
Doctorate of Philosophy
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**The Properties of the
Recombinant Fructosyltransferase of
*Streptococcus salivarius***

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1999

STATEMENT OF AUTHORSHIP

The research presented in this thesis was undertaken at the Institute of Dental Research (Department of Oral Biology) at the United Dental Hospital of Sydney between July 1994 and June 1999. The experiments are the original work of the author, except where indicated, and have not been submitted, in whole or in part, for any other degree.



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PREFACE

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Published Papers:

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Conference Presentations:

Song, D. D., Jacques, N. A. 1996. Purification of the β -D-fructosyltransferase of *Streptococcus salivarius* expressed in *Escherichia coli*. *Aust. Microbiologist* 17: A110.

Song, D. D., Jacques, N. A. 1998. Analysis of Aspartic acid 312 in the 'Sucrose Box' of the β -D-fructosyltransferase of *Streptococcus salivarius*. *6th Pacific Rim Biotech. Conf. and BioExpo '98*. p267.

Song, D. D., Rathsam, C., Jacques, N. A. 1999. Molecular and kinetic analysis of the β -D-fructosyltransferase of *Streptococcus salivarius*. *Proc. 5th Aust. Conf. Molec. Analysis Bact. Path.* p51

Song, D. D., Rathsam, C., Jacques, N. A. 1999. Molecular and kinetic analysis of the β -D-fructosyltransferase of *Streptococcus salivarius*. *Abstr. XIV Lancefield Int. Symp. Streptococci Streptococcal Dis.* P10.19.

Song, D. D., Jacques, N. A. 1999. Characterisation of the fructosyltransferase of *Streptococcus salivarius*. *Aust. Dent. J.* (in press).

SUMMARY

The fructosyltransferases (Ftfs) of oral streptococci polymerise the fructosyl moiety of sucrose to form fructan, a key storage polysaccharide implicated in the cariogenicity of dental plaque. The *ftf* gene coding for the cell-associated Ftf of *Streptococcus salivarius* ATCC 25975 had previously been cloned and sequenced. However, at the commencement of this study, little was known of its enzymic properties or its mechanism of catalysis.

Following the development of a procedure involving a series of chromatographic steps, the recombinant Ftf of *S. salivarius* ATCC 25975 was purified to electrophoretic homogeneity from an *Escherichia coli* lysate. The purified Ftf was found to be a monomeric protein which was processed at the same N-terminal site as in its natural host, *S. salivarius*.

Kinetic analysis revealed that the Ftf of *S. salivarius* ATCC 25975 catalysed the transfer of the fructosyl moiety of sucrose to a number of acceptors including water, glucose, sucrose and oligofructan. The reaction proceeded by a Ping Pong mechanism involving the formation of a putative covalent fructosyl-enzyme intermediate. The rate constants of the transfructosylation reaction were determined.

Two highly conserved aspartic acid residues in the Ftf of *S. salivarius*, Asp³¹² and Asp³⁹⁷, were altered by site-directed mutagenesis and the resulting effects on the kinetic properties and conformation of the protein determined. Asp³¹² appeared to play an important role in protein structure, possibly by hydrogen bonding to other amino acids in the region and thereby stabilising a β -turn in the protein. Asp³¹² might also be involved in the binding between the enzyme and the fructosyl acceptor or, alternatively, located close to the binding site of the fructosyl acceptor.

The aspartic acid, Asp³⁹⁷ in the Ftf of *S. salivarius*, was found to form part of a triplet motif, RDP, that was conserved in 60 β -fructosyltransferase and β -fructosyl-hydrolases of both bacterial and plant origin. Asp³⁹⁷ appeared to serve as the catalytic nucleophile forming the fructosyl-enzyme intermediate in the Ftf of *S. salivarius*. Comparison of current available data along with recent modelling predictions led to the hypothesis that all β -fructosyltransferase and β -fructosyl-hydrolases may share a common molecular basis for their mode of catalysis.

CONTENTS

STATEMENT OF AUTHORSHIP	(i)
ACKNOWLEDGEMENT	(ii)
PREFACE	(iii)
SUMMARY	(iv)
CONTENTS	(v)
ABBREVIATIONS	(xi)
1 FRUCTOSYLTRANSFERASES	1
1.1 Fructosyltransferases of Oral Streptococci	1
1.1.1 Microbial Colonisation, Dental Caries and Extracellular Polysaccharides	1
1.1.2 Structure of Fructans Produced by Oral Streptococci.	4
1.1.3 Purification and Properties of Streptococcal Fructosyltransferases	6
1.1.4 Genetic Characterisation of Streptococcal Fructosyltransferases	8
1.1.5 Domain Structure of Streptococcal Fructosyltransferases	10
1.1.6 Key Amino Acids of Streptococcal Fructosyltransferases	11
1.1.7 Cell Surface Attachment of the Fructosyltransferase of <i>Streptococcus</i> <i>salivarius</i>	14
1.2 Other Bacterial Fructosyltransferases	15
1.2.1 Purification and Properties of Bacterial Fructosyltransferases	15
1.2.2 Fructosyl-Transfer Reactions Catalysed by Fructosyltransferases	18
1.2.3 Environmental Effects on Fructan Yield	20
1.2.4 Key Amino Acid of Bacterial Fructosyltransferases	21
1.3 Plant Fructosyltransferases	22
1.3.1 Structure of Plant Fructans	22
1.3.2 Fructosyltransferases Involved in Plant Fructan Biosynthesis	23
1.3.2.1 <i>Sucrose:Sucrose 1-Fructosyltransferase (1-SST) and Fructan:</i> <i>Fructan 1-Fructosyltransferase (1-FFT)</i>	23
1.3.2.2 <i>Fructan:Fructan 6G-Fructosyltransferase (6G-FFT)</i>	24
1.3.2.3 <i>Invertase</i>	24
1.3.2.4 <i>Sucrose:Fructan 6-Fructosyltransferase (6-SFT)</i>	25
1.3.3 The Role of Fructans in Plants.	25

1.3.4	Molecular Characterisation of Plant Fructosyltransferases	26
1.3.5	Expression of Bacterial Fructosyltransferases in Plants	26
1.4	Commercial Uses for Fructans	28
1.4.1	Applications in the Food Industry	28
1.4.1.1	<i>Fructans as Healthy Foods</i>	28
1.4.1.2	<i>Fructans as a Source of Fructose</i>	29
1.4.2	Medical Applications for Fructans	29
1.4.2.1	<i>Effects of Fructans on Blood and Hepatic Lipid Content</i>	29
1.4.2.2	<i>Inulin as a Marker of Renal Function</i>	30
1.4.3	Uses of Fructans in the Organic Chemical Industry	30
1.4.4	Other Applications	31
1.5	Aims of the Research	31
2	ROUTINE METHODS	32
2.1	Reagents, Chemicals and Enzymes	32
2.2	Bacterial Strains and Phagemids	33
2.3	Media and Growth Conditions	33
2.4	DNA Manipulations	35
2.4.1	Rapid Isolation and Purification of Phagemid DNA by the Boiling-Lysis Method	35
2.4.2	Small Scale Isolation of High Purity Phagemid	35
2.4.3	Large Scale Isolation and Purification of Phagemid DNA	36
2.4.4	Spectrophotometric Determination of Nucleic Acid Concentration	37
2.4.5	Precipitation of DNA by Ethanol	37
2.4.6	Precipitation of Oligonucleotides by Ethanol	37
2.4.7	Digestion of DNA with Restriction Endonucleases	37
2.4.8	Gel Electrophoresis of DNA on Agarose Gels	38
2.4.9	Preparation of Cells for Electroporation	38
2.4.10	Transformation by Electroporation	39
2.4.11	DNA Sequencing	39
2.5	Protein Manipulations	39
2.5.1	Protein Assay	39
2.5.2	Assay for Fructosyltransferase Activity	40
2.5.3	SDS-PAGE Gel Electrophoresis	40
2.5.4	Non-Denaturing PAGE Electrophoresis	41

2.5.5	Detection of Proteins on SDS-PAGE or Non-Denaturing PAGE Gel with Silver Staining	41
2.5.6	Detection of Fructosyltransferase Activity in SDS-PAGE Gels	42
2.5.7	Electroblotting of Proteins from SDS-PAGE onto PVDF Membranes. . .	42
2.6	Data Processing	42
3	PURIFICATION OF THE RECOMBINANT FRUCTOSYL-TRANSFERASE OF <i>STREPTOCOCCUS SALIVARIUS</i>	43
3.1	Introduction	43
3.1.1	Protein Purification Using Fusion Protein Expression Systems	43
3.1.2	Protein Purification by Chromatographic Means.	44
3.2	Materials and Methods	45
3.2.1	Phagemids, Bacterial Strains and Growth Conditions	45
3.2.2	Columns and Chromatographic Systems	45
3.2.3	Expression of Recombinant Fructosyltransferase in <i>E. coli</i> at Various Stages of Growth	46
3.2.4	Evaluation of Cell Disruption Methods for Maximising Recombinant Fructosyltransferase Recovery from <i>E. coli</i>	46
3.2.5	Ammonium Sulfate Fractionation of <i>E. coli</i> Lysates	47
3.2.6	Hydroxyapatite (HA) Chromatography	48
3.2.6.1	<i>Packing of the Hydroxyapatite Column</i>	48
3.2.6.2	<i>Protein Separation by Hydroxyapatite Chromatography</i>	48
3.2.7	Ion Exchange Chromatography (IEC)	49
3.2.8	Gel Filtration Chromatography	49
3.2.9	Monitoring of the Purification Process	50
3.2.10	M_r Estimation of the Fructosyltransferase	50
3.2.11	Electroblotting and N-terminal Sequencing of Fructosyltransferase	50
3.3	Results	51
3.3.1	Effect of Cell Growth on the Level of Activity of Fructosyltransferase	51
3.3.2	Evaluation of Cell Disruption Methods for Maximising Recombinant Fructosyltransferase Recovery from <i>E. coli</i>	51
3.3.3	Ammonium Sulfate Fractionation of <i>E. coli</i> Lysates	55
3.3.4	Hydroxyapatite Chromatography of <i>E. coli</i> Lysates	56

3.3.5 Resource-Q Ion Exchange Chromatography of Pooled Fructosyl- Transferase Fractions from Hydroxyapatite Chromatography	56
3.3.6 Sephacryl S-300 Gel Filtration of Pooled Fractions Containing Fructosyltransferase Activity from Ion Exchange Chromatography	64
3.3.7 M_r of the Purified Fructosyltransferase	64
3.3.8 N-Terminal Amino Acid Analysis of the Purified Fructosyltransferase . .	72
3.4 Discussion	72
4 KINETIC ANALYSIS OF THE FRUCTOSYLTRANSFERASE OF <i>STREPTOCOCCUS SALIVARIUS</i>	75
4.1 Introduction	75
4.2 Materials and Methods.	77
4.2.1 Preparation of <i>S. salivarius</i> Ftf	77
4.2.2 Measurement of D-Glucose and D-Fructose for Determination of Fructosyltransferase Activity	77
4.2.2.1 <i>Principle of the Methodology</i>	77
4.2.2.2 <i>Procedure</i>	78
4.2.3 Examination of the Enzymic Properties of the Fructosyltransferase of <i>S. salivarius</i> and the Mechanism of the Transfructosylation Reactions . .	79
4.2.3.1 <i>pH Dependence of the Fructosyltransferase Activity</i>	79
4.2.3.2 <i>Effect of Temperature on Fructosyltransferase Activity</i>	79
4.2.3.3 <i>Effect of Metal Ions on Fructosyltransferase Activity</i>	79
4.2.3.4 <i>Analysis of the Reaction Products Produced by the Fructosyltransferase</i>	80
4.2.4 Calculation of Initial Velocity Measurements for the Fructosyltransferase	80
4.2.4.1 <i>Transfructosylation from Sucrose to Sucrose: Sucrose Hydrolysis and Fructan Synthesis</i>	80
4.2.4.2 <i>Transfructosylation from Sucrose to Glucose: the Exchange Reaction</i>	81
4.2.5 Examination of the Binding between Fructosyltransferase and its Fructan Product	81
4.2.6 Analysis of Data	82
4.3 Results	82

4.3.1	Effects of pH, Temperature and Metal Ions on Fructosyltransferase Activity	82
4.3.2	Mechanism of Transfructosylation	85
4.3.3	Derivation of Kinetic Equations for the Fructosyltransferase Using the King and Altman Procedure	89
4.3.3.1	<i>Transfructosylation from Sucrose to Sucrose: Sucrose Hydrolysis and Fructan Synthesis</i>	89
4.3.3.2	<i>Transfructosylation from Sucrose to Glucose: the Exchange Reaction</i>	92
4.3.4	Determination of the Kinetic Parameters of the Transfructosylation Reactions Catalysed by Fructosyltransferase of <i>S. salivarius</i>	93
4.3.4.1	<i>Transfructosylation from Sucrose to Sucrose: Sucrose Hydrolysis and Fructan Synthesis</i>	93
4.3.4.2	<i>Transfructosylation from Sucrose to Glucose: the Exchange Reaction</i>	101
4.3.5	Examination of the Binding between Fructosyltransferase and its Fructan Product	104
4.4	Discussion	104
5	SITE-DIRECTED MUTATIONAL ANALYSIS OF KEY ASPARTIC ACID RESIDUES IN THE FRUCTOSYLTRANSFERASE OF <i>STREPTOCOCCUS SALIVARIUS</i>	110
5.1	Introduction	110
5.2	Materials and Methods	112
5.2.1	Oligonucleotide Synthesis	112
5.2.2	Site-Directed Mutagenesis	112
5.2.2.1	<i>Phosphorylation of Oligonucleotide Primers</i>	112
5.2.2.2	<i>Denaturation</i>	112
5.2.2.3	<i>Annealing</i>	115
5.2.2.4	<i>Synthesis of the Mutated DNA Strand</i>	115
5.2.2.5	<i>Primary Selection</i>	115
5.2.2.6	<i>First Transformation</i>	116
5.2.2.7	<i>Secondary Selection and Transformation</i>	116
5.2.2.8	<i>Final Selection and Transformation</i>	116

5.2.2.9	<i>Isolation and Characterisation of the Mutated Phagemids</i>	116
5.2.3	Expression and Purification of the Mutated Fructosyltransferases.....	117
5.2.4	Effect of pH on Enzyme Activity.....	117
5.2.5	Kinetic Analysis of the Mutated Fructosyltransferases.....	117
5.2.6	Tryptophan Emission Fluorescence Spectroscopy.....	117
5.3	Results	118
5.3.1	Isolation of the Site-Directed Mutants.....	118
5.3.2	Expression and Purification of the Site-Directed Mutated Fructosyltransferases.....	118
5.3.3	Effect of pH on the Activities of the Mutated Fructosyltransferases....	129
5.3.4	Analysis of the Fructan Products of the Mutated Fructosyltransferases Expressed at 37°C.....	131
5.3.5	Effect of Amino Acid Replacement on the Kinetic Properties of the Mutated Fructosyltransferases.....	131
5.3.6	Effect of Culture Temperature on the Activity of the Mutated Fructosyltransferases, D312I and D397S, Expressed in <i>E. coli</i> NM 522	144
5.3.7	Effects of Site-Directed Mutations on the Folding of Fructosyltransferase.....	144
5.4	Discussion	145
6	DISCUSSION	153
	BIBLIOGRAPHY	174

ABBREVIATIONS

%	percentage
%T	percentage concentration of polyacrylamide gel
1-FFT	fructan:fructan 1-fructosyltransferase
1-SST	sucrose:sucrose 1-fructosyltransferase
3-D	3 dimension
6G-FFT	fructan:fructan 6G-fructosyltransferase
6-SFT	sucrose:fructan 6-fructosyltransferase
A	acceptor
Å	angstrom
A ₂₆₀	absorbance at 260 nm
A ₂₈₀	absorbance at 280 nm
A ₃₄₀	absorbance at 340 nm
A ₅₉₅	absorbance at 595 nm
A ₆₀₀	absorbance at 600 nm
ADP	adenosine 5'-diphosphate
ANGIS	Australian National Genomic Information Service
ATP	adenosine 5'-triphosphate
bp	base pair
Bq	becquerel
C-1	carbon at position 1
C-2	carbon at position 2
C-6	carbon at position 6
CBE	conduritol B epoxide
Ci	curie
cm	centimetre
cpm	counts per minute
C-terminus	carboxyl-terminus
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate

ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	a mixture of four types of deoxynucleotide, deoxyadenosine 5'-triphosphate (dATP), deoxyguanosine 5'-triphosphate (dGTP), deoxycytidine 5'-triphosphate (dCTP) and deoxythymidine 5'-triphosphate (dTTP)
DP	degree of polymerisation
dsDNA	double-stranded DNA
DTT	dithiothreitol
E	enzyme
EDTA	ethylenediaminetetraacetic acid
F	fructose
F-6-P	D-fructose-6-phosphate
F _n	n number of fructosyl group (n ≥ 1)
Fru	fructose
Ftf	fructosyltransferase
<i>ftf</i>	gene coding for fructosyltransferase
<i>g</i>	force of gravity
G	glucose
g	gram
G-6-P	D-glucose-6-phosphate
G6P-DH	glucose-6-phosphate dehydrogenase
GFR	glomerular filtration rate
Glc	glucose
Gtf	glucosyltransferase
h	hour
HA	hydroxyapatite
HDL	high density lipoprotein
HK	hexokinase
HMF	hydroxymethylfurfural

IEC	ion exchange chromatography
k	kilo
<i>k</i>	kinetic constant
K_{cat}	catalytic constant
K_m	Michaelis constant
K_r	the slope of the line $\{\text{Log}R_f = f(\%T)\}$
l	litre
LB medium	Luria-Bertani medium
LDL	low density lipoprotein
M	molar
mA	milli-ampere
mg	milli-gram
min	minute
ml	milli-litre
mM	milli-molar
M_r	relative molecular weight
NADH	1, 4-dihyronicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	1, 4-dihyronicotinamide adenine dinucleotide phosphate
nm	nanometre
nM	nanomolar
N-terminus	amino-terminus
°C	degree celsius
ORF	open reading frame
PAGE	polyacrylmide gel electrophoresis
PAS reaction	periodic acid Schiff reaction
PGI	phosphoglucose isomerase
pH	degree of acidity
pI	isoelectric point
PMSF	phenylmethylysulphonyl fluoride
PVDF	Polyvinylidenedifluoride

r^2	regression coefficient
R_f	relative mobility
r_G	initial rate of exchange of the [U- ¹⁴ C]-labelled glucose
RNA	ribonucleic acid
RNase	ribonuclease
s	second
S	sucrose
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
Suc	sucrose
SUPAMA	Sydney University and Prince Alfred Macromolecular Analysis Centre
TEMED	N, N, N', N'-tetramethylethylenediamine
TLC	thin layer chromatography
Tris	trizma base
U	unit of enzyme activity
U- ¹⁴ C-	uniformly carbon 14 isotope
UPRTase	uracil phosphoribosyltransferase
UV	ultraviolet
v	velocity
V	volts
v/v	volume per volume
W	watt
w/v	weight per volume
WHO	World Health Organisation
Y	yield of fructan
μ	micro-
μF	micro-farad (unit for capacitance)

CHAPTER 1

FRUCTOSYLTRANSFERASES

Fructosyltransferases (Ftfs) are a group of enzymes catalysing the transfer of the fructosyl moiety of sucrose to other acceptor molecules. Many fructan-synthesising enzymes, such as the levansucrase of *Bacillus subtilis*, the enzymes produced by oral streptococci and those produced by plants, are Ftfs. According to Lewis *et al.* (1993), fructan is any compound where one or more fructosyl-fructose bonds constitute the majority of the linkages. Fructan can be a large polymer with a high degree of polymerisation (DP) or a small oligomer such as the dimeric inulobiose or levanbiose. Levan is the name given to a fructan that has mostly or exclusively β -(2 \rightarrow 6) fructosyl-fructose linkages, while inulin is the name given to a fructan with mostly or exclusively β -(2 \rightarrow 1) fructosyl-fructose linkages. In both cases glucose may be present but is not necessary (Lewis *et al.*, 1993). In early research fructansucrase was usually used to name the enzyme synthesising fructans of either levan- or inulin-type, while levansucrase and inulinsucrase were used for enzymes producing levan- and inulin-type fructans respectively (Rosell and Birhed, 1974; Scales *et al.*, 1975; Figures and Edwards, 1979; Dedonder, 1966).

1.1 Fructosyltransferases of Oral Streptococci

1.1.1 Microbial Colonisation, Dental Caries and Extracellular Polysaccharides

The oral cavity harbours a complex microbial community. The unique features of the mouth (an open-ended environment with hard mineralised tissue, the teeth, in direct contact with soft epithelial tissues) provide a habitat for the largest and most diverse population of microorganisms anywhere in the body. The temperature is maintained close to 37°C and the pH is buffered close to neutrality by saliva. The source of nitrogen and carbon for the metabolism of microorganisms are continuously supplied by saliva in forms of salivary proteins and glycoproteins. Normally the microbial community in the oral cavity is relatively stable, but under certain circumstances, microbial biofilms may cause major dental diseases such as dental caries and

periodontitis. These diseases are of significant economic and social importance throughout the world. It is not surprising, therefore, that the environmental factors that affect the microbial colonisation of the mouth and the mechanisms leading to the onset of the dental diseases have been the subject of considerable research.

The ability of microorganisms to adhere to surfaces is an important ecological determinant, especially in the mouth where swallowing and flow of saliva rapidly removes non-adherent microorganisms (Gibbons, 1984). The firmly adherent accumulation of bacterial biofilms on the teeth is known as dental plaque and this has been identified as the aetiological agent of dental caries and periodontal disease. Because of the shearing action of saliva, dental plaque is restricted to the more sheltered sites such as pits and fissures found on the occlusal surfaces of the molar teeth or at the gingival margin.

Dental caries is a localised destruction of tooth enamel or cementum and if allowed to develop will progress to involve the underlying dentine. Its onset requires a susceptible tooth surface, bacteria, a fermentable carbohydrate and time (Parfitt, 1956). It has been widely accepted that dental caries can be attributed to acid production by oral microorganisms found in dental plaque (Hogg, 1990; Jacques, 1993). These acids may be trapped at the tooth surface by the overlying dental plaque for sufficient time to affect the solubility of the hydroxyapatite crystals of the tooth enamel or cementum. Although the use of various fluorides in the water supply, mouthwashes and toothpastes have greatly reduced the incidence of dental caries, it remains a serious problem in many countries (Anonymous, 1986 and 1994).

The nutrients required for oral microbial metabolism are continuously supplied by saliva and intermittently from exogenous foodstuffs in the diet. Sucrose is a major dietary carbon source for humans in many western countries (Glinsmann *et al.*, 1986). Studies have shown that sucrose is one of the major dietary agents in the aetiology of dental caries (Rugg-Gunn, 1989).

Based on animal studies and cross-sectional human studies, it is clear that oral streptococci are associated with dental caries (Loesche, 1986). Oral streptococci such as the mutans streptococci and *Streptococcus salivarius* produce a wide variety of sucrose-metabolising enzymes, which appear to be responsible for the critical role of sucrose in cariogenesis. Ftf's and glucosyltransferases (Gtf's) that synthesise fructans and glucans respectively are two of the major sucrose-metabolising

enzymes. These extracellular enzymes, existing either free or bound to the cell surface, can utilise the relatively high free energy of hydrolysis of sucrose to form their respective polysaccharides without the need for the input of energy in the form of ATP or other high energy intermediates (Jacques, 1993).

Fructan was the first polysaccharide identified in dental plaque (Niven, *et al.*, 1941). The large M_r ($>10^6$) and solution viscosity of fructan (Birkhed *et al.*, 1979; Ehrlich *et al.*, 1975) make it hard for the fructan to diffuse from the dental plaque matrix (Manly and Richardson, 1968). The importance of fructans in the aetiology of dental caries is often associated with extracellular glucans (Hardie, 1986; Gehring, 1981; Montville *et al.*, 1978). Gibbons and Nygaard (1968) surveyed a number of cariogenic and non-cariogenic oral streptococci and found that Ftf's were present along with Gtf's in the culture fluid of more than one species of *Streptococcus*. While the formation of water-insoluble glucans has been shown to be a critical factor in dental plaque formation by cross-linking bacteria and by interacting with receptors within the acquired pellicle to provide a mechanism by which the dental plaque biofilms adheres to the tooth surface (Hamada and Slade, 1980; Gibbons, 1983), fructans have been found to enhance the formation of glucan-based bacterial biofilms on tooth surfaces and serve as a reserve of extracellular carbohydrate (Gibbons, 1968).

Even though the amount of fructan represents only a small percentage (~5%) of the total polysaccharides in dental plaque, its rate of synthesis is higher than that of glucan in both plaque and saliva (Wood, 1967; Rölla *et al.*, 1983). The rapid accumulation of fructans in dental plaque following consumption of sucrose has been demonstrated with human volunteers (Gold *et al.*, 1974). The low level of fructan in dental plaque is due to its rapid hydrolysis by bacterial fructanases (Wood, 1967; Manly and Richardson, 1968; Walker *et al.*, 1983; Jacques *et al.*, 1985; Takahashi *et al.*, 1985; Burne *et al.*, 1987, 1996). It has been shown that following the exhaustion of dietary carbohydrate, the expression of fructanases could be up-regulated by fructan (Burne *et al.*, 1995). The fructose released from the hydrolysis of fructan is transported by the phosphoenolpyruvate-dependent phosphotransferase systems (Gauthier *et al.*, 1984) into the bacterial cell and subsequently catabolised to lactic acid or other acids, giving rise to prolonged periods of low pH in dental plaque and the subsequent demineralisation of tooth enamel. (Loesche, 1986; Jacques, 1993).

1.1.2 Structure of the Fructans Produced by Oral Streptococci

Fructans produced from sucrose in the oral cavity were originally ascribed to *S. salivarius* (Niven *et al.*, 1941; Wood, 1967). *S. salivarius* was first described by Andrewes and Horder (1906). It has been shown that *S. salivarius* could be isolated in great numbers from dental plaque on teeth when sucrose consumption increased (Carlsson, 1968) even though this species adhered poorly to tooth surfaces and was often found in the highest numbers on epithelium, especially the dorsum of the tongue (Gibbons and van Houte, 1975). Recent refined taxonomic studies have confirmed that *S. salivarius* is the dominant *Streptococcus* on the dorsum of the tongue (Milnes *et al.*, 1993b) and the numbers of this species persisting in dental plaque is larger than that previously believed (Milnes *et al.*, 1993a). *S. salivarius* is non-haemolytic and can be readily identified on sucrose-containing solid growth medium by its appearance as large mucoid colonies due to the production of extracellular fructan (Niven *et al.*, 1941). *S. salivarius* has been shown to produce fissure and root surface caries in mono-infected rats (Kelstrup and Gibbons, 1970) as well as in human, which is a problem of growing concern with an aging population (Ravald, 1994). *S. salivarius* has been classified into the high-caries category, along with the members of the mutans streptococci (Drucker *et al.*, 1984) and attempts have been made to study the fructans produced by this species in order to uncover the mechanism of its pathogenicity (Wood, 1967; Ebisu *et al.*, 1975; Stivala *et al.*, 1975; Seymour *et al.*, 1979a, b; Marshall and Weigel, 1980a, b; Garszczyński and Edwards, 1973; Jacques and Wittenberger, 1981; Jacques, 1984; Pitty and Jacques, 1987; Pitty *et al.*, 1989; Giffard *et al.*, 1993; Rathsam *et al.*, 1993; Jacques, 1993; Rathsam and Jacques, 1998; Song and Jacques, 1999).

The structure of the fructan produced by *S. salivarius* was characterised as a levan in which the D-fructofuranose units were joined by a β -(2 \rightarrow 6) linkage with β -(2 \rightarrow 1) branch points. The average repeating-unit contains 8-11 β -D-fructofuranose residues (Ebisu *et al.*, 1975; Stivala *et al.*, 1975; Seymour *et al.*, 1979a, b; Marshall and Weigel, 1980a, b). The linkage between the fructosyl unit in levan is shown in Figure 1.1. The branch point β -(2 \rightarrow 1)-bonds of levan are readily broken by acid hydrolysis prior to hydrolysis of the main chains (Lauren *et al.*, 1975).

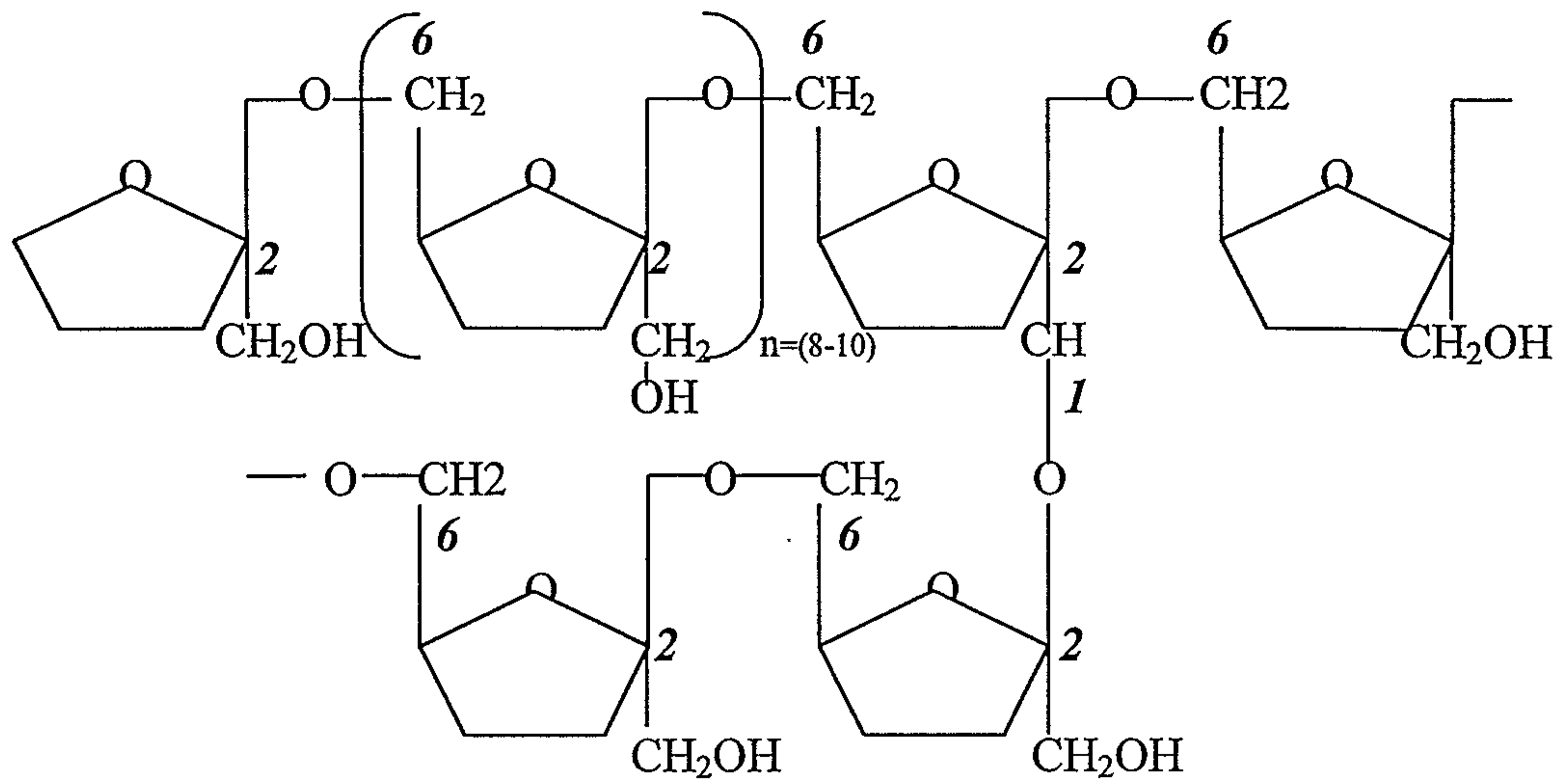


Figure 1.1: The levan structure of the fructan produced by *S. salivarius*.

