

**PURIFICATION AND CHARACTERIZATION OF
FRUCTOSYLTRANSFERASE FOR THE SYNTHESIS OF
SHORT-CHAIN FRUCTO-OLIGOSACCHARIDES AND
INVESTIGATION INTO THEIR ANTI-CARCINOGENIC
PROPERTIES**

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

ALUWANI NEMUKULA

January 2008

ABSTRACT

There is a growing attention in the synthesis of fructo-oligosaccharides (FOS) due to their excellent bio-functional and health-promoting properties. The current production processes are limited to chemical hydrolysis reactions of plant extracts, which are often associated with several drawbacks. In this study, fructosyltransferase (FTase) and polygalacturonase (PGase) activities, present in a commercial enzyme preparation (Pectinex® Ultra SP-L) sourced from *Aspergillus aculeatus*, have been separated and fully purified by anion-exchange and size-exclusion chromatography. The FTase possesses fructosyl transfer activity for FOS synthesis and the PGase has pectin hydrolytic activity. Fructosyltransferase is a single-band protein with a molecular weight of 85 kDa, whereas PGase is a distinct protein of 40 kDa. The temperature and pH optima of FTase were 60 °C and 6.0, with a half-life of 8 h; while that for PGase were 40 °C and 6.0, respectively. FTase was slightly inhibited in the presence of Ni^{2+} , Mg^{2+} and urea; but PGase was more susceptible to divalent ions such as Ca^{2+} , Ba^{2+} and Mn^{2+} . The kinetic parameters (K_m and V_{max}) of FTase for the hydrolysis of β -(2→1) linkages from sucrose were 752.3 mM and $120.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$, respectively; whereas the same parameters for pectin hydrolysis by PGase were $13.0 \text{ mg} \cdot \text{mL}^{-1}$ and $263 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$, respectively. The purified FTase was able to transfer fructosyl residues from sucrose, synthesizing the corresponding chains of FOS. PGase was relatively stable at 40 °C ($t_{1/2} > 3 \text{ h}$), depolymerizing the pectin backbone while releasing the inulins from within the chicory roots. Analysis of various mixtures of FOS by mass spectrometry, HPLC and ^1H -NMR was undertaken. Results indicated that MS with electrospray ionization and ^1H -NMR are capable of providing relative quantitative

data of the FOS present in the mixtures. The pharmaceutical effects of various sc-FOS (0.5%, v/v) and SCFA (0.3%, v/v) on certain bacterial enzymes (β -glucuronidase, urease and β -glucosidase) associated with the formation of carcinogens were also studied. These enzyme activities were not directly influenced by the sc-FOS, but were found to be remarkably decreased by SCFA, pointing toward the prebiotic effect of FOS in intestinal microflora modulation.

ACKNOWLEDGMENTS

I would like to express my appreciation to the following people:

Professor Chris Whiteley, my supervisor, for his inspiring suggestions and providing guidance throughout the duration of this study.

Members of Lab 309 (2006/2007) in the Biochemistry, Microbiology & Biotechnology Dept., Rhodes University; for sharing ideas, discussions and help.

I am greatly indebted to Dr Chamunorwa Togo, for his ingenious passion and tireless efforts in checking references, facts, faulty constructions and stylistic abominations; and for finding extra time when I needed advice on all things biochemical.

Friends – Habtamu Dugo, Thompho Rashamuse, Nathaniel Sebitso, Puso Phetwe and James Short, for their generous friendship and support during good and turbulent times.

Johan Fourie and Andy Soper for their quick and expedient sample analysis on Mass Spectrometry and Nuclear Magnetic Resonance spectroscopy.

My parents, for their encouragement and unconditional love; and also for allowing me the opportunity to explore the world around us.

Mbulu Mulaudzi, for believing in me more than I believe in myself.

The National Research Foundation (NRF) and LIFE**lab** for their financial support.

And last, but not least, our God, in whom I trust, and to whom all things are known.

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LIST OF ABBREVIATIONS

$\leq_x$:	less than or equal to x (where x is a given number)
%:	percent
μL :	microlitre
μm :	micrometre
μmol :	micromole
AIDS:	acquired immuno-deficiency syndrome
ANOVA:	analysis of variance
BSA:	bovine serum albumin
CD:	Crohn's disease
CHD:	coronary heart disease
COSY:	correlation spectroscopy
D_2O :	deuterium oxide
DEAE:	diethyl-amino ethylene
DNS:	di-nitrosalicylic acid
DP:	degree of polymerization
Endo-I:	endo-inulinase
ESI-MS:	electro-spray ionization mass spectrometry
Exo-I:	exo-inulinase
FTase:	fructosyltransferase
FOS:	fructo-oligosaccharides
GRAS:	generally regarded as safe
h:	hour
HCl:	hydrochloric acid
HDL~C:	high-density lipoprotein-cholesterol
HFS:	high fructose syrups
HIV:	human immunodeficiency virus
HMG-CoA:	hydroxyl-methyl glutarate-coenzyme A
HMOs:	human milk oligosaccharides
HPLC:	high-performance liquid chromatography
HSDH:	hydroxy steroid dehydrogenase
IBD:	inflammatory bowel disease
IEC:	ion-exchange chromatography
IOS:	inulo-oligosaccharides
IRS:	insulin resistance syndrome
IUB:	International Union of Biochemistry
IUPAC:	International Union of Pure and Applied Chemistry
k_{cat} :	enzyme turnover number
kDa:	kilodalton
K_m :	Michaelis-Menten constant
LDL~C:	low-density lipoprotein-cholesterol
M:	molar
Min:	minute
mL:	millilitre
mM:	millimolar
$(\text{NH}_4)_2\text{SO}_4$:	ammonium sulphate
nm:	nanometre

NMR:	nuclear magnetic resonance
PAGE:	poly-acrylamide gel electrophoresis
PEG:	poly(ethylene) glycol
<i>Pers. Comm.:</i>	personal communication
PGA:	polygalacturonic acid
PGase:	polygalacturonase
pH:	pondus hydrogenii
pI:	isoelectric point
pKa:	pH at which an acid is half dissociated
PNP:	<i>p</i> -nitrophenol
ppm:	parts per million
RT:	room temperature
s:	second
SCFA:	short-chain fatty acids
Sc-FOS:	short-chain fructo-oligosaccharides
SDS:	sodium dodecyl sulphate
SEC:	size-exclusion chromatography
S.E.M:	standard error of the mean
sp.:	species
TEMED:	<i>N,N,N',N'</i> -tetramethyl-ethylenediamine
TTPA:	tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt
UC:	ulcerative colitis
v/v:	volume per volume
w/v:	weight per volume

CHAPTER 1

LITERATURE REVIEW

1 LITERATURE REVIEW

1.1 Introduction

Fructo-oligosaccharides (FOS) have received so much attention recently because of their excellent bio-functional properties; particularly as prebiotic compounds. However, the health and wellbeing of man has been, for a long time, constantly challenged by pathogens. The intestinal environment faces a host of these challenges, although a number of defence mechanisms exist, which include the epithelial lining of the intestinal wall and other functional barriers able to defend the host against invasion and colonization (Bosscher *et al.*, 2006). This is attributed to the fact that there are numerous bacterial species that reside in the gastrointestinal tract, some of which are normal intestinal flora whereas others are potential pathogens. It is estimated that about 500 different bacterial species reside in the human intestinal environment (Gibson, 1999; Losada and Olleros, 2002).

Under normal circumstances, the commensal microflora play an important role by providing a frontline of defence against their pathogenic counterparts. The concept of beneficial microbes dates back to the early 1900's when Dr. Ellie Metchnikoff observed that the long life of Bulgarian peasants was a result of their intake of fermented milk and milk products (Keen *et al.*, 1999). Metchnikoff associated the intake of cultured, sour milks with increased longevity. Immediately after his observation, the concept of beneficial commensal microbes became widely accepted, and was later confirmed in infants during their neonatal immune development by Baar and Bickel (1964). They found that infants fed with human milk had low incidence of diarrhoea and other illnesses compared with infants that were fed with infant formula. This was due to the presence of non-digestible oligosaccharides in human milk; which are referred to as human milk oligosaccharides (HMOs) (Kunz *et al.*, 2000; Coppa *et al.*, 2001; Bosscher *et al.*, 2006).

Following these observations, the term functional food was introduced in Japan in the 1980's and basically refers to food ingredients that offer specific bodily functions in addition to being nutritious and provide health benefits beyond the ordinary food

products (Hasler, 1996). Oligosaccharides promote healthy colonization of the gut system by stimulating the growth of normal intestinal microflora such as *Lactobacilli* and *Bifidobacteria* (Chaturvedi *et al.*, 2001; Euler *et al.*, 2005). The IUB-IUPAC nomenclature defines oligosaccharides as non-digestible polymers with a degree of polymerization (DP) of between 2 and 10 monosaccharide units. These compounds are resistant to digestion by mammalian enzymes in the upper gastrointestinal tract, and are therefore selectively metabolized by gut bacteria.

There are two different types of carbohydrates that are found in nature, namely structural and non-structural carbohydrates. Oligosaccharides (prebiotics) belong to a group of non-structural carbohydrates known as fructans. They essentially serve as energy-rich compounds for metabolism and energy storage in living organisms; for example glucose, fructose, sucrose, raffinose, starch and inulin. Structural carbohydrates are found as part of the cell-wall and cytoskeleton in many organisms; for example chitin, peptidoglycan, pectins and cellulose (Van Hijum *et al.*, 2004).

This thesis focuses on the purification and characterization of enzymes for the synthesis of fructan compounds known as FOS; it further investigates their pharmaceutical properties against certain carcinogenic enzyme activities.

1.2 The Concept of Probiotics and Prebiotics

The concept of probiotics as first observed by Metchnikoff in 1908 is driving today's research, product development and offering range of new functional products. As a result, the use of functional food products containing live cultures or probiotics has increased rapidly in the recent past (Roberfroid, 2000; Zubillaga *et al.*, 2001). Probiotics are defined as live feed supplement bacteria which when ingested, beneficially affect the host. These beneficial bacteria are considered to prevent the growth of putrefactive flora through competitive availability of nutrients, acidification of the medium, production of proteins with antibiotic activity and other useful and scavenging substances (Losada and Olleros, 2002; Delzenne, 2003). Some of the most common probiotic strains include *Lactobacilli* such as *Lactobacillus acidophilus*, *L. fermentum*, *L. casei*, *L. reuteri*, *L. salivarius*, *L. brevis*, *L. bulgaricus*; Gram-positive cocci such as *Lactococcus lactis* subsp.

cremoris, *Enterococcus faecium*; and *Bifidobacteria* such as *Bifidobacterium bifidum*, *B. adolescentis*, *B. animalis*, *B. infantis*, *B. longum* and *B. thermophilum*. In a healthy colon these beneficial bacteria outnumber the pathogenic ones, thereby favourably altering the intestinal flora balance, aiding good digestion and boosting immune function (Delzenne, 2003). The viability of these probiotics in the gastrointestinal tract is, however, influenced by a number of factors (Table 1.1). In view of these critical factors, some researchers have attempted to genetically engineer bacterial strains resistant to the acidic environment in the stomach and to develop microencapsulation technology that would protect these bacteria in the gastrointestinal tract (Chung *et al.*, 1999; Kaplan and Hutkins, 2000; Crittenden *et al.*, 2001).

Table 1.1 **Survival requirements of probiotics in the human colon** (adapted from Gibson and Fuller, 2000)

Factors influencing the use and viability probiotics
Resistance to the acidic environment of the stomach and the biliary salts
High rate of proliferation and affinity for adherence to the intestinal wall
Competitive effect for availability of nutrients
Production of metabolites deleterious to pathogens (e.g. SCFA)
Modulation of metabolic activity (e.g. inactivation of pro-carcinogens)
Immuno-modulation (i.e. production of mucins and bacteriocins)
Must satisfy GRAS (generally regarded as safe) conditions for safety of use

However, it is now believed that the key factor is the ability of these probiotics to selectively metabolize or grow on dietary fibre. This led to the coining of the term prebiotic; which refers to a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of beneficial bacteria in the colon, thus leading to improved host health (MacFarlane and McBain, 1999). The fact that the human gastrointestinal tract is a highly complex ecosystem consisting of many species of bacteria means that the task of maintaining optimal balance in the gut is rather complicated (Tuohy *et al.*, 2007). In addition, survival of probiotics in the human body after ingestion is often physiologically unfeasible due to a number of factors including dietary patterns, stress, age, drugs and other

exogenous and endogenous factors of the host. As a result, the use of external supplements in the form of prebiotics has become more desirable and efficient for re-establishing probiotics equilibrium. Also, due to the potential synergy between probiotics and prebiotics, the development of food products containing the combination of these ingredients has become widespread, and these are referred to as synbiotics. However, prebiotics such as FOS are considered to exert beneficial effects from a nutritional and therapeutic point of view, in addition to just re-establishing equilibrium (Fig. 1.1). For this reason, they have emerged as suitable candidates for health improvement because they involve administration of non-viable entities and therefore overcome survival limitations in the upper gastrointestinal tract (Collins and Gibson, 1999).

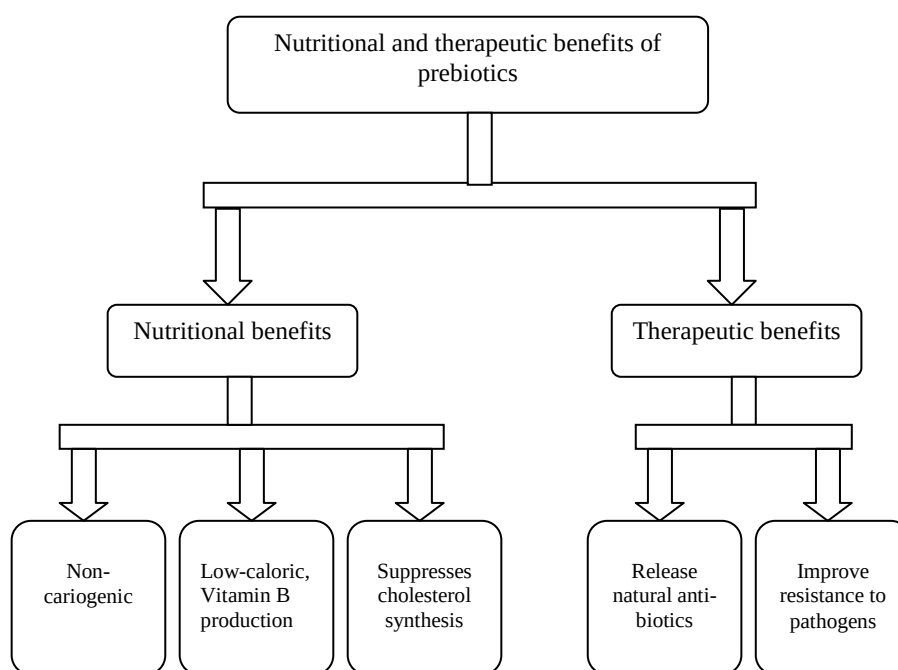


Figure 1.1 Nutritional and therapeutic benefits of prebiotics (adapted from Losada and Olleros, 2002)

Although there are different types of oligosaccharides that are classified as fructans (Table 1.2), FOS and inulin are known to possess many bio-functional and health-promoting properties (Roberfroid and Delzenne, 1998). This has been substantiated by many *in vitro* and *in vivo* studies confirming the production of essential metabolites and other micronutrients upon their fermentation by colonic bacteria (Cummings and McFarlane, 1997; Naidu *et al.*, 1999; Van Loo *et al.*, 1999).

Table 1.2 Different types of oligosaccharides (Murphy, 2001)

Type of oligosaccharide	Source	Industrial production process
Fructo-oligosaccharides	Fruits and vegetables (Garlic, banana, onion etc)	Synthesized from sucrose
Galacto-oligosaccharides	Breast milk	Hydrolysis from inulin
Lactulose		Synthesis from lactose
Inulo-oligosaccharides	Chicory root	Inulin hydrolysis
(Iso)malto-oligosaccharides	Maltose and dextran	Hydrolysis from dextran
Xylo-oligosaccharides		Polyxylans hydrolysis
Soy-oligosaccharides	Soybean	
Cyclodextrin	Vegetables (potato)	Synthesis from starch

1.3 Bio-functional Fructans

1.3.1 Inulin

Inulin is a reserve carbohydrate, which consists of a linear chain of β -(2 $\rightarrow$ 1) linked fructose residues with a terminal glucose moiety (Fig. 1.2). It is a naturally occurring polymer with an average DP of 20 fructose units, and is also classified as an oligosaccharide (Bacon and Edelman, 1951; Gibson and Roberfroid, 1995). Inulin is also a very suitable source for the production of FOS when strong endo-inulinase (Endo-I) activity is exerted compared to exo-inulinase (Exo-I) activity (Yun *et al.*, 1997).

Inulins are abundantly derived from chicory (*Cichorium intybus* L), but are also found in other plants such as Jerusalem artichoke, garlic, asparagus, dahlia and onion. *Cichorium intybus* L is a vivacious plant species whose tuberous roots serve as a storage organ for inulin (Fig. 1.3). It belongs to the Asteraceae family of plants and is native of Europe, west and central Asia, north and southern Africa and South America (Figueira *et al.*, 2004).

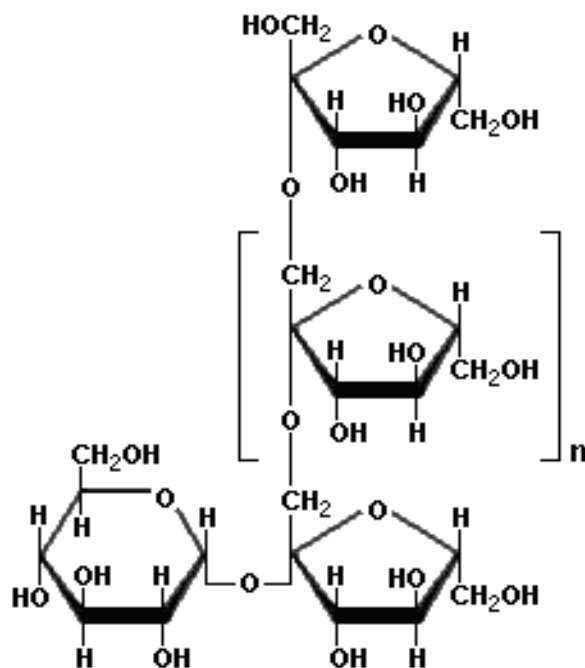


Figure 1.2 Inulin chemical structure, where n = approx. 20 fructose residues (Zamora, 2005).



