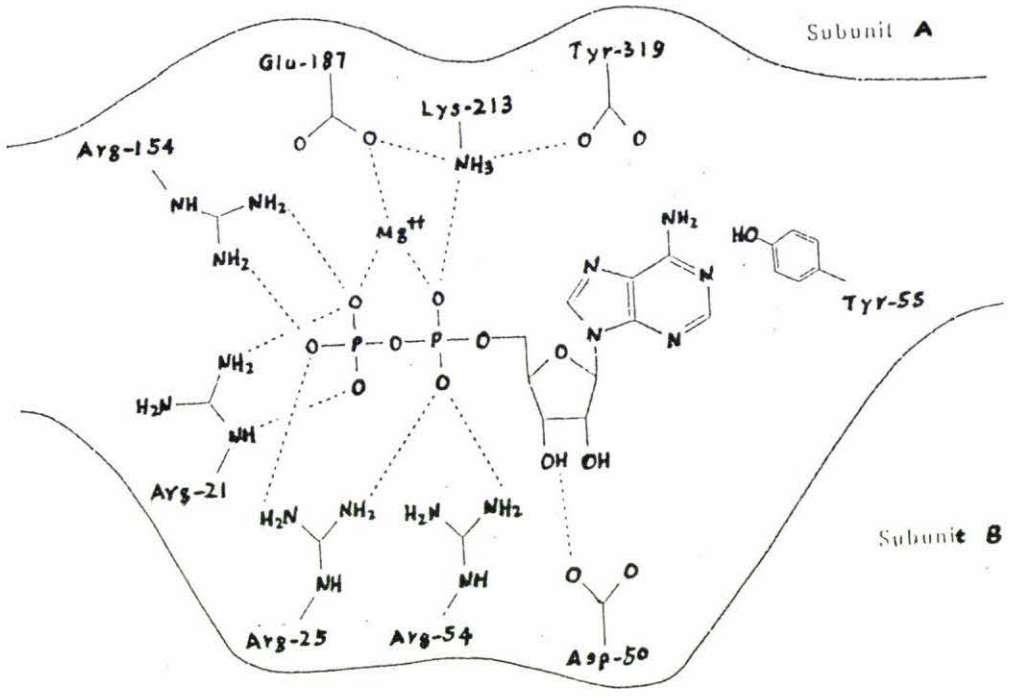
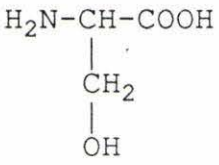


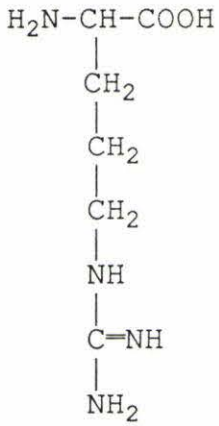
FIGURE 42: SIMPLIFIED SCHEM AT EC PFK EFFECTOR SITE.
ATP binds between two subunits.
(From Lau and Fersht, 1987).

residue in binding of the effectors. Arg54 which hydrogen bonds to α-phosphate group of effectors in EC PFK changes to Val in BS PFK and its function is replaced by Arg211 in BS PFK. In SL PFK, residue 54 is Ser whose side chain is too short to form hydrogen bond with α-phosphate group of effectors as Arg54 in EC PFK does (See the structures below).

Serine



Arginine



Residue Lys211 in SL PFK could have the same function as Arg54 in EC PFK. The side chain of Lys211 in SL PFK could extend out of its original position to approach the α-phosphate group of effectors as shown in figure 43.

5.2.6 Comparison of the subunit interaction in bacterial PFKs

The residues involved in subunit interaction are all conserved in bacterial PFKs from EC, BS and SL (Table 22) This is understandable because the mutation which affects the association of subunits of one enzyme could lead to the loss of the enzyme function.

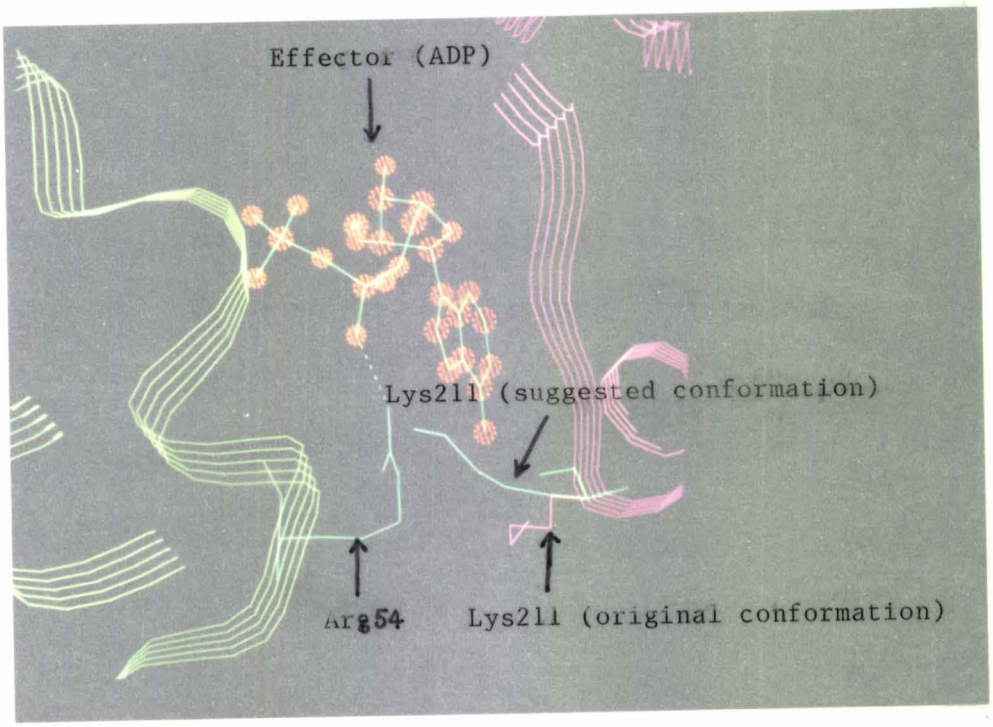


FIGURE 43: Computer-graphic view of the effector sites of EC PFK from the computer program FRODO showing the possibility that Lys211 replaces Arg54 in function in SL PFK.

Table 22: Residues involved in subunit association in bacterial PFKs

	12	63	156	160	252
*EC	Asp	Arg	Thr	His	Arg
*BS	Asp	Arg	Thr	His	Arg
SL	Asp	Arg	Thr	His	Arg

* From Hellinga and Evans, 1985

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