The levans elaborated by *S. salivarius* have a high M_r of $5-30 \times 10^6$ and possess multiple-branched chains as shown in Figure 1.2. As such the native levans are arborescent, highly branched, near-spherical structures having greatest density at their inner centre and lowest towards their periphery. These levans are water-soluble and behave hydrodynamically as compact particles with spherical symmetry, a property that results in their relatively low intrinsic viscosity (Stivala *et al.*, 1975).

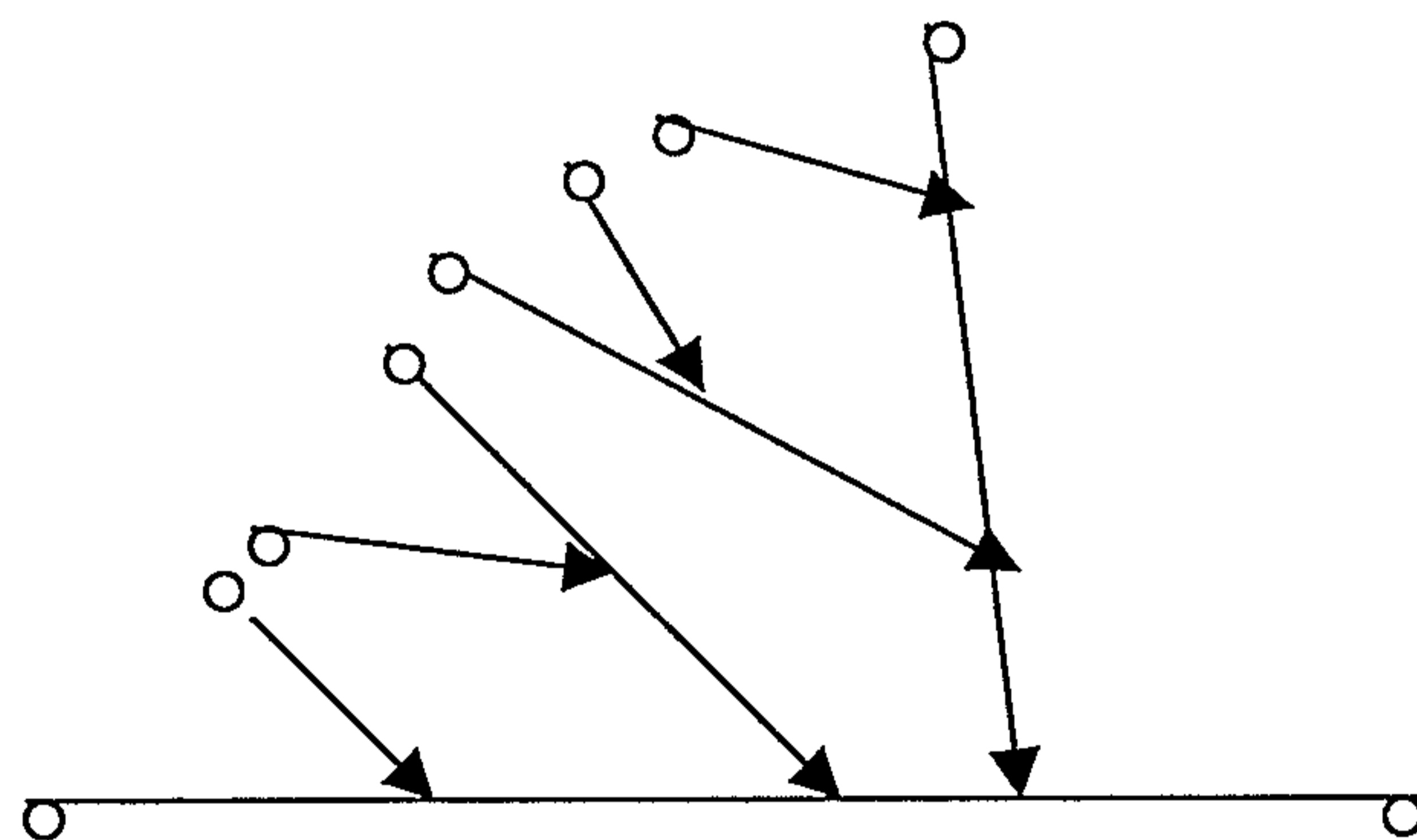


Figure 1.2: The arborescent structure of a multiple-branched chain levan (after Marshall and Weigel, 1980b).

The highly branched and spherical structure of the levan produced by *S. salivarius* allows it to be retained in dental plaque as a poorly-diffusible carbohydrate reserve while being effectively hydrolysed by exo-hydrolases (fructanases) (Da Costa and Gibbons, 1968; Ehrlich *et al.*, 1975; Marshall and Weigel, 1980b). Hydrolysis probably occurs at the more accessible outer branch points (Ehrlich *et al.*, 1975; Marshall and Weigel, 1980 a, b; Stivala and Zweig, 1981; Khorramian and Stivala, 1982).

Fructans are also synthesised from sucrose *in vivo* by the mutans streptococci (Yakushiji *et al.*, 1984). The fructans produced by *Streptococcus mutans* and numbers of other mutans streptococci, *Streptococcus rattus* and *Streptococcus cricetus* have been shown to be of predominantly inulin-type structures consisting of β -(2 \rightarrow 1)-D-fructofuranosidic linkages with β -(2 \rightarrow 6)-branch points (Baird *et al.*, 1973; Ebisu *et al.*, 1975; Birkhed *et al.*, 1979; Sato *et al.*, 1984). The inulin-type fructans elaborated by *S. mutans* have a similar M_r to that synthesised by *S. salivarius*, but are insoluble in cold water (Rosell and Birkhed, 1974; Carlsson, 1970). The insolubility may be attributable to the predominant β -(2 \rightarrow 1) linkages between D-fructose residues (Ebisu *et al.*, 1975). Jacques *et al.* (1985) have found that the rate of hydrolysis of β -(2 \rightarrow 6) linkages by *S. mutans* fructanases is 30 times higher than that of β -(2 \rightarrow 1) fructans. These authors suggested that the apparent inulin-type fructan of *S. mutans* could be due to the preferential hydrolysis of some of the β -(2 \rightarrow 6) linkages by the fructanases elaborated by this bacterium leaving β -(2 \rightarrow 1) linked fructans intact rather than the secretion of an Ftf that was inherently different from that produced by *S. salivarius* (Jacques *et al.*, 1985). However this hypothesis has not been experimentally investigated and seems at odds with recent information obtained from the cloned gene product (see 1.1.3 below).

1.1.3 Purification and Properties of Streptococcal Fructosyltransferases

Hehre (1945) was the first to show that fructan production was due to an extracellular enzyme synthesized by *S. salivarius*. A number of other strains of oral streptococci also have been found to produce extracellular Ftf's and attempts have been made to purify these enzymes.

An extracellular Ftf was found to be produced constitutively by *S. mutans* JC2 isolated from human dental plaque and grown on glucose (Carlsson, 1970). The

enzyme was extracted from the culture supernatant by hydroxyapatite adsorption and purified 200-fold to give a 10% overall yield following hydroxyapatite chromatography and isoelectric focusing. The Ftf exhibited optimal activity around pH 6 and at 40°C, and had a pI of 4.2 (Carlsson, 1970). The enzyme was inhibited by EDTA and activated by Ca²⁺ ions. The enzyme activity was completely abolished in the presence of 0.1 mM Hg²⁺ and was reduced to 40% by 1 mM Pb²⁺, Ag⁺, Ni²⁺ or Cu²⁺ ions (Carlsson, 1970). The high *M_r* fructan produced was described as inulin type with 5% β-(2→6) linked branches (Rosell and Birkhed, 1974).

The constitutive extracellular Ftf produced by *S. mutans* FA1 was co-purified with a Gtf at each step of ammonium sulfate precipitation, gel filtration and DEAE-cellulose chromatography (Scales *et al.*, 1975). The two enzymes were finally separated after addition of the nonionic detergent Tween 80 to break the hydrophobic interaction that bound the complex together (Figures and Edwards, 1979). Two forms of Ftf were identified with pIs of 3.0 and 1.7 respectively. Both forms showed maximum activity at around pH 6.0 and a *K_m* value of 55 mM for sucrose.

The Ftf of *S. mutans* GS-5 was isolated as a recombinant enzyme (Sato and Kuramitsu, 1986). Plasmid pSS22 containing the *ftf* gene of *S. mutans* GS-5 was expressed in *Escherichia coli* and the Ftf that was formed was purified to near homogeneity after ammonium sulfate precipitation, gel filtration, anion-exchange chromatography and preparative SDS-PAGE. The purified Ftf exhibited one protein band of 63,000 after SDS-PAGE and was capable of synthesising inulin-like fructan without the need of an oligofructan primer. Storage of the purified enzyme resulted in its degradation to active lower *M_r* species as small as 59,000.

In contrast to *S. mutans*, the Ftfs of *S. salivarius* are usually cell-associated. The cell-bound Ftf of *S. salivarius* SS2 was released by extracting the cell membrane fragments with 8 M lithium chloride (Garszczynski and Edwards, 1973). The enzyme was purified 23-fold for a 60% overall yield by gel-filtration and DEAE-cellulose chromatography. The final product, free of Gtf activity, exhibited optimal activity at pH 5.6. The Ftf possessed a *M_r* of 34,500 and a pI of 5.2. EDTA inhibited enzyme activity and the inhibition could be reversed by the addition of Mg²⁺. The apparent *K_m* was 63 mM for Mg²⁺ and 17 mM for sucrose.

The Ftf of *S. salivarius* ATCC 25975 is also cell-bound (Jacques and Wittenberger, 1981) and early on it was suggested that it might be associated with

the cytoplasmic membrane as its rate of expression was shown to be related to the rate of membrane lipid synthesis (Pitty and Jacques, 1987). The cell-associated Ftf was shown to be subject to proteolysis following its inhibition by free radicals generated by NADH oxidase (Jacques and Wittenberger, 1981; Abbe *et al.*, 1986). The addition of histidine, cysteine or Ca^{2+} prevented this proteolytic inactivation (Jacques and Wittenberger, 1981). Subsequent studies suggested that Ca^{2+} is a necessary cofactor for the cell-bound Ftf and K_m values for Ca^{2+} and sucrose of $18 \pm 2 \mu\text{M}$ and $12.0 \pm 0.1 \text{ mM}$ respectively were determined for the cell-bound enzyme (Jacques, 1984). Later studies revealed that in the presence of sucrose, the cell-associated Ftf was released from the cell surface and secreted into the culture medium. However, there have been no reports of the isolation and purification of the cell- and fructan-free Ftf of *S. salivarius* ATCC 25975.

1.1.4 Genetic Characterisation of Streptococcal Fructosyltransferases

The *ftf* gene coding for Ftf was isolated from a plasmid library of *S. mutans* chromosomal DNA (Sato and Kuramitsu, 1986) and subsequently sequenced (Shiroza and Kuramitsu, 1988). Sequence analysis revealed two small open reading frames (ORF1 and ORF2) upstream of the *ftf* gene and one downstream open reading frame (ORF3) which was transcribed in the opposite direction to that of the *ftf* gene (Shiroza and Kuramitsu, 1988). The intact *ftf* gene is AT-rich and codes for a 797-amino-acid protein with a predicted M_r of 87,600. The *ftf* gene is preceded by an inverted repeat region similar to that found upstream of the levansucrase gene of *B. subtilis*. In *B. subtilis* the inverted repeat region is considered to be involved in the regulation of levansucrase expression (Steinmetz *et al.*, 1985). Another inverted repeat was also found downstream of the *S. mutans ftf* gene. It was suggested that this stem-loop structure might act as a transcription terminator for the *ftf* gene and also for the downstream ORF3 (Shiroza and Kuramitsu, 1988).

A typical Gram-positive signal sequence containing a basic region followed by a hydrophobic region was found in the N-terminus of the deduced amino acid sequence of the *ftf* gene of *S. mutans*. The deduced amino acid sequence of the N-terminus and the central region of the *ftf* gene showed high homology to the corresponding domains of the levansucrase of *B. subtilis* (Shiroza and Kuramitsu, 1988). While the Ftf of *S. mutans* synthesised fructans with inulin linkages, the

levansucrase of *B. subtilis* produced levans. The molecular basis for this difference has not been elucidated.

The polypeptide encoded by ORF1 of *S. mutans* is of unknown function. However, ORF2 and ORF3 were proposed to code for regulatory proteins involved in Ftf expression (Shiroza and Kuramitsu, 1988). A later study, however, showed that ORF3 encoded a Ftf regulatory protein that bound specifically to the upstream region of the *ftf* promoter containing the inverted repeat structure (Shibata and Kuramitsu, 1996). Binding was not observed when the inverted repeat structure was removed. It was therefore suggested that the ORF 3 gene product might regulate Ftf expression by interacting with the inverted repeat region upstream of the *ftf* promoter (Shibata and Kuramitsu, 1996).

The *ftf* gene of *S. salivarius* was isolated after partial digestion of the chromosomal DNA of *S. salivarius* with *Sau3A* and cloned into the *Bam*HI sites of the λ -bacteriophage, λ L47.1 (Pitty *et al.*, 1989). The recombinant λ -bacteriophage, λ A-39, encoded a Ca^{2+} -dependent Ftf. The *ftf* gene was sub-cloned into phagemid pIBI31 (Giffard *et al.*, 1993; Rathsam *et al.*, 1993) and the DNA sequence determined (Rathsam *et al.*, 1993). Like that of *S. mutans*, the *ftf* gene of *S. salivarius* is also AT-rich but codes for a longer protein of 969 amino acid with a predicted M_r of 103,976. The *ftf* gene is preceded by a putative ribosome binding site and a promoter site and followed by a potential transcription terminator similar to that found in *S. mutans* and bacilli. The deduced amino acid sequence showed similarity to the levansucrase of *B. subtilis* (Steinmetz *et al.*, 1985) and the Ftf of *S. mutans* (Shiroza and Kuramitsu, 1988). Similar to the Ftf of *S. mutans*, only one cysteine residue was found in the entire protein (Rathsam *et al.*, 1993) which is consistent with other observations that extracellular proteins from oral streptococci contain little or no cysteine (Shiroza *et al.*, 1987; Shiroza and Kuramitsu, 1988).

An insertion sequence-like domain designated IS1611, was found upstream of the *ftf* gene of *S. salivarius*. The deduced amino acid sequence of the ORF encoded by IS1611 showed clear similarity with two transposases encoded by the *E. coli* insertion sequence IS30 and the clindamycin-resistant transposon Tn4551 (Giffard *et al.*, 1993). Both IS30 and Tn4551 have been shown to increase the expression of downstream genes (Smith, 1987; Neuwald and Stauffer, 1990). However, whether IS1611 regulates the *ftf* gene of *S. salivarius* in the same way has

not been investigated.

Two other ORFs were found downstream of the *ftf* gene of *S. salivarius*. The deduced amino acid sequences of these two ORFs were homologous to the uracil phosphoribosyltransferase (UPRTase) of *Saccharomyces cerevisiae* and the Clp family of ATP-dependent proteases (Giffard *et al.*, 1993). Whether the *FURI* homologue in *S. salivarius* encodes a UPRTase is not known since no other *FURI* homologue has been reported in prokaryotes. In contrast, *Clp* homologues are often found close to genes encoding surface or extracellular protein in a range of bacteria (Gottesman *et al.*, 1990). It has been suggested that the product of the *Clp* homologue might be related to the release of the *S. salivarius* cell-bound Ftf from the cell surface (Giffard *et al.*, 1993).

1.1.5 Domain Structure of Streptococcal Fructosyltransferases

Based on the biochemical and molecular analyses, seven domains have been defined in the Ftf of *S. salivarius* (Rathsam and Jacques, 1998) as shown in Figure 1.3. The Signal Domain contains a signal sequence of 31 amino acids, of which the comparable regions are also found in the Ftf of *S. mutans* and the levansucrases of *B. subtilis* and *Bacillus amyloliquefaciens*. The Propeptide Domain contains 78 amino acids, which may or may not be removed during the secretion of the enzyme (Rathsam, Song and Jacques, unpublished observations). The Catalytic Domain contains 433 amino acids and is highly homologous to the similar regions of the Ftf of *S. mutans* and the levansucrases of bacilli. Two domains of unknown functions designated as Spacer Domains have been identified, one on the N-terminus of the Catalytic Domain and other on the C-terminus. The Hydrophilic Wall-associated Domain is proline-glycine-threonine-serine-rich and is not found in the secreted extracellular Ftfs. This domain has been shown to stabilise the surface attachment of the enzyme to *S. salivarius* cells (Rathsam and Jacques, 1998). The C-terminal Hydrophobic Domain consists of 34 amino acids with a positively charged tail. Although it is not found in the levansucrases of bacilli, the hydrophobic domain is conserved in the C-termini of other Gram-positive surface-bound proteins and has also been shown to be necessary for the attachment of the Ftf of *S. salivarius* to the cell surface (Rathsam and Jacques, 1998).

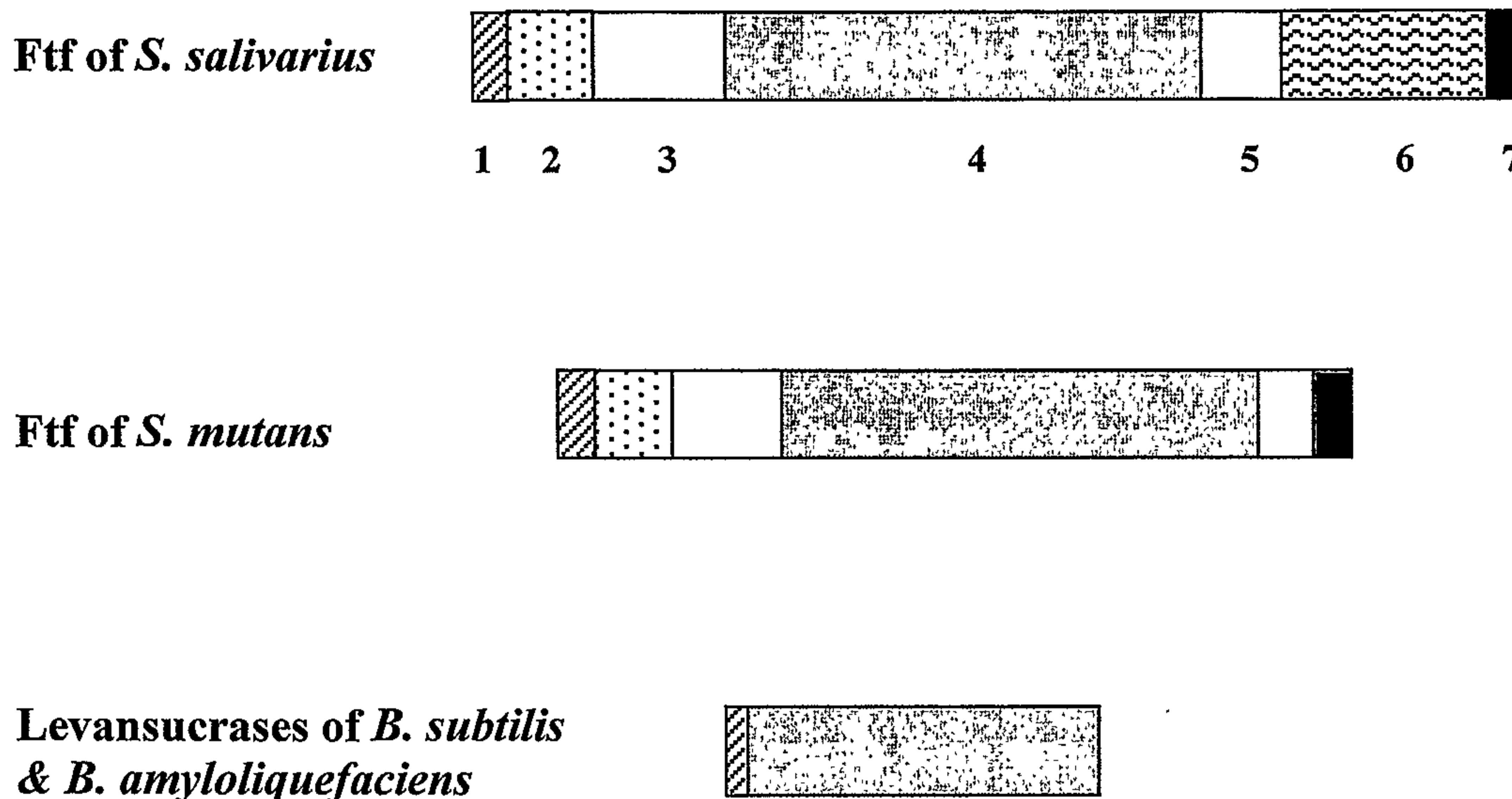


Figure 1.3: Domain structure of four known Ftfs.

The domain structure for the Ftf of *S. salivarius* ATCC 25975 is shown as: **1**, Signal Domain (M³¹ – S⁷⁸); **2**, Propeptide Domain (D¹ – S⁷⁸); **3**, Spacer Domain (S⁷⁹ – K¹⁹⁷); **4**, Catalytic Domain (Q¹⁹⁸ – D⁶³¹); **5**, Spacer Domain (W⁶³² – K⁷⁰⁴); **6**, Hydrophilic Wall-associated Domain (P⁷⁰⁵ – N⁸⁸²); **7**, Hydrophobic Domain (S⁸⁸³ – K⁹¹⁷). The shading shows the position of equivalent domains in the other three enzymes.

1.1.6 Key Amino Acids of Streptococcal Fructosyltransferases

Sequence alignment of the Ftf of *S. salivarius* with that of *S. mutans* and the levansucrases of *B. subtilis* and *B. amyloliquefaciens* revealed several regions of high identity (Figure 1.4) within the catalytic domain (Rathsam *et al.*, 1993). The key amino acid residues identified in the levansucrase of *B. subtilis*, Arg³³¹ and Gly³⁶⁶ (*B. subtilis* numbering, Chambert and Petit-Glatron, 1991; Petit-Glatron *et al.*, 1990), are also conserved in the Ftf of *S. salivarius* at position 513 and 555 respectively (Figure 1.4). Arg³³¹ is believed to act as a proton donor in catalysis and Gly³⁶⁶ is believed to be important in folding of the levansucrase of *B. subtilis* (Chambert and Petit-Glatron, 1991; Petit-Glatron *et al.*, 1990). Whether the corresponding amino acids in the Ftf of *S. salivarius*, Arg⁵¹³ and Gly⁵⁵⁵, also play similar roles has not been elucidated.

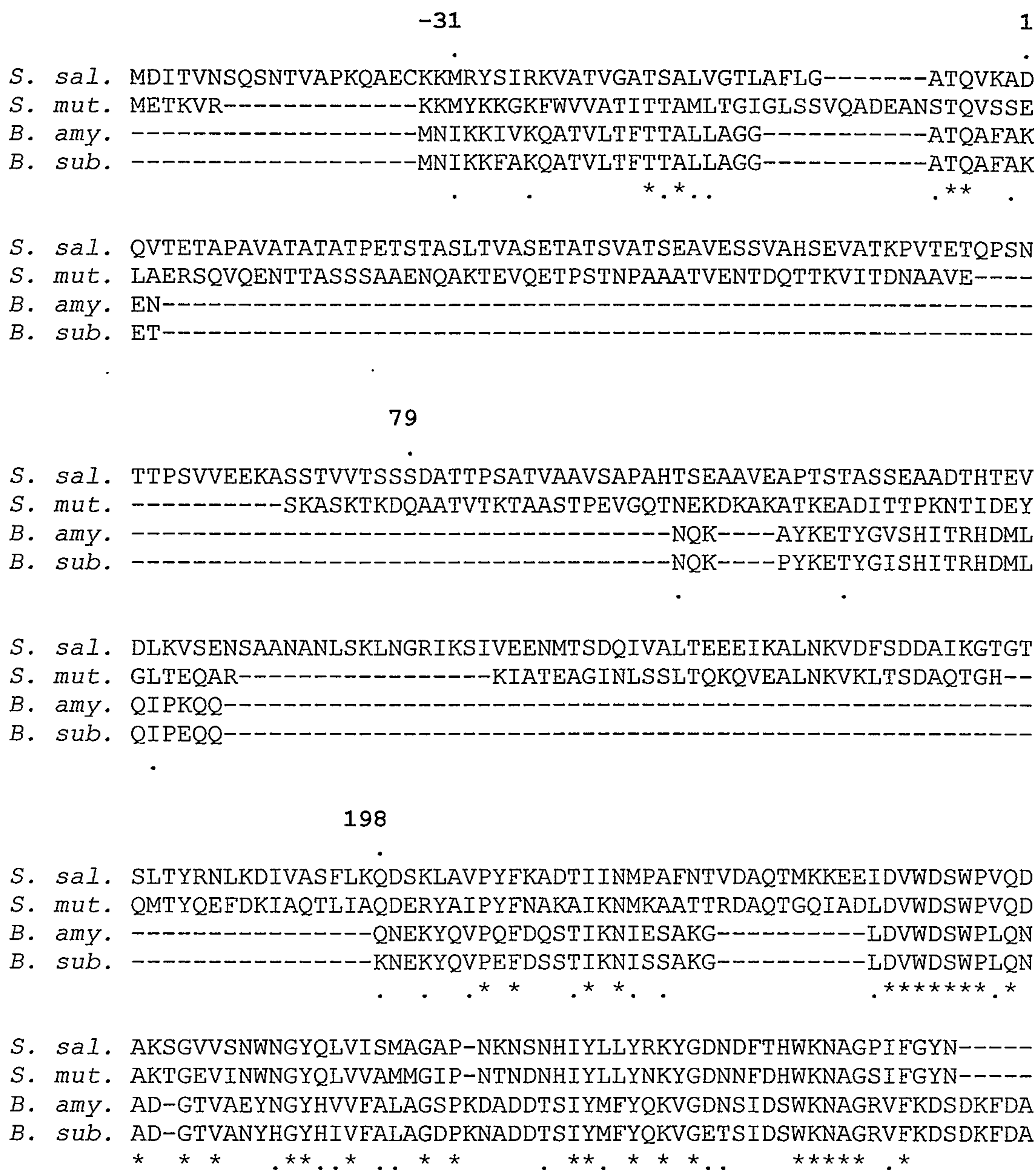


Figure 1.4: Multiple sequence alignment of Ftf s from *S. salivarius* (*S. sal.*), *S. mutans* (*S. mut.*), *B. subtilis* (*B. sub.*) and *B. amylo liquefaciens* (*B. amy.*).

The numbers on the top of the sequence indicate the N-terminal residues for each of the defined domains of *S. salivarius* (1.1.5). The symbols (*) and (.) indicate identical and highly conserved amino acid respectively. The amino acids, Arg and Gly, which have been identified in the levansucrase of *B. subtilis* and the ‘sucrose box’ structure are highlighted.

S. sal. ----ALEDDQQWSGSATVNSDGSIQLYYTKNDTSGGKLNWQQLASATLNLAVENDEVVIK
S. mut. ----ETPLTQEWSGSATVNEDGSLQLFYTKVDTSDKNSNNQRLATATVNLGFDDQDVRIK
B. amy. NDPILKDQTQEWSGSATFTSDGKIRLFYT--DYSGKHYGKQSLTTAQVNVSKSDDTLKIN
B. sub. NDSILKDQTQEWSGSATFTSDGKIRLFYT--DESGKHYGKQTLTTAQVNVASDSSLNIN
 * .***** . ** ..*.* * * . * * . * . * . *

S. sal. SVENDHILFGG--DNYHYQSYPKFMSTFDDDHNDGNPDRTDNYCLRDPHI IEDN-GSRY
S. mut. SVENDKVLTPEGVMAYHYQSYQQWR-----STFTGADNIAMRDPHVIEDENGDRY
B. amy. GVEDHKTI FDG--DGKTYQNVQQFID-----EGNYTSGDNHTLRDPHYVEDK-GHKY
B. sub. GVEDYKSI FDG--DGKTYQNVQQFID-----EGNYSSGDNHTLRDPHYVEDK-GHKY
 ** . . ** . ** .***** .** * .*

S. sal. LIFESNTGDEN-YQGEKQIYKWSNYGGDDAFNLKSFLNIVNNKHLYNLASWANGSIGILK
S. mut. LVFEASTGTEN-YQGEDQIYNFTNYGGSSAYNVKSLFRFLDDQDMYNRASWANAAIGILK
B. amy. LVFEANTGTENGYQGEESLFNKAYYGGGTNFFRKESQKLOQS-AKKRDAELANGALGIE
B. sub. LVFEANTGTEDGYQGEESLFNKAYYKSTSFRRQESQKLLQS-DKKRTAELANGALGMIE
 * .** . ** * **** .. . ** . . * ** ..*..

S. sal. LDDNEKNPSVAELYTPLVTSHMVTDEVERPSVVKMGGKYLLFTASRINKSTDAEGTVAAR
S. mut. LKGDKKTPEVDQFYTPLLSSTMVSELERPNVVKLGDKYLLFTASRLNHGSNNDANKAN
B. amy. LNNDY---TLKKVMKPLITSNTVTDEIERANVFKMNGKWYLLFTDSR---GSK---MTID
B. sub. LNDDY---TLKKVMKPLIASNTVTDEIERANVFKMNGKWYLLFTDSR---GSK---MTID
 * . ** . * * .** .** * * . * * . * **** ** .

S. sal. EAVGDDVVMLGFVSDSLRGKYRPLNGSGVVLTA SVPADWRTSTYSYYAVPVEGSSDTLLV
S. mut. EVVGDNVVMLGYVSDQLTNGYKPLNNSGVVLTA SVPADWRTATYSYYAVPVAGSSDTLLM
B. amy. GINSNDIYMLGYVSNLSLTGPYKPLNKTGLVLQMG LDPNDVTFTYSHFAVPQAKG-NNVVI
B. sub. GITSNDIYMLGYVSNLSLTGPYKPLNKTGLVLKMDLDPNDVTFTYSHFAVPQAKG-NNVVI
 . *** .** * . * .*** .* .** . * *** .***

632

S. sal. TSYMTNRGGIAGAENKSTWAPSFLIKMNADDTTEVLPKMTNQGDWIWDKSSSESLVHVGDO
S. mut. TAYMTNRNEVAGKGNSTWAPSFLIQVLPDGTTKVLAEMTQQGDWIWDEPSRTTDTVGTL
B. amy. TSYMTNRGFFEDK--KATFGPSFLMNIKGNKTSVVKNSILEQG-----
B. sub. TSYMTNRGFYADK--QSTFAPSFLNIKGNKTSVVKDSILEQG-----
 * .***** . * **** . * * . * *

705

S. sal. NSAKLPNE-DENVDYAVSGYGLKPHTYPTVDGSTGVSEAHGVLTVTVKDGKDKKADKPE
S. mut. DTAYLPGENDGYIDWNVIGGYGLKPHTPGQYQPTVPSTPIHTDDIISFEVSFDGHL----
B. amy. -----
B. sub. -----

S. sal. TPVSPTEGNHSVDDKTNKPGTSKPADNNQPSADKEDKPTNPTNPDSPARTFPFYGDHSN
S. mut. -----VIKPVKVNND SAGRIDQSRNSGG-----
B. amy. -----
B. sub. -----

S. sal. DNSSNDHHVAVPVK PSTGDSVGD RRPVAQAAEIATPVPKTIVATGPTVPTNTVKEESVT
S. mut. -----SLNVAFNVSAGGNISVKPSQKSINNTKET---
B. amy. -----QLTVN-----
B. sub. -----QLTVNK-----
 *

Figure 1.4 (continued)

```

S. sal. ETEAPKPKVSEEKVVQSHGVVDKANEVTKSDESSKGNNTKVAAKLATTPKTPSDSEGSNSNI
S. mut. -----KKAHHVSTKQKGN-----SFF
B. amy. -----
B. sub. -----

S. sal. LSILATIFAAIASLALLGYGLVTGKIHLPPK
S. mut. AALLALFSAFCVSIKFK-----
B. amy. -----
B. sub. -----

```

Figure 1.4 (continued)

Another highly conserved region within these enzymes is the ‘sucrose box’ structure, SGSA---D---LYYT, surrounding an aspartic acid residue at position 312 of the Ftf of *S. salivarius* (Rathsam *et al.*, 1993). The ‘sucrose box’ has also been found in other enzymes catalysing the transfer of fructose from sucrose such as sucrose-6-phosphate hydrolase (Sato and Kuramitsu, 1988) which otherwise show little homology to the Ftfs (Rathsam *et al.*, 1993). However, the actual role of the ‘sucrose box’ structure in catalysis has not been determined for any Ftf or sucrose hydrolysing enzyme.