Figure 1.3 Photograph of chicory plant (*Cichorium intybus* L) and its roots. (Reproduced with permission: from en.wikipedia by Lorsh, 2004).

Among the sources of inulin, chicory roots and Jerusalem artichoke tubers contain the highest composition of inulin per plant distribution (Fig. 1.4). However, at harvest, chicory roots contain 75% inulin on a dry matter basis, much more than Jerusalem

artichoke. In addition to this, chicory is a more reliable source of inulin than Jerusalem artichoke because crop yield is often less variable from year to year, especially for agricultural and market production purposes (Wilson *et al.*, 2004).

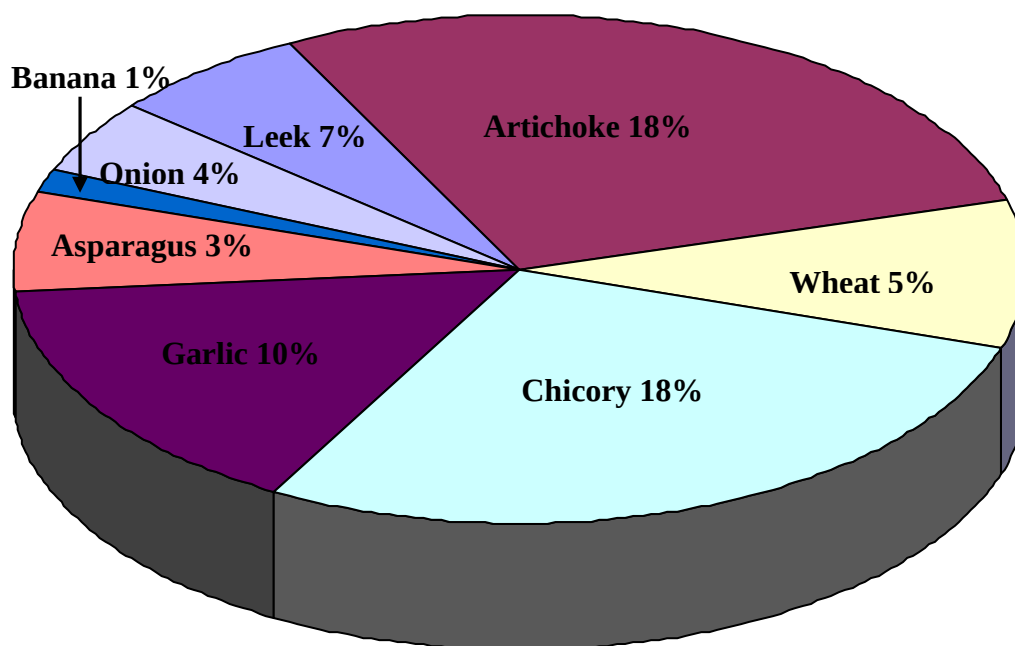


Figure 1.4 Inulin distributions from various sources (Roberfroid, 1998).

Despite its abundant availability at low cost and health properties such as lowering incidence of heart attack, the commercial interest of inulin is surpassed by that of FOS. Fructo-oligosaccharides have attracted so much commercial interest and are now marketed in countries such as Japan, the United States, and many European countries. This is due to the fact that FOS are preferentially metabolized by *Lactobacilli* and *Bifidobacteria*, thus rapidly increasing the production of short-chain fatty acids (SCFA) - acetate, butyrate, lactate and propionate; than any other substrates (Flickinger *et al.*, 2000). These SCFA provide the host with many physiologic properties, for example butyrate is an important respiratory fuel for the colonocytes. Propionate may inhibit HMG-CoA reductase, the rate-limiting step in cholesterol synthesis (Wong *et al.*, 2006).

1.3.2 Fructo-oligosaccharides

Fructo-oligosaccharides are produced as reserve carbohydrates and found in trace amounts in many fruits, plants and vegetables such as chicory, wheat, banana, dahlia and asparagus (Van Loo *et al.*, 1995; Roberfroid, 2000; Tanriseven and Aslan, 2005). They are also designated as short-chain fructo-oligosaccharides (sc-FOS) and consist of β -(2 $\rightarrow$ 1)-linked D-fructose residues with a terminal α -(1 $\rightarrow$ 2)-D-glucose moiety (Fig. 1.5). They have an average DP of 5 fructose residues (Roberfroid *et al.*, 1998). Commercially, these compounds are produced by the enzymatic transfer of a fructosyl residue from substrates such as sucrose using a fructosyltransferase (FTase) (EC.2.4.1.9) or through optimized inulin hydrolysis using inulinases (Fujishima *et al.*, 2005; Tanriseven and Aslan, 2005).

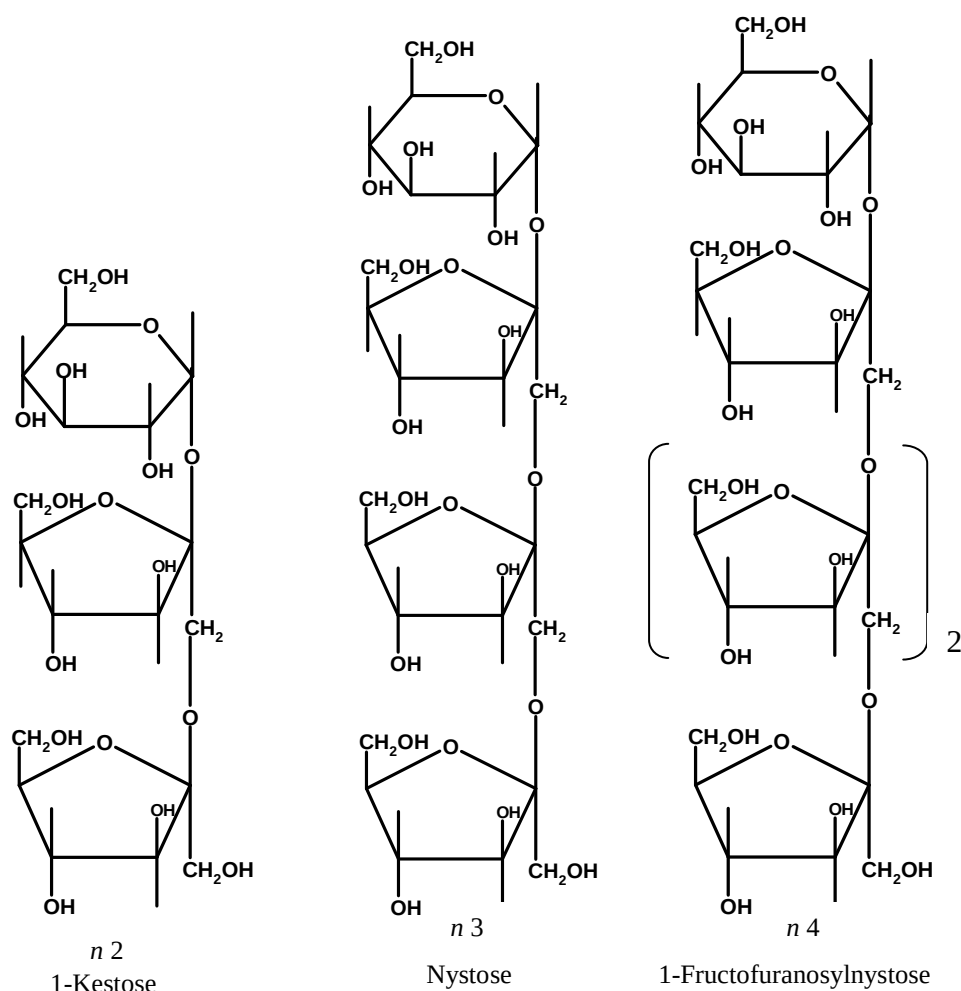


Figure 1.5 Chemical structures of various fructo-oligosaccharides (FOS) (Yun, 1996). $n = 2, 3$ or 4 indicates the number of fructose moieties in the molecule.

Fructo-oligosaccharides of different chain-lengths are fermented at different rates, and as a result are considered to offer specific bio-functional and bifidogenic properties; the most cited being anti-cariogenicity and low caloric (Roberfroid, 2000; Bosscher *et al.*, 2006).

1.4 Beneficial Properties of Fructo-oligosaccharides

1.4.1 Bifidogenic Effect

From a human body perspective, the digestive system handles all carbohydrates in more or less the same way – it breaks them down into simple sugars (usually glucose), enough to enter the bloodstream because it is most easily accessible and the cells are designed to use it as a universal energy source (Donovan, 2006). However, there is an exception with FOS because of the β -configuration in the anomeric C2 of their fructose residues. The human digestive enzymes (α -glucosidases, sucrases and maltases) are specific for α -glycosidic linkages and therefore cannot hydrolyze the β -configuration glycosidic linkages (Roberfroid, 2000). As a result, these compounds pass through the upper gastrointestinal tract into the large intestine without being metabolized. In the colon, FOS are selectively metabolized by beneficial bacteria (Gibson and Roberfroid, 1995; Bornet *et al.*, 2002). The result is a selective proliferation of *Bifidobacteria* and *Lactobacilli* and a subsequent reduction in the number of other pathogenic bacteria such as *Clostridia*, *Bacteriodes*, *Staphylococci* and *Fusobacteria* (Fig. 1.6) (Roberfroid, 1998; Gomes and Malcata, 1999; Gibson 1999).

This phenomenon is known as the “prebiotic effect” and it favourably alters the balance in the gut microflora populations by conferring several health benefits (Saier and Mansor, 2005; Katz and Novak, 2006). Consequently, the role of FOS has been the subject of high interest in the last decade, mainly because of their untapped health-promoting properties (Seeberger and Werz, 2007).

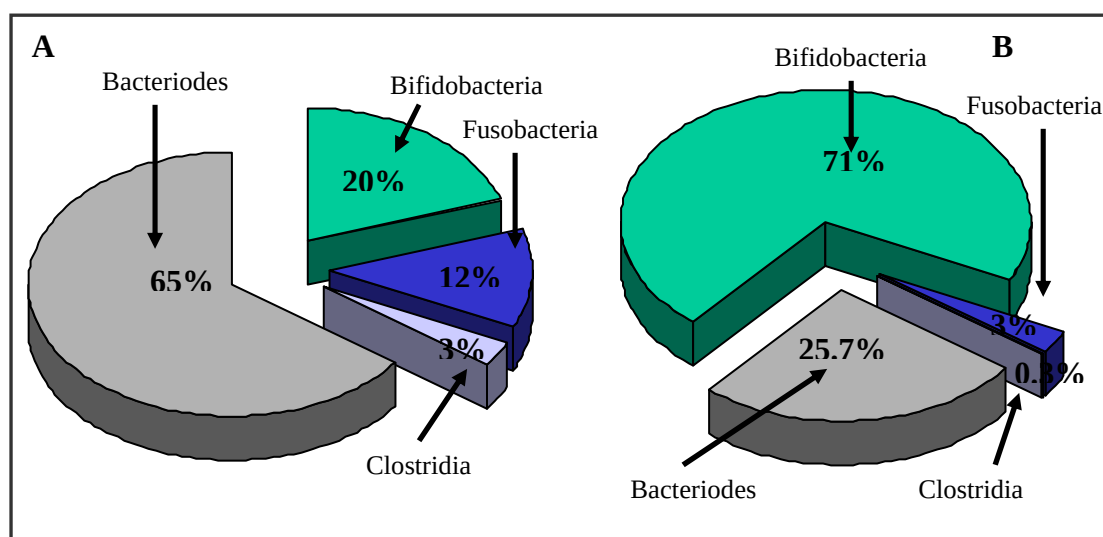


Figure 1.6 Bifidogenic effect of sc-FOS: (A) before sc-FOS intake, (B) after sc-FOS intake (Roberfroid, 1998).

1.4.2 Mineral Absorption

Apart from favourably altering the gut microflora balance, FOS have been shown to confer other physiologic benefits such as synthesis of B-complex vitamins and mineral absorption, amongst others (Mitsuoka, 1990; Prapulla *et al.*, 2004) (Fig. 1.7). Furthermore, FOS have low degree of sweetness (one-third) compared to that of sucrose, a traditional sweetener, and thus are suitable for use in diabetic supplements (Niness, 1999; Bornet *et al.*, 2002).

The distal part of the colon plays a pivotal role in mineral absorption; this is a result of FOS fermentation and the associated production of other essential metabolites such as SCFA. Butyrate serves as an energy source for colonocytes and also promotes the absorption of essential minerals such as Ca^{2+} and Mg^{2+} , and to a less extent, Zn^{2+} , Cu^{2+} and Mn^{2+} (Mitsuoka, 1990; Losada and Olleros, 2002). In particular, calcium precipitates acidic lipids (bile acids) as calcium soaps, thereby protecting the colon epithelium against possible toxic effects.

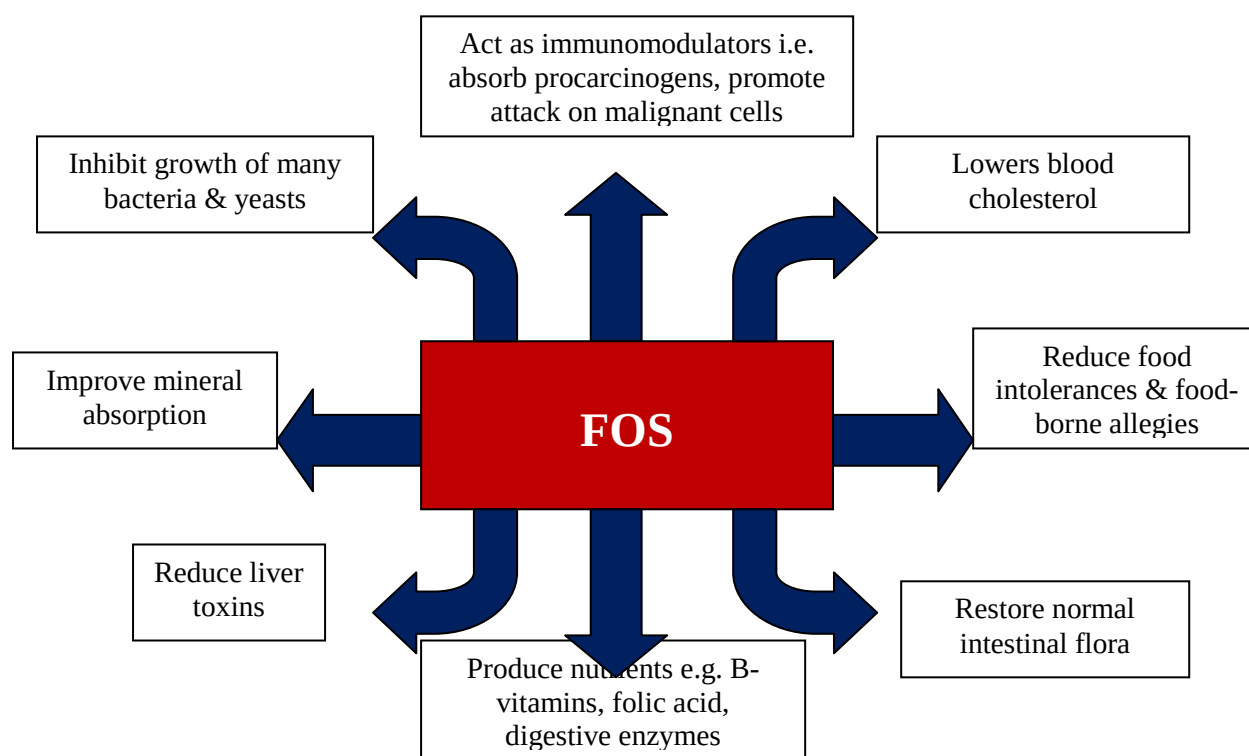


Figure 1.7 Health benefits exerted by FOS after fermentation by intestinal microflora (adapted from Mitsuoka, 1990).

In addition to the effect of calcium, a substantial number of investigations have shown an increase in Mg^{2+} absorption in both human and animal subjects after FOS intake (Delzenne *et al.*, 1995; Brouns and Vermeer, 2000; Coudray *et al.*, 2003). Improved Mg^{2+} absorption has also been shown to increase bone Mg^{2+} content, and is consequently an important factor for the reduction of osteoporosis risks (Rude and Gruber, 2004). Magnesium levels can also help ease asthma symptoms in young people, as a study by Abrams *et al.* (2007) suggests.

While the effect of FOS fermentation on Zn^{2+} levels is still unclear, other scientists have proposed a possible relation between Zn^{2+} levels and FOS intake (Lopez *et al.*, 1998; Scholz-Ahrens *et al.*, 2007). A study by Ducros *et al.* (2005) has shown that FOS supplementation in marginally Zn^{2+} deficient humans significantly enhanced Zn^{2+} levels. Zinc has also been shown to possess antioxidant properties, especially the prevention of lipid peroxidation at very low doses (Girotti *et al.*, 1985). Increased Zn^{2+} levels have also been shown to exert anti-hyperglycemic effect which may help

to regulate sugar cravings; suggesting that FOS might be crucial to normal control of diabetes and body weight (Patrice, 2003).

1.4.3 Lactose Intolerance Alleviation

Lactose intolerance is caused by a deficiency of the enzyme β -galactosidase (lactase), and individuals lacking this enzyme suffer from abdominal discomfort when they consume milk or other dairy products (Vesa *et al.*, 2000). The fermentation of FOS in the gastrointestinal tract results in the increased amount of *Lactobacilli* (Fahey *et al.*, 2002; Swanson *et al.*, 2002). *Lactobacilli* provide the enzyme β -galactosidase which hydrolyzes lactose and convert it to lactic acid. In this way, lactic acid plays a role in colon cancer suppression by deconjugating bile salts, thereby interrupting entero-hepatic circulation of carcinogenic bile salts (Hirayama and Rafter, 1999; Soomro *et al.*, 2002).

1.4.4 Anti-hepatic Encephalopathy

Bifidobacteria and *Lactobacilli*, after FOS fermentation, produce SCFA and other antagonistic substances creating an intestinal environment which is not conducive for the growth of putrefactive organisms such as *Helicobacter pylori* (Midolo *et al.*, 1995; Collado *et al.*, 2005). This results in lower intestinal urease levels and consequently lower blood ammonia levels. Hepatic encephalopathy is a neurologic disorder associated with liver failure due to elevated ammonia blood levels (Lockwood *et al.*, 1979; Ong *et al.*, 2003). This condition occurs when the enzyme urease from *H. pylori* acts on amino acids, urea and other nitrogenous compounds leading to the increased production of ammonia.