1.1.7 Cell Surface Attachment of the Fructosyltransferase of *S. salivarius*

Unlike the Ftf of *S. mutans* and the levansucrases of *B. subtilis* and *B. amyloliquefaciens*, which are secreted extracellular enzymes, the Ftf of *S. salivarius* is cell-bound. Comparison of the peptide sequence of these enzymes revealed that the C-terminal hydrophilic proline-glycine-threonine-serine-rich domain (Figure 1.3) is unique to *S. salivarius* (Rathsam *et al.*, 1993). The Hydrophobic Domain is not found in the levansucrases of bacilli but is present in many other Gram-positive surface-bound polypeptides such as the M protein of *S. pyogenes* (Hollingshead *et al.*, 1986). However the cell wall sorting signal of these proteins, a consensus pentapeptide motif ‘LPXTG’ at the N-terminus of the Hydrophobic Domain, is not found in the Ftf of *S. salivarius* (Rathsam and Jacques, 1998). Mutation analysis of the C-terminal domains of the Ftf of *S. salivarius* has indicated that both the Hydrophilic Wall-associated Domain and the Hydrophobic C-terminal Domain are required for stable

attachment of the enzyme to the cell surface. The Hydrophobic Domain anchors the protein to the plasma membrane. The Hydrophilic Wall-associated Domain containing turn-promoting proline-glycine residues may allow the C-terminal wall-associated region to span the cell wall by randomly intercalating throughout the peptidoglycan-carbohydrate-teichoic acid matrix. The high serine-threonine content could further stabilise such an association by forming hydrogen bonds to the constituents of the cell wall (Rathsam and Jacques, 1998).

1.2 Other Bacterial Fructosyltransferases

Apart from oral streptococci, fructans synthesis and the production of Ftfs have been reported in a number of other bacteria. Cell-free enzymic synthesis of levan was first reported in 1942 by Aschner *et al.*, who described the occurrence of an extracellular, inducible levansucrase from *B. subtilis* and a constitutive levansucrase-containing extract from a strain of *Aerobacter*. Since then, Ftfs have been identified in a wide variety of microorganisms including the Gram-negative bacteria, *A. diazotrophicus* (Hernandez *et al.*, 1995), *Acetobacter levanicum* (Hestrin *et al.*, 1943; Hestrin and Avineri-shapiro, 1944), *Erwinia amylovora* (Gross *et al.*, 1990), *Erwinia herbicola* (Cote and Imam, 1989), *Gluconobacter oxydan* (Elisashivili, 1980), *Pseudomonas syringae* (Hettwer *et al.*, 1995), *Rhanella aquatilis* (Ohtsuka *et al.*, 1992), *Zymomona mobilis* (Lyness and Doelle, 1983) and the Gram-positive bacteria, *B. subtilis* (Hestrin *et al.*, 1943; Dedonder, 1966; Gonzy-Treboul *et al.*, 1975), *B. amyloliquefaciens* (Mantsala and Puntala, 1982), *Bacillus natto* (Takahama *et al.*, 1991), *Bacillus polymyxa* (Hestrin *et al.*, 1943; Han, 1989) *Bacillus stearothermophilus* (Li *et al.*, 1997), *S. salivarius* (Hehre, 1945; Jacques, 1984, 1993), *S. mutans* (Sato *et al.*, 1984), *S. rattus* (Baird *et al.*, 1973; Ebisu *et al.*, 1975), *S. cricetus* (Birkhed *et al.*, 1979) and *Actinomyces viscosus* (van der Hoeven *et al.*, 1976; Pabst, 1977; Pabst *et al.*, 1979). The Ftfs of Gram-negative bacteria are usually constitutively expressed and secreted. In contrast, the expression and secretion of the Gram-positive Ftfs is usually substrate-induced.

1.2.1 Purification and Properties of Bacterial Fructosyltransferases

Levansucrases of bacilli are the most extensively studied Ftfs. An extracellular sucrose-induced levansucrase has been purified from *B. subtilis* BS5 by Dedonder's

laboratory (Dedonder, 1966). The enzyme shows optimal activity at pH 6.0 and a K_m for sucrose of 27 mM. Addition of the metal ions, Fe^{3+} , Al^{3+} , or Zn^{2+} increased the heat stability of the enzyme (Dedonder, 1966). The average M_r determined by three different methods, sedimentation equilibrium, SDS-gel electrophoresis and gel filtration, was 54,000 (Gonzyl-Treboul *et al.*, 1975).

Several mutants of *B. subtilis* have been isolated, in which the extracellular levansucrase is expressed constitutively (Lepesant and Dedonder, 1969). This made it possible to prepare a large quantity of purified enzyme with relative ease. An X-ray crystallographic analysis of the levansucrase purified from the constitutive mutant *B. subtilis* BS5C4 has been determined and a model of the enzyme constructed to 3.8 Å resolution (LeBrun and van Rapenbusch, 1980). The molecule was shown to be an elongated prolate ellipsoid, with the dimensions 26 x 32 x 117 Å and a narrow region near the centre, which may allow some degree of flexibility around the active site region.

Levansucrases of *A. levanicum* (*E. herbicola*) have been isolated from three different sources; a cell-free extract of an autolysed culture (Hestrin *et al.*, 1943), homogenates of cells (Ebert and Schenk, 1968) and the media containing the secreted enzyme (Cote and Imam, 1989). The resulting enzymes differed largely in M_r and the K_m value for sucrose, probably due to the different techniques used to obtain them (Cote and Ahlgren, 1993).

A. viscosus is commonly associated with dental plaque and produces a constitutive and extracellular levansucrase (Snyder *et al.*, 1967; van der Hoeven *et al.*, 1976; Pabst, 1977, Pabst *et al.*, 1979). A levansucrase has been purified from the culture supernatant of *A. viscosus* Ny1 by DEAE-anion exchange chromatograph and isoelectric focusing with a 165-fold increase in specific activity (van der Hoeven *et al.*, 1976). The purified enzyme exhibited maximal activity at pH 6.8 and 45°C. Metal ions, Ca^{2+} and Mg^{2+} were found to activate the enzyme, whereas Hg^{2+} and Cu^{2+} inhibited it.

Pabst (1977) also purified a levansucrase from the culture supernatant of *A. viscosus* T-14V following ammonium sulfate precipitation, DEAE-cellulose chromatography, gel filtration and two hydroxyapatite chromatographic steps with a 37,000-fold increase in specific activity. The levansucrase possessed a native M_r of 220,000 according to gel filtration chromatography and consisted of subunits of M_r

80,000 as determined by SDS-PAGE (Pabst, 1977). In contrast to the levansucrase purified by van der Hoeven *et al.* (1976), maximal activity was observed at pH 6.0 and 37°C and no activation was observed with the addition of Ca²⁺ or Mg²⁺. The K_m for sucrose was estimated to be 12 mM (Pabst, 1977). The fructan product was a branched levan of a high M_r of 10⁸ (Pabst, 1977). A later study discovered that *A. viscosus* produced both a soluble extracellular and a cell wall-associated levansucrase. The solubilised cell wall-associated form released from the cell surface by lysozyme digestion appeared to be identical to the native soluble extracellular form (Pabst *et al.*, 1979). It has also been shown that the levansucrase of *A. viscosus* remains tightly bound to its levan product (Pabst, 1977) but not to exogenously added levan (Pabst *et al.*, 1979). It has not been determined whether the levansucrase purified by van der Hoeven *et al.* (1976) and that by Pabst (1977) are inherently different enzymes or that the differences in their enzymic properties are due to experimental error as the gene(s) encoding this enzyme(s) have not been cloned and the recombinant enzyme(s) analysed.

The plant pathogenic bacterium, *P. syringae*, produces a levan as a protective spacer between the plant cell wall and the bacteria in order to inhibit the recognition and initiation of a hypersensitive response by the host plant (Hettwer *et al.*, 1995). The extracellular levansucrase of *P. syringae* pv. Phaseolicola has been purified from the cell supernatant by chromatography on TMAE-Fraktogel and butyl-Fraktogel. The purified enzyme shows a M_r of 45,000 on SDS-PAGE and M_r of 68,000 by gel filtration. Optimal activity is observed at pH 5.8 to 6.0 and 60°C. The enzyme shows high tolerance to denaturing agents, proteases, and repeated freezing and thawing (Hettwer *et al.*, 1995).

A. diazotrophicus, a nitrogen-fixing bacterium associated with sugar cane, secretes a levansucrase which is expressed constitutively and constitutes more than 70% of the total protein secreted by strain SRT4 (Hernandez *et al.*, 1995). The purified enzyme has a M_r of 58,000 and pI of 5.5. The enzyme shows optimal activity at pH 5.0 and is inhibited by Hg²⁺. Addition of Ca²⁺ or the metal chelator EDTA does not affect enzyme activity (Hernandez *et al.*, 1995).

An extracellular levansucrase is also secreted by *Z. mobilis*, a fermentative bacterium that efficiently produces ethanol from glucose. Since the level of levansucrase expression by this organism is very low even when induced by sucrose,

the purification of this enzyme has not been reported. However, the gene that codes for this enzyme has been cloned and sequenced (Song *et al.*, 1993; Kyono *et al.*, 1995).

1.2.2 Fructosyl-Transfer Reactions Catalysed by Fructosyltransferases

Unlike the streptococcal Ftf's, detailed kinetic studies have been performed with the levansucrase of *B. subtilis* and the mechanism of catalysis elucidated. The transfructosylation reaction catalysed by this levansucrase is a multiple-reaction in which sucrose or raffinose acts as the fructosyl donor and substances possessing alcoholic (hydroxyl) groups such as water, sucrose, glucose or oligolevan can serve as the fructosyl acceptor according to the following scheme (Dedonder, 1966):



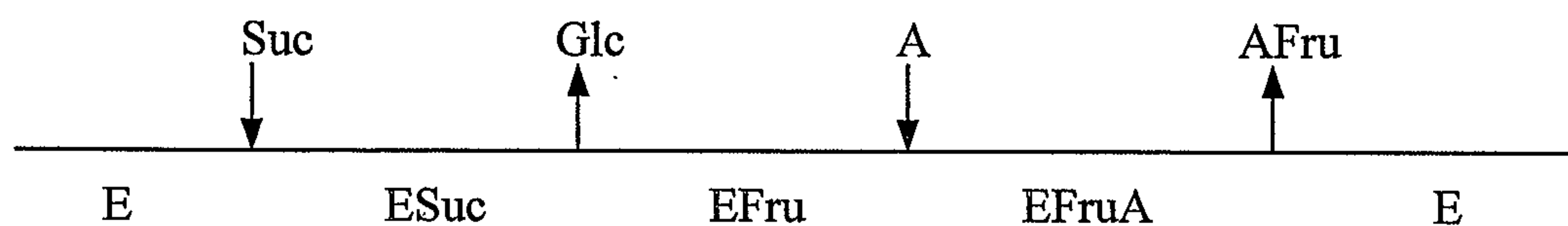
Levansucrase can hydrolyse small levans, but not inulin and the hydrolysis is stopped at branch points.

An exchange reaction is also observed when levansucrase is mixed with sucrose and [U-¹⁴C]-labelled glucose (Dedonder, 1966):



Based on these findings, it has been concluded that levansucrase transfers a fructosyl residue from sucrose to the primary alcohol at C-6 of the fructosyl residue at the non-reducing end of a growing levan chain possibly via a fructosyl-enzyme intermediate complex (Dedonder, 1966).

The mechanism of the reaction catalysed by the levansucrase of *B. subtilis* was further investigated by Chambert *et al.* (1974) by careful analysis of the initial velocities of the fructosyl transfer reactions. The results strongly supported a Ping Pong mechanism involving the participation of a fructosyl-enzyme intermediate. Thus the enzyme reaction can be described using the short-hand notation of Cleland (Cleland, 1973) as:



where E, Suc, Glc, Fru and A represent the enzyme, glucose, fructose and acceptor respectively (Chambert and Gonzyl-Treboul, 1976a). A multiple chain elongation mechanism has been proposed for the extension of the levan chain. It has been argued that the fructosyl residues are added randomly to all molecules of levan acceptor rather than successively to the same molecule of the levan (Chambert *et al.*, 1974).

The proposed fructosyl-enzyme intermediate in the Ping Pong reaction was isolated from the reaction mixture of enzyme and sucrose by Chambert and Gonzyl-Treboul (1976a). Proteolytic hydrolysis of the trapped complex resulted in the identification of an aspartic acid residue as the amino acid to which the fructosyl residue attached. The lability of the fructosyl-aspartate bond under mild alkaline conditions suggested that the fructosyl residue was linked through an ester bond involving the β -carboxyl of the aspartic acid residue (Chambert and Gonzyl-Treboul, 1976a).

The Ping Pong mechanism was further confirmed by thermodynamic and kinetic studies (Chambert and Gonzyl-Treboul, 1976b). A simplified method to evaluate the kinetic constants of the exchange and hydrolytic reaction was developed and the resulting data (Chambert and Gonzyl-Treboul, 1976b) was in close agreement with those predicted by a theoretical approach (Chambert *et al.*, 1974).

The thermodynamic results revealed that the high energy of the glycosidic linkage of sucrose is preserved in the fructosyl-enzyme intermediate. The entropy of activation for both exchange and hydrolytic reactions had approximately the same value (Chambert and Gonzyl-Treboul, 1976b) indicating that the orientation requirements of the reacting molecules and groups are of the same order of magnitude for the two activation processes. However, the enthalpy of the activation for the process of transfructosylation to water was greater than that to glucose (Chambert and Gonzyl-Treboul, 1976b), suggesting that the energy barrier to be overcome for the activated water-fructosyl-enzyme complex was higher than that for the activated glucose-fructosyl-enzyme complex. Based on these findings, two

different hypotheses were proposed (Chambert and Gonzyl-Treboul, 1976b). First, the enzyme possesses two separate and independent acceptor sites, a water site and a glucose site that could be the general sugar acceptor site as well. According to this hypothesis the transfructosylation to water and to glucose would be different, not only due to the nature of the acceptor but also due to the reacting groups of the enzyme which assist these chemical events. The second model suggests that the enzyme only possesses one acceptor site, but the binding of glucose or water to this common site would be thermodynamically different (Chambert and Gonzyl-Treboul, 1976b).

The mechanism by which levan synthesis is catalysed by the levansucrase of *B. subtilis* was investigated by Yamamoto *et al.* (1985) using raffinose as the substrate. The kinetic and chemical results indicated that the fructofuranoside chain of levan was extended by accepting a fructosyl residue at C-6 of the terminal fructofuranosyl residue by forming the linkage between C-2 and C-6. It was proposed that the enzyme strongly bound to the growing end of the levan chain and the fructosyl residues were added one at a time onto the levan (Yamamoto *et al.*, 1985).

The levansucrase secreted by *A. diazotrophicus* SRT4 is the only other Ftf whose kinetic properties have been studied in detail. The enzyme catalyses fructosyl transfer from sucrose by a Ping Pong mechanism involving the formation of a transient fructosyl-enzyme intermediate in a similar manner to the levansucrase of *B. subtilis* (Hernandez *et al.*, 1995). Both enzymes can synthesise fructan from sucrose alone when the sucrose concentration is ≥ 50 mM. Most of the kinetic parameters of the two enzymes are of the same magnitude, except that the levansucrase of *A. diazotrophicus* produces large amounts of 1-kestotriose and kestotetraose from sucrose, while the levansucrase of *B. subtilis* can synthesise fructan with more than 15 DP without accumulation of small oligofructans (Hernandez *et al.*, 1995).

1.2.3 Environmental Effects on Fructan Yield

Chambert and Petit-Glatron (1989) discovered that increasing the concentration of organic solvent, such as 1,4-dioxane, acetone or acetonitrile, in the reaction mixture of levansucrase of *B. subtilis* resulted in an increase in fructan yield. In the presence of 60% acetonitrile, the hydrolase activity of the enzyme was completely inhibited

and the yield of fructan reached 100%. The K_m values for sucrose and raffinose were unaffected but the k_{cat} values were increased 5 fold (Chambert and Petit-Glatron, 1989). Immobilisation of the levansucrase on hydroxyapatite was also found to enhance the polymerase action at the expense of sucrose hydrolysis. It was proposed that this change was due to the restriction of the availability of water when the enzyme was adsorbed onto the hydroxyapatite surface (Chambert and Petit-Glatron, 1993).

1.2.4 Key Amino Acids of Bacterial Fructosyltransferases

The nucleotide sequences of the *ftf* genes of several bacteria have been elucidated. These include *S. salivarius* (Rathsam *et al.*, 1993), *S. mutans* (Shiroza and Kuramitsu, 1988), *B. subtilis* (Steinmetz *et al.*, 1985), *B. amyloliquefaciens* (Tang *et al.*, 1990), *E. amylovora* (Geier and Geider, 1993), *Z. mobilis* (Song *et al.*, 1993; Kyono *et al.*, 1995), *A. diazotrophicus* (Arrieta *et al.*, 1996), *B. stearothermophilus* (Li *et al.*, 1997) and most recently *P. syringae* (Hettwer *et al.*, 1998). However, identification of the key amino acids of these Ftf's has only been explored in the levansucrase of *B. subtilis*.

Following the discovery that the levansucrase of *B. subtilis* preferentially maintains its polymerase activity in the presence of high concentrations of organic solvents, Chambert and Petit-Glatron (1991) demonstrated that the polymerase and hydrolase activities of the enzyme could be separately modulated by site-directed mutagenesis. Mutation of an arginine residue at position 331 to a histidine resulted in an enzyme of much lower polymerase activity. Substitution of Arg³³¹ with Lys, Ser or Leu caused the enzyme to lose the ability to synthesise levan from sucrose alone. The mutated enzymes could only catalyse the formation of the trisaccharide, kestose, similar to the sucrose:sucrose fructosyltransferase of plants (Chambert and Petit-Glatron, 1991).

The secretion of the levansucrase of *B. subtilis* has been characterised as a two-step process. The first step involves the proteolytic cleavage of the N-terminal signal sequence to give a mature membrane-associated form and the second, rate limiting step, involves the secretion and concomitant conformational change of the resulting mature form (Petit-Glatron *et al.*, 1987). The second step of the secretion has been found to be coupled to the continuous uptake of Fe³⁺ by the bacteria (Chambert and

Petit-Glatron, 1988). Studies on the *in vitro* refolding of the levansucrase following its denaturation by urea revealed that the rate of refolding was greatly enhanced by Fe^{3+} whereas the ion chelator, EDTA, prevented correct folding (Chambert *et al.*, 1990). Like Fe^{3+} , addition of Ca^{2+} ions has also been shown to promote the refolding of levansucrase at physiological temperature and pH. In the absence of either of these ions, the levansucrase would be predominantly in an unfolded form (Chambert and Petit-Glatron, 1990). It has been proposed that the energy required for the vectorial diffusion of the protein from the membrane phase to the external aqueous phase is provided by the energy change accompanying the folding process and the efficiency of such a coupling could be strongly dependent on the rate of the folding process (Chambert *et al.*, 1990). Consequently changes in the external concentration of any effector, such as Fe^{3+} or Ca^{2+} , which modulates protein folding, also affects the efficiency of the second step in the secretion of the active form of the enzyme.

Mutational analysis of the levansucrase has shown that substitution of the glycine at position 366 with Asp or Val reduces the level of secretion of levansucrase from *B. subtilis* (Petit-Glatron *et al.*, 1990). The refolding process mediated by Fe^{3+} or Ca^{2+} was not observed in these mutated enzymes. However mutation of Gly³⁶⁶ to Ser did not affect the secretion or refolding properties of the enzyme. It was therefore proposed that two requirements were essential for the secretion of the enzyme. The first, a structural flexibility of the protein, was indicated by the important role of Gly³⁶⁶ in the folding process, and the second, a structural stability under exocellular conditions, was indicated by the involvement of the metal ions, Fe^{3+} or Ca^{2+} as essential cofactors for coupling the folding and translocation processes (Petit-Glatron *et al.*, 1990).

1.3 Plant Fructosyltransferases

1.3.1 Structure of Plant Fructans

Fructans are found in about 15% of flowering plant species (Hendry and Wallace 1993), such as in the prevalent and evolutionally advanced orders of Asterales (chicory and Jerusalem artichoke), Liliales (tulip and onion) and Poales (wheat and barley) (Hendry, 1993). Unlike bacterial fructans that are usually of high DP in the order of 10^5 (M_r of 10^7) (Ebskamp *et al.*, 1994), most plant fructans have much lower DP of 5-60 (M_r of $1-10 \times 10^3$ Da). There are five major classes of fructans in plants

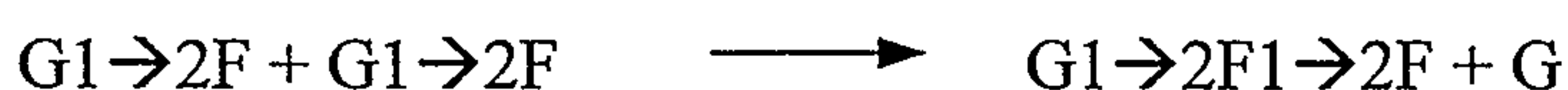
which can be distinguished according to their structure. Apart from the three types found in bacteria, inulin of β -(2 \rightarrow 1) linkage, linear levan of β -(2 \rightarrow 6) linkage and mixed linkage type levan of both β -(2 \rightarrow 1) and β -(2 \rightarrow 6) linkages, there are also two types belonging to the neokestose-series (neoserries). One is the inulin neoserries, with a fructosyl residue on both the carbon 6 and carbon 1 of a glucose molecule producing a polymer with β -(2 \rightarrow 1) linked fructosyl residues on either end of a sucrose molecule. The other is the levan neoserries, with β -(2 \rightarrow 1) and β -(2 \rightarrow 6) linked fructosyl residues on either end of the sucrose molecule (Vijn *et al*, 1997).

1.3.2 Fructosyltransferases Involved in Plant Fructan Biosynthesis

Fructan synthesis takes place in the plant vacuole. Unlike bacterial Ftf's, which are multifunctional and capable of utilising a wide range of fructosyl donors and fructosyl acceptors (eg. the levansucrase from *B. subtilis*), plant Ftf's require more specific fructosyl donors and acceptors.

1.3.2.1 Sucrose:Sucrose 1-Fructosyltransferase (1-SST) and Fructan:Fructan 1-Fructosyltransferase (1-FFT)

In plants, linear inulins are thought to be synthesised from sucrose by the concerted action of at least two Ftf's (Edelman and Jefford 1968; Pollock and Cairns, 1991). Edelman and Jefford (1968) have proposed a model for the synthesis of inulin in Jerusalem artichoke. 1-SST initiates fructan biosynthesis by transferring a fructosyl residue from a sucrose molecule to another, forming 1-kestose as follows where the numbers indicate the glucosyl or fructosyl carbon atoms that participate in the bonding:



1-FFT catalyses the fructosyl transfer between two oligofructoses:

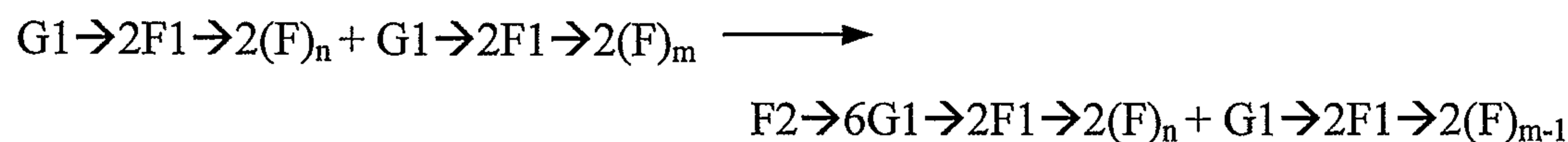


It has been shown that *in vitro* incubation of purified 1-SST and purified 1-FFT from *Helianthus tuberosus* with sucrose is sufficient for the production of a series of

inulins with high DP (Koops and Jonker, 1996; Luscher *et al.*, 1996). The trisaccharides produced by 1-SST serve as the fructosyl donors for 1-FFT and thus permit the progressive elongation of acceptor inulin chains.

1.3.2.2 Fructan:Fructan 6G-Fructosyltransferase (6G-FFT)

For the production of neoseris, a third enzyme is needed (Shiomi, 1981 and 1989; Wiemken *et al.*, 1995). 6G-FFT catalyses the fructosyl transfer from a oligofructose to the C-6 of the glucose moiety of another oligofructose (Shiomi, 1981) as shown in the following reaction:



It has been shown that 6G-FFT is the key enzyme in the formation of the inulin neoseris in onion. Introduction of onion 6G-FFT into chicory plants, which normally only produce inulin, results in the production of an inulin neoseris. Therefore, for synthesis of inulin neoseris, three enzymes, 1-SST, 1-FFT and 6G-FFT are probably necessary (Vijn *et al.*, 1997).

1.3.2.3 Invertase

Invertases (β -fructofuranosidases) catalyse sucrose hydrolysis:



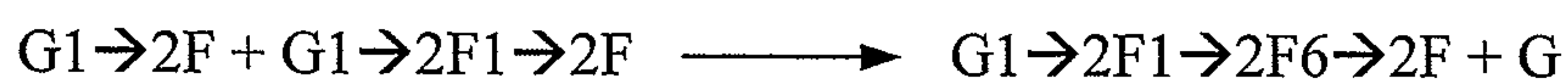
Plant invertases are also known to act as Ftf's under certain conditions (Straathof *et al.*, 1986; Cairns and Ashton, 1991; Pollock and Cairns, 1991) forming kestose from sucrose, the first step in fructan synthesis that is also catalysed by 1-SST (Edelman and Jefford, 1968; Pollock and Cairns, 1991). It was therefore thought at one stage that the 1-SST activity in plants was carried out by invertases (Pollock and Cairns, 1991). This doubt was soon resolved after Obenland and colleagues isolated three soluble invertases from barley leaves (Obenland *et al.*, 1993). These enzymes hydrolysed sucrose into its constituent monosaccharides and also appeared to be able to act as 1-SST when supplied with high concentration of sucrose. The Ftf activity of these enzymes was inhibited by pyridoxal in the same way as their invertase activity, suggesting these enzymes were different to the 1-SST isolated from barley leaves which was insensitive to pyridoxal inhibition (Obenland *et al.*, 1993).

1.3.2.4 Sucrose:Fructan 6-Fructosyltransferase (6-SFT)

The biosynthesis of β -(2 \rightarrow 6) linked fructans in barley is catalysed by another Ftf, 6-SFT (Simmen *et al.*, 1993). The purified enzyme exists in two similar isoforms with indistinguishable catalytic properties, both consisting of two subunits with M_r of 49,000 and 23,000 (Sprenger *et al.*, 1995). With sucrose as the sole substrate, the enzyme may hydrolyse sucrose to glucose and fructose and produce 6-kestose as the main oligomer as shown in the following reaction.



However, when the enzyme is incubated with sucrose and 1-kestose, it acts mainly as a 6-SFT forming bifurcose as shown in the following reaction while its sucrose:sucrose 6-fructosyltransferase and sucrose hydrolytic activities are suppressed (Sprenger *et al.*, 1995).



Functional expression of a 6-SFT from barley (Sprenger *et al.*, 1995) in transgenic tobacco, a plant naturally unable to form fructans, results in the synthesis of the trisaccharide kestose and a series of unbranched fructans of β -(2 \rightarrow 6) linkages (Sprenger *et al.*, 1997). Expression of 6-SFT in chicory, a plant naturally producing only unbranched inulin type fructans, results in the additional synthesis of the β -(2 \rightarrow 6) linked fructan, bifurcose (Sprenger *et al.*, 1997).

1.3.3 The Role of Fructans in Plants

Fructans serve as important carbohydrate storage compounds as alternatives to, or in addition to, starch in many plant species (Hendry, 1993). Both sucrose level and fructan biosynthesis are highly regulated processes, with a role for fructan as the temporary storage product for assimilated carbon reserves. In particular, fructan accumulation is important when plant growth or cell expansion is limited by environmental influences, or as a consequence of seasonal growth or perennation (Jenkins *et al.*, 1996). Fructan metabolism is believed to help in controlling sucrose content within the vacuole thus regulating carbohydrate partitioning and osmoregulation (Housley and Pollock, 1993). Reducing the sucrose content by fructan synthesis has an advantage over the hydrolysis of sucrose by invertase in that

it does not result in a dramatic change in osmotic potential or reducing power (Housley and Pollock, 1993). When and where osmotic increases are desirable, hydrolysis of fructan can yield free fructose to create turgor (Schnyder and Nelson, 1989). Fructan is also considered to play an important role in protecting plants against drought and cold environments (Hendry, 1993). For instance, transgenic fructan-accumulating tobacco has been shown to possess improved tolerance under drought stress (Pilon-Smits *et al.*, 1995).

1.3.4 Molecular Characterisation of Plant Fructosyltransferases

A number of enzymes involved in plant fructan biosynthesis have been cloned and sequenced, including the 6-SFT from barley (Sprenger *et al.*, 1995), 6G-FFT from onion (Vijn *et al.*, 1997), 1-SST and 1-FFT from Jerusalem artichoke (van der Meer *et al.*, 1998), and 1-SST and the acid invertase from onion (Vijn *et al.*, 1998). Comparison of the deduced amino acid sequence of barley 6-SFT with the sequences of various β -fructosyl-hydrolases and Ftf's of both plant and bacterial origin has revealed seven highly conserved domains (Figure 1.5), for which bacterial Ftf's are only homologous to domain C and D (Sprenger *et al.*, 1995). However, no experimental investigations have been carried out to elucidate the roles of these highly conserved domains.

The deduced amino acid sequences of 6G-FFT from onion and 1-SST and 1-FFT from Jerusalem artichoke show 52-61% identity to plant invertases in contrast of 48-50% identity to barley 6-SFT (Vijn *et al.*, 1997; van der Meer *et al.*, 1998; Vijn *et al.*, 1998). It has been proposed that all plant *ftf* genes might have evolved from invertase genes by small mutational changes (Sprenger *et al.*, 1995; Vijn *et al.*, 1997; van der Meer *et al.*, 1998; Vijn *et al.*, 1998).

1.3.5 Expression of Bacterial Fructosyltransferases in Plants

Most bacterial Ftf's are either extracellular or cell-bound. In contrast, plant Ftf's are usually found in the vacuoles. The gene of the levansucrase of *B. subtilis* has been introduced into tobacco plants, which do not normally synthesise fructans, after the presequence of the gene was removed and the resulting truncated gene fused with a yeast vacuolar targeting signal sequence (Ebskamp *et al.*, 1994). The resulting transgenic plants accumulated fructans and showed no phenotype difference

Origin	Enzyme	Highly Conserved Domains							
Barley	6-SFT	MSDPNG	HMFYQ	WGH	LSGSM	DFRDP	EWECI	GK-FYA	
Mung bean	Inv	MNDPNG	HFFYQ	WGH	WTGSA	DFRDP	MWECV	GI-FYA	
Carrot	Inv	MNDPNG	HLFYQ	WGH	WTGSA	DFRDP	MWECV	GI-YYA	
Tomato	Inv	MNDPNG	HFFYQ	WGH	WTGSA	DFRDP	MWECV	GK-YYA	
Carrot	cwInv	INDPNG	HFFYQ	WAH	RSGSA	AFRDP	MWECV	GN-FYA	
<i>E. coli</i>	Inv	MNDPNG	HAFYQ	WGH	FSGSA	HFRDP	MWECV	GHDFYA	
<i>S. mutans</i>	ScrB	LNDPNG	NLFYQ	WIH	YSGSA	HFRDP	MIECP	GFEFYA	
<i>B. subtilis</i>	SacC	MNDPNG	HLFYQ	WGH	FSGSA	DFRDP	VWECV	GRDFYA	
<i>K. marxianus</i>	Inu	MNDPNG	HLYYQ	WGH	FSGSM	NFRDP	QYECV	GKDYYA	
<i>S. cerevisiae</i>	Inv	MNDPNG	HLYFQ	WGH	YSGSA	QFRDP	QYECV	GKDYYA	
<i>A. niger</i>	Inv	IGDPCL	HVGFL	-SS	FDGS-	AFRDP	TWAGR	GFSSYA	
<i>B. subtilis</i>	SacB	LQNAD-	HIVFA	YQK	WSGSA	TLRDP	L-RTA	YMLGYV	
<i>S. mutans</i>	SacB	VQDAKT	QLVVA	YNK	WSGSA	AMRDP	MYNRA	VMKGYV	
	Region	A	B	B'	C	D	E	F	

Figure 1.5: Comparison of the deduced amino acid sequence of barley 6-SFT with the sequences of various β -fructosyl-hydrolases and Ftf's (Sprenger *et al.*, 1995). Inv, invertase; cw, cell wall; ScrB, bacterial invertase; SacC, levanase; Inu, inulinase; SacB, levansucrase. *K. marxianus*, *Kluyveromyces marxianus*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *A. niger*, *Aspergillus niger*.

compared with the wild type plants. The fructan synthesised by the transgenic plant has similar size and properties to the fructan produced by *B. subtilis*. The level of fructan accumulation ranged from 3-8 % of the dry weight (Ebskamp *et al.*, 1994). However, no levansucrase mRNA or protein could be detected in the transgenic plants. This could be due to the low expression level of the levansucrase in tobacco (Ebskamp *et al.*, 1994).

Expression of bacterial *ftf* gene in different subcellular compartments of plants and the resulting effects on plant development were investigated by Röber *et al.* (1996). The gene of *E. amylovora* levansucrase was introduced into apoplast, vacuole and cytosol in tubers of potato plants. Accumulation of fructan was only observed in the case of apoplastic and vacuolar targeting and the M_r and structure of the fructan was identical to the levan produced by *E. amylovora*. The vacuole is the preferred organelle for high DP fructan synthesis, whilst high DP fructan production in the apoplast has detrimental and lethal effects on plants (Röber *et al.*, 1996).

The *ftf* gene encoding the Ftf of *S. salivarius* has also been engineered and transferred to tobacco (Snow *et al.*, 1994) and to white clover with significant accumulation of fructan (~ 1% dry weight) (Jenkins *et al.*, 1996). High level expression of the *ftf* gene resulted in slow growth of the plants and the leaves became necrotic on some plants, while low to moderate expression of the *ftf* gene did not show any obvious phenotype change (Jenkins *et al.*, 1996).

1.4 Commercial Uses for Fructans

1.4.1 Applications in the Food Industry

1.4.1.1 Fructans as Healthy Foods

Fructans are beneficial and healthy ingredients of human food. Due to their β -(2 \rightarrow 1) or β -(2 \rightarrow 6) glycosidic linkages, fructans can not be digested by acid hydrolysis in the stomach or by enzymes in the stomach and small intestine of man (Oku *et al.*, 1984; Nilsson *et al.*, 1988; Hosoya *et al.*, 1988). Thus they reach the large intestine undigested and unabsorbed, where they are fermented by colonic bacteria, particularly the non-putrefying bifidobacteria (Modler *et al.*, 1990). Yamada *et al.* (1990) has shown that ingestion of short chain fructans does not cause any increase in the plasma concentrations of glucose and fructose or in the insulin response which is normally observed after the intake of sucrose. Low DP oligofructose molecules are sweet tasting and have been exploited for the production of natural low caloric sweeteners. The small portion of glucose in the fructan molecule makes it an ideal carbon source for diabetics.

Many studies have shown that ingestion of fructans improves the colonic microflora and reduces digestive problems such as constipation (Hidaka *et al.*, 1986). Short chain fructans are utilised by almost all bifidobacteria species but not by undesirable putrefactive bacteria such as *Clostridium perfringens*, *Clostridium difficile* and *E. coli*. Administration of small fructans to constipated patients (50-90 years old) for 14 days resulted in a significant increase in the number of bifidobacteria in their faeces (Hidaka *et al.*, 1991). Eleven out of fifteen patients treated with short chain fructans gained relief from their constipation. It was suggested that the alleviation of constipation by non-digestible fructans was partly due to the high osmotic pressure of short-chain fatty acids produced by the intestinal bacteria and that this pressure accelerated peristaltic movement (Hirayama *et al.*, 1993).

As a consequence of the many beneficial aspects of fructans, there is an increasing interest in fructan-containing foods. Fructans of various sizes are also commercially available in Europe and Japan for use as food additives, such as Raftilose and Raftiline from Raffinerie Tirlemontoise S. A., Tienen, Belgium and Neosugar from Meiji Seika Kaisha Ltd., Kawasaki, Japan (Incoll and Bonnett, 1993).

1.4.1.2 Fructans as a Source of Fructose

Excessive consumption of sucrose causes obesity and is of concern to diabetics. Fructose, the sweetest natural sugar known, is about 20-30% sweeter than sucrose. Due to its lower energy content, slow absorption and quick metabolic transformation, fructose has a beneficial physiological effect on people suffering from obesity or diabetes. As a result, fructose has become an increasingly popular sweetener in many food products, primarily at the expense of sucrose. The cost of the fructose mainly depends on the cost of the enzyme, glucose isomerase.

Another alternative source of fructose is fructan. In Hungary, there are government and food industry supported projects for the cultivation and processing of Jerusalem artichoke with its high fructan content (72-80% dry weight) (Barta, 1993). The Jerusalem artichoke concentrate is hydrolysed by inulinases. The resulting high-fructose syrup can be used in the production or preparation of food of reduced energy content for diabetics, such as canned fruits and fruit juices, sweet bakery products, confectionery jellies, cocoa drink and puddings etc. Fructose syrup made from fructans can contain at least 96% fructose. Crystalline fructose can also be produced using fructans as a primary source of fructose (Barta, 1993).

1.4.2 Medical Applications for Fructans

1.4.2.1 Effect of Fructans on Blood and Hepatic Lipid Content

Hidaka *et al.* (1991) discovered that short chain fructans could improve the blood lipid composition in hyperlipidaemia. Serum total cholesterol, HDL (high-density lipoprotein)-cholesterol, triglycerides and apolipoproteins were monitored before and after administration of short chain fructans to hypercholesteromic patients. Total cholesterol and triglycerides remained unchanged and apoprotein E significantly increased. The daily intake of fructans reduced the LDL (low-density lipoprotein)-cholesterol level thus lowering total serum cholesterol concentration (Hidaka *et al.* 1991; Hirayama *et al.*, 1993).

Ethanol and dexamethasone, a synthetic glucocorticoid and potent analog of cortisol, have a detrimental effect on the liver. Animal tests have shown that chicory, with its high fructan content (up to 72-80% dry weight), has the beneficial effect of keeping the lipid profile of the liver, especially the total lipids and triglycerides, to near normal levels if given in feed during ethanol and dexamethasone injections (Gupta *et al.*, 1993). Feeding chicory roots also helps in the recovery of liver damage subsequent to dexamethasone injections. However, the molecular factor responsible for this hypotriglyceridemic effect of chicory roots is not known (Gupta *et al.*, 1993), although it might be due to the fructans in the chicory roots since ingestion of fructans has been shown to improve the blood lipid composition by increasing apoprotein E and lowering LDL-cholesterol level (Hidaka *et al.* 1991).

1.4.2.2 Inulin as a Marker of Renal Function

Methods involving radioactive isotopes have been used in the measurement of renal function. However, use of radioactive isotopes may not always be permitted and patients are more and more unwilling to be exposed to any unnecessary radiation. Inulin is an ideal substance to quantify glomerular filtration rate (GFR), a key parameter of renal function. Inulin is filtered freely, not secreted or reabsorbed by tubules, has no plasma binding or toxic side effects and does not give rise to any allergic adverse reactions (Gretz *et al.*, 1993). GFR measurement using inulin used to be tedious. The availability of the ready-for-injection inulin solution, Inutest[®], overcomes the difficulty in inulin handling and a number of simplified methods of GFR determination with inulin have now been developed (Gretz *et al.*, 1993), making inulin a convenient and useful marker of renal function.

1.4.3 Uses of Fructans in the Organic Chemical Industry

Raw materials for the organic chemical industry mainly come from the by-products of refining crude oil, coal or gas and thus are very sensitive to price fluctuations and availability. To escape from this dependence, attempts have been made to open up alternative sources of raw materials for the chemical industry. Fructans can be readily converted to the basic chemical hydroxymethylfurfural (HMF) by a number of different processes (Kunz, 1993). HMF can be utilised to produce a wide range of chemical intermediates or end-products of potential industrial application due to its

various functionalities, a hydroxyl group, two aldehyde groups and a furan ring with aromatic character and a diene-like structure (Kunz, 1993).

In theory, the products of esterification of HMF with organic acids could be used as selective solvents in the chemical industry or as antibacterial compounds in pharmaceuticals and food. HMF could also be used in the production of resins with the advantages of lowering energy costs, allowing a more convenient manufacturing process, and lowering the amount of residual monomers such as phenol and formaldehyde in the end product (Kunz, 1993). The polyamides produced from HMF are comparable with the well-known terephthalic acid- and isophthalic acid-based polyamides Kevlar[®] and Nomex[®]. Another important potential use of HMF is in the production of Zantac[®] (Glaxo, UK), an anti-ulcer drug and the first prescription drug to exceed 1 billion US dollar annual sales. Zantac[®] contains the structural feature of HMF and is conventionally produced from furfural. The intermediate in the synthetic pathway can also be produced from HMF in a more economic one-step reaction. However the price difference between HMF and furfural still limits the usage of HMF in this large scale pharmaceutical application (Kunz, 1993).

1.4.4 Other Applications

Longo and Combes (1995) described the application of Ftf in protein modification. In their experiment, Ftfs were used to chemo-enzymatically link glycosides onto the surface of lysozyme. The properties of lysozymes with variable lengths of glycoside side chain were studied to determine any differences brought about by the glycosylation. It was shown that glycosylation of the enzyme surface resulted in an increase in the global hydrophilicity and a decrease in thermal stability of the modified enzyme. The hydrolytic activity of the glycosylated lysozyme on the substrates *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid decreased markedly with increases in the length of the attached glycosidic chain. This application of Ftfs opens a novel strategy for research on glycosylation, one of the most important protein modifications known.

1.5 Aims of the Research

The aims of this study were to investigate the enzymic properties, the catalytic mechanism and the active site residues in the Ftf of *S. salivarius*.

CHAPTER 2

ROUTINE METHODS

This chapter describes methods used routinely during this research. Experimental methods that were developed during the course of this study are described in the relevant chapters.

2.1. Reagents, Chemicals and Enzymes

All chemicals were of analytical reagent grade except for those indicated below:

Ampicillin, tetracycline, cesium chloride, deoxyribonuclease (DNase) I, lysozyme, ribonuclease (RNase) A: Boehringer Mannheim, Sydney, Australia.

Tryptone, yeast extract, bacteriological agar: Oxoid, Hampshire, England.

[U-¹⁴C-fructosyl]-labelled sucrose, [U-¹⁴C]-labelled sucrose, [U-¹⁴C]-labelled glucose, [U-¹⁴C]-labelled fructose, [³⁵S]-dATP: Dupont NEN, Boston, Mass. U.S.A.

Electrophoresis gel stain solution: Gradipure Ltd, Sydney, Australia.

Coomassie[®] Plus Protein Assay Reagent: Pierce, IL USA

Agarose NA, bind-silane, repel-silane: Pharmacia Biotech, Melbourne, Australia.

Acrylamide, adenosine 5'-triphosphate (ATP) (sodium salt), ammonium persulphate, Bis-Tris, bromophenol blue, calcium chloride, dithiothreitol (DTT), ethidium bromide, ethylenediaminetetraacetic acid disodium salt (EDTA), formaldehyde, fructose, glucose, L-histidine, β -mercaptoethanol, *N,N'*-methylene-bis-acrylamide, periodic acid, piperazine sodium salt, phenylmethylsulphonyl fluoride (PMSF),

potassium phosphate dibasic, potassium phosphate monobasic, raffinose, Schiff's reagent, sodium azide, sodium dodecyl sulphate (SDS), sodium fluoride, sucrose, *N,N,N',N'*-tetramethylethylenediamine (TEMED), thiomersal, Triton X-100, Trizma base (Tris): Sigma, Sydney, Australia.

Restriction enzymes, λ DNA-*Hind*III digest or λ DNA-*Hind*III digest/ ϕ X-174RF DNA-*Hinc*II digest molecular weight markers, T4 nucleotide kinase, T4 DNA polymerase and T4 ligase were obtained either from Boehringer Mannheim (Sydney, Australia), Promega Corporation (Sydney, Australia), New England Biolabs (Queensland, Australia) or Pharmacia Biotech (Melbourne, Australia).

Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia Biotech, Melbourne, Australia), using Pharmacia chemicals or purchased from Auspep Ltd (Melbourne, Australia) or Beckman Instruments (Sydney, Australia).

Broad range SDS-PAGE standards and gel filtration chromatography standards were both obtained from Bio-Rad (Sydney, Australia). Non-denaturing-PAGE standards were purchased from Sigma (Sydney, Australia).

2.2 Bacterial Strains and Phagemids

Strains and vectors used in these studies are listed in Table 2.1.

2.3 Media and Growth Conditions

Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) (5 g yeast extract l⁻¹, 10 g tryptone l⁻¹, 10 g NaCl l⁻¹), supplemented with 100 µg ampicillin ml⁻¹, or '2xYT' medium (10 g yeast extract l⁻¹, 10 g tryptone l⁻¹, 5 g NaCl l⁻¹) (Sambrook *et al.*, 1989) as appropriate.

Table 2.1. Bacterial Strains and Phagemids

Bacteria or Phagemid	Description ^a	Source/Reference
Bacteria		
<i>Streptococcus salivarius</i> ATCC 25975	(no description)	Hamilton, 1968.
<i>Escherichia coli</i> NMS22	F' lacI _q (lacZ) M15 proA ⁺ B ⁺ /supE thi Δ(lac-proAB) Δ (hsdMS-mcrB)5 (r _k ⁻ m _k ⁻ McrBC ⁻)	Gough and Murray, 1983
<i>Escherichia coli</i> BMH71-18 <i>mutS</i>	thi supE Δ (lac-proAB) [mutS::Tn10][F' proAB lacI _q Z ΔM15]	Clonetech
Phagemids		
pIBI31	Ap ^r , f1 origin replication, β-galactosidase, T3 and T7 polymerase promoters	IBI Corp.
pKRK1969	Ap ^r ; Ftf ⁺ ; 3.29 kbp insert of <i>S. salivarius</i> chromosomal DNA in pIBI31	Rathsam <i>et al.</i> , 1993
pKRK1801	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Dpn</i> I site; <i>fif</i> coding for D312N	This study (Table5.1)
pKRK1802	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Dpn</i> I site; <i>fif</i> coding for D312E	This study (Table5.1)
pKRK1803	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Ava</i> I site; <i>fif</i> coding for D312S	This study (Table5.1)
pKRK1804	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Sly</i> I site; <i>fif</i> coding for D312K	This study (Table5.1)
pKRK1805	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Dpn</i> I site; <i>fif</i> coding for D312I	This study (Table5.1)
pKRK1806	pKRK1969; <i>Mlu</i> I site → <i>Bsr</i> I; new <i>Bgl</i> II site; <i>fif</i> coding for D397S	This study (Table5.1)

^a Ap^r, ampicillin resistance

2.4 DNA Manipulations

2.4.1 Rapid Isolation and Purification of Phagemid DNA by the Boiling-Lysis Method

Phagemid DNA was isolated from *E. coli* essentially by the method of Holmes and Quigley (1981). A single colony of *E. coli* NM522 hosting phagemid pKRK1969 was inoculated into 3 or 6 ml of LB medium containing the appropriate antibiotics (e.g. 100 µg ampicillin ml⁻¹) and shaken at 37°C for 16 h. The overnight culture was centrifuged (4,000 g, room temperature 18-22°C, 5 min), the cell pellet resuspended in 200 µl of STET buffer [8% (w/v) glucose, 5% (v/v) triton X-100, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH8.0] and 10 µl of lysozyme/RNase Mix (10 mg lysozyme ml⁻¹, 1 mg RNaseA ml⁻¹, 50 mM Tris-HCl pH8.0) was added to the cell suspension. The mixture was heated at 100°C for 3 min before centrifugation (12,000 g, 18-22 °C, 10 min). The supernatant was transferred to a fresh microcentrifuge tube, 200 µl of 5.0 M ammonium acetate and 400 µl of isopropanol added and the precipitate recovered by centrifugation (12,000 g, 18-22°C, 2 min). The pellet was rinsed with 70% (v/v) ethanol and dried under vacuum before resuspending in 50-100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.4.2 Small Scale Isolation of High Purity Phagemid DNA

High purity phagemid DNA was prepared using WizardTM Plus Minipreps (Promega Corp. USA.). A single colony of *E. coli* was inoculated into 10 ml of LB medium containing the appropriate antibiotics (e.g. 100 µg ampicillin ml⁻¹) and shaken at 37°C for 16 h. The overnight culture was centrifuged (4,000 g, 18-22°C, 5 min), the cell pellet resuspended in 400 µl of Solution I (50 mM Tris-HCl pH7.5, 10 mM EDTA, 100 µg RNase A ml⁻¹) before the addition of 400 µl of Solution II [0.2 M NaOH, 1% (w/v) SDS] and mixing by inverting the tube 4 times. The lysate was neutralised by addition of 800 µl of Solution III (1.32 M potassium acetate pH 4.8), mixed by inverting the tube 4 times and centrifuged (12,000g, 18-22°C, 20 min). The supernatant was transferred to the WizardTM minicolumn/syringe assembly (i.e. a WizardTM minicolumn attached to the syringe barrel of a 3 ml sterile syringe) containing 1 ml of the resuspended WizardTM Miniprep resin. The supernatant was

filtered through the resin by gently pushing the slurry into the minicolumn with the syringe plunger. The minicolumn was then washed twice with 2 ml of Column Wash Solution [80 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5, 40 μ M EDTA, 55% (v/v) ethanol] and centrifuged (12,000 g, 18-22°C, 2 min) to dry the resin. The minicolumn was transferred to a fresh sterile microcentrifuge tube, 50 μ l of water or TE buffer applied to the minicolumn and after 1 min was centrifuged (12,000 g, 18-22°C, 20 s) to elute the DNA.

2.4.3 Large Scale Isolation and Purification of Phagemid DNA

Phagemids were purified from *E. coli* by the alkaline lysis method essentially as described by Sambrook *et al.* (1989). *E. coli* was grown overnight at 37°C with vigorous shaking in 500 ml LB medium with the appropriate antibiotic (e.g. 100 μ g ampicillin ml⁻¹). Cells were harvested by centrifugation (4,000 g, 18-22°C, 15 min), resuspended in 10 ml solution I (2.4.2) and incubated at 18-22°C for 5 min. Solution II (20 ml; 2.4.2) was added to the suspension and left to stand on ice for 10 min. Subsequently, 15 ml of solution III (2.4.2) was added and the resulting suspension incubated on ice for a further 10 min. Bacterial debris was removed by centrifugation (48,000 g, 4°C, 30 min) and the DNA precipitated by the addition of two volumes of ethanol. The DNA was recovered by centrifugation (12,000 g, 18-22°C, 30 min) and dissolved in 16 ml of TE buffer pH 8.0. Cesium chloride was added to a final concentration of 1 g ml⁻¹ before the addition of 1.6 ml of ethidium bromide solution (10 mg ml⁻¹). The cesium chloride solution was transferred to an ultracentrifuge tube and the remainder of the tube filled with light paraffin oil. After centrifugation (200,000 g, 20°C, 40 h), the lower band consisting of closed circular phagemid DNA was removed with a hypodermic needle and syringe. The ethidium bromide was extracted with equal volumes of water-saturated 1-butanol and the resulting aqueous phase dialyzed against several changes of TE buffer pH 8.0. The DNA was then precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 4.8 and 2.5 volumes of ethanol. The ethanol precipitate was recovered by centrifugation (16,000 g, 18-22°C, 5 min) and resuspended in TE buffer pH 8.0.

2.4.4 Spectrophotometric Determination of Nucleic Acid Concentration

The nucleic acid concentration was determined spectrophotometrically (Sambrook *et al.*, 1989). The absorbance of a DNA sample (5 μ l) was obtained at 260nm and 280nm in a Pharmacia GeneQuant DNA/RNA Calculator. An A_{260} of 1.000 corresponded to approximately 50 μ g ml⁻¹ for dsDNA and 40 μ g ml⁻¹ for ssDNA or RNA. The A_{260}/A_{280} ratio gave an estimate of the purity of the sample as pure preparations of DNA and RNA have a ratio of 1.8 and 2.0 respectively.

2.4.5 Precipitation of DNA by Ethanol

DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol or 1 volume of 5 M ammonium acetate and 4 volume of ethanol at -70°C for 30 min. The DNA was recovered by centrifugation (12,000 g, 18-22°C, 5 min), washed with 70% (v/v) ethanol and dried under vacuum. However DNA or oligonucleotides that were to be phosphorylated were not precipitated by the second method in order to avoid ammonium contamination.

2.4.6 Precipitation of Oligonucleotides by Ethanol

Oligonucleotides were precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.2, 3.5 volumes of ethanol and an appropriate amount of 1 M MgCl₂ to a final concentration of 0.01 M, before being chilled at -70°C for 30-60 min and centrifuged (12,000 g, 18-22°C, 30 min). The supernatant was removed and the precipitate washed with 70% (v/v) ethanol and dried under vacuum. For oligonucleotides of low concentration, 1 volume of 1-butanol was added to the oligonucleotide solution, the solution vortexed and then centrifuged (4000 g, 18-22°C, 30 s) and the top layer of butanol discarded. The butanol extraction was repeated several times until the concentration of oligonucleotides was above 200 μ gml⁻¹.

2.4.7 Digestion of DNA with Restriction Endonucleases

Restriction endonuclease digestion of DNA was routinely carried out in 40 μ l volumes using the restriction buffer and conditions supplied by the manufacturer. Incubation was carried out for 3 h at the optimal temperature required for the particular restriction endonuclease. Heating to 80-85°C for 10 min was routinely

used to inactivate the restriction endonuclease prior to agarose gel electrophoretic analysis of the digested DNA (2.4.8).

2.4.8 Gel Electrophoresis of DNA on Agarose Gels

DNA was separated on 0.8% (w/v) agarose gels prepared in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.0), using either the 50 ml 'mini-gel' or 150 ml 'maxi-gel' system (Hoefer Scientific Instruments, California, U.S.A.). Samples up to 40 μ l were mixed with 4 μ l of loading dye (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF, 30% [v/v] glycerol). *Hind*III digested bacteriophage- λ was generally used as M_r standards. Electrophoresis was carried out in TBE buffer at either 100-150 V for 1-4 h or 30 V for 16 h. Gels were stained in ethidium bromide solution (5 μ g ml⁻¹) for 30 min before being destained in water for 30 min. Gels were routinely photographed on a UV transilluminator (Novex Australia Pty. Ltd., Sydney, Australia) with a polaroid camera fitted with a Kodak Wratten 23A orange filter using type 667 Polaroid film (Polaroid Australia Pty. Ltd., Sydney, Australia).

2.4.9 Preparation of Cells for Electroporation

Cells that were to be electroporated were prepared by a method derived from the instruction manual for the 'Gene-Pulser' unit (BioRad, Sydney, Australia). One litre of LB medium was inoculated with a 1% (v/v) overnight culture of the relevant strain of *E. coli* grown at 37°C in the same medium. This culture was then grown at 37°C with vigorous shaking to an A_{600} of 0.500 (\pm 0.030), at which time the culture was chilled on ice (15 to 30 min) before centrifugation (4000 g, 4°C, 15min). The pelleted cells were resuspended in 200 ml of ice-cold 10% (v/v) glycerol and incubated on ice for 20 min before centrifugation (3000 g, 4°C, 10 min). The cell pellet was resuspended in 20 ml of ice-cold 10% (v/v) glycerol and incubated for another 20 min before being centrifuged (1200 g, 4°C, 10 min) and resuspended in 2 ml ice-cold 10% (v/v) glycerol. This suspension was dispensed into 60 μ l aliquots and stored at -70°C until required.

2.4.10 Transformation by Electroporation

An aliquot of cells (60 μ l) that had been prepared for electroporation (2.4.9) was thawed on ice, 5 μ l of DNA (0.05-0.2 μ g) added and the mixture of cells and DNA transferred to a ice-cold electroporation cuvette. The 'Gene Pulser' (BioRad, Sydney, Australia) was set at 25 μ F and the 'Pulse Controller' (BioRad, Sydney, Australia) to 200 Ω . A pulse of 2.4 kV was applied to those cuvettes with a 0.2 cm electrode gap and 1.6 kV to cuvettes with a 0.1 cm electrode gap. Ice cold 2xYT medium (1 ml; 2.3) was immediately added and the cell suspension was transferred to a 28 ml McCartney bottle and incubated at 37°C with shaking (150 rpm) for 1 h before the addition of selective medium or plating on selective agar.

2.4.11 DNA Sequencing

Sequence determinations were carried out using WizardTM Plus Minipreps purified dsDNA (2.4.2) by the chain-termination method of Sanger *et al.* (1977). All sequencing reactions used the Pharmacia T7 sequencing kit (Pharmacia Biotech, Melbourne, Australia) according to the manufacturer's instructions using custom-synthesized oligonucleotide primers and [³⁵S]-dATP.

Sequencing reactions were run using a Hoefer Poker Face I Nucleic Acid Sequencer (Hoefer Scientific Instruments, California, U.S.A.). Sequencing gels [8% (w/v) acrylamide, 28% (w/v) urea] were pre-run at 110 W for at least one hour. Three loadings were applied to the gel, at 0 h, 2 h, and 3.5 h, with a total running time of approximately 4.5 h. After loading, the gel was run at 10 W until the loading dye moved into the gel (approximately 5 min), then the power was increased to 110W. Gels were fixed with a solution containing 10% (v/v) methanol and 10% (v/v) glacial acetic acid before drying and subjecting to autoradiography.

2.5 Protein Manipulations

2.5.1 Protein Assay

The concentration of protein was determined using the Bradford method (Bradford, 1976) with Coomassie[®] Plus Protein Assay Reagent (Pierce, IL USA). Bovine serum albumin (BSA) standards of various concentrations (0, 1, 2, 4, 8, 12, 16, 20 and 24

$\mu\text{g ml}^{-1}$) were assayed at the same time as the unknown samples. Sample (and standard) solutions of 1.0 ml were mixed with 1.0 ml of protein assay reagent and the absorbance was measured against the reagent blank at 595 nm within 30 min. A standard curve was prepared by plotting the average A_{595} of each BSA standard vs the concentration of BSA. The unknown protein concentration was then calculated from the standard curve.

2.5.2 Assay for Fructosyltransferase Activity

Ftf activity was expressed as the polymer-forming activity determined by the formation of radioactively labelled fructan at 37°C (Jacques & Wittenberger, 1981). The assay reaction was initiated by the addition of 0.5 ml of 40 mM [^{14}C -fructosyl]-labelled sucrose (1.8 kBq ml^{-1}) into a 1.5 ml of reaction mixture containing 100 mM potassium phosphate buffer pH 6.0, 10 mM NaF, 0.1 mM histidine, 1 mM CaCl_2 and an appropriate amount of Ftf. At the end of 60 min the reaction was stopped by transferring the mixture to 6 ml of absolute ethanol and filtering through 2.5-cm Whatman GF/B glass fibre filters (Whatman International Ltd., Maidstone, England) under vacuum. Reaction tubes and filters containing the trapped precipitated fructan were each washed three times with 5 ml of 75 % (v/v) ethanol. The dried filters were suspended in Ultima-Gold[™] scintillation solution (Packard Instrument Co., USA) and counted in a liquid scintillation spectrometer (Beckman LS 9000, Beckman Instruments, Sydney, Australia). One unit of enzyme activity (U) was defined as the amount of Ftf that catalysed the incorporation of 1 μmol of the fructose moiety of sucrose into 75 % (v/v) ethanol-insoluble polysaccharide per min.

2.5.3 SDS-PAGE Gel Electrophoresis

SDS-PAGE gels were run on a Hoefer SE 600 system (Pharmacia Biotech, Melbourne, Australia) or a Bio-Rad Protean II xi cell (Bio-Rad, Sydney, Australia) according to the method of Laemmli (1970). Samples of proteins (20-200 μl) were solubilized at 95°C in a third the volume of SDS sample buffer (0.2 M Tris, 40% [v/v] glycerol, 4% [w/v] SDS, 20% [v/v] β -mercaptoethanol, 0.001% [w/v] bromophenol blue) for 5 min before applying to the gel. Broad Range SDS-PAGE molecular weight markers (1 μl , Bio-Rad, Sydney, Australia) were similarly treated

prior to application to the gel. For the Hoefer SE 600 system, the gels were run at 15 mA until the tracking dye passed through the stacking gel when the current was increased to 30 mA, or alternatively, gels were run at 60 V overnight for 16 h. For the Bio-Rad Protean II xi cell, gels were run at 200 V until the loading dye moved out of the gel (~1 h). The gels were then fixed in 10% (w/v) trichloroacetic acid for 30 min and stained with 'Gradipure' stain overnight, before destaining in 5% (v/v) glacial acetic acid. In some cases, the gels were silver-stained or activity-stained as described below (2.5.5).

2.5.4 Non-Denaturing PAGE Electrophoresis

Non-denaturing PAGE gels were run on a Bio-Rad Protean II xi cell according to the method of Laemmli (1970) as described above (2.5.3) except that no SDS was used in the PAGE gel or the loading buffer and the samples were incubated with the loading buffer at room temperature (18-22°C) for 30 min before loading on the gel. Non-denaturing PAGE M_r markers were used. After electrophoresis, gels were subjected to silver staining as described below (2.5.5).

2.5.5 Detection of Proteins on SDS-PAGE or Non-Denaturing PAGE Gel with Silver Staining

After electrophoresis, gels were washed in Milli-Q water (Millipore, Sydney, Australia) for 5 min before being fixed in 200 ml of ethanol/acetic acid/water (40:10:50, by volume) for 30 min. After fixing, the gels were shaken in ethanol/acetic acid/water (10:10:180, by volume) for 1-16 h, washed in Milli-Q water for 5 min, soaked in 0.5 M sodium acetate containing 1% (v/v) glutaraldehyde for 30 min, washed in Milli-Q water 3 x 10 min and stained in 150 ml of silver-staining solution for 30 min (2 ml of concentrated ammonia added to 28 ml of 0.1 M NaOH followed by slow addition of 5 ml 20% [w/v] AgNO_3 with mixing and then 115 ml of Milli-Q water). After a further 4 x 10 min washes in Milli-Q water, the gels were developed in 200 ml of 0.05% (w/v) citric acid solution containing 0.1% (v/v) formaldehyde until the protein bands could be seen (1-15 min). Acetic acid (5%, v/v) was then added to stop the colour development.