Under normal circumstances, ammonia is absorbed and detoxified in the liver. However, in patients suffering from liver failure, the detoxification process is impaired and the ammonia levels rise in the circulating blood. The increased proliferation of *Bifidobacteria* and *Lactobacilli* has also been found to be effective in decreasing intestinal urease levels and consequently suppressing hepatic coma and precoma (Mitsouka *et al.*, 1987; Gmeiner *et al.*, 2000). This explains the usefulness of administering FOS in the treatment of hepatic encephalopathy.

1.4.5 Therapeutic Effect

Intestinal disorders such as inflammatory bowel diseases (IBD), ulcerative colitis (UC), Crohn's disease (CD), aphthous stomatitis and glossitis are also caused by a deficiency in B-vitamins due to the imbalance in intestinal microflora (Andoh and Fujiyama, 2006). Prebiotics such as FOS are considered to modulate this balance by quantitatively altering the composition of the microflora. This is mainly because in a healthy intestinal environment, *Bifidobacteria* and *Lactobacilli* often produce essential metabolites and other nutrients such as B-vitamins that are necessary for normal bowel function (Rasic, 1983; Andoh and Fujiyama, 2006). Also, new research indicates that vitamins can significantly improve the fertility of both men and women (McKay *et al.*, 2000; Costello and Osrin, 2003).

The use of orally administered capsules containing both FOS and *Lactobacillus acidophilus* has also been used in patients suffering from various forms of migraine headaches (Redillas and Solomon, 2000). Although successful in 60-70% of the cases, FOS prophylaxis was found to reduce the severity of the headaches. Moreover, no adverse side effects were observed.

Diarrhoea is one of the most common symptoms of intestinal disturbance. Many studies have shown the positive effects of FOS combined with probiotics on various forms of diarrhoea such as antibiotic-induced diarrhoea, traveller's diarrhoea, HIV/AIDS diarrhoea and rotavirus-induced diarrhoea (Duffy *et al.*, 1994; Saavedra *et al.*, 1994; Yasui *et al.*, 1995; O'Sullivan and Kullen, 1998). An article published by the Nutrition Information Centre University of Stellenbosch (NICUS) also hypothesizes that FOS may be used therapeutically in patients suffering from HIV/AIDS. This hypothesis is based on the restoration of gut microecological balance, offering antimicrobial resistance and boosting immune function.

1.4.6 Alternative to Antibiotic Treatment

A number of studies have proven that the microflora balance in the gut is disturbed by treatment with antibiotics (Alestig *et al.*, 1983; O'Sullivan and Kullen, 1998). The consequences include: depletion of beneficial microflora and the re-establishment of pathogens in the gastrointestinal tract with subsequent invasion of the gut system

causing inflammatory, immunologic, neurologic and endocrinologic disorders (Cummings and MacFarlane, 1991; Duffy, 2000). Administering FOS along with antibiotics is considered to prevent this anomaly.

In addition, antibiotic resistance among some of the potent pathogens such as *Clostridia*, *Salmonella* and *E. coli* is becoming widespread due to the excessive use of these compounds in medicine and in animal feed. Consequently, the pressure to remove antibiotics from animal feeds and medicine has left a need for safe alternatives that can significantly minimize the levels of these pathogens (Hileman, 2001). Fructo-oligosaccharides have shown potential as alternatives to antibiotics (Wang and Gibson, 1993; Monsan and Paul, 1995).

1.4.7 Modulation of Serum Cholesterol levels

A variety of cardiovascular disorders such as coronary heart disease, and colorectal cancer, are often associated with elevated serum cholesterol levels (low-density lipoprotein-cholesterol, LDL~C) (Elaine and Feldman, 2002). When the intestinal environment is colonized by pathogens, the microbial 7- α -dehydroxylase and cholesterol dehydrogenase activities are increased resulting in the formation of bile salts and phospholipids which help in the absorption of cholesterol (Pool-Zobel *et al.*, 2001). In a balanced intestinal microbiota, *Bifidobacteria* and *Lactobacilli* deconjugate the bile salts to form bile acids which are further hydrolyzed by hydroxy steroid dehydrogenase (HSDH) and thereby inhibit micelle formation. When the bile salts and LDL~C levels are reduced, cholesterol is removed from plaque thereby leading to regression of heart disorders and colon cancer.

1.4.8 FOS and Insulin Resistance Syndrome

Insulin resistance syndrome (IRS) occurs in many forms, and the most severe form is known as Type 2 diabetes (Hotta *et al.*, 2001). This condition is also associated with a 2 to 4-fold increase in coronary heart disease (CHD) mortalities (Reaven, 2002). In trying to find a suitable mechanism for the connection between IRS and CHD risk, serum lipid levels (particularly triglyceride levels) and small dense LDL particles (both oxidation and glycation prone) are considered to be important indicators of CHD in Type 2 diabetes individuals (Reaven, 2002).

Similarly, in searching for the potential beneficial effect of prebiotics on IRS and CHD, there are several postulated mechanisms by which FOS and other prebiotics regulate plasma lipid and glucose homeostasis. These include, SCFA production, which in turn, affects enterohepatic circulation of cholesterol and bile acids. Precipitation of bile acids and reduced enterohepatic cholesterol circulation contribute to low incidence of CHD, IRS and colorectal cancer. Although the exact mechanism by which FOS influence glucose metabolism is unclear; a possible role of SCFA has been suggested (Venter *et al.*, 1990; Bornet *et al.*, 2004). Among the SCFA produced, propionate in particular, was shown to affect hepatic glucose metabolism in rats and in healthy human subjects. Propionate is also a gluconeogenesis inhibitor and has been shown to inhibit gluconeogenesis from lactate and to stimulate glycolysis in isolated hepatocytes (Anderson and Bridges, 1984).

Thus, the possibility that FOS as a sugar substitute could satisfy the desire for dietary sweetness suffered by many diabetics, and also lower both insulin and plasma triglyceride levels, would mark the much needed use of FOS for major diseases such as diabetes, obesity and colon cancer.

1.4.9 Immune System Modulation

Some researchers have shown that the ingestion of FOS stimulated intestinal IgA and the production of cytokines by blood mononuclear cells (Hosono *et al.*, 2003; Manning and Gibson, 2004). Roller *et al.* (2004) also indicated that prebiotic (FOS) treatment demonstrates putative effects such as phagocytosis on immune function modulation.

The production of SCFA, after FOS metabolism, may also modulate the immune system with the concomitant production of mucins, which are glycoproteins that are secreted on the surface of the gut by epithelial cells having a role as a barrier to intestinal infection (McGuckin *et al.*, 2007). As a result, the combination of prebiotics and probiotics has been the subject of many recent investigations and is postulated to play a pivotal role in maintaining good intestinal health, particularly against colon cancer (Reid *et al.*, 2003; Roos and Katan, 2004).

1.4.10 Anti-carcinogenic Effect

There is ample research evidence supporting the association between carcinogenic bacterial enzyme activities and colon cancer as a result of low fibre diet (Burns and Rowland, 2000; Pool-Zobel and Sauer, 2007). The mechanisms underlying this association can probably be explained by an increased amount of cytotoxic compounds such as secondary bile acids – deoxycholic acid (DCA), lithocholic acid (LA), and other pro-carcinogens. Secondary bile acids induce cell proliferation in the colon, and are therefore believed to promote colon carcinogenesis (Pool-Zobel *et al.*, 2001). However, intervention studies have shown that it is possible to reduce the concentration of bile acids by administering a diet high in fibre (Peters *et al.*, 2003). This effect is mainly attributed to an increase in the bioavailability of minerals such as calcium, which is thought to protect against cell damage in the colon (Fuchs, 2002).

The main anti-carcinogenic property of FOS appears to be the production of SCFA and subsequent reduction of intestinal bacterial enzyme activities produced by pathogenic microorganisms. Putrefactive colonic bacteria produce enzymes such as β -glucuronidase, urease and azoreductase which convert pro-carcinogens into carcinogens (Reddy, 1998; Teitelbaum and Walker, 2002). *Bifidobacteria* and *Lactobacilli*, through their competitive inhibition and production of unfriendly acidic environment, suppress the metabolic activity of these putrefactive enzymes and in this manner, reduce the formation of carcinogens in the colon. Moreover, SCFA play a role in normal cell proliferation and apoptosis of altered cells (Fig 1.8).

Although most of the above beneficial properties of FOS have been extensively demonstrated in many animal and human studies (Gibson and Roberfroid, 1995; Pool-Zobel *et al.*, 2001); a clear correlation between FOS and their anti-carcinogenic properties is yet to be fully investigated.

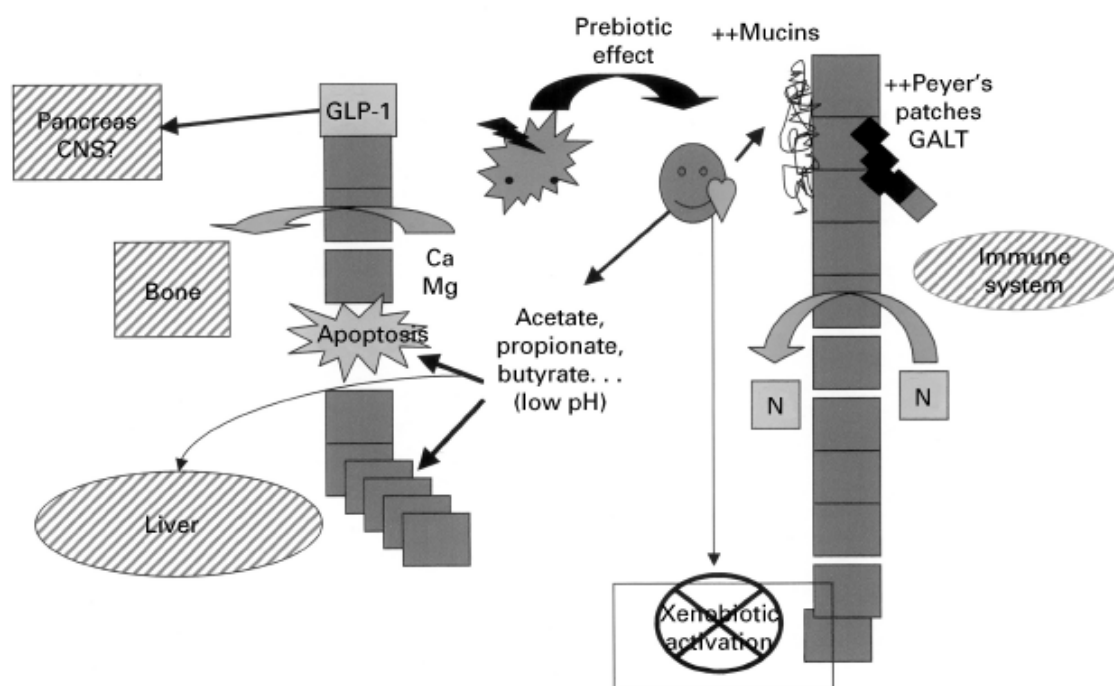


Figure 1.8 Illustration of the physiological effects in the gastrointestinal tract after FOS intake (adapted from Delzenne, 2003).

1.5 Fructo-oligosaccharides and Colon Cancer

Colon cancer is one of the leading causes of cancer mortalities in industrialized societies, and it is usually caused by a combination of hereditary, environmental and dietary factors, and the absence of physical activity (Potter, 1999; Gill and Rowland, 2002). Amongst these factors, diet is considered to have the most profound effect (Gibson and Roberfroid, 1995). This is attributed to the fact that a fine balance is established in the colon between the normal flora and the pathogenic bacteria after dietary fibre fermentation. Any changes in that balance inevitably results in the disturbance of the colonic environment (Fooks *et al.*, 1999). While it is known that the increase in beneficial bacteria favourably alters the colonic environment; in contrast, the proliferation of putrefactive bacteria may pose a risk factor for the development of colon cancer (Hirayama and Rafter, 1999).

Thus, the current hypothesis is that supplementing the diet with prebiotics such as FOS increases the density and the metabolic activity of the beneficial microflora in the colon, which in turn, improves bowel function and further protects the

gastrointestinal tract against colon carcinogenesis (Wolf *et al.*, 1996; Fuller and Gibson, 1997; Roberfroid, 2000; Sarkar, 2007).

Currently, what is lagging behind is the mechanism for the controlled synthesis of long-chain FOS. Recent data obtained with sc-FOS show that the production pattern of SCFA varies with the DP of each FOS (MacFarlane and MacFarlane, 2007). The proportion also changes with the duration of oligosaccharide intake and is an important factor in the enhancement of the prebiotic effect, i.e. the increase in *Lactobacilli* and *Bifidobacteria* (Tuohy *et al.*, 2003).

1.6 Synthesis of Fructo-oligosaccharides

1.6.1 Chemical Synthesis

1.6.1.1 Acid-catalyzed hydrolysis

Fructo-oligosaccharides can be obtained from inulin through acid-catalyzed hydrolysis (Sharma and Gill, 2007). This method has received great interest as it involves relatively inexpensive and widespread substrates such as inulin. However, the downstream processing and separation of the hydrolysis products is costly and thus makes this chemical method uneconomical. Furthermore, this method results in coloring of inulin hydrolysates and by-product formation in the form of difructose or oligosaccharide anhydrides (Van Damme and Derycke, 1983). In addition, most of the hydrolysis products such as fructose and other low DP sugars are not stable at low pH and are therefore easily degraded (Sharma and Gill, 2007). Also, there is an inherent difficulty in controlling the formation of sc-FOS with a defined and specific chain-length when using this method.

1.6.1.2 Automated solution-phase synthesis

Another chemical method includes the automated solution-phase oligosaccharide synthesis (Seeberger and Werz, 2007). The synthesis occurs on the basis of the relative reactivity of several monosaccharide ‘building blocks’, where a computer program known as Optimizer, selects the appropriate monosaccharides as well as the manner in which they should be assembled in the reaction vessel. According to

Seeberger and Werz (2007), each monosaccharide building block is synthesized at a multi-gram scale and can be used for the assembly of various sugar targets. Compared to oligonucleotide and peptide synthesis, glycosidic linkage formation occurs mostly at low temperatures such as -20°C and requires a reaction vessel that can be cooled.

To facilitate coupling reactions to completion and to obtain high yields, excess building blocks (5 -10 fold molar excess) are added to the reaction vessel. After completion of the assembly reaction, the fully synthesized oligosaccharide is cleaved from solid support and is purified and its structure analyzed (Fig 1.9). A mixture of oligosaccharides has been synthesized within a day or few hours using this automated method. However, this Optimizer method has been applied well for oligosaccharides up to six monosaccharide units, and yet it requires an extensive set of building blocks. Another drawback of this method is the difficulty in adding certain monosaccharides, and the double bond in linking moieties that restrict the deprotection conditions (Seeberger and Werz, 2005). Furthermore, this automated synthesis is not currently being performed by many researchers mainly because the synthesis instrument is not yet commercially available.

1.6.2 Enzymatic Synthesis

1.6.2.1 Inulinases

Inulin has been extensively studied as the main source for the production of high-fructose syrup (HFS) and inulo-oligosaccharides (IOS), through enzymatic hydrolysis by either sole action of exo-inulinase (β -D-fructosidase, EC 3.2.1.80) or the synergistic action of Exo-I and Endo-I (2,1- β -D-fructan frutanohydroxylase, EC 3.2.1.7) (Zhang *et al.*, 2004). However, the use of Exo-I enzyme is undesirable since it only gives rise to low molecular weight sugars such as fructose and glucose (Zhengyu *et al.*, 2004). On the other hand, efforts to utilize Endo-I for the synthesis of oligosaccharides seem ineffective due to the difficulty in getting a selective endo-inulinase free from hydrolytic activities (Nakamura *et al.*, 1997; Wang, 2000).

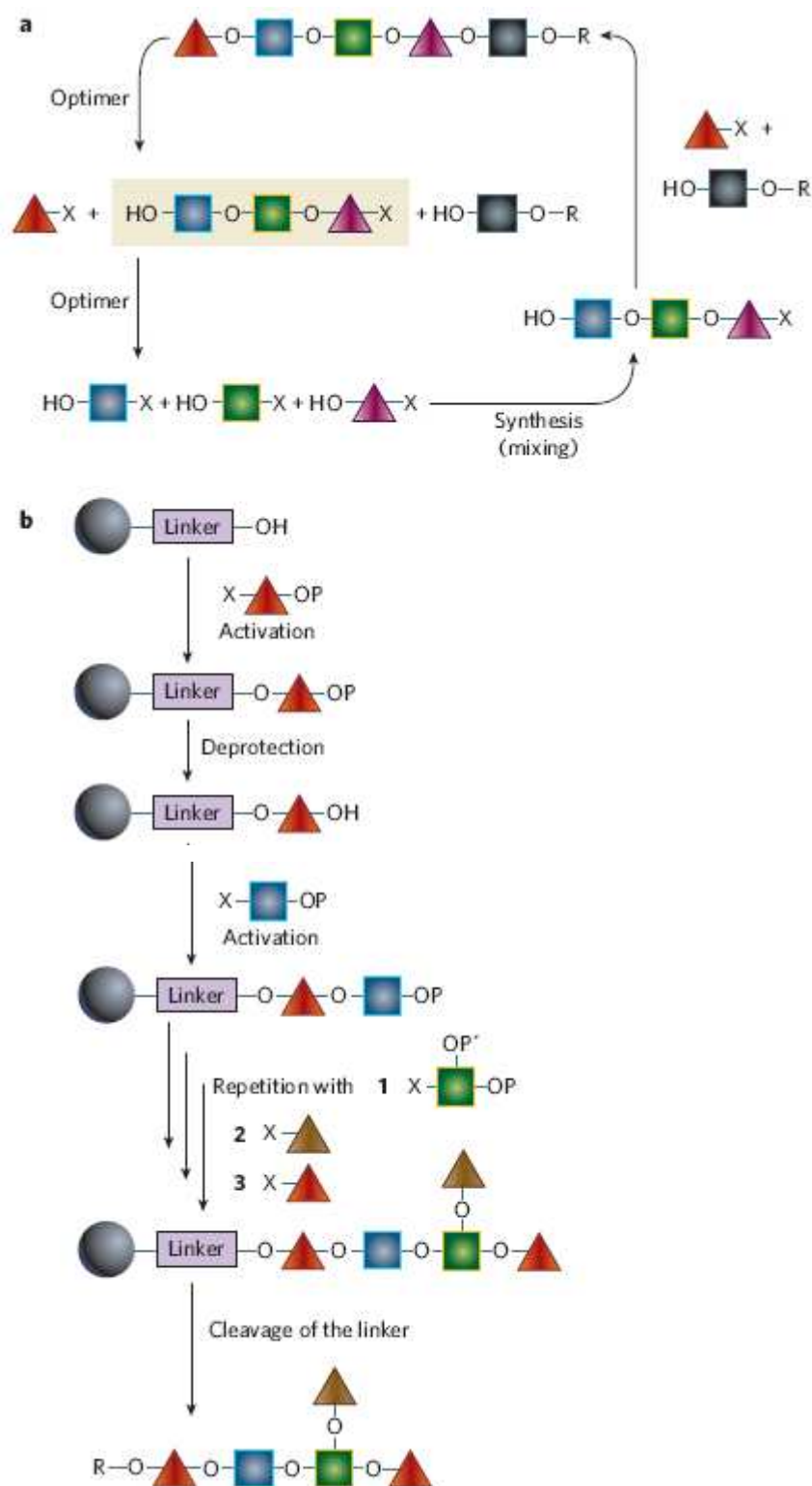


Figure 1.9 A schematic representation of the oligosaccharide sequence for automated oligosaccharide assembly (adapted from Seeberger and Werz, 2007).

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1.6.2.2 Extraction from plants

Fructo-oligosaccharides can be produced by extraction from plant sources through enzymatic hydrolysis of polysaccharides (such as pectin and cellulose) that are abundantly found in plant cell walls (Yun, 1996; Esteban *et al.*, 2000). Polygalacturonase (PGase, EC 3.2.1.67), is an enzyme usually produced by *Aspergillus*, *Mucor* and *Erwinia* species, and catalyzes the depolymerization of high molecular weight polymers such as pectin. Polygalacturonase may also extract low DP sugars that are stored as reserve carbohydrates after the depolymerization of high molecular weight glucans (Narbad *et al.*, 2006). Therefore, the production and hydrolysis of an enzyme-extracted oligosaccharide such as inulin holds promise for FOS synthesis, especially in the presence of hydrolytic enzymes such as endo-inulinases or exo-inulinases.

1.6.2.3 Fructosyltransferases

Fructosyltransferases (EC.2.4.1.9) catalyze the synthesis of defined chain-length FOS from sucrose. In the presence of efficient acceptors such as 1-kestose or nystose, these enzymatic reactions may lead to the formation of high molecular weight FOS (Tanriseven and Aslan, 2005).

The sources of FTase may be classified into two categories: one is plants such as onion, asparagus and dahlia (Darbyshire and Henry, 1981; Shiomi *et al.*, 2004); the other source is micro-organisms such as fungi (*Aspergillus* sp.) and bacteria (*Bacillus* sp.). The types of FTases produced by plants are known as invertases, whereas those produced by micro-organisms are termed levansucrases (Van Hijum, 2004). Fungal

FTases are also included in the glycoside hydrolase (GH) family 68 of the sequence-based classification of glycosidases. They are proposed to have a three-dimensional structure similar to that of levansucrase (LsdA, EC 2.4.1.10) (Fig. 1.10), which converts sucrose into FOS and levan (Martínez-Fleites *et al.*, 2005).

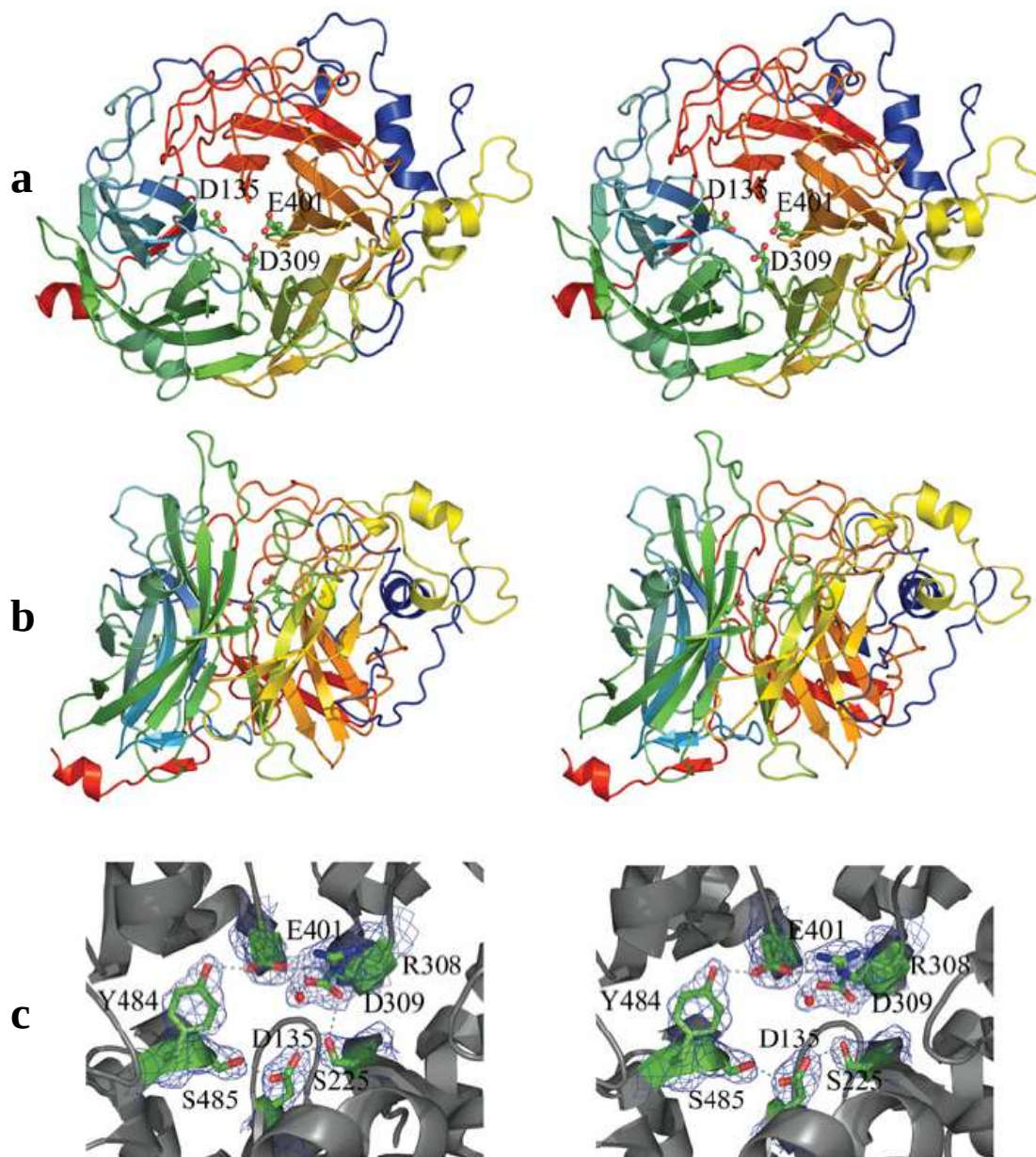


Figure 1.10 Three-dimensional structure of levansucrase (a bacterial FTase) from *G. diazotrophicus*. Superior (a) and lateral (b) stereo views of the five-bladed β -propeller fold. Catalytic residues Asp135, Asp309 and Glu401 are shown in ball-and-stick representation. (c) Stereo view of the electron density map (contoured at 1σ level) 'carved' around catalytic residues and other residues involved in the hydrogen-bond (broken lines) network at the active site. These structures were prepared with PYMOL (adapted from Martínez-Fleites *et al.*, 2005).

The sucrose-binding domain of *Aspergillus* FTase is also considered not to be significantly different from that of levansucrase (depicted in Fig. 1.11). However, the contrast is that levansucrase may also catalyze the transfer of the fructosyl residue, from sucrose, to a variety of acceptors including water (sucrose hydrolysis), glucose (exchange), sucrose (oligofructoside synthesis) and fructan (polymerase reaction) as indicated in Figure 1.14 (Martínez-Fleites *et al.*, 2005).

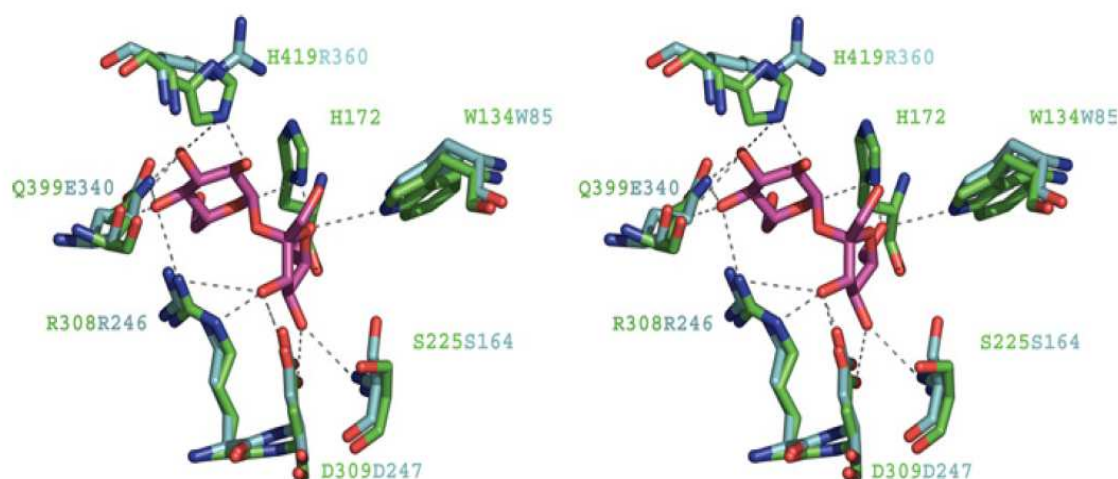


Figure 1.11 Sucrose-binding domain of levansucrase from *G. diazotrophicus* and *B. subtilis*. This Figure shows an overlap of the co-ordinates of the Bs levansucrase E342A -sucrose complex (Protein Data Bank accession code 1PT2) with the Gv levansucrase, LsdA. Potential hydrogen-bonding interactions are represented by broken lines. LsdA residues are bond-coloured green and Bs levansucrase are turquoise. Labels of depicted residues follow the same colour pattern (adapted from Martínez-Fleites *et al.*, 2005).

Aspergillus FTases are produced as extracellular enzymes. Recently, there have been attempts to produce FTases through various methods such as immobilization of fungal or bacterial cells onto polymers (Tanriseven and Aslan, 2005; Csanádi and Sisak, 2006; Polakovič *et al.*, 2006) and also from fungal cells in bioreactors (Vaňková *et al.*, 2005). The latter is very promising although still hampered with several limitations. This process produces a powdery purified FTase according to the separation steps illustrated in Fig 1.12. But apart from the difficulty in optimizing the operating conditions, the operating costs of this production method are very high, making it economically not feasible and undesirable.

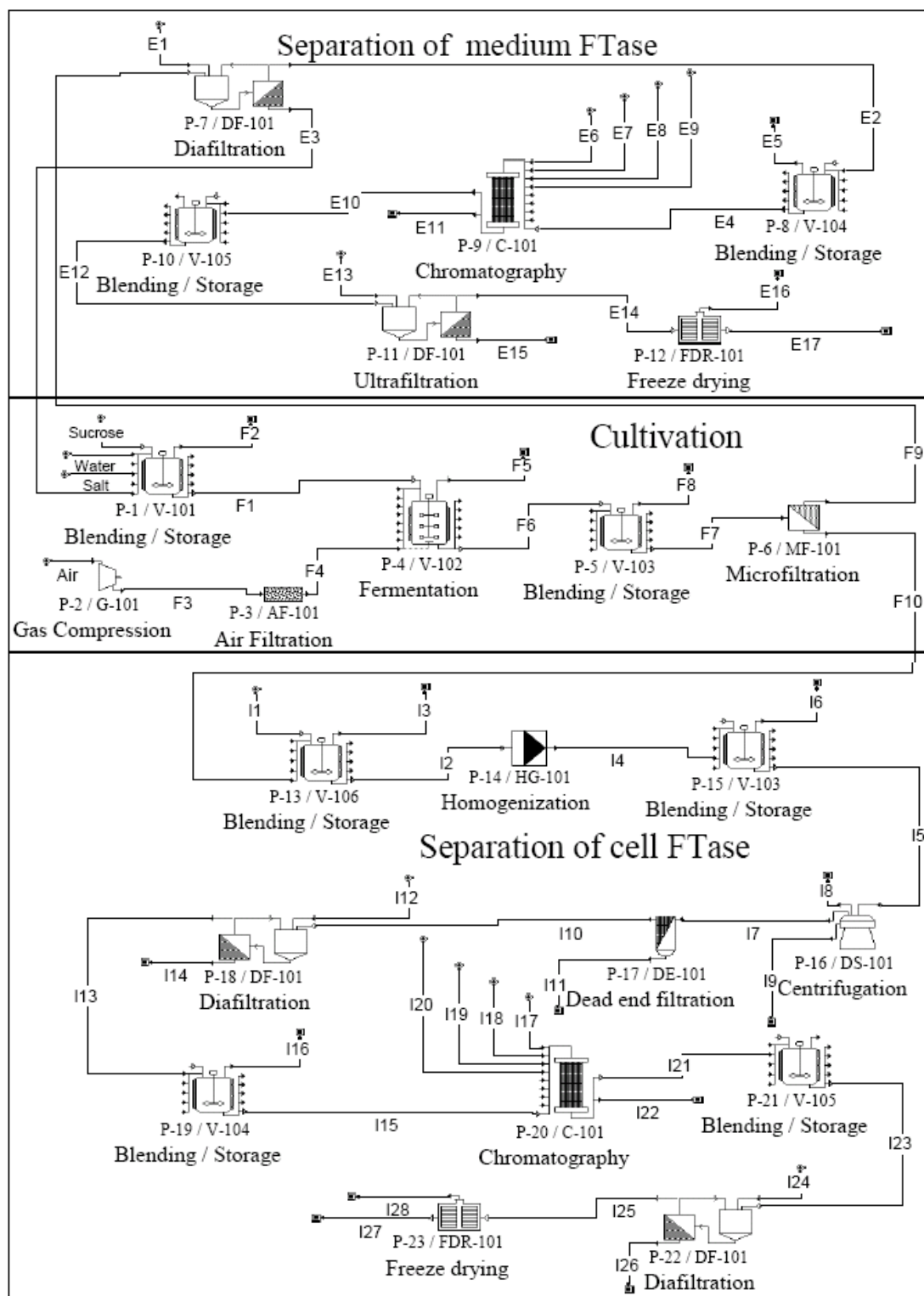


Figure 1.12 An illustration of the industrial process for the production of FTase. Numbers indicate the labels of the Type of Process / Equipment unit (adapted from Vaňková *et al.*, 2005).

In an attempt to maximize FOS synthesis, some researchers have investigated the cloning of FTase genes and the identification of important amino acid residues in

these enzymes, but even these approaches could not provide avenues for the synthesis of higher DP sugars (Rehm *et al.*, 1998; Heyer and Wendenburg, 2001). Also, in the quest of defining the actual three-dimensional structure of the enzyme, the crystallization of *Aspergillus* FTases still defies the crystallographers, probably due to the large size (± 118 kDa) and the instability of the protein subunits (Monchois *et al.*, 1999). These current gaps necessitate for the complete purification and biochemical characterization of FTase in order to understand the catalytic mechanism for producing long-chain FOS with novel properties.

Ideally, in the presence of sucrose, FTase cleaves sucrose at the β -(2 $\rightarrow$ 1) position and uses the energy released (between glucose and fructose) to couple a fructosyl residue to another acceptor molecule such as sucrose or 1-kestose, thereby growing the fructan chain (Fig. 1.13). In subsequent reactions, the enzyme elongates the chain in the same way, and the reaction is accompanied by the release of a glucose molecule as a by-product (Fig. 1.14). The actual DP of specific chain-length FOS depends on the catalytic properties of the enzyme and the availability of an efficient chain-ending acceptor. This means that, in the presence of adequate amount of substrate and the absence of inhibitory factors, FTase may catalyze the synthesis of variable long-chain FOS.

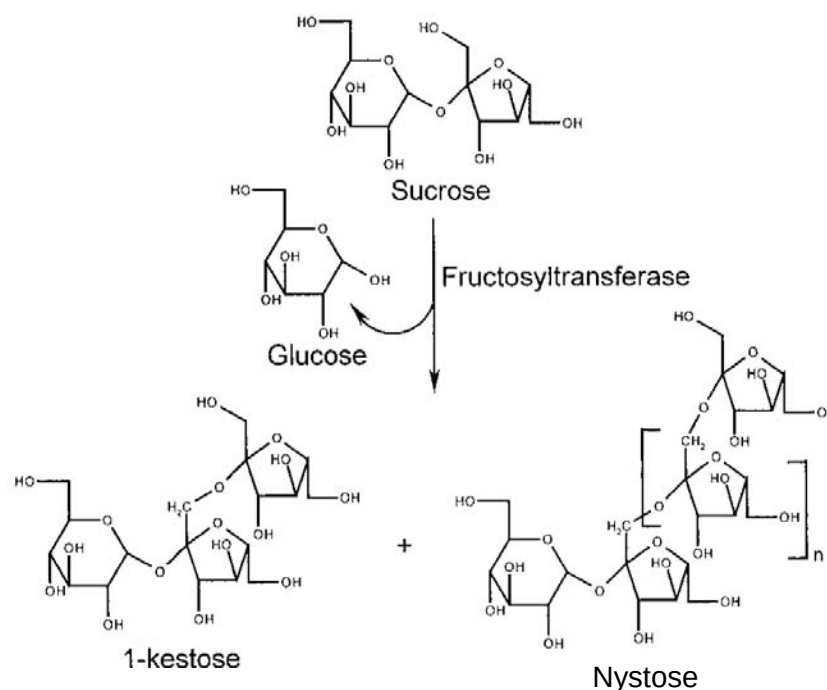


Figure 1.13 Enzymatic synthesis of FOS from sucrose by FTase (adapted from Tanriseven and Gokmen, 1999).