2.5.6 Detection of Fructosyltransferase Activity in SDS-PAGE Gels

Ftf activity in SDS-PAGE gels was determined by a periodic acid Schiff (PAS) reaction (Pitty *et al.*, 1989). After 3 x 30 min washes in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl₂, the SDS-PAGE gels were incubated at 37°C for 1-16 h in 50 mM sodium phosphate buffer (pH 6.5) containing 1% (w/v) sucrose, 1% (v/v) Triton X-100, 0.01% (w/v) thiomersal, 1 mM CaCl₂ and 0.02% dextran T-10. PAS staining was then carried out following the fixation of the gel in 75% (v/v) ethanol for 30 min. Essentially, the gel was shaken for 30 min in a 5% (v/v) glacial acetic acid solution containing 0.7% (w/v) periodic acid followed by a further 3 x 20 min washes in a 5% (v/v) glacial acetic acid solution containing 0.2% (w/v) sodium metabisulphite. Following these washes, the gel was placed in Schiff's reagent for 60 min, followed by 3 x 30 min washes in a 40% (v/v) methanol solution containing 0.5% (w/v) potassium metabisulphite and 5% (v/v) acetic acid. The gel was then destained by repeating washes in a 40% (v/v) methanol solution containing 5% (v/v) glacial acetic acid.

2.5.7 Electroblothing of Proteins from SDS-PAGE onto PVDF Membranes

Electrotransfer of proteins from SDS-PAGE gels to PVDF (Polyvinylidene difluoride) membranes (0.2 µm, Bio-Rad) was carried out on a Hoefer TE Series Transphor electrophoresis unit (Pharmacia Biotech, Melbourne, Australia) following the instructions of Speicher (1997). The transfer buffer contained 10 mM Tris base, 100 mM glycine, 10 % (v/v) methanol and 0.1 mM thioglycolate. The electroblotting was carried out at a constant current of 250 mA for 1.5 h.

2.6 Data Processing

The Sigma Plot program (version 3.06, SPSS Inc., Chicago, U.S.A.) was used to construct all linear, non-linear and bar graphs.

CHAPTER 3

PURIFICATION OF THE RECOMBINANT FRUCTOSYLTRANSFERASE OF *STREPTOCOCCUS SALIVARIUS* *

3.1 Introduction

Oral streptococci produce extracellular fructans and glucans through the activity of Ftfs and Gtfs respectively. These polysaccharides are important cariogenic polymers in dental plaque (1.1.1). Fructans produced from sucrose in the oral cavity were originally ascribed to *S. salivarius* (1.1.2). Although the structure of the fructan produced by *S. salivarius* has been intensively studied, there have been few studies on the Ftf that produces this polysaccharide. This chapter describes the isolation and purification of the Ftf of *S. salivarius* ATCC 25975 prior to the study of its enzymic properties.

Most bacterial Ftfs are extracellular and have been isolated from culture media (1.1.3 and 1.2.1). The Ftf of *S. salivarius* ATCC 25975, however, is cell-bound. It is released from the cell surface and secreted in the presence of sucrose (1.1.3). As a result, the Ftf prepared from the culture supernatant of *S. salivarius* would be expected to contain a certain amount of fructan that was synthesised by the Ftf when sucrose was added to initiate its secretion from the cell surface. The successful cloning of the *ftf* gene of *S. salivarius* into the *E. coli* phagmid, pIBI 31 (1.1.4), however, made it possible to express the Ftf protein in *E. coli* in a soluble form in the absence of added sucrose.

3.1.1 Protein Purification Using Fusion Protein Expression Systems

A number of fusion protein expression systems have been developed, such as the QIAexpress system from QIAGEN (Melbourne, Australia) and the IBI FLAG expression system from Integrated Sciences (Sydney, Australia). With these systems, the gene encoding the protein of interest can be cloned into the expression vector

* Some of the results presented in this chapter have been published (Song and Jacques, 1997, 1999a).

containing a sequence coding for an 'affinity tag'. As a result, the protein of interest can be co-expressed with the 'affinity tag' either attached to the N-terminus or C-terminus. The fusion protein can thus be purified efficiently by affinity chromatography utilising the specific binding of the 'affinity tag' to an appropriate affinity column. Purification using fusion protein expression systems is usually time-efficient and leads to a high yield of the required protein. The Ftf of *S. salivarius*, however, is processed at both its N- and C-terminus (Rathsam and Jacques 1998). Thus purification by a fusion protein expression system was not considered to be a viable alternative for the isolation of the recombinant Ftf of *S. salivarius*.

3.1.2 Protein Purification by Chromatographic Means

Adsorption chromatography, ion-exchange chromatography (IEC) and gel filtration chromatography have been widely used in protein purifications. Gel filtration chromatography differentiates proteins by size. The resins in the column are beads with pores with a fixed range of diameters. The column has two measurable liquid volumes, the external volume consisting of the liquid between the beads, and the internal volume consisting of the liquid within the pores of the beads. Large proteins are excluded from the internal volume and equilibrate only with the external volume and therefore emerge first from the column, while small proteins can access both the external and internal volumes and emerge later.

IEC separates proteins according to their charge. Proteins are ampholytes and the net charge on the proteins is pH dependent. Therefore the pH of the buffer used in IEC is important for the resolution of a mixture of proteins. Positively (negatively) charged proteins pass through the anion (cation) IEC column, while negatively (positively) charged proteins bind to the anion (cation) exchanger. The stronger the electrostatic interaction between the fixed charges on the column and the protein, the higher the concentration of counter-ions required to elute the protein from the column.

Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$, is a form of calcium phosphate. The mechanism for protein adsorption to and desorption from HA is different from that of IEC. Thus HA has unparalleled selectivity and resolution and can be used to separate proteins shown to be homogeneous by other chromatographic and electrophoretic techniques. The mechanism of protein-HA interaction has been proposed to be a

complex process (Gorbunoff and Timasheff, 1984; Gorbunoff, 1984a, b). Amino and carboxyl groups act differently in the adsorption of proteins to HA. When the column is equilibrated with phosphate buffer, amino groups bind to HA through non-specific electrostatic interactions resulting from their positive charge and the general negative charge on the HA column. Carboxyl groups, however, act in two ways. First, they are repelled electrostatically from the negative charge of the column. Second, they bind specifically by complexation to calcium sites on the column, forming clusters of the form, $\text{HACa}-(\text{OOC}-\text{Protein})$. Elution of basic proteins results from either charge screening with ions such as F^- , Cl^- , ClO_4^- , SCN^- , or PO_4^{3-} , or from specific displacement by Ca^{2+} and Mg^{2+} ions which complex with column phosphates and neutralise their negative charge. Acidic proteins are eluted by displacement of their carboxyl groups from HA calcium sites by ions which form stronger complexes with Ca^{2+} than do the carboxyl groups themselves, such as F^- or PO_4^{3-} . In this study a low phosphate concentration was used for protein adsorption and a high phosphate concentration for elution.

3.2 Materials and Methods

3.2.1 Phagemids, Bacterial Strains and Growth Conditions.

Phagemid pKRK1969 contains the gene coding for the Ftf of *S. salivarius* ATCC 25975 (Table 2.2). *E. coli* NM522 (2.2) harbouring pKRK1969 was grown with shaking (150 rpm) at 37°C in Luria-Bertani media (2.3) supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$).

3.2.2 Columns and Chromatographic Systems

The HA resin, Bio-Gel[®] HTP was purchased from Bio-Rad (Sydney, Australia). The empty C16/20 column (1.6 x 20 cm), prepacked Resource-Q ion exchange column (1ml) and prepacked Hiprep 26/60 Sephacryl S-300 High Resolution column (2.6 x 60 cm) were supplied by Pharmacia Biotech (Melbourne, Australia). The peristaltic pump P-1, connecting tubing and FPLC system were also obtained from Pharmacia Biotech.

3.2.3 Expression of Recombinant Fructosyltransferase in *E. coli* at Various Stages of Growth

An overnight culture (16 h) of *E. coli* NM522 harbouring pKRK1969 was used as a 1% (v/v) inoculum for 300 ml of LB medium containing 100 μg ampicillin ml^{-1} . The culture was grown at 37°C in a shaking incubator to early exponential phase (~1.5 h). Aliquots of the culture (5 ml) were removed at various intervals and the A_{600} determined. The cells from each aliquot were harvested by centrifugation (4,000 g, 4°C, 10 min) and resuspended in 1 ml of ice-cold cell lysis buffer (10 mM potassium phosphate buffer pH 6.5, 1 mM CaCl_2 [to stabilise Ftf activity, 1.1.3], 10 mM MgCl_2 [as a cofactor for DNase], 100 μg lysozyme ml^{-1} , 100 μg DNase I ml^{-1} , 100 μg PMSF ml^{-1} [as a protease inhibitor] and 0.05% [w/v] NaN_3 [to inhibit bacterial growth]). The cells from each aliquot were lysed on ice in a 5 ml glass test tube using a sonicator (Branson Sonic Power, Danbury, USA) for a total sonication time of 3 min (40 W, 30 s with 30s-cooling periods). The cell lysates were centrifuged (12,000 g, 4°C, 30 min) and the supernatants (0.9 ml) retained as the source of Ftf, from which 300 μl was used to determine enzyme activity as previously described (2.5.2).

3.2.4 Evaluation of Cell Disruption Methods for Maximising Recombinant Fructosyltransferase Recovery from *E. coli*

LB medium (5 x 1 litre) containing 100 μg ampicillin ml^{-1} was inoculated with 1% (v/v) of an overnight culture of *E. coli* NM522 harbouring pKRK1969 and incubated at 37°C in a shaking incubator to an A_{600} of 0.700. The cells were harvested and resuspended in 125 ml of cell lysis buffer as described above (3.2.3). Aliquots of the cell suspension (10 ml) were disrupted using four different protocols including sonication and three different methods of glass bead disruption (0.5 g acid-washed glass beads [0.10-0.11 mm diameter; Sigma, Sydney, Australia] ml^{-1}) as follows:

- (i) *Sonication*: the cells were sonicated on ice in 20 ml glass sonication tubes (possessing three circulation cooling arms) using a Branson Sonic Power sonicator (40 W, 30 s with 30 s cooling periods) for a total sonication time of 2 min, 4 min or 6 min.
- (ii) *Grinding cells with glass beads*: cell suspensions were ground with a pestle and mortar at 4°C for 10 min, 20 min or 30 min.

- (iii) *Stirring cells with glass beads*: cell suspensions were stirred with glass beads at 4°C in a covered 50 ml beaker on a magnetic stirrer for 10 min, 20 min or 30 min with the stirring speed adjusted just high enough to swirl the glass beads.
- (iv) *Vortexing cells with glass beads*: cell suspensions were vortexed at 4°C with glass beads in a 30 ml glass test tube on the lowest speed that allowed vortexing to occur. Vortexing was continued for 1 min, 2 min or 3 min.

In each case, after cell disruption, the cell lysate was centrifuged (12,000 g, 4°C, 30 min) and the supernatant retained as the source of Ftf activity. The total protein content and the specific activity of the Ftf were determined for each extract (2.5.1, 2.5.2). The glass beads were reused after washing with 200 mM HCl using the following protocol:

- (i) Two volumes of 200 mM HCl was added to the used glass beads and stirred for 30 min using a magnetic stirrer at a speed just high enough to swirl the glass beads. After stirring, the glass beads were allowed to settle and the excess solution decanted.
- (ii) Step (1) was repeated 4 times.
- (iii) The acid washed glass beads were washed with deionised water until the pH approached neutrality.
- (iv) The glass beads were dried at 40-50°C prior to storage for subsequent re-use.

3.2.5 Ammonium Sulfate Fractionation of *E. coli* Lysates

The clear cell lysate (90 ml in a 500 ml beaker), obtained by stirring a cell suspension with acid-washed glass beads for 30 min (3.2.4), was mixed with 10 ml of 1 M potassium phosphate buffer pH 6.5 to give a higher buffering strength (100 mM) to resist the acidification effect of ammonium sulfate, and chilled to 4°C. Solid ammonium sulfate (10.6 g) was added slowly to the 100 ml lysate with stirring to give a 20% saturated solution. The mixture was left stirring for another 30 min at 4°C and the 20% ammonium-sulfate-saturated precipitate recovered by centrifugation (12,000 g, 10 min, 4°C). The supernatant was subjected to further rounds of salting out by successively creating 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% and 80% saturated ammonium sulfate solutions (Englard and Seifter, 1990). The precipitates

successively obtained from each fractionation were re-dissolved in 1 ml of 10 mM potassium phosphate buffer pH 6.5 and dialysed against the same buffer to remove the excess ammonium sulfate before assaying for Ftf activity and protein content.

3.2.6 Hydroxyapatite (HA) Chromatography

3.2.6.1 Packing of the Hydroxyapatite Column

The HA powder (3.2.2) was mixed with 6 parts of starting buffer, 10 mM potassium phosphate pH 6.5, pre-heated at 80°C. The mixture was swirled gently then settled for 10 min at 80°C before decanting the cloudy upper level and the fines at the top of the settled bed. Suspending, settling and decanting was repeated 3 times before the HA was finally resuspended in an equal volume of buffer at room temperature (18 - 22°C) prior to packing of the column. The Pharmacia C 16/20 column (1.6 x 20 cm) was fixed vertically, connected to a peristaltic pump through the bottom outlet and pre-filled with 1/2 bed volume of the starting buffer (18 - 22°C). The HA suspension was swirled gently while pouring into the column and allowed to settle with a flow rate of 0.5 ml min⁻¹. Addition of HA suspension was continued before the HA in the column was totally settled to ensure the even bedding of the HA resin. After packing, the column was equilibrated with 6 bed volumes of starting buffer (4°C) at the same flow rate of 0.5 ml min⁻¹ before loading of the sample.

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3.2.6.2 Protein Separation by Hydroxyapatite Chromatography

The clear lysate containing Ftf activity was dialysed over night (16 h) at 4°C against sterile 10 mM potassium phosphate buffer pH 6.5 before being loaded at a constant flow rate of 0.5 ml min⁻¹ onto the equilibrated HA column at 4°C. The column was washed with 10 mM potassium phosphate buffer pH 6.5 until the A₂₈₀ became steady (~90 ml) prior to elution of Ftf activity with a 10 - 200 mM gradient of potassium phosphate buffer pH 6.5. Aliquots of 3 ml were collected during the elution. The HA column was regenerated by washing with 3 bed volumes of 1 M potassium phosphate buffer pH 6.5 before equilibrating with 10 mM potassium phosphate buffer pH 6.5.

3.2.7 Ion Exchange Chromatography (IEC)

IEC was performed at room temperature (18 - 22°C) using a strong anion exchanger, a Resource-Q column (1 ml), which was connected to a FPLC system. The flow rate was kept constant at 1 ml min⁻¹.

HA fractions containing Ftf activity that had been eluted with 35-70 mM potassium phosphate buffer pH 6.5, were pooled and dialysed against sterile 20 mM piperazine-HCl buffer pH 5.2. The dialysed pooled samples were then loaded onto the Resource-Q column that had been equilibrated with the same buffer. The column was washed with 20 mM piperazine-HCl buffer pH 5.2 until the A₂₈₀ became stable. Proteins were eluted with a 0 - 20 mM NaCl gradient in piperazine-HCl buffer pH 5.2 and 2-ml aliquots collected. Strongly bound proteins were subsequently eluted with 1 M NaCl in 20 mM piperazine-HCl buffer pH 5.2.

Two other buffer systems, 50 mM Tris-HCl pH 7.4 and 20 mM Bis-Tris-HCl pH 6.0 were initially trialled with the NaCl gradient in order to determine the best pH conditions for elution and purification of Ftf by IEC.

The Resource-Q column was stored in 20% (v/v) ethanol between uses after being cleaned by reverse flow using successive 5-ml aliquots of 1 M NaCl, 1 M NaOH, 1 M HCl and 1 M NaCl with 5 ml water rinses between each cleaning step.

3.2.8 Gel Filtration Chromatography

The resource-Q fractions containing Ftf activity that were eluted by 14 - 20 mM NaCl were pooled, dialysed against sterile water and freeze-dried at -50°C. The lyophilised proteins were resuspended in 500 µl of 50 mM potassium phosphate buffer pH 6.5 containing 150 mM KCl and further fractionated at room temperature (18 - 22°C) on a Sephacryl S-300 HR gel filtration column connected to a FPLC system. The same buffer was used to elute the protein and flow rate was kept constant at 0.5 ml min⁻¹. All solutions were de-gassed before applying to the column and 2-ml aliquots were collected.

The Sephacryl S-300 HR column was washed with a half column volume of 0.2 M NaOH at 0.5 ml min⁻¹ followed by at least 2 column volumes of Milli-Q water until the pH of the eluate reached neutrality. The column was stored in 20% (v/v) ethanol when not in use.

3.2.9 Monitoring of the Purification Process

At each stage of the purification, the polymer-forming activity of the Ftf fractions was quantified as previously described (2.5.2). The specific activity was determined by measuring the concentration of protein in each fraction (2.5.1). The degree of purity of the Ftf samples was also monitored by SDS-PAGE using 9% (w/v) polyacrylamide gels as previously described (2.5.3). Apart from the Coomassie blue staining and silver staining of SDS-PAGE gels (2.5.5), activity staining was also used in some cases (2.5.6).

3.2.10 M_r Estimation of the Fructosyltransferase

The M_r of the denatured and native forms of Ftf were determined by discontinuous SDS-PAGE and non-denaturing PAGE respectively (2.5.3, 2.5.4). The M_r of the denatured form of the Ftf was calculated from the calibration curve of $\text{Log } M_r$ vs relative mobility, R_f , of the standards. Four different concentrations (%T) of non-denaturing polyacrylamide gel, 8%, 10%, 12% and 14%, were used. The M_r of the native form of the Ftf was determined from the calibration curve of $-\text{Log } K_r$ vs $\text{Log } M_r$ of the standards, where K_r is the slope of the straight line of $\text{Log } R_f$ of the standard proteins vs %T (Speicher, 1997). The M_r of the native form of the Ftf was also determined by gel filtration. In this instance, the M_r was calculated from the calibration curve of the elution volumes of the standard proteins vs $\text{Log } M_r$ of the standards.

3.2.11 Electrophoretic and N-Terminal Sequencing of Fructosyltransferase

After running a purified sample of Ftf protein (10 μg) on a 9 % SDS-PAGE (9 cm x 14 cm) for 5 h at 200 V to check its purity, the Ftf was electroblotted onto a PVDF membrane (Bio-Rad, Sydney, Australia) at 4°C as previously described (2.5.7). The Ftf on the PVDF membrane was stained for 15 s with amido black reagent consisting of 0.5 % (w/v) amido black, 25 % (v/v) isopropanol and 10 % (v/v) acetic acid, followed by destaining with 6 x 5 min washes with milli-Q water. The PVDF membrane was air-dried at room temperature (18 – 20°C) overnight before the Ftf band was excised. N-terminal sequencing of the protein was carried out at the Biomolecular Resource Facility, Australian National University, Canberra, Australia.

3.3 Results

3.3.1 Effect of Cell Growth on the Level of Activity of Fructosyltransferase

In order to determine the best time to harvest *E. coli* expressing the recombinant Ftf, Ftf activity was monitored during cell growth as described above (3.2.3). Ftf activity reached a maximum at $A_{600} = 0.700$, beyond which it started to decrease even though the cell density continued to increase (Figure 3.1). This could be due to the action of proteases when the cells autolysed at higher densities. As a result of this finding, all subsequent Ftf preparations were obtained from cells harvested at $A_{600} = 0.700$. The protease inhibitor PMSF was added to the cell lysis buffer. Less than 5% decrease in Ftf activity was observed after the crude lysate had been stored at 4°C for 6 weeks suggesting that 10 mM phosphate buffer pH 6.5 containing 1 mM CaCl_2 , 100 μg PMSF ml^{-1} and 0.05% [w/v] NaN_3 is an appropriate stabilising buffer for the Ftf protein.

3.3.2 Evaluation of Cell Disruption Methods for Maximising Recombinant Fructosyltransferase Recovery from *E. coli*

In order to determine the best way to lyse *E. coli* to recover active recombinant Ftf, several different methods of mechanical disruption were compared (Figure 3.2). Although sonication (6 min), grinding (10 min), bead-vortexing (2 min) and bead-stirring (30 min) gave comparable yields of protein, the bead-stirring method gave a higher specific activity for the Ftf (Figure 3.2). Bead-stirring was the only lysis condition that gave virtually constant specific Ftf activity irrespective of the time interval of cell lysis. This implied a lower denaturing shear force with this method. The SDS-PAGE analysis of the proteins were indistinguishable irrespective of the cell lysis method used (Figure 3.3).

While sonication and bead-vortexing are not practical for large-scale cell disruption, there was no such limitation to the bead-stirring method. When cell suspensions of 200 ml volume (i.e. from 8 l culture) were disrupted using this method, the total protein yield was 1.32 ± 0.40 mg protein ml^{-1} ($n = 8$; range 1.02 - 1.96 mg protein ml^{-1}) and the Ftf specific activity was 1.46 ± 0.21 U (mg protein) $^{-1}$ ($n = 8$; range 1.16 - 1.76 U [mg protein] $^{-1}$).

It was concluded that bead-stirring was a simple, gentle, efficient and inexpensive method for disrupting *E. coli* cells for the recovery of recombinant

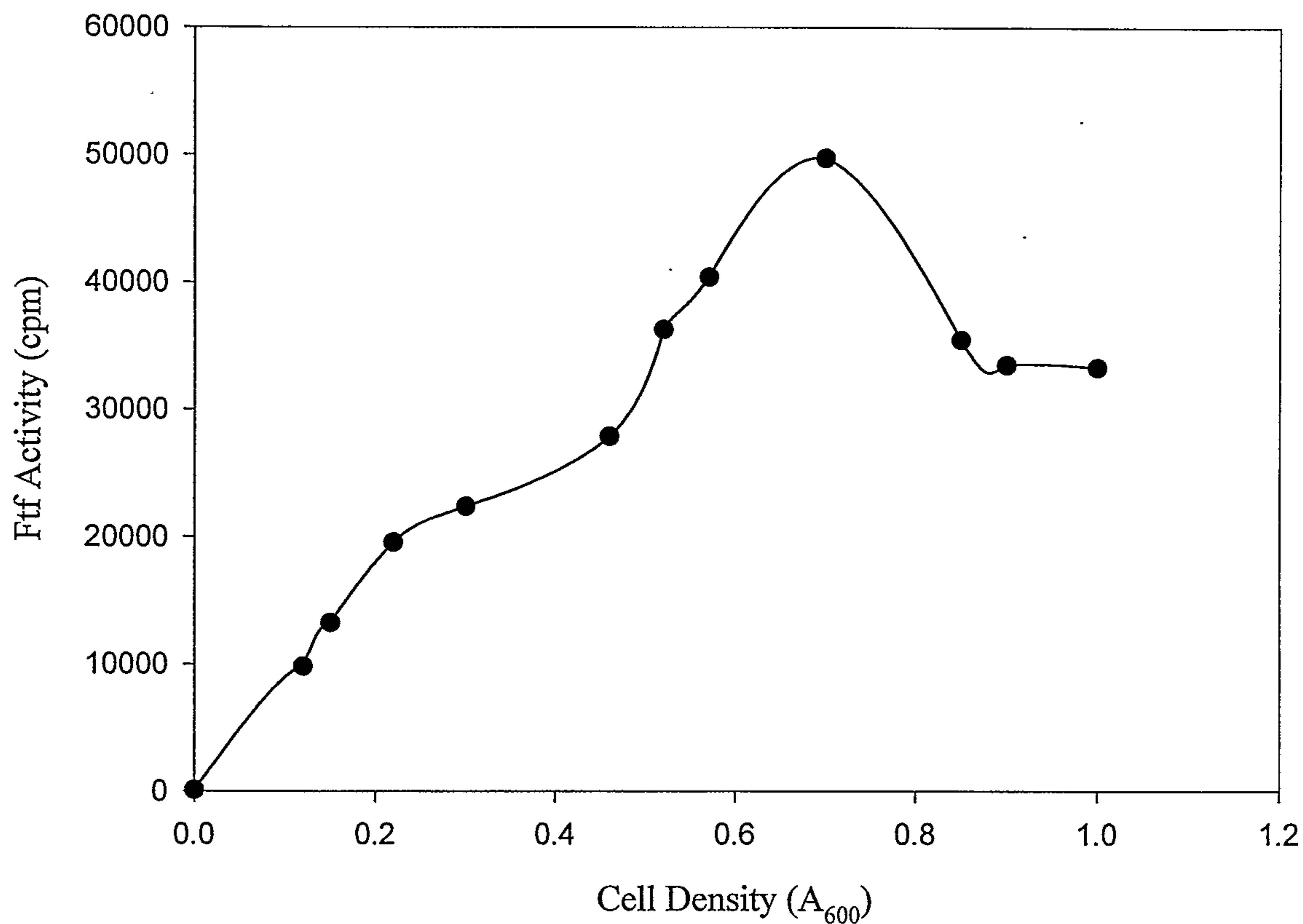


Figure 3.1: Recovery of recombinant Ftf activity at various stages of growth of *E. coli* NM522. The activity was determined by measuring the amount of [U- 14 C-fructosyl]-labelled fructan produced by the Ftf obtained from 5 ml of culture.

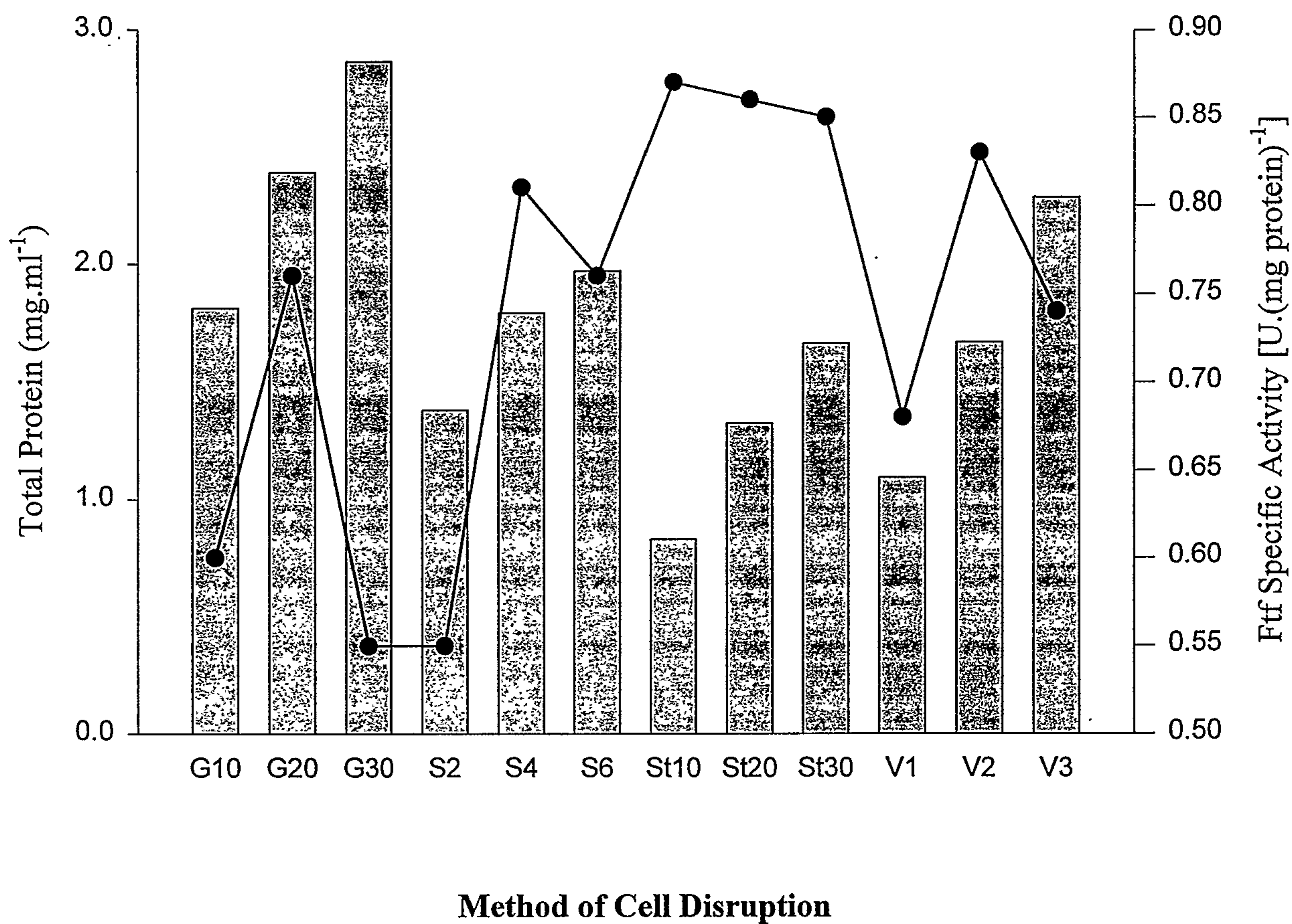


Figure 3.2: Total protein (\square) and Ftf specific activity (\bullet) in the soluble fractions of *E. coli* NM522 harbouring plasmid pKRK1969 following cell disruption using different techniques. S2, S4 and S6, sonication for 2, 4 and 6 min respectively; G10, G20 and G30, grinding with a pestle and mortar for 10, 20 and 30 min respectively; St10, St20 and St30, bead-stirring for 10, 20 and 30 min respectively; V1, V2 and V3, bead-vortexing for 1, 2 and 3 min respectively.

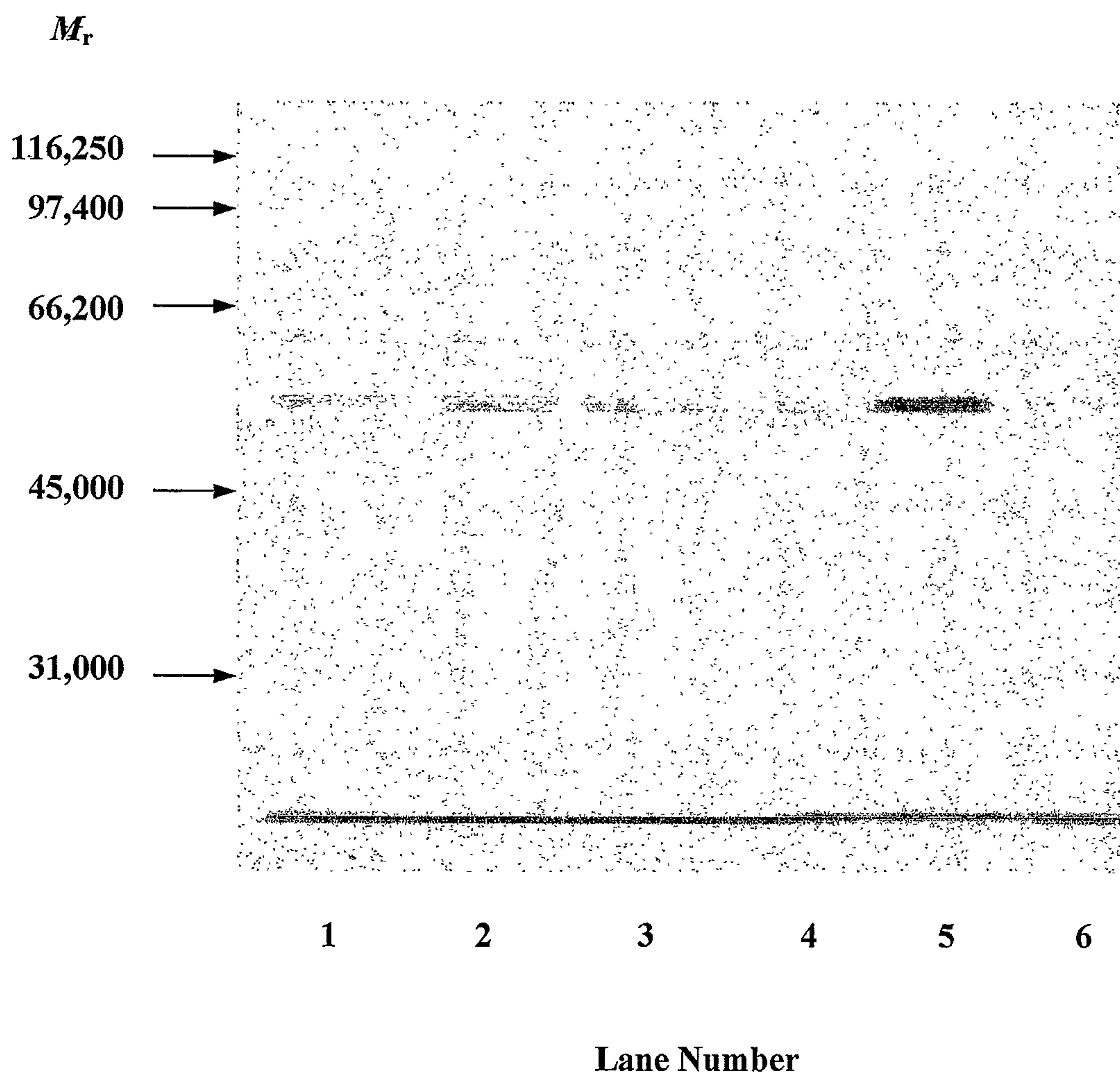


Figure 3.3: SDS-PAGE analysis of cell lysates obtained using different mechanical disruption techniques following Commassie blue staining. Lane 1, sonication 4 min; Lane 2, bead-grinding 30 min; Lane 3, bead-stirring 30 min; Lane 4, bead-vortexing 1 min; Lane 5, bead-vortexing 2 min; Lane 6, bead-vortexing 3 min.

proteins without the need for specialised equipment. This technique has been published (Song and Jacques, 1997) and was used routinely as a first step in both small scale and larger scale Ftf purification in this study.

3.3.3 Ammonium Sulfate Fractionation of *E. coli* Lysates

Fractionation with ammonium sulfate was carried out as described (3.2.5) with the Ftf activity and protein content of each fraction being compared (Table 3.1). Although fine fractionation steps (5% increments) were followed, Ftf activity was found in all the fractions and none of the fractions showed a significantly improved specific activity compared with the cell lysate (Table 3.1). The recovery of Ftf activity was also very low. Loss of activity could be due to protein denaturation during the salting out procedure. It was concluded that purification of Ftf by ammonium sulfate fractionation was impractical and therefore this method was not pursued.

Table 3.1: Protein content and Ftf activity obtained by ammonium sulfate fractionation.

(NH ₄) ₂ SO ₄ Fraction (% Saturation)	Specific Ftf Activity [U (mg protein) ⁻¹]	Total Protein (mg)	Protein Yield (%)	Total Activity (U)	Activity Yield (%)
Lysate	2.39	35.600	100	85.203	100
20	2.85	0.149	0.42	0.425	0.50
25	2.61	0.132	0.37	0.345	0.40
30	1.88	0.249	0.70	0.468	0.55
35	1.93	0.412	1.16	0.794	0.93
40	2.82	1.086	3.05	3.059	3.59
45	1.97	1.738	4.88	3.425	4.02
50	1.10	3.046	8.56	3.341	4.51
55	1.00	2.934	8.24	2.931	4.70
60	0.32	2.854	8.02	0.920	1.08
80	0.02	9.020	25.34	0.192	0.23
80 Sup*	0	3.128	8.79	0	0

* Supernatant of 80% saturation.

3.3.4 Hydroxyapatite Chromatography of *E. coli* Lysates

Proteins were eluted from the HA column with a potassium phosphate gradient (3.2.6.2) and 3-ml aliquots collected. The major peak of Ftf activity was eluted with 35-70 mM potassium phosphate buffer pH 6.5 (Figure 3.4) and the specific activity of these fractions ranged from 3.13 to 7.52 U (mg protein)⁻¹. These fractions were pooled and retained for further purification. The recovery of Ftf activity and protein was 74% and 22% respectively with a 3-fold increase in the specific activity to 5.5 U (mg protein)⁻¹ from that of the cell lysate.

The protein profile of the various fractions was determined by SDS-PAGE and Coomassie blue staining (Figure 3.5a). Activity staining of the SDS-PAGE gel revealed that the major protein band(s) possessing Ftf activity had a M_r of approximately 100,000 (Figure 3.5b). Residual activity was also found with some small fragments which presumably were the degraded fragments of the Ftf (Figure 3.5b).

3.3.5 Resource-Q Ion Exchange Chromatography of Pooled Fructosyltransferase Fractions from Hydroxyapatite Chromatography

IEC using 20 mM piperazine-HCl, pH 5.2 gave good separation of the Ftf from other contaminating proteins (Figure 3.6 and 3.7). About 80% of the contaminating proteins passed through the column without binding under this pH condition. The Ftf activity bound only weakly to the column and was readily eluted with 14-20 mM NaCl (Figure 3.6). The fractions containing Ftf activity (95-118; Figure 3.6) were pooled. The recovery of Ftf activity and protein from the column was 31% and 4.2% respectively, for an increase in Ftf specific activity to 58 U (mg protein)⁻¹.

The other two buffer systems of higher pH values of pH 6.0 and 7.4 that were evaluated were unable to separate proteins of M_r 100,000 (Figures 3.8 and 3.9) of which Ftf was but one (Figure 3.5b). Thus these alternative pH conditions were not appropriate for IEC of the recombinant Ftf.

3.3.6 Sephacryl S-300 Gel Filtration of Pooled Fractions Containing Fructosyltransferase Activity from Ion Exchange Chromatography

The pooled and concentrated Resource-Q fractions (500 μ l) were further fractionated on a Sephacryl S-300 column. The majority of the Ftf activity was found in the first

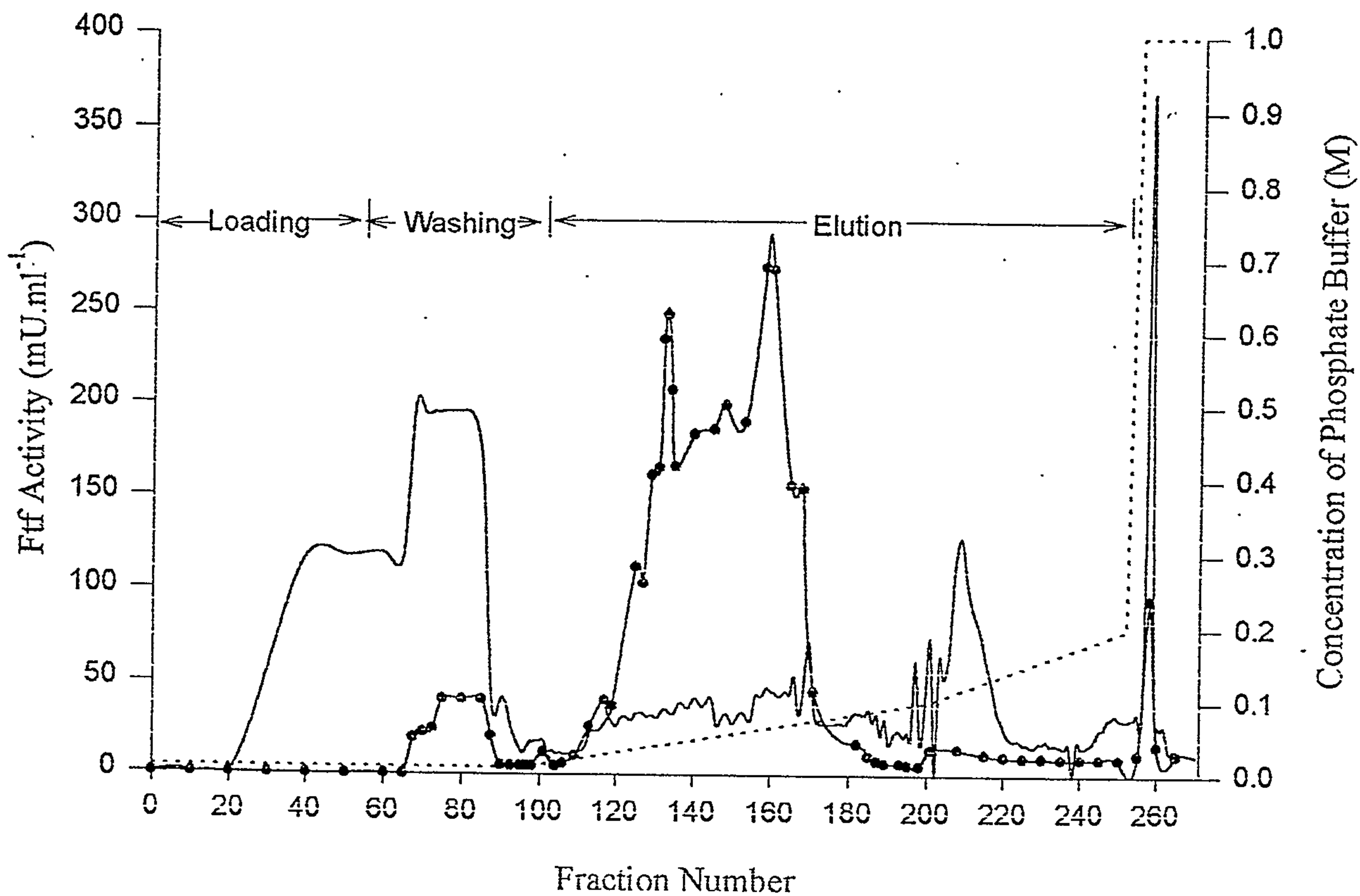


Figure 3.4: Typical hydroxyapatite chromatographic profile of an *E. coli* lysate.

(—) A₂₈₀ (arbitrary units); (—●—) polymer forming activity measured using [U-¹⁴C-fructosyl]-labelled sucrose; (·····) concentration gradient of phosphate buffer.

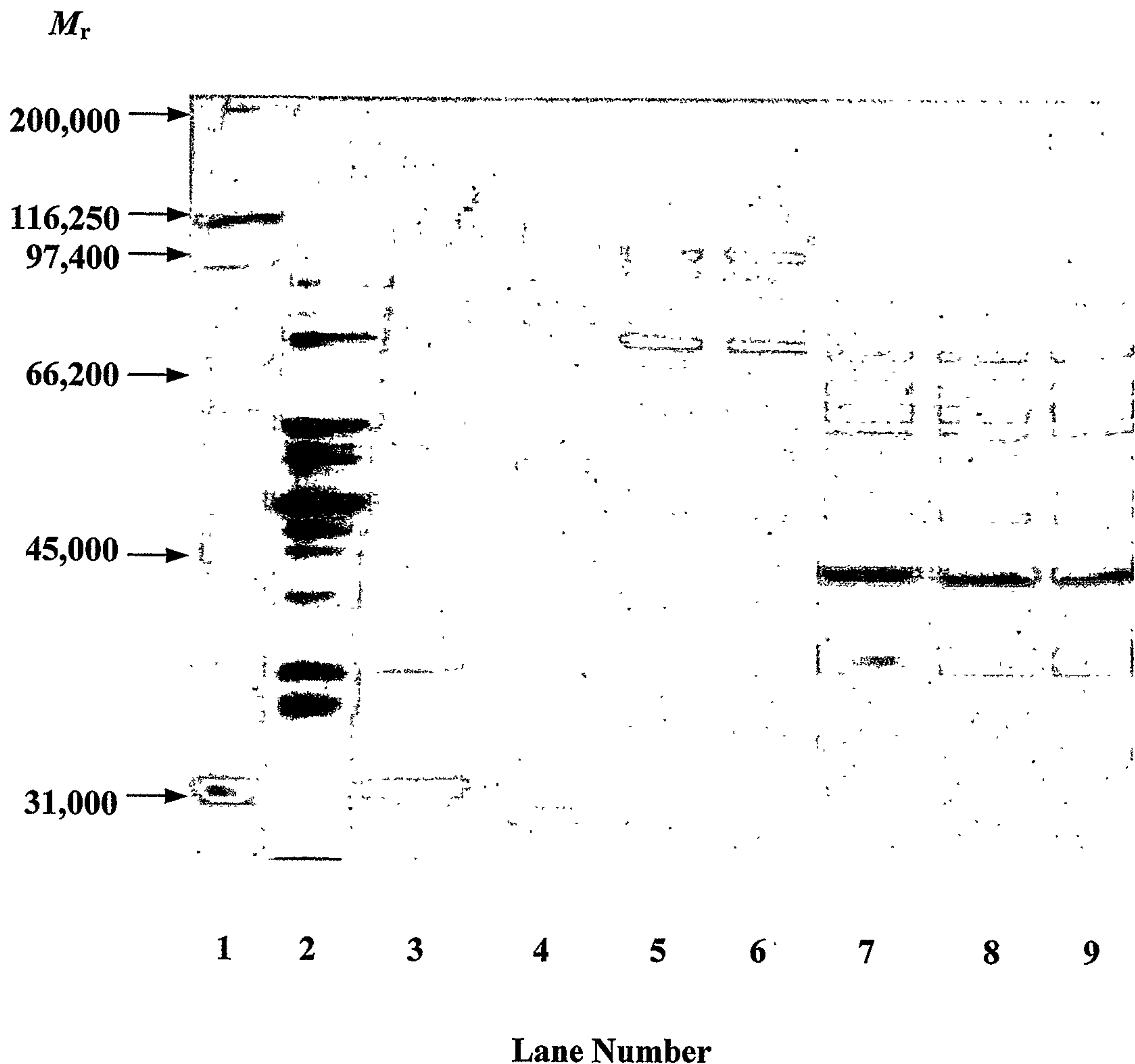


Figure 3.5a: SDS-PAGE analysis of Ftf fractions obtained by HA chromatography following Coomassie blue staining (cf. Figure 3.4). **Lane 1**, broad range M_r markers: Myosin (200,000), β -galactosidase (116,250), Phosphorylase B (97,400), Serum albumin (66,200), Ovalbumin (45,000) and Carbonic anhydrase (31,000).; **Lane 2**, cell lysate; **Lane 3**, initial column eluate (washing with 10 mM phosphate buffer pH 6.5); **Lanes 4-6**, fraction number 130, 140 and 160 eluted with 35-70 mM potassium phosphate buffer pH 6.5; **Lanes 7-9**, fraction number 185, 200 and 210 eluted with 70-200 mM potassium phosphate buffer pH 6.5.

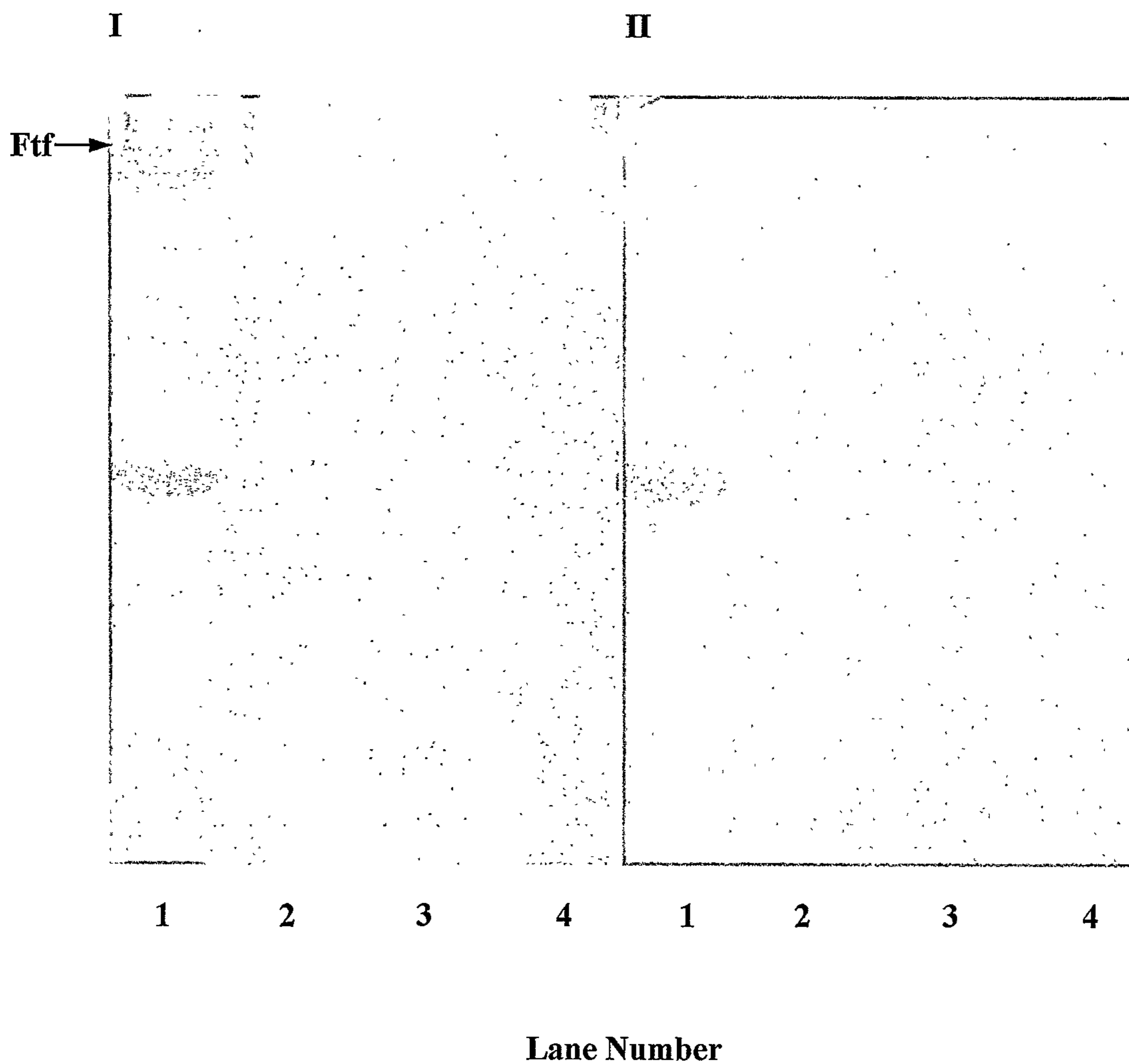


Figure 3.5b: SDS-PAGE analysis of Ftf fractions from HA chromatograph following staining for Ftf activity (cf. Figure 3.4). (I), activity staining with raffinose. (II), activity staining without raffinose, i.e. non-specific staining. Lane 1, cell lysate; Lanes 2-4, fraction number 130, 140 and 160, eluted with 35-70 mM potassium phosphate buffer pH 6.5. Bands detected on (I) but not on (II) indicate proteins possessing Ftf activity. In addition to the intact Ftf, some degraded fragments of the Ftf also were stained by raffinose as shown in Lanes 3 and 4 on (I).

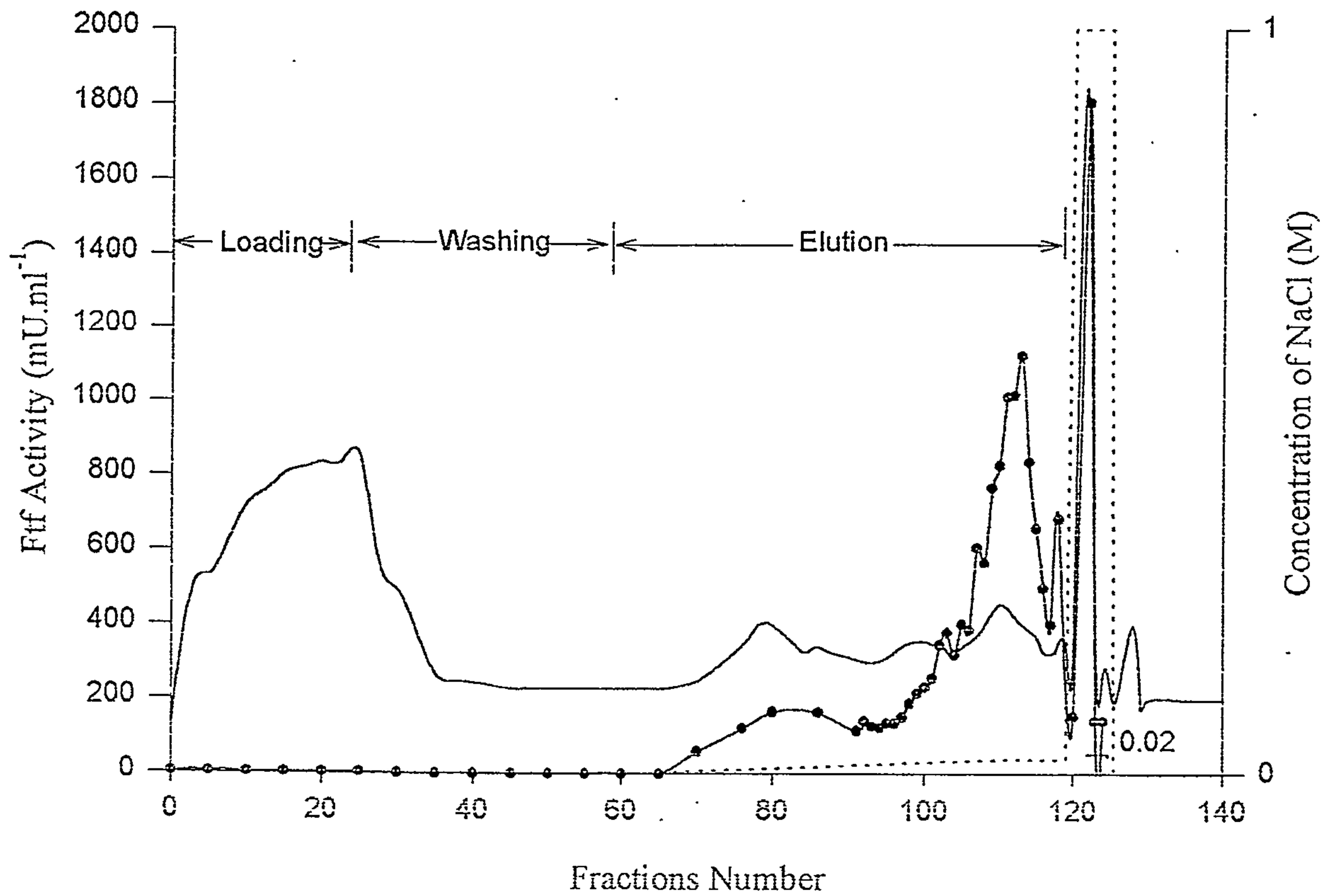


Figure 3.6: Typical Resource-Q ion exchange chromatographic profile of pooled Ftf fractions from HA chromatography using the 20 mM piperazine-HCl pH 5.2 buffer system. (—) A₂₈₀ (arbitrary units); (—●—) polymer forming activity measured using [U-¹⁴C-fructosyl]-labelled sucrose; (·····) NaCl concentration gradient.

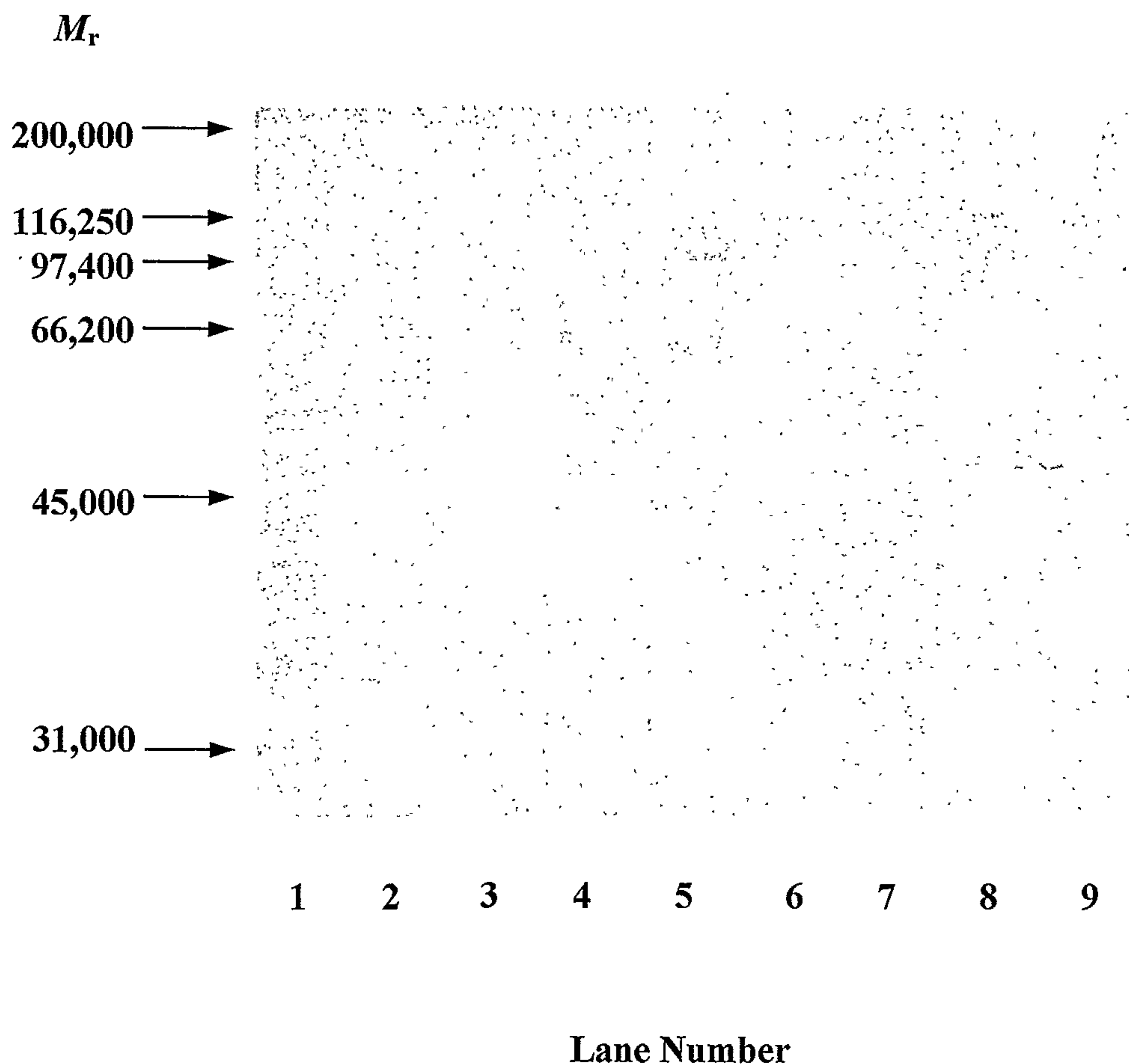


Figure 3.7: SDS-PAGE analysis of fractions containing Ftf activity eluted from the Resource-Q ion exchange column using the 20 mM piperazine-HCl pH 5.2 buffer system following Coomassie blue staining (cf. Figure 3.6). Lane 1, Ftf sample applied to column; Lane 2, initial column eluate (washing with starting buffer); Lanes 3-4, fraction number 80 and 85 eluted with 0-14 mM NaCl; Lanes 5-9, fraction number 100, 105, 110, and 115 eluted with 14-20 mM NaCl.

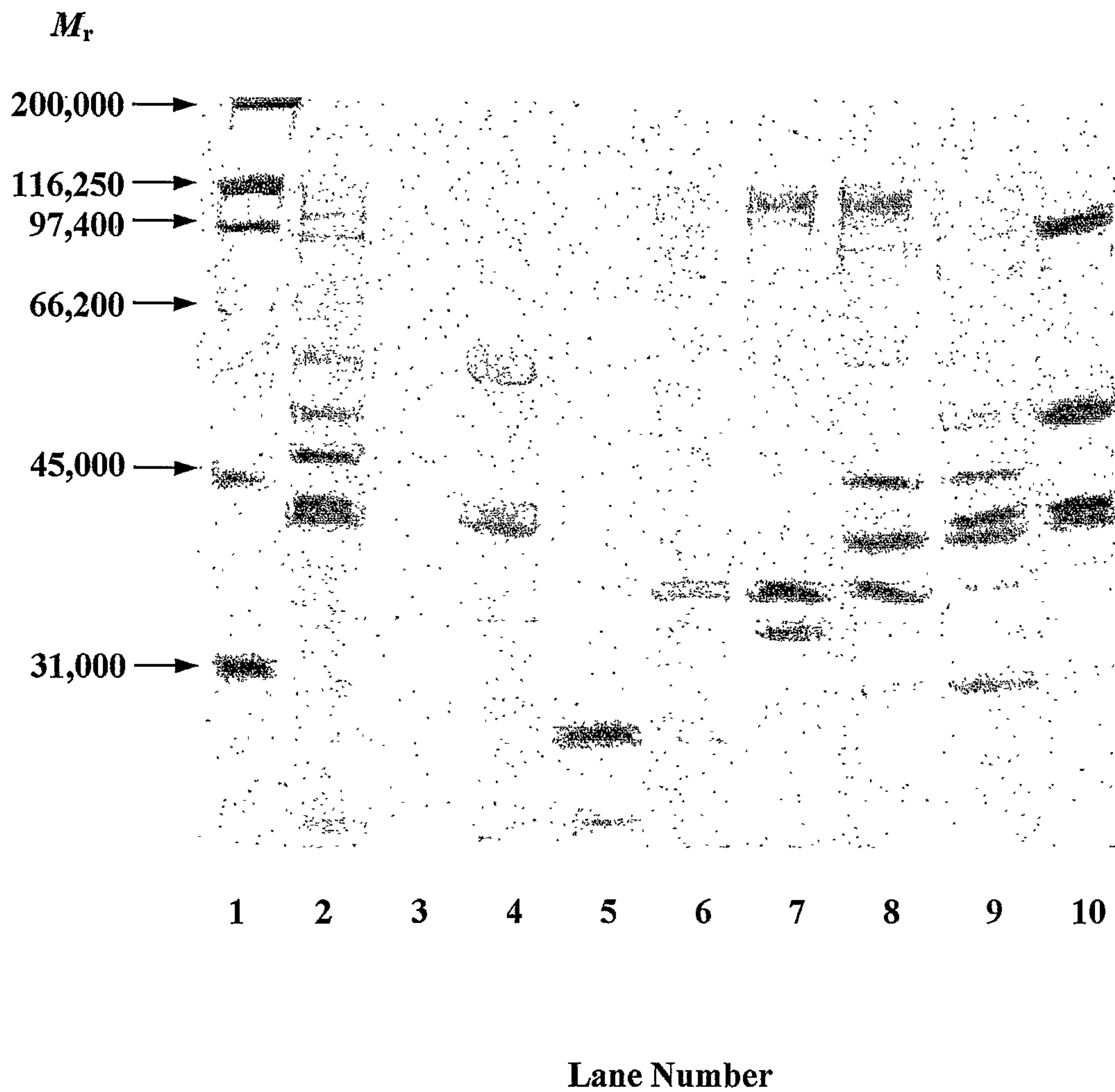


Figure 3.8: SDS-PAGE analysis of fractions containing Ftf activity eluted from a Resource-Q ion exchange column using the 50 mM Tris-HCl pH 7.4 buffer system following Coomassie blue staining. **Lane 1**, broad range M_r markers (cf. Figure 3.5a); **Lane 2**, Ftf sample applied to column; **Lane 3**, initial column eluate (washing with the starting buffer); **Lanes 4-10**, selected protein peaks eluted with the NaCl gradient.