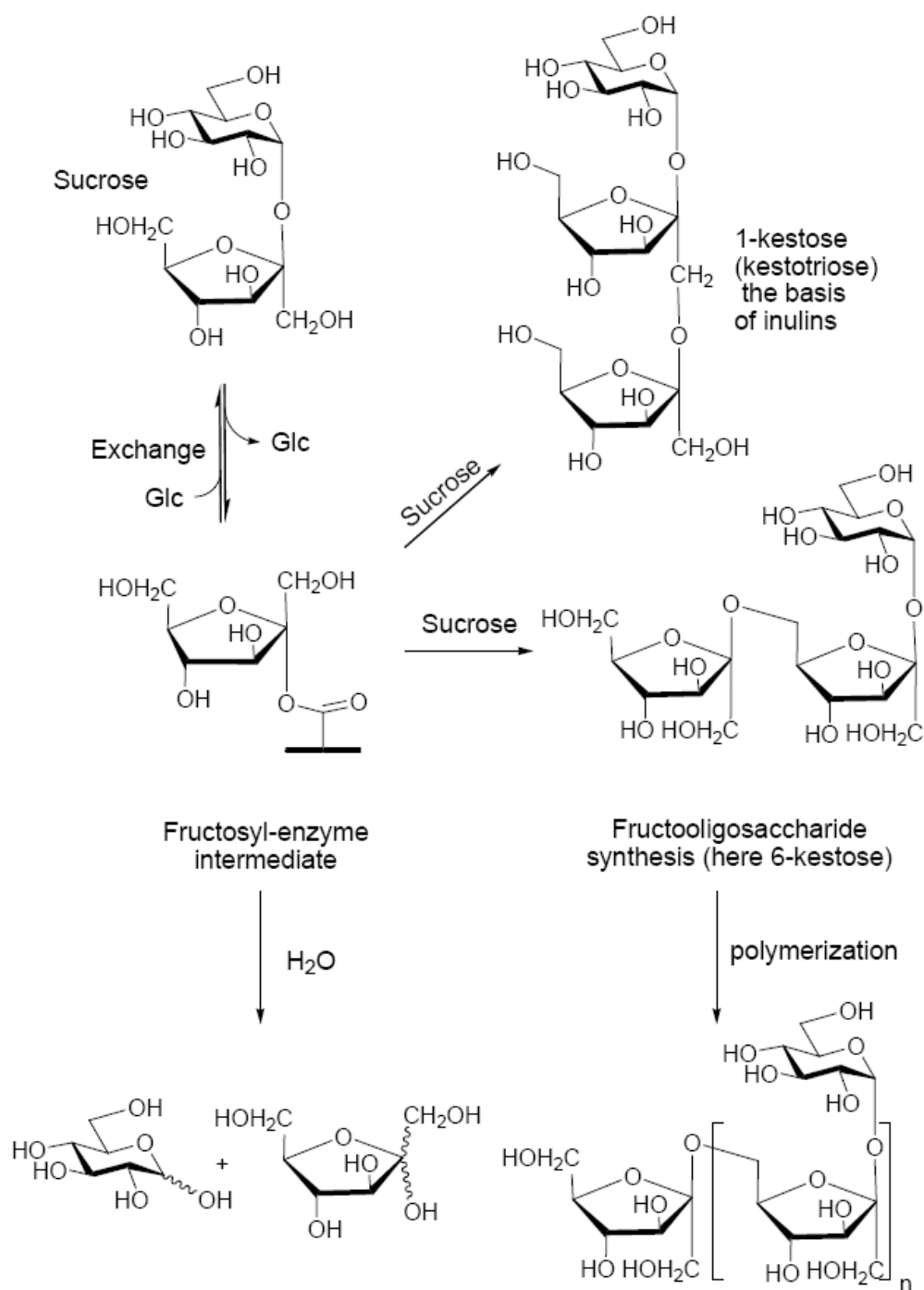


Figure 1.14 Reactions catalyzed by FTase to elongate the FOS chain (adapted Martínez-Fleites *et al.*, 2005).

The commercial enzyme preparation, Pectinex[®] Ultra SP-L derived from *Aspergillus aculeatus*, contains several enzymes such as β -galactosidase, cellulase, pectinase, and among these, fructosyltransferase activity. This crude enzyme preparation has been used for the production of FOS from sucrose (Prapulla *et al.*, 2005). However, since it

is a crude preparation with a mixture of several enzymes including hydrolases, it is very difficult to obtain defined chain-length FOS.

Substantiating the importance for a specific FTase activity, recent studies show that the production pattern of SCFA varies with the type of FOS; and is an important factor in the enhancement of the prebiotic effect, i.e. the increase in *Lactobacilli* and *Bifidobacteria* (Tuohy *et al.*, 2001). Therefore, by virtue of these potential health properties; detailed purification and characterization of the specific enzymes for the synthesis of FOS is crucial and remains to be established.

The aims of the present research are to develop an industrially applicable enzymatic method for the production of defined chain-length FOS using *Aspergillus aculeatus* as an enzyme source, and also to investigate the pharmaceutical potential of these FOS as anti-carcinogenic compounds.

1.7 Research Hypothesis

The synthesis of short-chain fructo-oligosaccharides of defined chain-length can be controlled by a thoroughly purified fructosyltransferase after characterization of the enzyme properties.

1.8 Objectives

This study investigates the avenues for the synthesis of FOS and their pharmaceutical properties by:

- Purifying and biochemically characterizing FTase from *Aspergillus aculeatus*
- Determining the catalytic parameters of FTase
- Purifying PGase for the extraction of inulin and comparing the FOS synthesis with inulin hydrolysis
- Employing various analytical techniques such as HPLC, ESI-MS and NMR to identify the synthesized FOS
- Elucidating the therapeutic and pharmaceutical properties of the synthesized FOS against certain carcinogenic enzyme activities.

CHAPTER 2

PURIFICATION AND CHARACTERIZATION
OF FRUCTOSYLTRANSFERASE

2 PURIFICATION AND CHARACTERIZATION OF FRUCTOSYLTRANSFERASE

2.1 Introduction

Fructosyltransferases, especially from microbial sources, represent an effective means for the synthesis of FOS with novel industrial and pharmaceutical applications (Antošová and Polakovič, 2001). However, since most enzymes are proteins, it is important to characterize them in terms of their kinetic properties prior to any fundamental application. But prior to characterization, there needs to be enzymes of high purity to better understand their specific mechanism and to ultimately develop processes that are incredibly efficient.

Despite numerous previous reports on the purification, characterization and industrial production of FTases from fungal and bacterial sources (L'Hocine *et al.*, 2000; Fujishima *et al.*, 2005; Vaňková *et al.*, 2005; Ghazi *et al.*, 2007), there are still no studies describing the successful purification and properties of FTase that can synthesize high DP sugars. Meanwhile, the synthesis of high DP FOS with novel prebiotic and physiological properties has evoked great interest in the medical, food and pharmaceutical industries. This, in turn, has fueled investigation and justifies the need to rigorously purify and characterize FTases for use as catalysts in order to produce products with new or highly specific functions. Also, the discovery of enzymes that can synthesize long-chain FOS from sucrose may provide an alternative to costly and polluting chemical methods such as acid-hydrolysis of inulin. Thus, it is the rationale of this chapter, based on the need to understand mechanistic influences of enzyme kinetics, pH, temperature, inhibitors, and activators on enzyme activity; to purify and characterize a tailored and specific FTase that can be used for FOS synthesis on industrial-scale.

2.2 Theory of techniques utilized

2.2.1 *Poly(ethylene) glycol Precipitation*

Poly(ethylene glycol) (PEG) is a water-soluble, inert polymer of cross-linked dextrans that is used for fractional precipitation of proteins and other macromolecules. When mixed with protein samples, PEG increases the viscosity of the solution and reduces the solubility of proteins, thus precipitating the protein according to its size (Plourde *et al.*, 1991). PEG is not influenced by the buffer conditions of the sample, and unlike acetone and other precipitating agents, it has little tendency to denature or otherwise interfere with proteins, even at high concentrations and increased temperatures (Ramshaw *et al.*, 1984). Another advantage of using PEG precipitation is that it is more rapid than acetone or $(\text{NH}_4)_2\text{SO}_4$ precipitation. The optimal PEG concentration is usually determined by means of an analytical precipitation curve. The protein samples are mixed with buffered solutions containing 5 – 40% (w/v) PEG and incubated at optimal temperature for 30 – 60 minutes (Thrash *et al.*, 1991). The samples are centrifuged and the remaining protein in the supernatant is assayed. This provides an estimate of the maximum concentration of PEG that can be added without losing much of the protein of interest, and also the minimum concentration of PEG required for bringing it out of solution. PEG is usually removed from the sample by ultrafiltration or in subsequent chromatographic steps. It is, however, worth noting that the performance of the chromatography column may be altered due to osmotic effects of PEG (Atha and Ingham, 1981; Thrash *et al.*, 1991).

2.2.2 *Ion Exchange Chromatography*

Ion-exchange chromatography is the most powerful chromatographic technique used for the separation and purification of proteins, nucleic acids and other charged biomolecules (Aquilar *et al.*, 2006). The ion exchange technique is most frequently used for its high resolving power, its simplicity and its high capacity for a wide range of biological molecules (Amersham Biosciences, 2007). The principle of ion exchange chromatography is based on the reversible binding of charged molecules to an immobilized ion exchange group of the opposite charge called the ion-exchanger. There are basically two different types of ion exchange groups namely, the cation exchanger and the anion exchanger (Fig. 2.1). A cation exchanger is a negatively-

charged matrix that binds to positively charged counter-ions (cations). Anion exchangers are positively-charged and therefore bind to negatively charged counter-ions (anions) in the ion exchange column. The matrix used for ion exchange comes in various forms based on its characteristics and may include inorganic compounds, polysaccharides and synthetic resins (Amersham Biosciences, 2007).

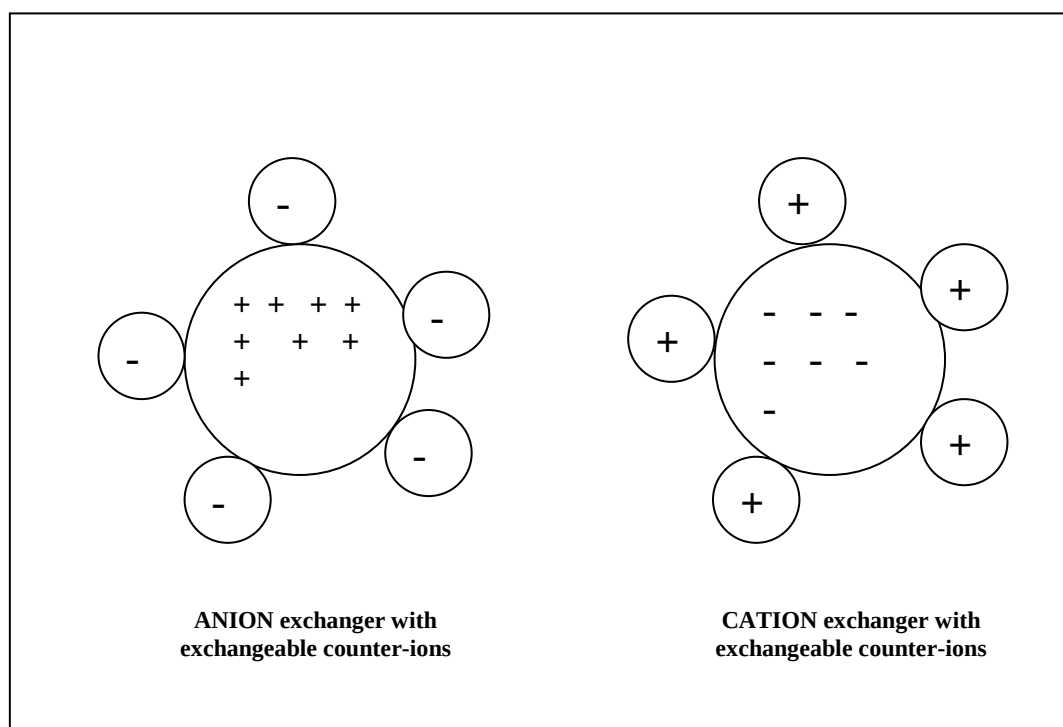


Figure 2.1 Ion exchanger groups (adapted from Amersham Biosciences Catalogue, 2007).

In principle, ion exchange separation occurs in five main steps. The first step is equilibration where the ion exchanger (or resin) is mixed with the ideal buffer solution to obtain a uniform state in terms of pH and ionic strength. This allows for the binding of the desired molecule when it is introduced into the column. The second step is sample application and binding (adsorption) of the desired molecule. In this step, the molecule of interest carrying the appropriate charge replaces the counter-ions and adsorb reversibly to the ion-exchanger matrix. After binding, unbound materials can be washed out from the ion exchanger using the starting buffer (Amersham Biosciences, 2007).

In the third step, the molecule of interest is eluted from the column by changing the elution buffer conditions. This normally involves a change in pH or increasing the ionic strength of the eluting buffer with a salt gradient. Desorption of the desired molecule is achieved by the introduction of an increasing salt concentration gradient, either through a step-wise or linear gradient elution. In this way, adsorbed molecules are eluted from the column in the order of their binding strength, with weakly bound molecules eluting first. The fourth and fifth steps are simply the removal, from the column, of all materials not eluted by the elution procedure and re-equilibration of the column for the next purification experiment.

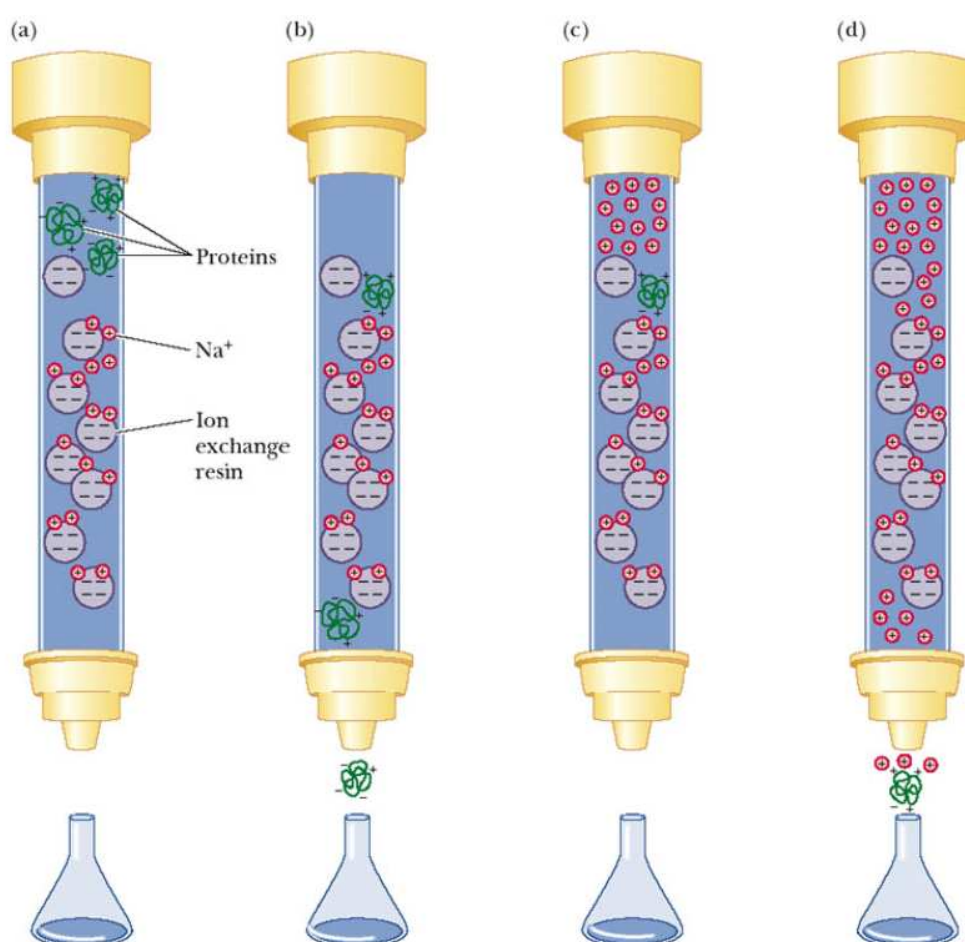


Figure 2.2 A schematic representation of IEC using a cation exchanger. (Source: Brooks/Cole – Thomson, 2006). (a) Proteins are applied to the column; The column resin is bound to Na⁺ counterions (small red spheres); (b) Proteins with a net negative charge pass through the column; (c) An excess of Na⁺ ions are added to the column; (d) The Na⁺ ions outcompete the bound proteins for the binding sites and the proteins of interest elute.

2.2.3 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is very a useful technique in the purification of larger molecules. The SEC technique is based on the selective permeation of the polymer for various macromolecules in and out of the mobile-phase filled pores of the column (Hedlund, 2004). The separation of molecules occurs as a function of size rather than molecular weight distribution such that larger molecules are excluded from the pores of the stationery phase, and hence elute first from the column. The elution time of the smaller molecules is longer as they are restricted inside the pores of the polymer, and therefore elute last from the column (Kirkland, 1979). Figure 2.3 illustrates the events that occur during size exclusion separation.

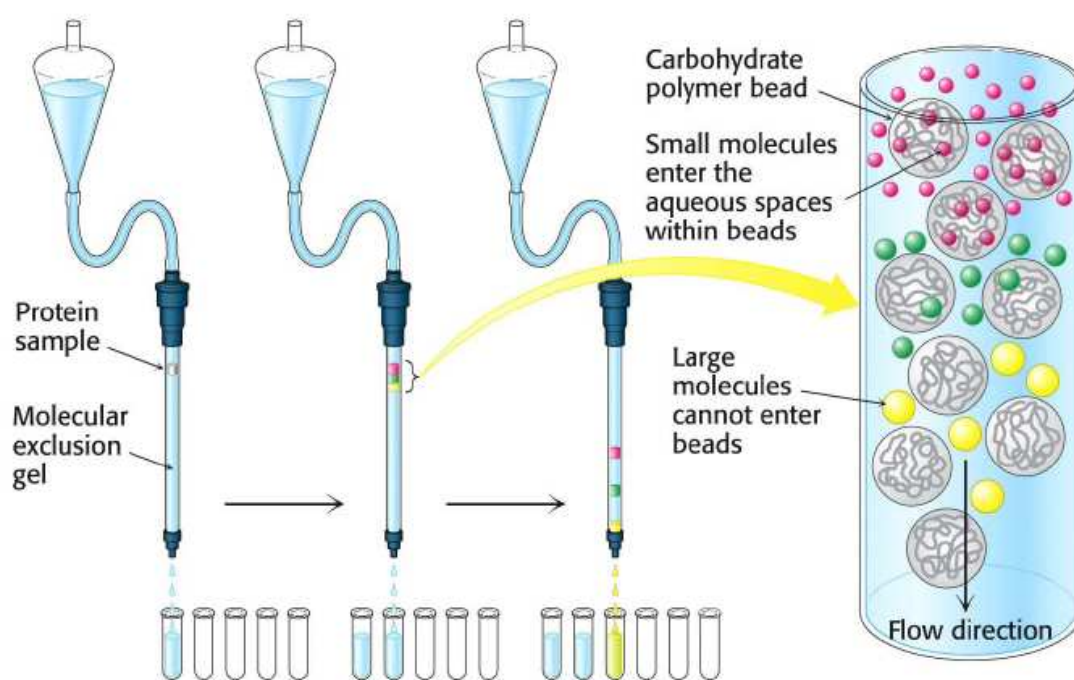


Figure 2.3 A schematic illustration of separation in SEC (adapted from Scholze, 2004).