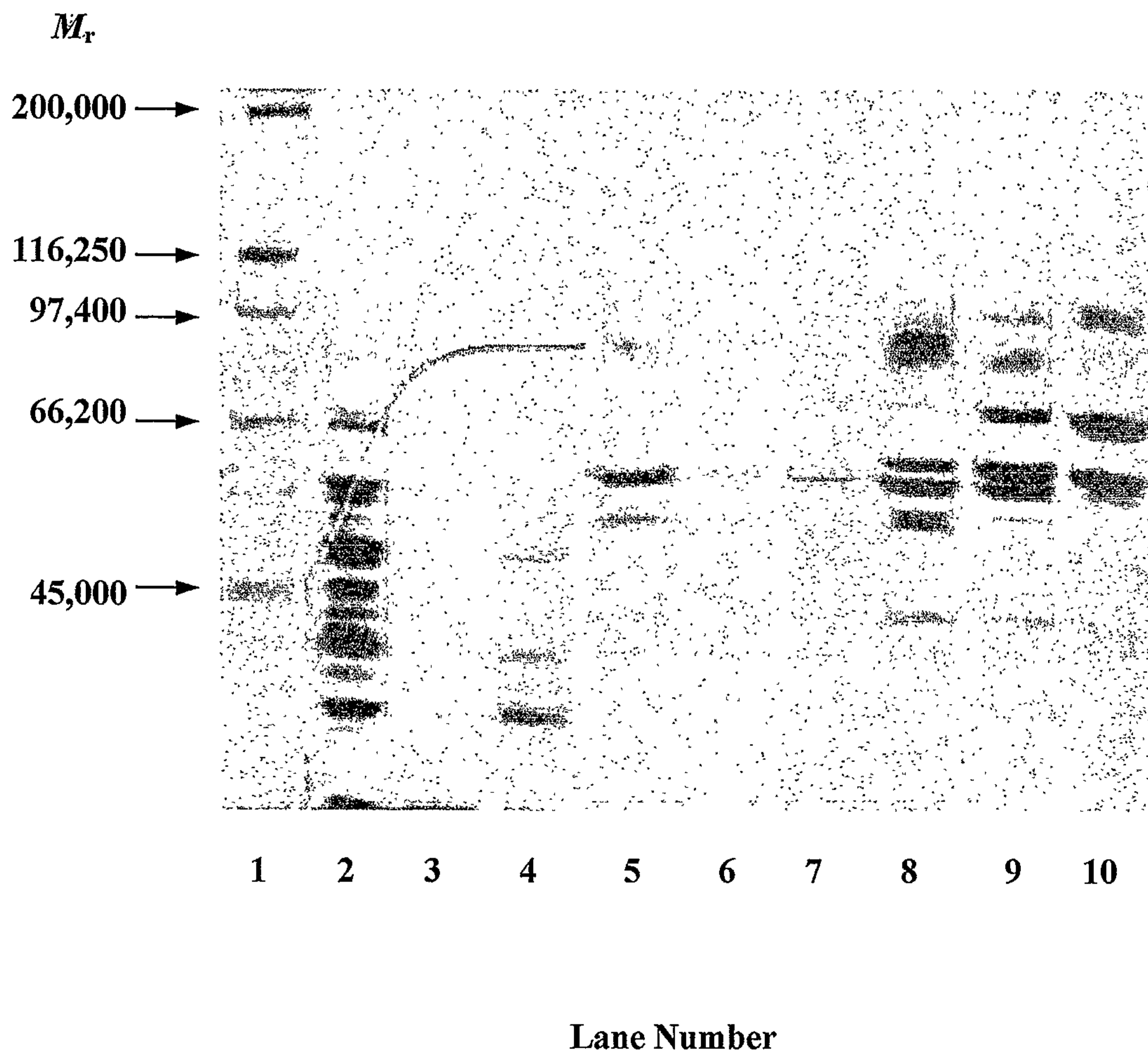


Figure 3.9: SDS-PAGE analysis of fractions containing Ftf activity eluted from a Resource-Q ion exchange column using the 20 mM Bis-Tris-HCl pH 6.0 buffer system following Coomassie blue staining. Lane 1, broad range M_r markers (cf. Figure 3.5a); Lane 2, Ftf sample applied to column; Lane 3, initial column eluate (washing with the starting buffer); Lanes 4-10, selected protein peaks eluting with the NaCl gradient.

protein peak eluted (fractions 70 - 90) as shown in Figure 3.10. Some Ftf activity was also found in the second peak (fractions 95 -120), which contained degraded Ftf proteins (Figure 3.11) corresponding to the lower bands in Figure 3.5b(I). The fractions of the first protein peak (fractions 70-90) were pooled and found to possess a specific Ftf activity of 58 U (mg protein)⁻¹. While the pooled fractions of the second peak showed a number of protein bands, those of the first peak showed only one band on SDS-PAGE after silver staining (Figure 3.11). The purified Ftf was freeze-dried at -50°C after being dialysed against deionised water. The lyophilised enzyme was stored at -20°C prior to further use. No significant decrease in activity was observed in the lyophilised enzyme when stored at -20°C for up to 12 months.

3.3.7 M_r of the Purified Fructosyltransferase

The apparent M_r of the denatured form of the purified Ftf was 125,400 when evaluated by SDS-PAGE (Figure 3.12). The M_r of the native enzyme estimated from gel filtration was 180,600 (Figure 3.13) and 102,000 from non-denaturing PAGE (Figure 3.14 a, b & c). The differences in measured M_r could be due to the various procedures used. The elution volume of a protein in a gel filtration column is dependent on its M_r as well as its shape. Although the 3-D structure of the Ftf of *S. salivarius* is not known, the levansucrase of *B. subtilis* has been shown to be an elongated prolate ellipsoid with the dimensions 26 x 32 x 117 Å (1.2.1). It is reasonable to presume that part of the structure of the Ftf of *S. salivarius* is similar to that of the levansucrase of *B. subtilis* based on the high homologies in the amino acid sequences of both enzymes (Figure 1.4). The highly hydrophilic proline-rich C-terminal extension of the Ftf of *S. salivarius* (1.1.5) may further extend the long axis of the protein. The M_r standard proteins used in the Sephacryl S-300 gel filtration, however, were globulin proteins. An elongated-shape protein has bigger dimensions than a globulin protein of the same M_r and is eluted earlier from the gel filtration column. As a result, the M_r 180,600 of the native Ftf determined by gel filtration using globulin protein standards could be an over-estimation of its real value. In contrast, although the Ftf and the M_r standard proteins used in the non-denaturing PAGE were also of different shapes, the effects of the protein shape have been calibrated by plotting Log R_f of each standard vs %T of the acrylamide gel. The K_r

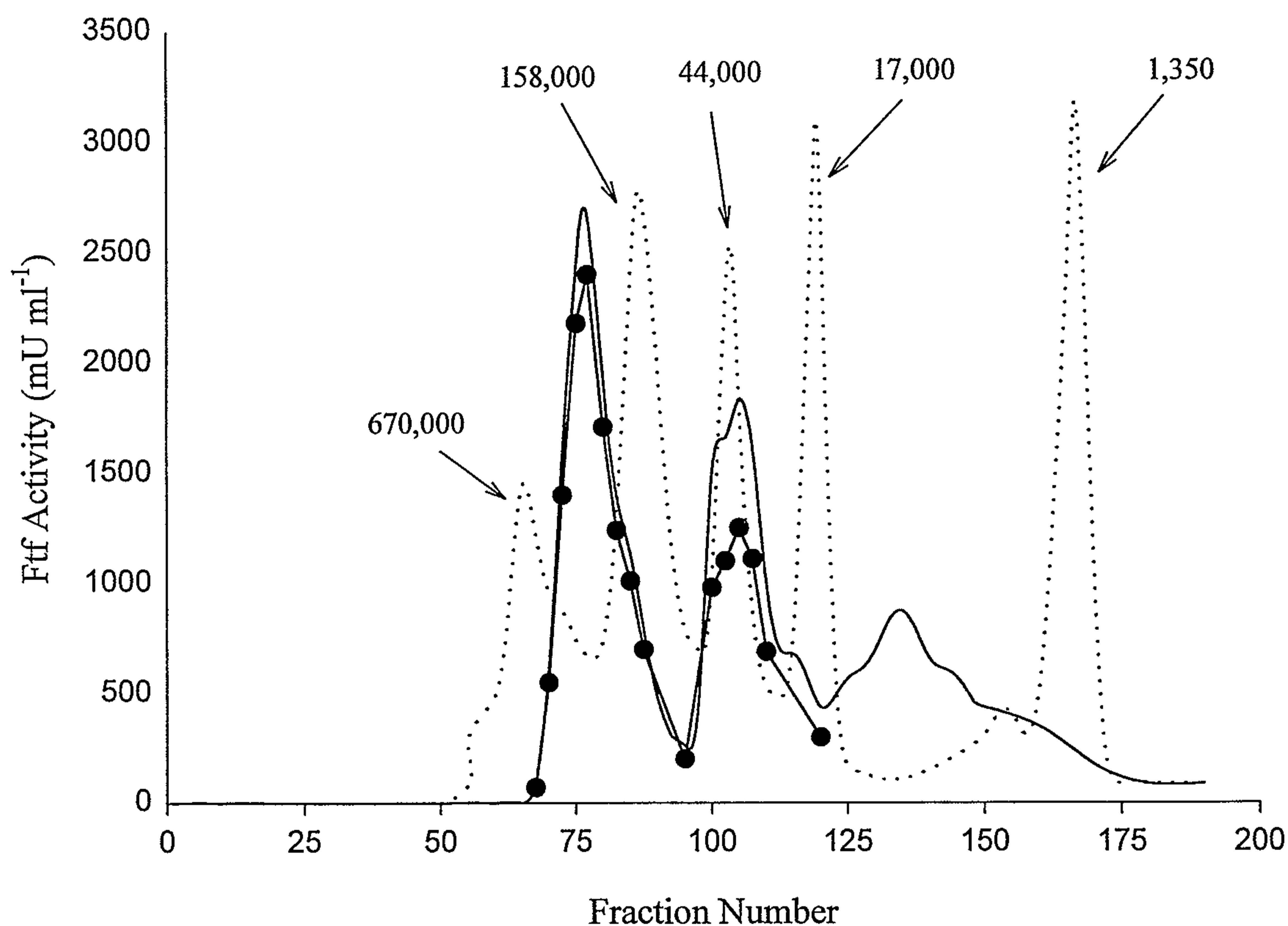


Figure 3.10: Typical Sephacryl S-300 gel filtration chromatographic profile of pooled Ftf fractions from IEC. (—) A_{280} (arbitrary units); (—●—) polymer forming activity measured using $[U-^{14}C]$ -fructosyl-labelled sucrose; (·····) M_r standards: thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000) and vitamin B-12 (1,350).

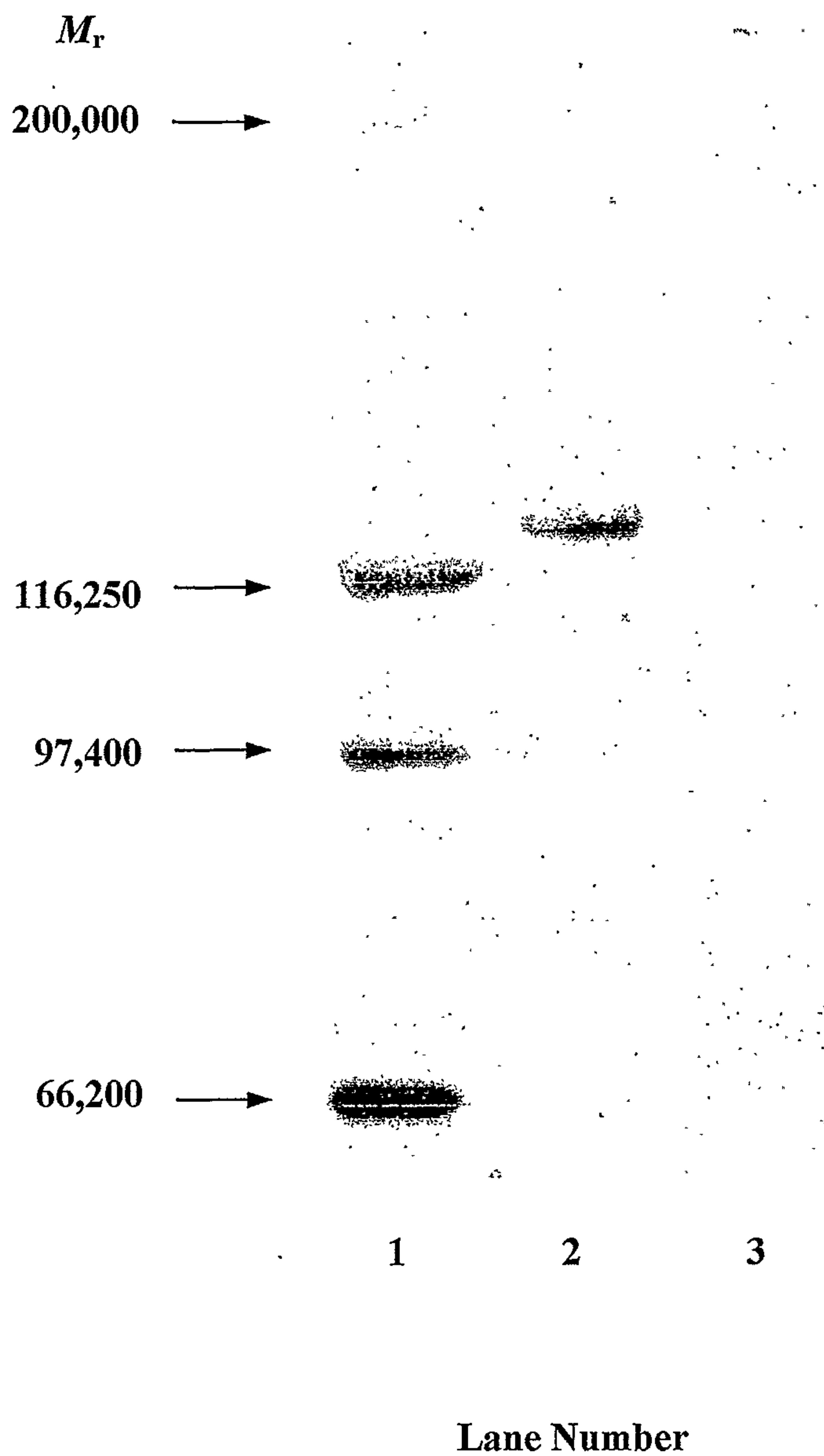


Figure 3.11: SDS-PAGE analysis of fractions from the Sephacryl S-300 gel filtration following silver staining. Lane 1, broad range M_r markers (cf. Figure 3.5a); Lane 2, pooled Ftf fractions of the first peak (cf. Figure 3.10); Lane 3, pooled fractions of the second peak (cf. Figure 3.10).

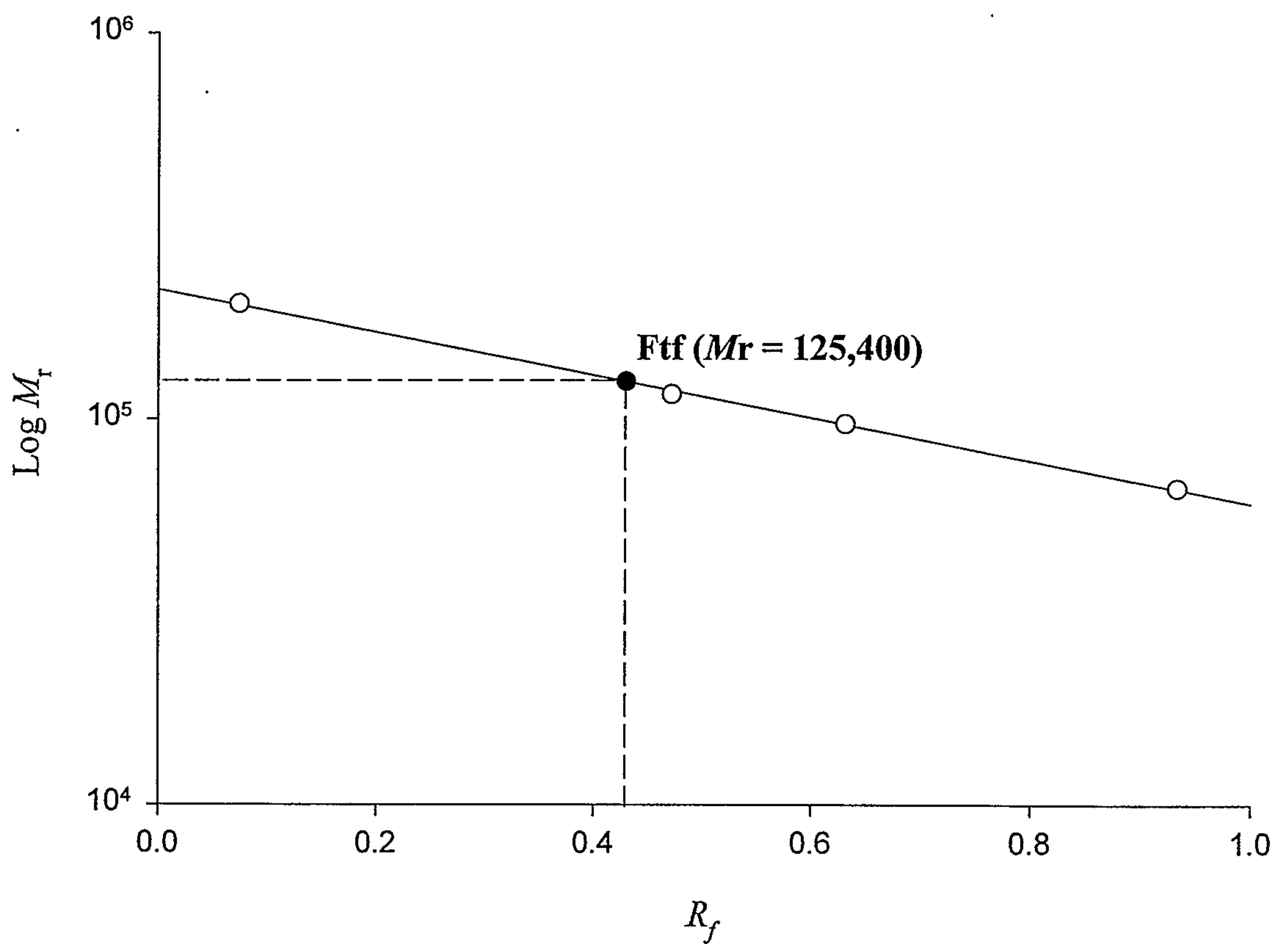


Figure 3.12: M_r calibration graph obtained from SDS-PAGE by plotting the $\text{Log } M_r$ vs the relative mobility of the standards, R_f (\circ). The graph ($r^2 = 0.999$) was used for estimating the M_r of the denatured form of the recombinant Ftf (\bullet). R_f values were measured from Figure 3.11.

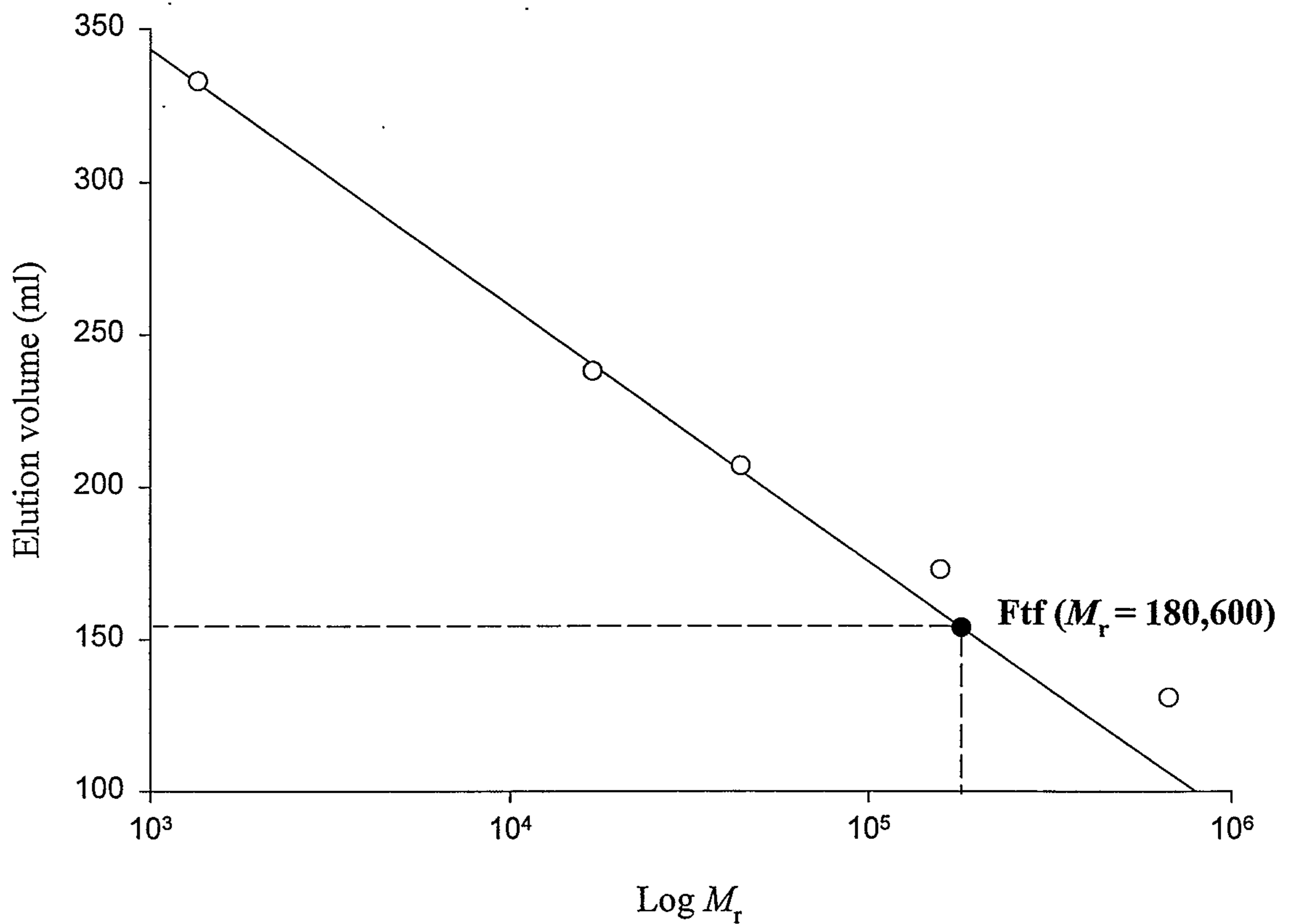


Figure 3.13: M_r Calibration graph for the Sephacryl S-300 column. The graph was obtained by plotting the protein elution volumes from Figure 3.10 vs the Log M_r of the five standards (○). The graph ($r^2 = 0.966$) was used for estimating the M_r of the native form of the recombinant Ftf (●).

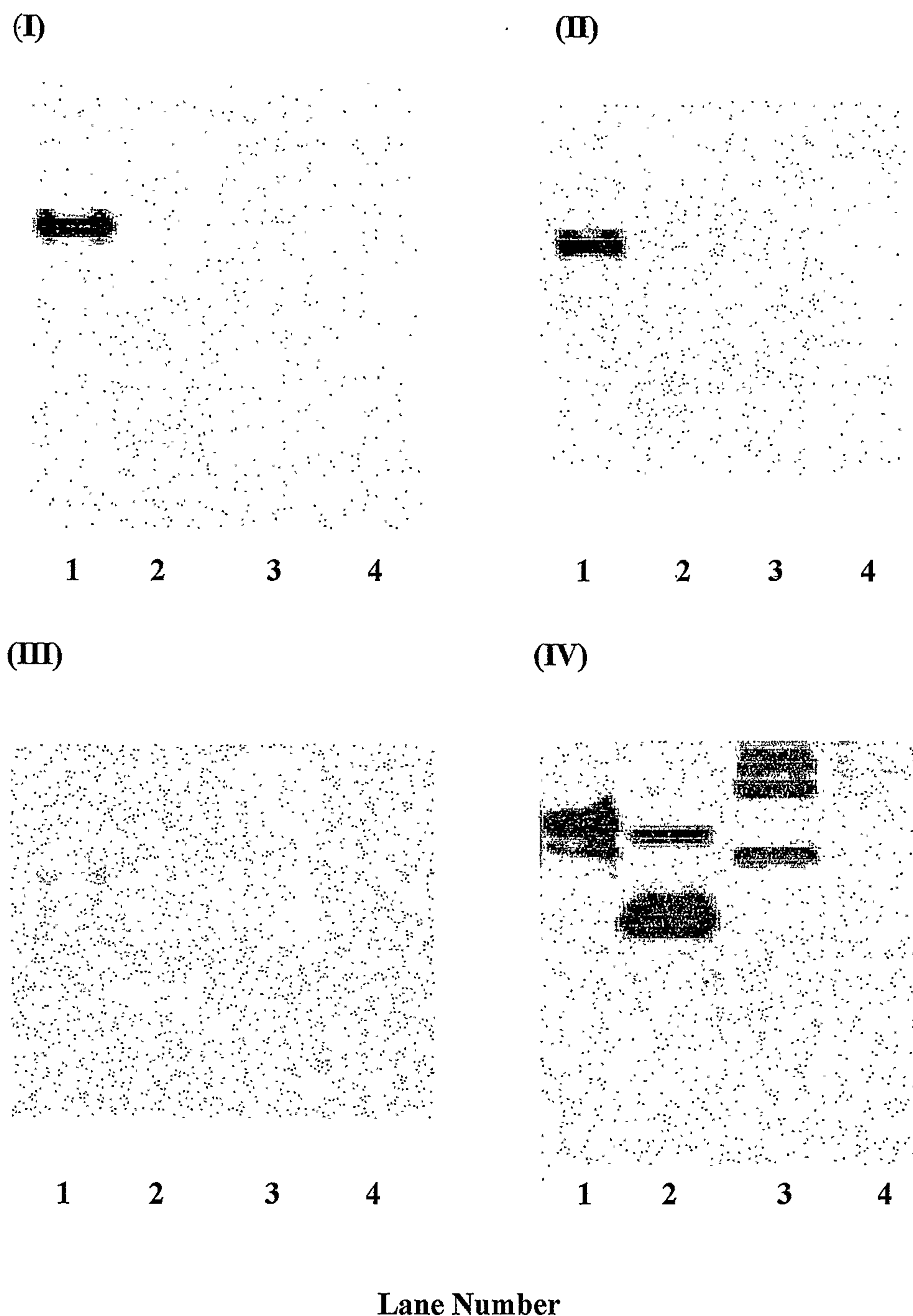


Figure 3.14a: Non-denaturing discontinuous PAGE analysis of standards and purified Ftf following silver staining. (I) 8% gel; (II) 10% gel; (III) 12% gel; (IV) 14% gel. Native M_r standards included: Lane 1, carbonic anhydrase (29,000); Lane 2, chicken egg albumin (45,000, 90,000); Lane 3, bovine serum albumin (66,000, 132,000). Lane 4, Ftf.

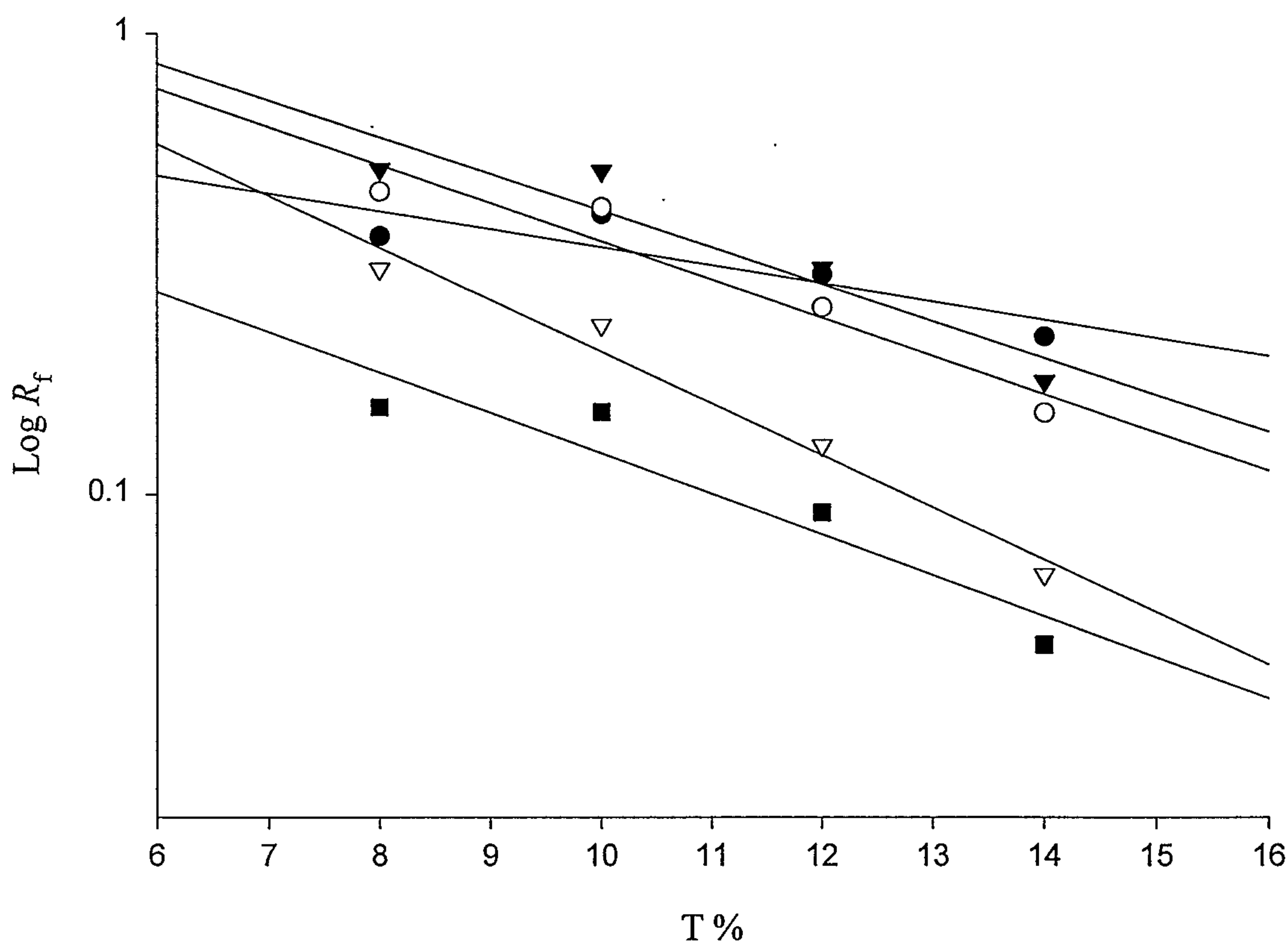


Figure 3.14b: Plot of the logarithm of the relative mobility, $\text{Log } R_f$, of the standard proteins against the concentrations of polyacrylamide gel (% T) for non-denaturing PAGE. R_f values were measured from Figure 3.14a. The slope of each line for the standard proteins ($r^2 = 0.860, 0.887, 0.890, 0.927, 0.973$), K_r , was determined from the graphs and used in plotting Figure 3.14c. (●) carbonic anhydrase 29,000; (○) chicken egg albumin 90,000; (▼) bovine serum albumin 66,000; (▽) bovine serum albumin 132,000; (■) Ftf.