2.2.4 Electrophoresis

Sodium dodecyl sulphate-polyacrlamide gel electrophoresis (SDS-PAGE), under denaturing conditions was used to examine the purity and to determine the molecular weight of proteins.

The electrophoresis technique is based on the migration of charged particles in an electric field (Laemmli, 1970). To enable the estimation of molecular weights, proteins are mixed with an anionic detergent (such as SDS), which binds to the protein at a constant ratio of 1.4 g to 1 g of protein to form rod-like complexes. The lengths of these complexes vary according to the molecular weights of the individual proteins. The predominant negative charge of the SDS gives all the protein/SDS complexes an overall negative charge, with the same charge to mass ratio.

The addition of a thiol reducing agent (such as 2-mercaptoethanol) to the protein/SDS complex breaks the disulfide bonds when boiled in the presence of excess SDS (Laemmli, 1970). This disruption of disulfide bonds dissociates the individual polypeptide subunits. When a gel system is placed in the appropriate buffer preparation, the introduction of an electric field will allow the complexes to migrate according to their molecular weights. Consequently, the molecular weights of the unknown subunits may then be determined by comparing their migration distances with those of proteins with known molecular weights (molecular standards).

As a precaution, SDS-PAGE only affords the separation of proteins according to their molecular weights on a preparative scale, and therefore does not allow re-use of proteins due to the difficulty of removing SDS and denaturation of proteins. Native (non-denaturing) PAGE has a distinct advantage over SDS-PAGE in that it allows the recovery of biological activity of the proteins being separated and can be applied to protein samples with other conjugates (e.g. glycoproteins and lipoproteins) (Suck *et al.*, 2004). The purification method is described below.

2.3 Materials and Methods

2.3.1 Reagents

The commercial enzyme preparation (Pectinex[®] Ultra SP-L) from *A. aculeatus* with fructosyltransferase activity (24 IU/mL) was kindly donated by Novozymes (South Africa). DEAE-Sephacel[®]; Sephacryl[®] S-200; Bradford reagent; dinitrosalicylic acid (DNS); Acrylamide; bis-acrylamide; Coomassie Brilliant Blue R-250 and *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED) were purchased from Sigma-Aldrich (South

Africa). Sodium dodecyl sulphate (SDS); poly(ethylene glycol) (PEG 20 000); sucrose; glucose; fructose and bovine serum albumin (BSA) were obtained from Merck Chemicals (South Africa). Molecular weight markers were from PEQGOLD™ (Erlangen, Germany). All other chemicals were of analytical grade and were obtained from readily available sources. In all the experiments, reagents were dissolved in Milli-Q water and all the experimental conditions throughout the purification, except where indicated, were performed at 0 – 4 °C.

2.3.2 Materials

All the chromatographic columns, Mini PROTEAN III Electrophoresis kit and the fraction collector (model 2110) were from Bio-Rad (South Africa). Bench-top centrifuges (Eppendorf 5810R and Eppendorf MiniSpin®); self-regulated temperature water bath (model 130) and pH meter (Inolab pH level 1) were purchased from Merck (South Africa). The PowerWaveX™ microplate reader was purchased from Bio-Tek Instruments (Vermont, USA).

2.3.3 Enzyme Assays

The reaction mixture of the FTase assay contained sucrose (300 µL, 30%, w/v) in phosphate buffer (20 mM, pH 6.0, 180 µL) and enzyme (20 µL). The mixture was incubated at 60 °C for 40 min in a water bath. The reaction was stopped by adding the DNS reagent (40%, 500 µL). The mixture was boiled for 5 minutes and the total reducing sugars released were measured at 540 nm using a PowerWaveX™ microplate reader. One unit of FTase was defined as the amount of enzyme liberating 1 µmol of reducing sugar (glucose or fructose) per minute under the above assay conditions. A standard curve with known glucose concentrations was constructed for calculating enzyme activity (Appendix A). All absorbance readings were performed in triplicate and are presented as the means ± S.E.M.

The total protein concentration for all the experiments was routinely determined according to the method of Bradford (1976). The assay was performed in a 96-well microplate. To each well, 5 µL of the unknown protein sample were added, followed by 245 µL of the Bradford reagent. The mixture was incubated at room temperature (RT) for 10 minutes, after which the absorbance was measured at 595 nm using a

PowerWaveX™ spectrophotometer. The concentration of the unknown samples was determined by referring the absorbance values ($A_{595\text{nm}}$) to the BSA standard curve (Appendix B).

2.3.4 Poly(ethylene glycol) Precipitation

Poly(ethylene glycol) 20 000 (30% w/v, 27 mL) was added to the crude protein sample (18 mL), and the mixture was stirred at 4 °C for 50 minutes to allow precipitation to occur. The mixture was centrifuged at $3000 \times g$ for 40 minutes using an Eppendorf centrifuge. The pellet was collected and re-suspended in phosphate buffer (20 mM, pH 6.5, 8.0 mL). Protein and activity assays were performed.

2.3.5 Anion-Exchange on DEAE-Sephacel®

The FTase sample from PEG precipitation was further purified by ion-exchange chromatography (IEC) on a Glass Econo-Column® (3 × 40 cm) packed with DEAE-Sephacel® anion-exchanger resin, with a bed volume (45 mL). The sample (5.0 mL) was applied onto the DEAE-Sephacel column previously equilibrated with phosphate buffer (20 mM, pH 6.5). The column was washed with the same buffer until the $A_{595\text{nm}}$ was at the baseline and then the adsorbed proteins were eluted with a linear salt gradient (0 – 0.5 M NaCl) in phosphate buffer (20 mM, pH 6.5) at a flow rate of 1.3 mL/min (3.5 mL per tube). The fractions were collected in a fraction collector and the total protein and enzyme activity assays were performed. The FTase-enriched fractions (8.0 mL) were pooled, dialyzed against phosphate buffer (20 mM, pH 6.5), concentrated by PEG using a 10 kDa cut-off membrane and subjected to further purification.

2.3.6 Size Exclusion on Sephacryl® S-200

The concentrated fractions (3.0 mL) from the DEAE-Sephacel column were then applied onto the Sephacryl S-200 column (2.2 × 65 cm) equilibrated in phosphate buffer (20 mM, pH 6.5). The column was washed with the same buffer until the $A_{595\text{nm}}$ remained at the base-line and then proteins were eluted with a linear NaCl (0 – 0.5 M) gradient in phosphate buffer (20 mM, pH 6.5) at a flow rate of 1.2 mL/min (2.4 mL per tube). Fractions were analyzed for total protein and FTase activity. Active fractions were pooled, dialyzed against distilled water, freeze-dried and stored at -20

°C. The purity and the molecular weight of the enzyme were analyzed by both native and SDS-PAGE.

2.3.7 *SDS and Native PAGE Analysis*

The separation of proteins by SDS-PAGE was performed with a few modifications from the method of Laemmli (1970). The apparatus were assembled according to the manufacturers' instructions. The gels were run at a constant voltage of 160 V. After the electrophoretic run was completed, both the stacking (5%) and the separating (12%) gels were routinely stained with Coomassie Brilliant Blue R-250.

The procedure for native PAGE was performed with 5% stacking gel and 8% separating gel at the same constant voltage of 160 V. After electrophoresis, the protein band was sliced out, re-suspended in minimal phosphate buffer (20 mM, pH 6.5, 1.0 mL) and incubated with sucrose (30%, w/v). The activity was assayed as described in 2.2.3.

2.3.8 *Effect of Temperature and thermal stability on FTase Activity*

The effect of temperature on FTase activity was determined at various temperatures (ranging from 20 °C to 70 °C) under the standard assay conditions (described in 2.3.3), except with 0.2 U/mL enzyme dosage, before stopping the reaction with the addition of DNS.

The temperature stability of the FTase was determined at the optimum temperature (60 °C). Aliquots were removed periodically (at 1 h intervals) and analyzed for activity for a maximum period of 12 h

2.3.9 *Effect of pH on the FTase activity*

To determine the pH optimum, the enzyme activity was assayed at different pH values (2.0 – 9.0) under the assay conditions described in section 2.2.3, except that phosphate buffer was replaced with McIlvaine buffer, at the optimum temperature of 60 °C.

2.3.10 Determination of the Kinetic Parameters

The enzyme (0.2 U/mL) was incubated with sucrose (100 – 500 mM) in phosphate buffer (20 mM, pH 6.0) at 60 °C. The kinetic parameters (K_m and V_{max}) of the purified FTase were determined by linear-regression analysis using the Hanes – Woolf plot. The k_{cat} value was calculated from the moles of the purified FTase using the equation in Appendix C.

2.3.11 Effect of metal ions and chemicals on FTase activity

The chemical susceptibility of the FTase was evaluated by incubating the enzyme with various (divalent and monovalent) metal ions, detergents and reducing agents prepared at 5 mM concentration under the optimal conditions (60 °C, pH 6.0).

2.4 Results and Discussion

2.4.1 Purification of FTase from *A. aculeatus*

The *Aspergillus aculeatus* FTase was first fractionated from the crude extract using PEG 20 000 (30%, v/v) and further purified to homogeneity by two successive chromatography steps on DEAE-Sephacel® and Sephacryl® S-200, respectively. The results of the purification procedure are summarized in Table 2.1. A number of fractions (28 – 34, 24.5 mL) with FTase activity were eluted from the DEAE-Sephacel column using a linear gradient (0 – 0.5 M NaCl) as shown in Figure 2.4. These active fractions were pooled, dialyzed against phosphate buffer (20 mM, pH 6.5) and concentrated by PEG to yield a 5 mL fraction.

Table 2.1 Purification table for FTase from *A. aculeatus*.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purif. fold	Yield (%)
Crude extract	15	118.5	345	2.91	1	100
PEG (30%)	8	37.6	113.6	3.02	1.04	33
DEAE-Sephacel	5	27	31	1.15	0.4	9
Sephacryl S-200	3	1.6	0.6	0.38	0.34	0.17

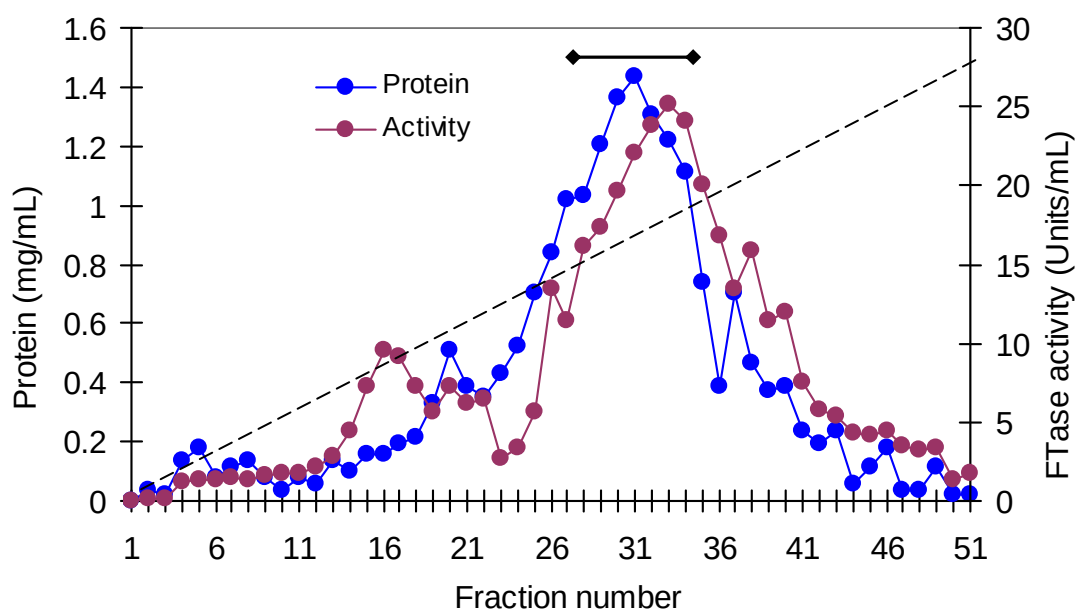


Figure 2.4 Elution profile of DEAE-Sephacel ion-exchange chromatography. Proteins eluted with linear (-----) NaCl gradient (0 – 0.5 M) in phosphate buffer (20 mM, pH 6.5); Flow rate, 1.3 mL/min (3.5 mL per tube). Fractions pooled indicated by: (◄—►).

The concentrated sample (3.0 mL) from the DEAE-Sephacel column was loaded onto the Sephacryl S-200 column and all recoverable proteins were eluted into four FTase-rich fractions (9 – 12) with a linear salt gradient (Fig. 2.5). However, the separation of proteins by size-exclusion chromatography led to a decrease of the overall FTase activity from 31 to 0.6 U. The enzyme was purified 0.34-fold; its specific activity was 0.38 U/mg, with a yield of 0.17%. This decrease in total activity and yield could be explained, in part, by the removal of one of the important subunits during subsequent purification. It could also be a result of the NaCl gradient used for elution which probably led to partial dehydration of the gel as well as dehydration of proteins resulting in large protein loss or partially denatured protein.

2.4.2 Confirmation of Purity and Molecular Weight: Native and SDS-PAGE

The purified FTase electrophoretically migrated as a single homogeneous band of approximately 85 kDa when examined on 12% SDS-PAGE gel (Fig. 2.6). However, native PAGE revealed three protein bands estimated at 85, 50 and 30 kDa,

respectively (Appendix D), although these bands may not necessarily be separate distinct proteins but rather a mixture of partially denatured protein.

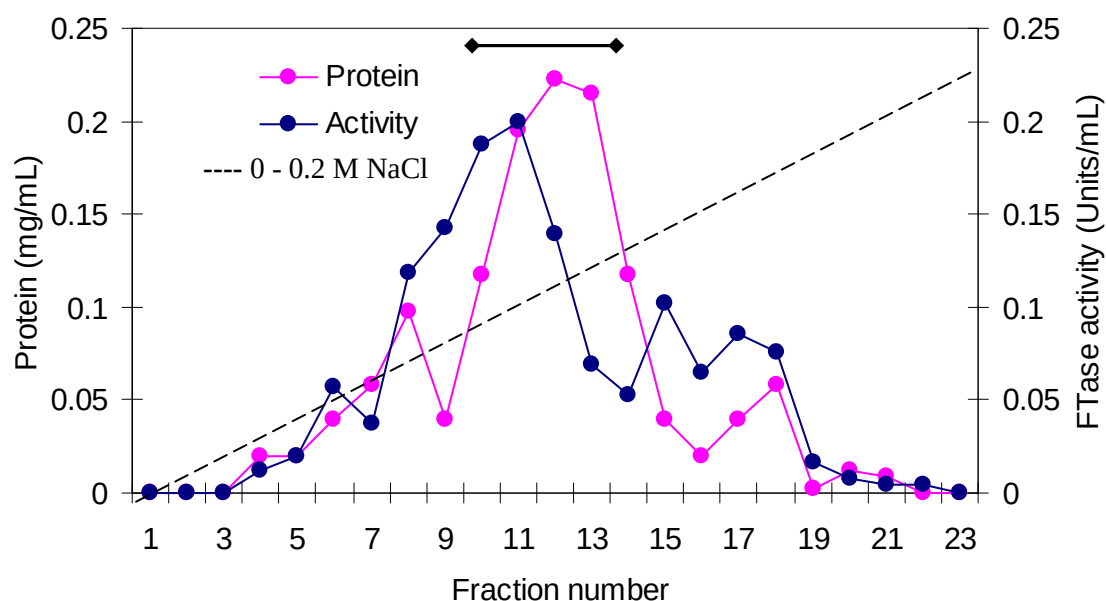


Figure 2.5 Size-exclusion chromatography elution profile on Sephacryl S-200: Linear NaCl (-----) gradient (0 – 0.2 M) in phosphate buffer (20 mM, pH 6.5); Flow rate, 1.2 mL/min (2.4 mL/tube). Fractions pooled: (◆—◆).

It is also probable that the three bands on native PAGE could be three species of the same protein with different charges due to glycosylation or phosphorylation. Also, bearing in mind the fact that most of the purified β -D-fructofuranosidases (EC 3.2.1.26) are glycoproteins with a raffinose and/or glucose residue in their carbohydrate moiety (Wallis *et al.*, 1997; L'Hocine *et al.*, 2000), this could be one such slight modification. Moreover, in their native form, β -D-fructofuranosidases tend to under go association-dissociation, resulting in the aggregation of the subunits to form oligomers (dimer, tetramer and hexamer). Since it is difficult to accurately determine the molecular size of a glycoprotein on a native gel (Harris and Angal, 1994), therefore if FTase was a dimeric protein, SDS-PAGE would have revealed it.

2.4.3 Physico-chemical Properties of FTase

The temperature and pH optima of the SEC purified FTase were determined at various ranges under the conditions described in 2.2.8 and 2.2.9, respectively. The pH

optimum for the FTase was found to be 6.0 (Fig. 2.7). This pH was similar to that of the FTase from *A. niger* reported by L'Hocine *et al.* (2000), as well as from *A. japonicus* reported by Hirayama *et al.* (1989).

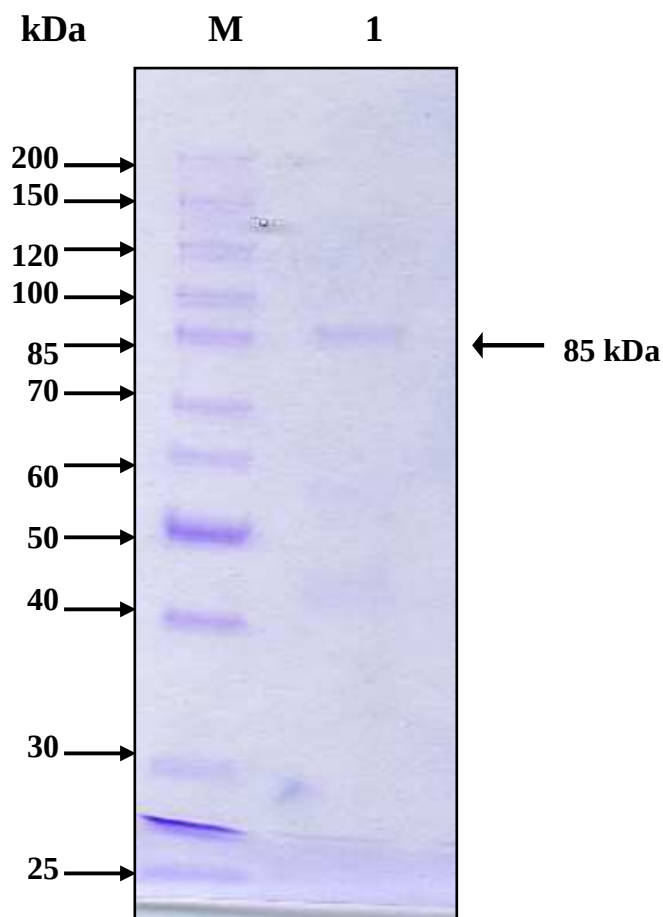


Figure 2.6 SDS-PAGE analysis of the SEC purified FTase. Lane 1, the purified FTase; Lane M, molecular weight markers. The stacking gel concentration was 5% and the separating gel was 12%. The gel was stained with Coomassie Brilliant Blue R-250 and destained with acetic acid:methanol: water (1:1:8, v/v).

The enzyme lost its activity rapidly at pH values above 6.0. Typically, an enzyme is expected to have at least 50% activity between the pH values equivalent to the pKa values of the crucial amino acid residues at the active site. Therefore, the decrease in FTase activity at pH values above 6.0 suggests that the pKa values of the crucial amino acids at the active site are in the range between 3.0 and 6.5. Thus, the most likely amino acids in the active site region could be aspartic acid, glutamic acid and histidine.

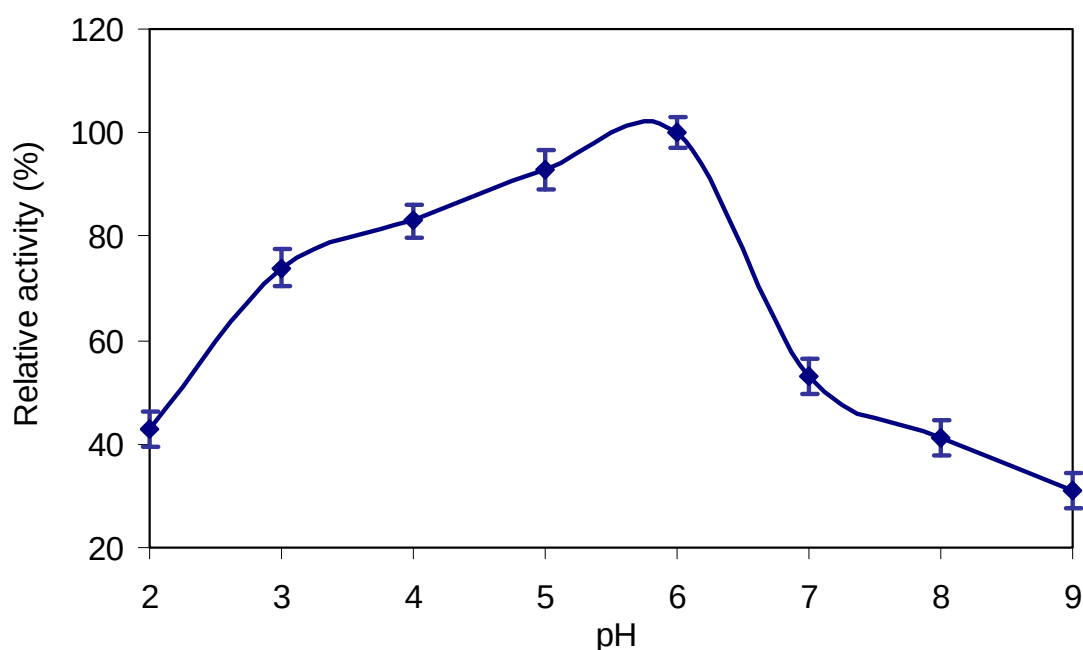


Figure 2.7 pH profile of the purified FTase from *A. aculeatus*. Error bars represent the means ($\pm$ S.E.M) of three trials.

Based on the results shown in figure 2.8, the purified FTase shows maximum activity at 60 °C with a half-life of 8 h. However, the enzyme rapidly lost its activity when assayed at temperatures above 60 °C. This optimum temperature of 60 °C is also closely comparable to that of other FTases from *A. japonicus* and *A. niger* strains (Park and Almeida, 1991; L'Hocine *et al.*, 2000). After 7 hours of incubation at 60 °C, more than 80% of the enzyme activity was retained (Fig. 2.9). However, incubation period longer than 8 hours rapidly decreased the enzyme activity (half-life, $t_{1/2}$ = 8 h). This data suggest that the prolonged (half-life) biocatalysis may be carried out at 60 °C for at least 8 hours.

The effect of various metal ions and other relevant compounds on FTase activity was determined relative to the control (without any added compounds), which was taken as 100% activity (0.2 U/mL). As shown in Table 2.2, Ca^{2+} , Fe^{2+} , Mn^{2+} and Cu^{2+} ions did not have any significant effect ($P > 0.05$) on FTase activity. Magnesium ions were required for the FTase enzyme activity. However, the heavy metal ions (Ag^{2+} , Zn^{2+} and Ni^{2+}) strongly inhibited the enzyme activity. This is mainly due to the fact that heavy metal ions disrupt disulfide bonds often formed by amino acids such as

histidine and cysteine. On adding 5 mM urea, EDTA and glycerol, the enzyme retained more than 95% of its activity.

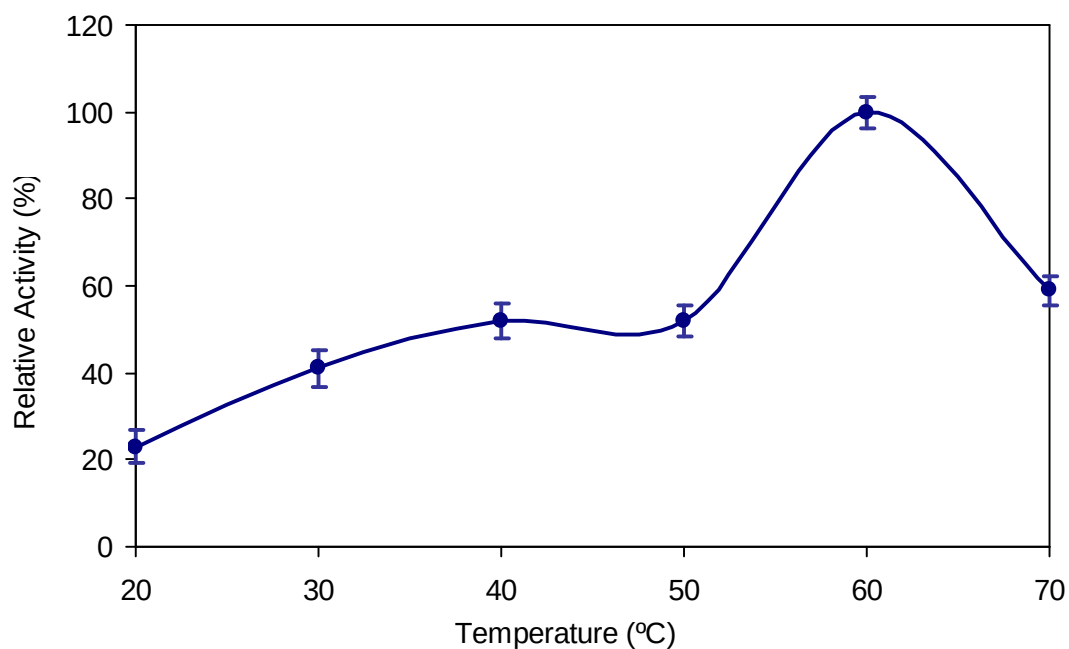


Figure 2.8 Temperature profile of the purified FTase from *A. aculeatus*. Error bars represent the means ($\pm$ S.E.M) of three trials.

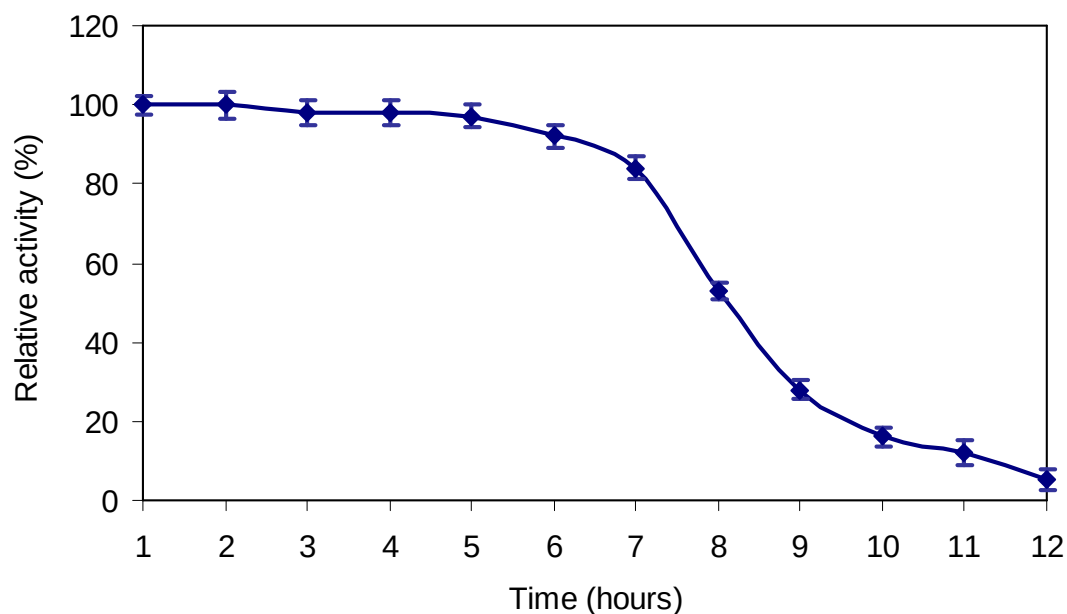


Figure 2.9 Temperature stability of the FTase from *A. aculeatus* at 60 °C.

Table 2.2 Effect of various metal ions and compounds on the activity of FTase from *A. aculeatus*.

Metal ions / Compound (5 mM)	Residual activity (%)
Control	100
ZnSO ₄	13
CuSO ₄	98
FeSO ₄	94
AgNO ₃	21
MnSO ₄	97
CaCl ₂	104
MgSO ₄	112
NiSO ₄	16
EDTA	107
Glycerol	83
Urea	98

2.4.4 Kinetic Behaviour of the FTase from *A. aculeatus*

The substrate specificities of the FTase were determined using sucrose (100 – 500 mM). Most of the β -fructofuranosidases (levansucrases and invertases) catalyze both sucrose hydrolysis and fructosyl transfer (FOS synthesis) in the presence of sucrose. The results from the Hanes-Woolf plot (Fig. 2.10) show that, for sucrose hydrolysis, the $V_{\max}$ and K_m are $120.5 \mu\text{mol} \cdot \text{min}^{-1}$ and $\pm 752.3 \text{ mM}$, respectively. However, the $V_{\max}$ value was calculated from the graph equation as it would not have been possible to estimate this value from the graph. The data on the graph indicates saturation kinetics. The turnover number (k_{cat}) and the catalytic efficiency (k_{cat}/K_m), calculated using the equations in Appendix C, were found to be 602.5 min^{-1} and $0.8 \text{ min}^{-1} \cdot \text{mM}^{-1}$, respectively.

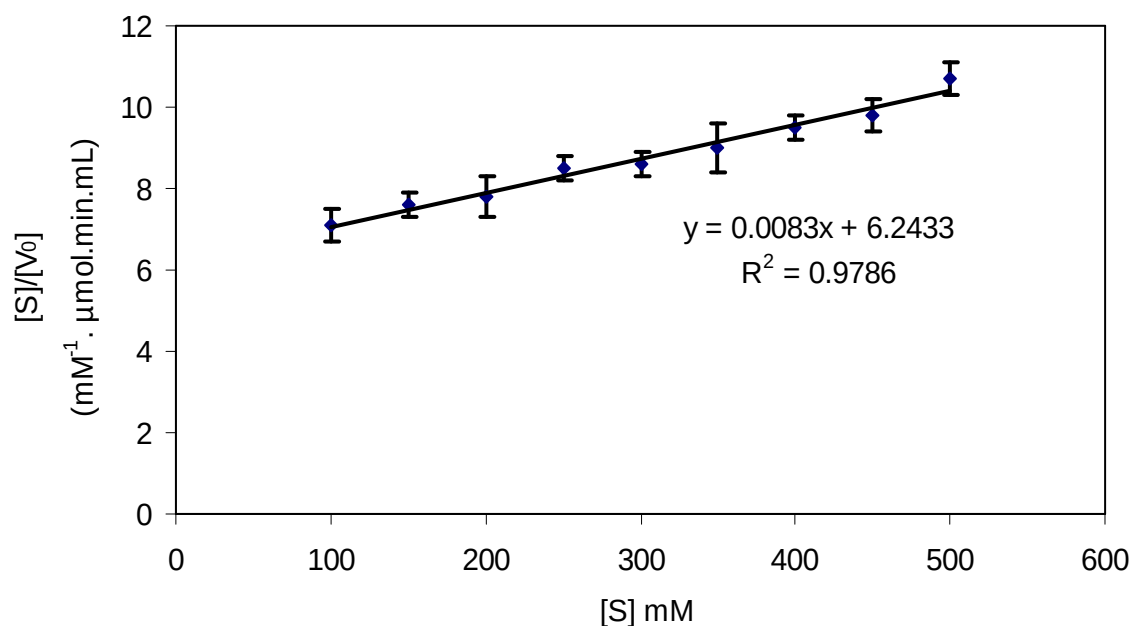


Figure 2.10 Hanes-Woolf plot for the determination of kinetic parameters ($V_{\max}$ and K_m) of the FTase from *A. aculeatus*.

The kinetic parameters for both hydrolysis and fructosyl transfer activity were determined in another independent study in our laboratory, and it was found that this FTase possesses a high transfer activity. Interestingly, the kinetic constant K_m (752.3 mM) also indicates high sucrose affinity as opposed to other reported values (44.38 mM and 88 mM) for FTase (L'Hocine *et al.*, 2000; Fujishima *et al.*, 2005). However, this high substrate affinity did not translate to the formation of higher DP FOS; a factor that may be attributed to the end-product (glucose) inhibition as observed in our laboratory.

2.4.5 Synthesis of short-chain Fructo-oligosaccharides

The purified FTase (0.24 U/mL) from *A. aculeatus* was tested for the synthesis of sc-FOS in another independent study in our laboratory (Mutanda and Whiteley, *pers. com.*). The HPLC analysis showed a maximum sc-FOS production of GF₂ (199 mM) and GF₃ (73.3 mM). There was no GF₄ or other higher DP compounds synthesized in all the experimental runs, except for the high glucose concentration (225 mM), which explains the low concentration of sc-FOS. It is also probable that part of the observed low FOS production could be the result of partial denaturing of the enzyme during subsequent purification.

2.5 Conclusion

The purified FTase was shown as a single-band protein on SDS-PAGE, suggesting that the enzyme could be a monomeric protein, although this is not always the case unless confirmed by other techniques such as activity gels (zymography). Native PAGE results suggest that the enzyme could be a glycoprotein with raffinose and/or glucose in its carbohydrate moiety, due to the hydrodynamic electrophoretic mobility of the protein. Most of the purified FTases, especially from *Aspergillus* species, are reported to be glycoproteins (L'Hocine *et al.*, 2000; Prapulla *et al.*, 2005). After the final purification step (Sephacryl S-200), the % recovery of the enzyme was too low (0.2%), which may have contributed to low FOS synthesis. The structural changes in the enzyme introduced by the purification procedure might have changed the binding conformations, resulting in lower accessibility of the substrates to the active site of the purified enzyme, and low FOS yield. For enzyme kinetics, the catalytic behaviour of the purified FTase did not fit well into the Michaelis-Menten plot but the pattern improved substantially when adjusted to the Hanes-Woolf plot. The enzyme showed a narrow range of pH (4.0 – 6.0), but was found to be highly stable at 60 °C with a half-life of 8 hours. The enzyme also showed a sufficiently broad chemical stability and a relatively higher catalytic activity, making it a potential biocatalyst for FOS synthesis under controlled reaction conditions.

Three main purification steps were used to successfully achieve the aims of this chapter, namely: PEG precipitation, anion exchange on DEAE-Sephacel and size-exclusion on Sephacryl S-200.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION
OF POLYGALACTURONASE

3 PURIFICATION AND CHARACTERIZATION OF POLYGALACTURONASE

3.1 Introduction

Polygalacturonases (PGases) belong to a group of pectin degrading enzymes known as pectinases. Within this group are enzymes such as pectin methylesterases, pectin lyases and pectate lyases (Dinischiotu *et al.*, 2007; Guebitz *et al.*, 2007). They are produced as extracellular enzymes by a wide variety of organisms such as bacteria, yeasts, fungi and even plants (Jayani *et al.*, 2005; Guebitz *et al.*, 2007). Their substrate (pectin) consists of α -(1 $\rightarrow$ 4)-linked D-galacturonic acid residues, and occurs as a structural material in the cell wall and middle lamellae of higher plants (Bhat *et al.*, 2003; Nakagawa *et al.*, 2004; Dinischiotu *et al.*, 2007). By virtue of its complex structure, pectin depolymerization is catalyzed by a number of pectinolytic enzymes, in which PGases play a major role. The physiological functions of PGases are still under investigation, however, industrially these enzymes are used in a number of different applications namely, fruit juice clarification, textile, oil extractions and paper making industries (Filho *et al.*, 2006). There are no studies yet on the application of PGases for the extraction of inulin from chicory roots.