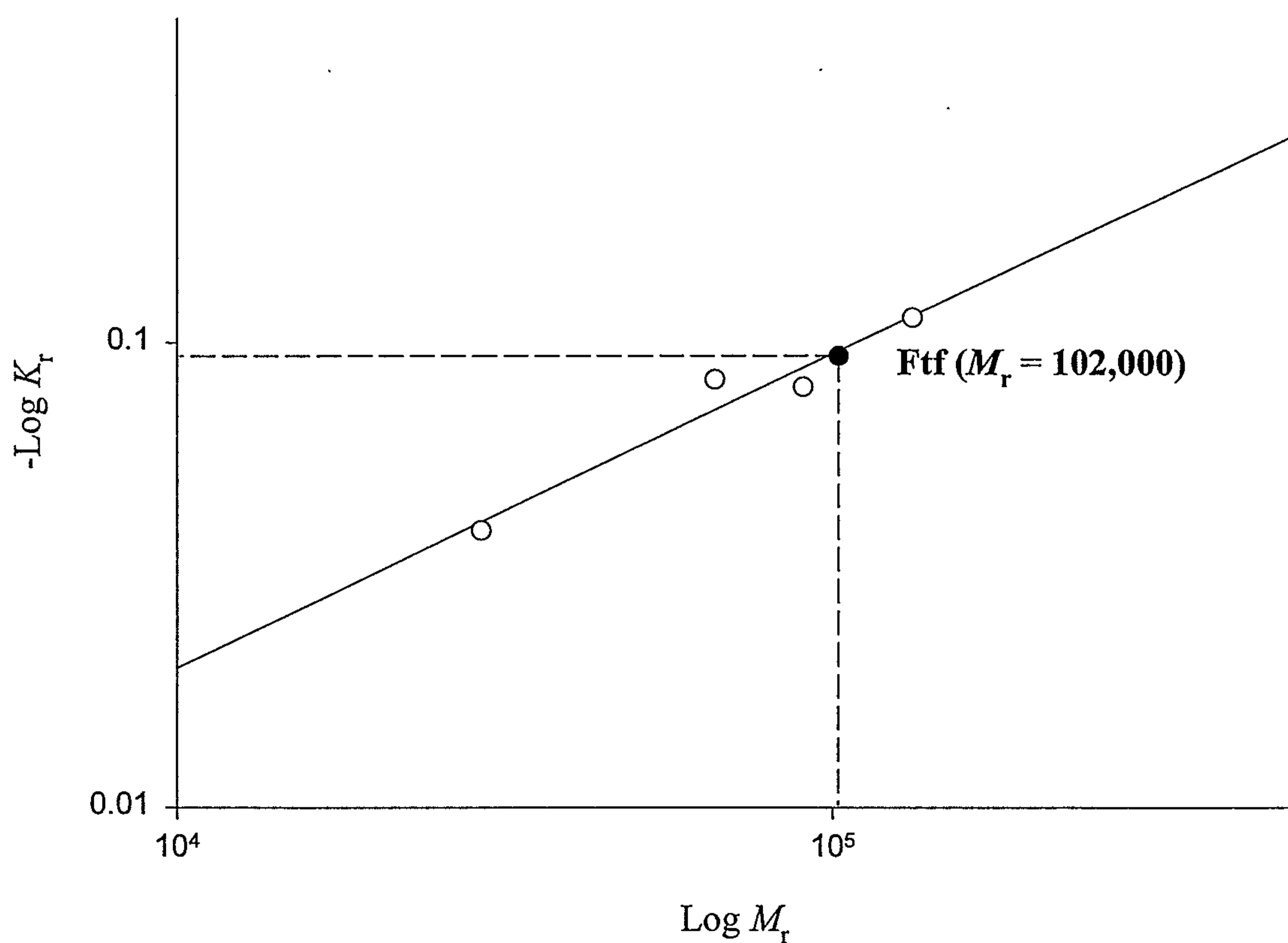


Figure 3.14c: Calibration curve of the native PAGE obtained by plotting the $-\text{Log } K_r$ against $\text{Log } M_r$ of the standards (\circ) ($r^2 = 0.944$) where K_r is the slope of the line in Figure 3.14b. The graph was used for evaluating the M_r of the native form of the recombinant Ftf (\bullet).

value obtained from this plot is only dependent on M_r . As a result, the value of 102,000 for the M_r of the native Ftf estimated from the calibration curve of $-\text{Log } K_r$ vs $\text{Log } M_r$ of the standards is more accurate. Comparing the value of M_r 102,000 of the native Ftf with the value of 125,400 for the M_r of the denatured Ftf determined by SDS-PAGE suggests that the native Ftf is a monomeric protein as the predicted M_r of the recombinant Ftf obtained from the deduced amino acid sequence is 98,545 (cf. 3.3.8 below).

3.3.8 N-Terminal Amino Acid Analysis of the Purified Fructosyltransferase

Results of N-terminal sequencing revealed that the Ftf expressed in *E. coli* was processed at TQVKA_DQVTET rather than the computer-predicted site, GTLAFL_GATQVK, previously published (Rathsam *et al.*, 1993). The same processing site was also identified in the native Ftf expressed by *S. salivarius* (Rathsam and Jacques, 1998). The theoretical pI of the Ftf devoid of the signal peptide is 4.86 and the M_r 98,545.

3.4 Discussion

A method for the purification of recombinant Ftf expressed in *E. coli* was developed. The efficiency of the whole procedure is summarised in Table 3.2 and the SDS-PAGE profile of the proteins obtained from each step are shown in Figure 3.15.

Table 3.2: Purification of the recombinant Ftf of *S. salivarius* expressed in *E. coli*.

Purification Step	Specific Activity (U [mg protein] ⁻¹)	Protein Yield (%)	Activity Yield (%)	Purification (fold)
Cell lysate	1.7	100	100	1
HA	5.5	22	74	3.2
Resource-Q	58	1.1	14.8	34.5
Sephacryl S-300	58	0.2	3	34.5

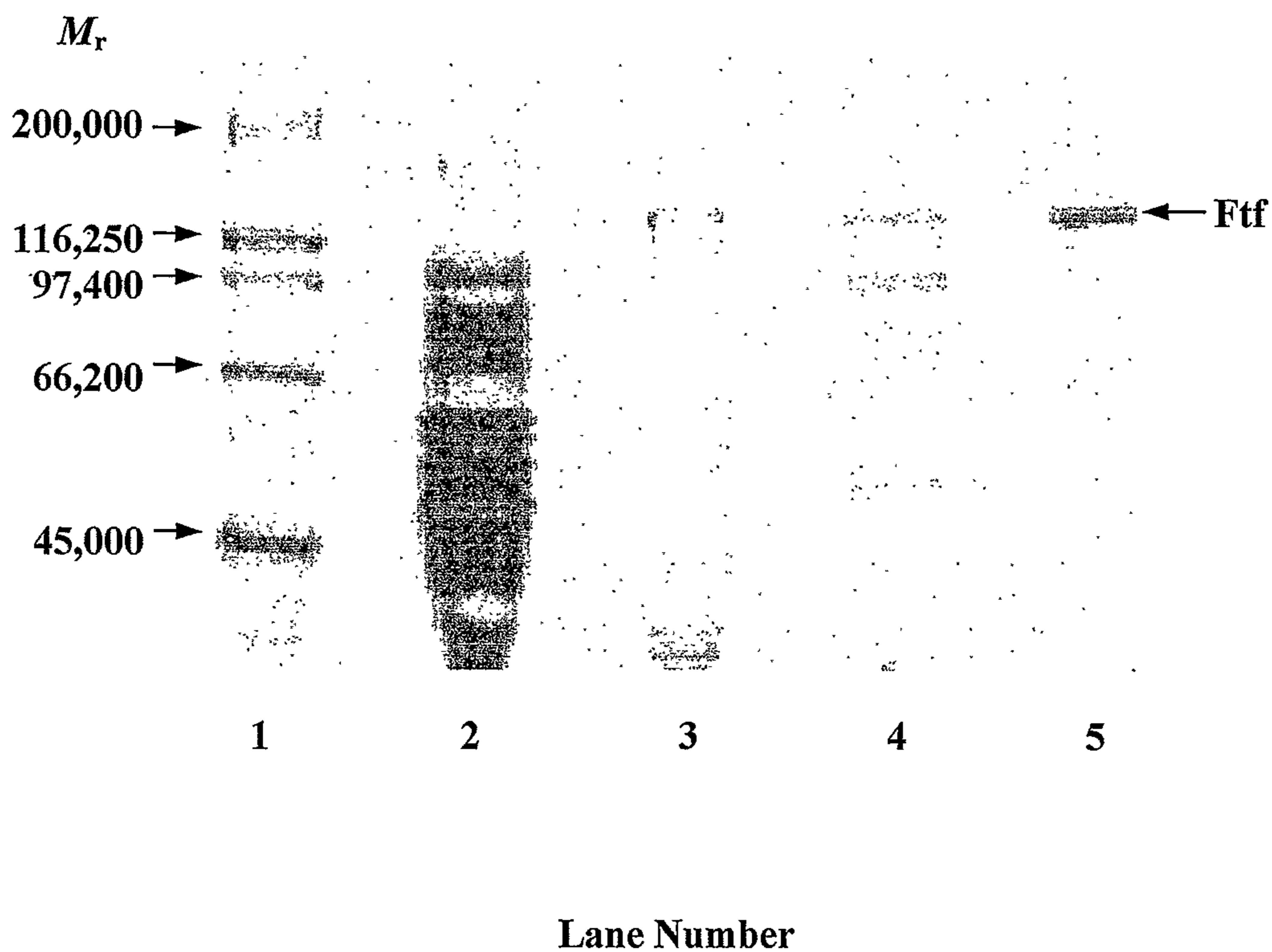


Figure 3.15: SDS-PAGE analysis of Ftf fractions from each purification step following silver staining. Lane 1, broad range M_r markers, (cf. Figure 3.5a); Lane 2, *E. coli* cell lysate; Lane 3, pooled HA fractions eluted with 35-70 mM potassium phosphate buffer pH 6.5; Lane 4, pooled Resource-Q fractions eluted with 14-20 mM NaCl in 20 mM piperazine-HCl pH 5.2; Lane 5, pooled Sephacryl S-300 fractions of the first protein peak.

The recombinant Ftf was processed at the same N-terminal site in *E. coli* as in its natural host, *S. salivarius*. Results obtained from the fractionation of *E. coli* cells suggested that the Ftf was anchored on the periplasmic side of the plasma membrane by its C-terminal hydrophobic and positively charged tail in a similar manner to that in the native organism (Rathsam *et al.*, unpublished observations). This might account for why the bead stirring method appears to be a more efficient way for cell disruption than the other methods used.

The combination of three conventional chromatographic procedures, HA adsorption chromatography, Resource-Q ion exchange chromatography and Sephacryl S-300 gel filtration chromatography successfully purified the Ftf from the *E. coli* cell lysate to electrophoretic homogeneity. The resultant specific Ftf activity was 58 U (mg protein)⁻¹. The overall yield of protein and enzyme activity were 0.2% and 3% respectively, for a purification factor of 35-fold.

The final gel filtration step removed the degradation products but gave no increase in specific activity. One explanation for this observation is that while the degraded fragments of Ftf have polymer-forming activity (Figure 3.5b), they may also have the same specific activity as the intact Ftf.

Electrophoretic analyses of the purified Ftf indicated that the Ftf is most likely a monomer. The value of the M_r , either determined by SDS-PAGE or non-denaturing PAGE (3.3.7) or predicted from the amino acid sequence of the Ftf of *S. salivarius* (3.3.8), is much bigger than the M_r of 34,500 previously reported for the Ftf of *S. salivarius* SS2 (Garszczyński and Edwards, 1973; 1.1.3). The 'Ftf' of strain SS2 was released from the cell surface by extracting the cell membrane with 8 M lithium chloride prior to purification by gel filtration and DEAE-cellulose chromatography. As has been shown in Figure 3.5b and Figure 3.11, degraded fragments of Ftf also exhibit some activity. The 'Ftf' purified from *S. salivarius* SS2 therefore may represent a degraded fragment of the intact Ftf protein which possesses only a third of the predicted M_r of 98,548 determined from the deduced amino acid sequence of the *ftf* gene from *S. salivarius* ATCC 25975.

The purified recombinant Ftf was used as an alternative source to the sucrose-released form from *S. salivarius* in all subsequent kinetic studies.

CHAPTER 4

KINETIC ANALYSIS OF THE FRUCTOSYLTRANSFERASE OF *STREPTOCOCCUS SALIVARIUS* *

4.1 Introduction

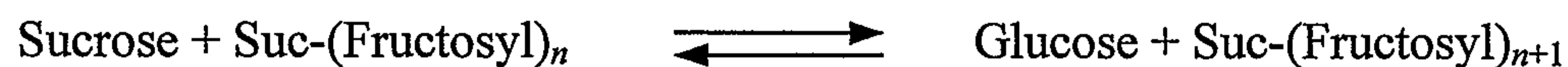
Amongst the family of β -D-fructosyltransferases only two bacterial Ftf's, the levansucrases of *B. subtilis* (Chambert *et al.*, 1974; Chambert and Gonzyl-Treboul, 1976a, b) and *A. diazotrophicus* (Hernandez *et al.*, 1995), have been studied in detail. These enzymes mainly catalyse the following reaction (1.2.2):



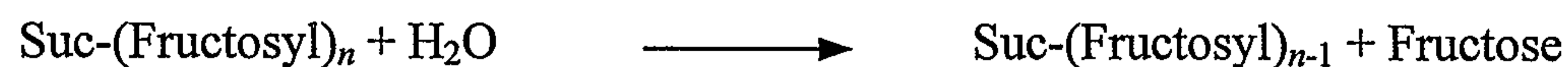
According to the experimental conditions, water, sucrose, monosaccharides, oligosaccharides and levan (fructan) may act as fructosyl acceptors (Dedonder, 1966). In the presence of sucrose alone the levansucrases form free fructose and glucose, oligo-fructans and levans:



Small levans accelerate the rate of transfructosylation from sucrose and increase the ratio of levans formed to free fructose:

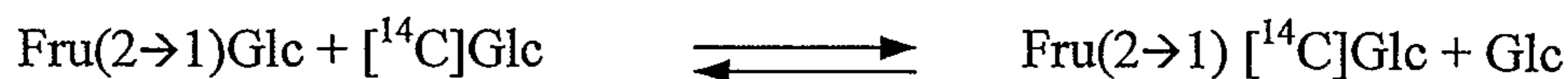


Levansucrases can also hydrolyse small levans:

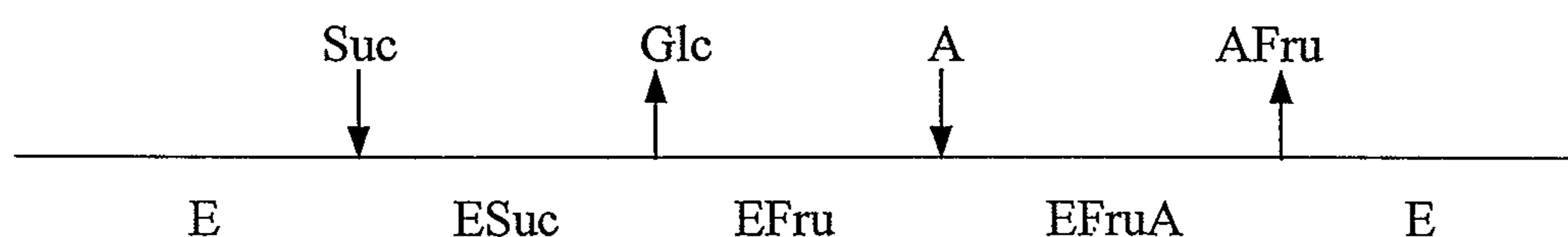


* Some of the results presented in this chapter have been published (Song and Jacques, 1999a).

In the presence of glucose, the levansucrases also catalyse the following exchange reaction between sucrose and glucose:



Kinetic analysis of the transfructosylation reactions catalysed by the levansucrase of *B. subtilis* led to the conclusion that the reaction proceeds via a Ping Pong mechanism involving the formation of a transient fructosyl-enzyme complex (1.2.2). The Ping Pong mechanism was confirmed by thermodynamic and kinetic studies (1.2.2). The existence of a fructosyl-enzyme intermediate was substantiated by the isolation of a fructosyl-enzyme complex from the reaction mixture of enzyme and sucrose by the quenching effect of a large decrease in pH (1.2.2). The mechanism of enzyme action can thus be described formally using the short-hand notation of Cleland (Cleland, 1973) as follows:



where E, Suc, Glc, Fru and A represent enzyme, sucrose, glucose, fructose and fructosyl acceptor respectively. The kinetic constants of the levansucrases of *B. subtilis* and *A. diazotrophicus* for each step of the Ping-Pong mechanism have been estimated (Chambert *et al.*, 1974; Chambert and Gonzyl-Treboul, 1976b; Chambert and Petit-Glatron, 1991; Hernandez *et al.*, 1995).

Unlike the levansucrases of *B. subtilis* and *A. diazotrophicus*, there have been no systematic studies of the kinetic properties of streptococcal Ftf. Earlier study showed that Ca^{2+} activated the cell-associated Ftf of *S. salivarius* (Jacques, 1984). The K_m for Ca^{2+} of the cell-associated Ftf in the presence of 10 mM sucrose was determined to be $18 \pm 2 \mu\text{M}$ and the K_m for sucrose in the presence of 1 mM Ca^{2+} as $12.0 \pm 0.1 \text{ mM}$. A later study, however, showed that the cell-bound Ftf was secreted from the cell surface and released into the culture media in the presence of sucrose (Milward & Jacques, 1990). Thus whether the above constants actually relate to the properties of the cell-free form is not known.

Successful purification of the recombinant Ftf of *S. salivarius* (Chapter 3) made it possible to investigate the kinetic mechanism of the enzyme without interference from cells or other protein contaminants. In this chapter the mechanism of the transfructosylation reactions catalysed by the Ftf of *S. salivarius* are examined and the kinetic properties of the enzyme analysed and compared with those of other Ftfs.

4.2 Materials and Methods

4.2.1 Preparation of *S. salivarius* Ftf

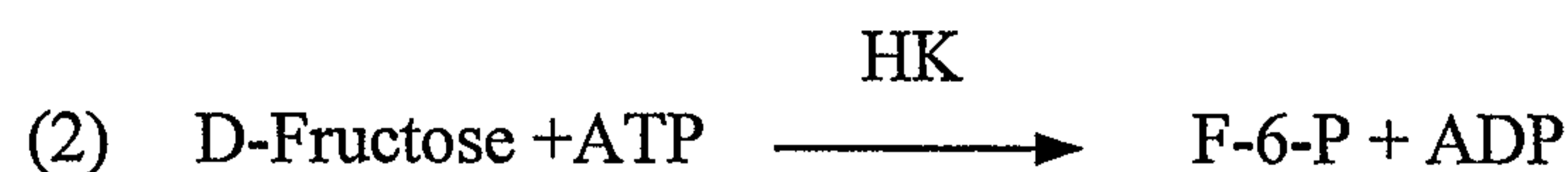
The recombinant Ftf of *S. salivarius* was isolated and purified from a cell lysate of *E. coli* NM522 harbouring plasmid pKRRK1969 encoding the *ftf* gene of *S. salivarius* (Table 2.1) by the combined chromatography procedure described in Chapter 3. The lyophilised enzyme was redissolved in 10 mM potassium phosphate buffer pH 6.0 prior to use.

4.2.2 Measurement of D-glucose and D-fructose for Determination of Fructosyltransferase Activity

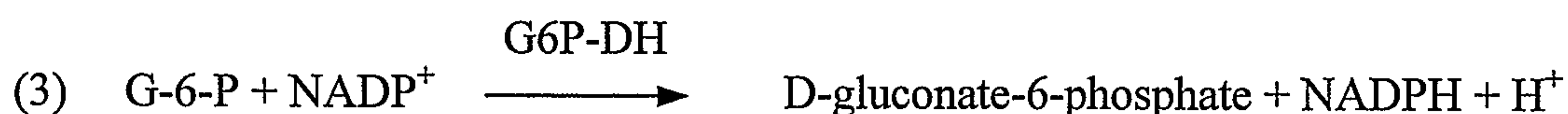
The amount of D-glucose and D-fructose formed from sucrose in the transfructosylation reaction catalysed by the Ftf of *S. salivarius* was quantified at 37°C using the TC D-Glucose/D-Fructose enzymatic assay kit supplied by Boehringer Mannheim GmbH (Mannheim, Germany) according to manufacturer's instructions except that comparisons were made with glucose and fructose standards assayed in the same manner at the same time.

4.2.2.1 Principle of the Methodology

D-Glucose and D-fructose are phosphorylated by the enzyme hexokinase (HK) and ATP to form D-glucose-6-phosphate (G-6-P) and D-fructose-6-phosphate (F-6-P) as shown in reactions 1 & 2:



In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by NADP to D-gluconate-6-phosphate with the formation of NADPH as shown in reaction 3:



The amount of NADPH formed in this reaction is stoichiometric to the amount of glucose in the sample. NADPH is measured by the increase in absorbance at 340 nm. On the completion of reaction 3 above, phosphoglucose isomerase (PGI) is added to convert F-6-P to G-6-P as shown in reaction 4:



G-6-P reacts in turn with NADP forming D-gluconate-6-phosphate and NADPH. The amount of NADPH obtained in this second reaction is therefore stoichiometric to the amount of D-fructose present in the sample.

4.2.2.2 Procedure

A sample or standard solution of glucose and fructose in 2 ml of water was mixed with 1ml of NADP-ATP reagent (supplied by Boeringer Mannheim GmbH, and consisting of triethanolamine buffer, pH 7.6 containing approximately 2.37 mg NADP ml⁻¹, 5.93 mg ATP ml⁻¹, MgSO₄ and stabiliser of unknown concentrations) in a 4.5 ml PMMA UV grade disposable cuvette (Crown Scientific, Sydney, Australia). The A₃₄₀ was measured after approximately 3 min (A₁), using a Beckman DU[®] 640 spectrophotometer (Beckman Instruments, Sydney, Australia) with a 6-cell holder controlled at a constant temperature of 37°C. Six samples were measured simultaneously. The reactions were initiated by the addition of 20 µl of suspension 1 (approximately 286 U HK ml⁻¹ and 143 U G6P-DH ml⁻¹) and rapidly mixing by pipetting. The A₃₄₀ was continuously measured with a read average time of 1.00 s and a time interval of 15.00 s until the end of the reactions when the A₃₄₀ did not increase any more with time (approximately 10 min). The maximum A₃₄₀ for each sample was recorded (A₂). The reactions were continued by the addition of 20 µl of suspension 2 (approximately 700 U PGI ml⁻¹) until the A₃₄₀ of each sample reached a

new maximum (A_3). The absorbance differences of A_2-A_1 and A_3-A_2 were calculated for both reagent blanks and samples (or standards). The reagent blank used for the standards was water, whereas that for the transfructosylation reactions from sucrose contained the same reaction mixture and sucrose concentration as in the sample but without any Ftf. The increase in absorbance due to D-glucose (A_G) and D-fructose (A_F) were calculated as follows:

$$A_G = (A_2 - A_1)_{\text{sample}} - (A_2 - A_1)_{\text{blank}}$$

$$A_F = (A_3 - A_2)_{\text{sample}} - (A_3 - A_2)_{\text{blank}}$$

The amount of glucose and fructose of an unknown sample was determined from the standard curve of A_G or A_F vs the concentration of glucose or fructose ($0-20 \mu\text{g ml}^{-1}$) respectively.

4.2.3 Examination of the Enzymic Properties of the Fructosyltransferase of *S. salivarius* and the Mechanism of the Transfructosylation Reactions

4.2.3.1 pH Dependence of the Fructosyltransferase Activity

In order to determine the effect of pH on Ftf activity, 25 nM Ftf was incubated at 37°C for 60 min with 10mM sucrose solution containing 10mM NaF, 0.1 mM histidine and 1 mM CaCl_2 in 100 mM potassium acetate buffer, pH 5.0 or 5.5, or 100 mM potassium phosphate buffer, pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 respectively. The reactions were stopped by the addition of 5 volumes of ethanol preheated to 80°C. After heating at 80°C for a further 15 min, the samples were evaporated to dryness *in vacuo* and redissolved in 2 ml of distilled water. The enzyme activity was then determined by measuring the amount of glucose released in the reaction (4.2.2).

4.2.3.2 Effect of Temperature on Fructosyltransferase Activity

Ftf was incubated with 10 mM sucrose for 60 min in the standard assay buffer (100 mM potassium phosphate buffer pH6.0, containing 10mM NaF, 0.1 mM histidine and 1 mM CaCl_2), at 25°C, 30°C, 37°C, 42°C or 50°C. The reactions were stopped and the enzyme activities measured as described above (4.2.3.1).

4.2.3.3 Effect of Metal Ions on Fructosyltransferase Activity

Ftf was incubated at 37°C with 10 mM sucrose in 100 mM potassium phosphate buffer containing 10 mM NaF and 0.1 mM histidine. In each of the reactions, 1 mM

of NaCl, KCl, CaCl₂, MgCl₂, HgCl₂, CuSO₄, Zn(CH₃COO)₂, FeSO₄, FeCl₃ or EDTA (disodium) was added. At the end of 60 min, the reactions were stopped and the enzyme activities analysed as described above (4.2.3.1).

4.2.3.4 Analysis of the Reaction Products Produced by the Fructosyltransferase

The reaction products produced by the Ftf were analyzed by both paper chromatography and thin layer chromatography (TLC). For both analyses, the 300 μl assay mix contained 50 mM [U-¹⁴C]-labelled sucrose (92.5 kBq ml⁻¹; 2.5 μCi ml⁻¹) in the standard assay buffer (4.2.3.2) and the reaction was started by the addition of Ftf to give a final concentration of 100 nM. Aliquots (20 μl) were transferred from the reaction mixtures at 0 s, 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 4 h into 100 μl ethanol pre-heated at 80°C and inactivated and dried as described above (4.2.3.1). Each sample was redissolved in 10 μl H₂O and a 5 μl aliquot analyzed by ascending paper chromatography on Whatman #3 filter paper (Whatman International Ltd., Maidstone, England) in a solvent system consisting of butan-1-ol / acetic acid / water (4:1:1, by volume) which was developed for 15 h. The reaction products were visualized by autoradiography after exposure to Hyper Film[™]-MP (Amersham Australia Ltd., Sydney, Australia) for 24 h. Another 5 μl aliquot from each time point was analyzed by TLC on pre-coated 20 x 20 cm silica-gel TLC-ready-foils (F1500, Schleicher and Schuell, FRG) as described by Cairns and Pollock (1988). Chromatograms were developed twice in a solvent consisting of butan-1-ol / propan-2-ol / water (3:12:4, by volume). The radioactive products were visualized by autoradiography as described above. Fructans with a degree of polymerization (DP) of more than 15 did not migrate in either system and remained at the application site (Cairns and Pollock, 1988).

4.2.4 Calculation of Initial Velocity Measurements for the Fructosyltransferase

4.2.4.1 Transfructosylation from Sucrose to Sucrose: Sucrose Hydrolysis and Fructan Synthesis

The ability of Ftf to hydrolyse and/or polymerise the fructosyl moiety of sucrose was determined at 37°C by measuring the amount of D-glucose and D-fructose produced by the enzyme (4.2.2). Each reaction mixture (2 ml) contained sucrose (0, 1, 2, 3, 5, 10, 20, 40, 50, 75, 100 or 500 mM) in standard assay buffer (4.2.3.2). Ftf was added

to a final concentration of 25 nM to start the reaction. Aliquots (50 or 100 μ l) were removed at 0.0, 0.5, 1, 2, 5, 10, 20, 30, 40, 50 and 60 min, quenched in ethanol and dried *in vacuo* as described above (4.2.3.1) before being redissolved in 2 ml water. The amount of glucose and fructose produced at each time point was then quantified at 37°C (4.2.2). The initial velocity for the rate of formation of glucose, v_G ($d[G]/dt$; defined as the molarity of glucose released per minute [$M \text{ min}^{-1}$]), and fructose, v_F ($d[F]/dt$), at each sucrose concentration was calculated from these data using the 'Sigma-Plot' program (2.6).

4.2.4.2 Transfructosylation from Sucrose to Glucose: the Exchange Reaction

The ability of Ftf to exchange the glucose moiety of sucrose was determined at 37°C in 50 μ l reaction mixtures containing 20, 40, 60, 100 or 200 mM sucrose and 50, 100, 120 or 200 mM [^{14}C]-labelled glucose (185 $\text{kBq}\cdot\text{ml}^{-1}$; 5 $\mu\text{Ci}\cdot\text{ml}^{-1}$) in standard assay buffer (4.2.3.2). Ftf was added to a final concentration of 250 nM. Aliquots of 10 μ l were removed at intervals of 0.5 or 1 min and quenched in ethanol and dried as described above (4.2.3.1). After being redissolved in 10 μ l of water, an aliquot of 5 μ l was analysed by ascending paper chromatography and the radioactive products identified by autoradiography. The radioactive products were excised from the paper, cut into strips, suspended in Ultima-Gold[™] scintillation solution (Packard Instrument Co., USA) and shaken at 4°C for 15 h before being counted with a liquid scintillation spectrometer [Beckman LS 9000]. Under these conditions the difference in quenching was found to be negligible. The initial rate of exchange, r_G , was defined as the molarity of the fructosyl moiety transferred from sucrose to glucose per minute [$M \text{ min}^{-1}$].

4.2.5 Examination of the Binding between Fructosyltransferase and its Fructan Product

After incubating the purified recombinant Ftf (250 nM) with 10 mM sucrose at 37°C for 30 min in 50 μ l of standard assay buffer (4.2.3.2), the reaction mixture was heated at 95°C for 5 min with SDS sample buffer and analysed by SDS-PAGE (2.5.3).

4.2.6 Analysis of Data

All experiments were repeated in triplicate. The 'Sigma Plot' program (version 3.06, SPSS Inc, Chicago, U.S.A.) was used for calculating both linear and non-linear plots. The same program was used to obtain linear regression coefficients (r^2).

4.3 Results

4.3.1 Effects of pH, Temperature and Metal Ions on Fructosyltransferase Activity

The influences of pH and temperature on Ftf activity were examined in order to define the best condition for subsequent kinetic studies. The results showed that Ftf activity reached a maximum at around pH 6.0 and 37°C (Figures 4.1 and 4.2).

The effects of different metal ions on the Ftf activity were also examined (Table 4.1). Addition of the metal ion chelator, 1 mM EDTA, reduced the enzyme activity by 83% consistent with the previous results that had indicated a role for Ca^{2+} in stabilising enzyme activity (Jacques, 1984; Milward and Jacques, 1990). This was confirmed by the addition of 1 mM Ca^{2+} which enhanced the activity by 50%. Neither Na^+ , K^+ nor Mg^{2+} , the metal ions other than Ca^{2+} used in the preparation of enzyme, affected Ftf activity.

Unlike the situation with the levansucrase of *B. subtilis* where Fe^{3+} serves to stabilise the enzyme (Chambert *et al.*, 1990; Chambert and Petit-Glatron, 1990), the Ftf of *S. salivarius* was partially inhibited by 1 mM Fe^{3+} as well as 1 mM Zn^{2+} . The heavy metals, Cu^{2+} and Hg^{2+} , at the same concentration of 1 mM inhibited the enzyme to a greater degree (Table 4.1). The mechanism by which the heavy metals inhibit the cell-free Ftf is not known. However, the Cu^{2+} -mediated inactivation of the cell-associated enzyme appears to depend on free-radical inactivation of the enzyme rather than competition with Ca^{2+} (Jacques, 1984; Abbe *et al.*, 1986).