Chicory roots are made up of abundant pectic substances with a molecular structure similar to that of pectin, which is methylated forming a linear backbone of α -(1 $\rightarrow$ 4)-linked D-galacturonic acid residues (Khashyap *et al.*, 2001; Bhat *et al.*, 2003). The hydrolysis of this pectic structure may be obtained by the sole or synergistic action of such enzymes as pectin esterases (EC 3.1.11.1), endo-(EC 3.2.1.15) and exo-(3.2.1.67) PGases (Soares *et al.*, 2001; Oliveira *et al.*, 2007). In these reactions, the PGase cleaves off the α -(1 $\rightarrow$ 4) linkages between the α -D-galacturonic acid residues, thereby depolymerizing the main-chain of the pectic structure in the chicory root cell wall. This enzymatic mechanism releases the abundant inulins from the chicory roots. However, in order to obtain high yields of these inulins, the purification and characterization of the PGase is therefore an essential exercise. This chapter describes the purification and characterization of a PGase present in the crude enzyme preparation (Pectinex[®] Ultra SP-L) sourced from *Aspergillus aculeatus*.

3.2 Materials and Methods

3.2.1 Reagents

The crude enzyme preparation (Pectinex[®] Ultra SP-L) from *A. aculeatus* was kindly donated by Novozymes (South Africa). The following reagents were obtained from Sigma-Aldrich (South Africa): Pectin (polygalacturonic acid); D-galacturonic acid; DEAE-Sepharose CL-6B; DEAE-Sepharcel[®]; Bradford reagent; di-nitrosalicylic acid (DNS); Acrylamide; bis-acrylamide; Coomassie Brilliant Blue R-250 and *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED). Sodium dodecyl sulphate (SDS); ammonium sulphate and bovine serum albumin (BSA) were obtained from Merck Chemicals (South Africa). PeqGold[™] molecular markers were purchased from PEQGOLD (Erlangen, Germany). All other chemicals were of analytical grade, obtained from readily available sources.

3.2.2 Materials and Equipment

The SnakeSkin[®] dialysis membrane (10 000 Da cut-off) was purchased from Pierce (CA, USA). The PowerWave[™] plate reader was obtained from Bio-Tek instruments (Vermont, USA). The water-bath (model 130); bench-top centrifuges (model 5180R and Eppendorf MiniSpin[®]) and pH meter (Inolab pH level 1) were obtained from Merck (South Africa). All the chromatographic columns; Electrophoresis kit (model 5214) and the fraction collector (model 2110) were from Bio-Rad (South Africa).

3.2.3 PGase Enzyme Assay

Polygalacturonase activity was determined by measuring the amount of reducing sugar (D-galacturonic acid) released from polygalacturonic acid (PGA) using the DNS method (Miller, 1959). The reaction mixture contained the PGA substrate (0.5% w/v) in sodium citrate buffer (50 mM, pH 5.5, 450 μ L) and enzyme (50 μ L). The mixture was incubated at 38 °C for 30 min, and was stopped by the addition of the DNS reagent (500 μ L). After boiling for 5 min, the mixture was cooled and its absorbance measured at 540 nm. The liberated reducing sugars were quantified against the D-galacturonic acid standard curve (Appendix E). One unit (IU) of enzyme was defined as the amount liberating 1 μ mol of D-galacturonic acid under the assay conditions

employed. All absorbance measurements were performed in triplicate and presented as the means $\pm$ S.E.M.

3.2.4 Protein Estimation

Protein concentration was routinely determined as described in section 2.3.3.

3.2.5 Dialysis of the Crude and Subsequent Protein Samples

The protein samples were dialyzed using a SnakeSkin[®] dialysis bag (10 000 Da cut-off) for the removal of unwanted small molecules. Prior to dialysis, the dialysis membrane was soaked in citrate buffer (50 mM, pH 5.5) for 5 min. A tubing clamp was used to close one end of the dialysis bag and the protein sample was pipetted into the bag. The tubing was closed off with another clamp and placed into in a large beaker with citrate buffer (50 mM, pH 5.5) at 4°C. Stirring of the buffer was carried out at the same temperature for 3 h, after which the dialyzed solution was removed from the bag. Enzyme and protein assays were subsequently performed as described before (section 3.2.3 and 2.3.3, respectively).

3.2.6 Concentration by PEG

For the concentration of large volumes of dilute protein solutions, dialysis against PEG (M_r 20 000), which has high osmotic pressure when in solution, was used. Samples were concentrated based on the principle that a water gradient- concentration is established between the protein solution within the dialysis membrane and the substance (PEG) on the outside of the membrane. Water and buffer ions gradually diffuse from the membrane, while proteins larger than the membrane's molecular weight cut-off limit, are retained. The resultant volume in which the protein is dissolved is thus reduced. The process in this experiment was terminated after 50 minutes when the required degree of concentration was achieved.

3.2.7 Ammonium Sulphate Precipitation

The dialyzed sample was precipitated using ammonium sulphate at 60% saturation. During precipitation, 7.8 g of $(\text{NH}_4)_2\text{SO}_4$ salt was added into a 20 mL crude enzyme solution. The mixture was stirred at 4°C for 40 min. After stirring, the mixture was centrifuged at $4000 \times g$ for 30 min. The pellet was collected and re-dissolved in citrate

buffer (50 mM, pH 5.5, 7.0 mL). The precipitate was dialyzed against the same (citrate) buffer to remove excess salt. The enzyme activity and protein assays were performed as described previously (section 3.2.3 and 2.3.3, respectively).

3.2.8 Anion Exchange on DEAE-Sepharose CL-6B

The dialyzed sample (6.0 mL) from $(\text{NH}_4)_2\text{SO}_4$ precipitation was applied onto a column (2.2×65 cm) of DEAE-Sepharose CL-6B equilibrated in citrate buffer (50 mM, pH 5.5). The column was washed with the same buffer until the $A_{595\text{nm}}$ remained at base-line. The elution of the protein of interest was performed with a linear salt gradient (0 – 0.5 M NaCl) in the same buffer at a flow rate of 1.0 mL/min (2.0 mL per tube). The enzyme-enriched fractions (12 – 20, 18 mL) were pooled, dialyzed against citrate buffer (50 mM, pH 5.5) and concentrated by PEG (20 000) to give a 6.0 mL sample. Protein and enzyme activity assays were performed as described before.

3.2.9 Anion Exchange on DEAE-Sephacel

The concentrated sample (5.0 mL) was applied onto a column (3×45 cm) of DEAE-Sephacel equilibrated with citrate buffer (50 mM, pH 5.5). The column was washed with the same buffer until the $A_{595\text{nm}}$ remained at base-line. The elution was carried out with a linear salt gradient (0 – 0.5 M NaCl) at a flow rate of 1.2 mL/min (2.4 mL per tube). The PGase-rich fractions (16 – 18, 7.2 mL) were pooled, dialyzed against citrate buffer (50 mM, pH 5.5) and concentrated by freeze-drying and stored at -20°C .

3.2.10 SDS-PAGE Analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). The stacking gel concentration was 5% and the resolving gel was 12%. The gel electrophoretic run was performed at 160 V for approximately 45 mins. Proteins were stained by Coomassie Brilliant Blue R-250 and destained with acetic acid: methanol: water (1:1:8, v/v). The molecular mass of the enzyme was estimated using the PeqGold™ protein markers.

3.2.11 Biochemical Characterization of PGase

The pH optimum was determined by assaying PGase activity at different pH values (3.0 – 8.0) in sodium citrate buffer as well as McIlvaine buffer (50 mM, pH 5.5) at 38°C . To

obtain the optimum temperature, the enzyme activity was assayed at various temperatures (20 – 70 °C) at pH 6.0. The effect of various metal ions and chemical reagents was assessed by incubating the enzyme (50 μ L, 11 U/mL) for 30 minutes at 38 °C with the compounds at 5 mM concentration. After the incubation, the enzyme activity was assayed by the standard assay as described in 3.2.3. Residual activity was expressed as percent (%) activity relative to the control (100% activity, 11 U/mL) obtained without addition of chemicals.

3.2.12 Kinetic Parameters of PGase

The initial reaction rates were determined using the Michaelis-Menten plot at the optimum conditions of the enzyme with pectin at various concentrations (0.4 – 21 mg/ml). The freeze-dried enzyme sample (23 U/mg) was reconstituted in 2.0 mL citrate buffer (50 mM, pH 6.0). The kinetic parameters (K_m and V_{max}) were calculated from the Lineweaver-Burk plot. The value of k_{cat} was calculated using the equation: $k_{cat} = V_{max} / [E_t]$, where $[E_t]$ is the enzyme concentration (11.5 U/mL).

3.3 Results and Discussion

3.3.1 Purification of PGase

The precipitation step was performed as a purification step to remove unwanted proteins and other substances, as well as concentrating the protein sample by resuspending the pellet in less volume of buffer. The best precipitation was achieved at 60% $(NH_4)_2SO_4$ saturation. For further purification, the re-constituted precipitate was dialyzed against citrate buffer (50 mM, pH 5.5) and subjected to a two-step chromatographic procedure on DEAE-Sepharose CL-6B and DEAE-Sephacel. Eight PGase-rich fractions (12 – 20, 18 mL) were identified after the DEAE-Sepharose chromatography (Fig. 3.1).

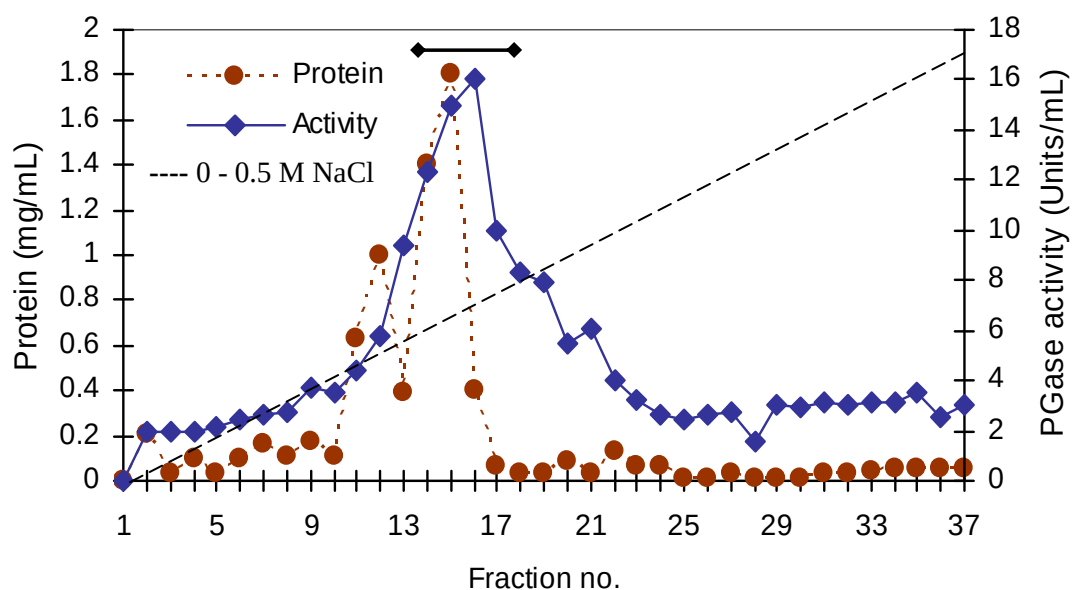


Figure 3.1 DEAE-Sepharose CL-6B elution profile of the PGase: Eluted with 0 – 0.5 M NaCl linear gradient in citrate buffer (50 mM, pH 5.5). Flow rate, 1.0 mL/min (2.0 mL/tube).

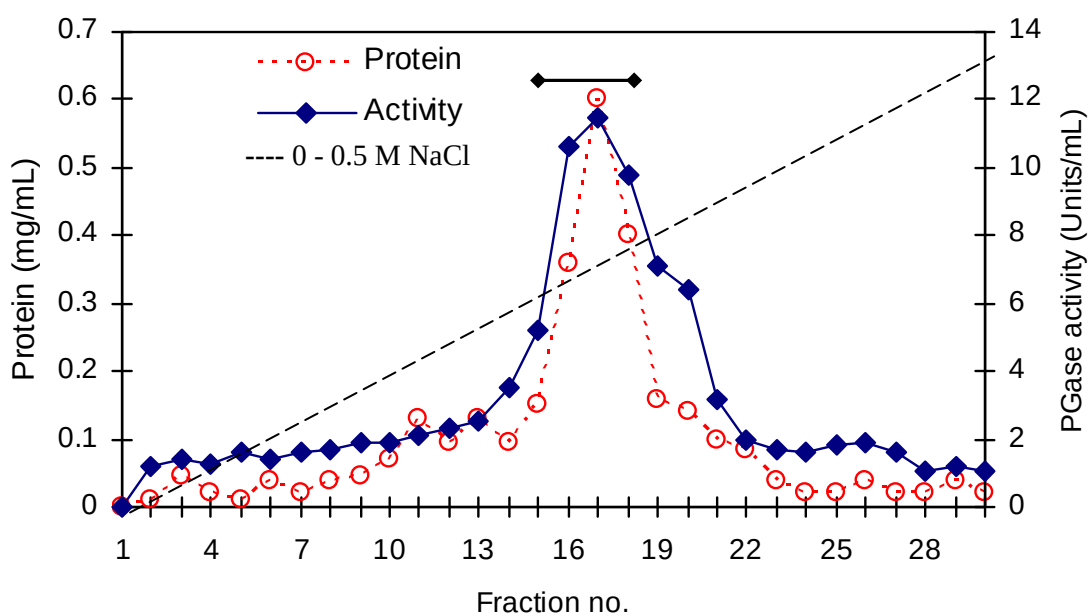


Figure 3.2 DEAE-Sepharcel elution profile of the PGase: Eluted with 0 – 0.5 M NaCl linear gradient in citrate buffer (50 mM, pH 5.5). Flow rate, 1.2 mL/min (2.4 mL/tube).

These fractions were pooled, dialyzed against citrate buffer (50 mM, pH 5.5), concentrated and further purified on DEAE-Sepharcel column. The DEAE-Sepharcel

chromatography resulted in three PGase-rich fractions (16 – 18, 7.2 mL) eluted with a linear NaCl (0 – 0.5 M) gradient (Fig. 3.2). These were pooled, dialyzed against citrate buffer (50 mM, pH 5.5), and used in the characterization studies

SDS-PAGE analysis of the purified protein revealed a single distinct band with a molecular weight estimated at 42 kDa (Fig. 3.3). This molecular weight was similar to those reported for other PGases from *Amycolata* sp., *Bacillus* sp. TS47, *Mucor* sp. and *Neurospora* sp. (Gummadi and Panda, 2003; Saad *et al.*, 2007); although in some cases there is variability depending on the source of the pectinolytic enzyme. However, one should bear in mind that a single protein band does not always suggest that the enzyme is a monomeric protein; even though in this case the band (M_r) is relatively comparable with other purified PGases (Dinischiotu *et al.*, 2007).

The enzyme was successfully purified to apparent homogeneity with a yield of 10% and a 9.7-fold purification (Table 3.1). The specific activity of the purified PGase was 28.3 U/mg. This level of specific activity is low when compared with other enzymes belonging to the EC 3.2.1.15, for example, the PGase from *A. niger* exhibited a specific activity of 539.1 after the final purification step (Dinischiotu *et al.*, 2007; Saad *et al.*, 2007).

Table 3.1 Purification of PGase from Pectinex® Ultra SP-L sourced from *Aspergillus aculeatus*

Purification Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purif. fold	Yield (%)
Crude extract	10	78	230	2.9	1	100
(NH ₄) ₂ SO ₄	8	24	168	7	2.4	73
DEAE-Sephacel	5	9	78	8.6	2.9	34
DEAE-Sephacel	2	0.8	23	28.3	9.7	10

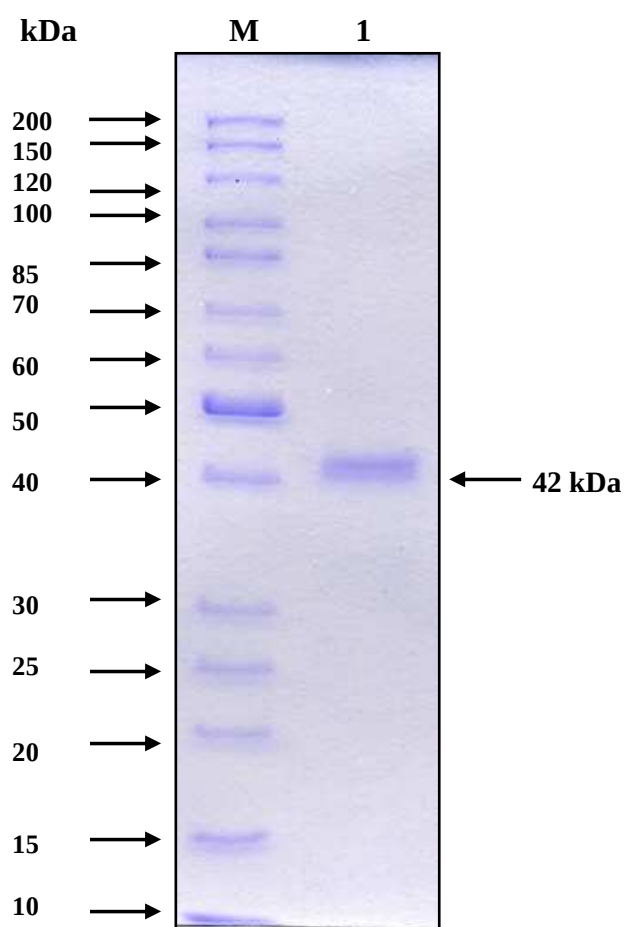


Figure 3.3 SDS-PAGE of the PGase from *Aspergillus aculeatus* after purification on DEAE-Sephacel ion-exchange chromatography. Lane 1, purified PGase; Lane M, molecular weight markers.

3.3.2 Biochemical Characterization of PGase

The purified enzyme showed a high degree of chemical stability upon addition of various metal ions and reagents. However, some differences in the susceptibility of the enzyme to mono and divalent cations such as Ca^{2+} , Ba^{2+} , K^+ , Na^+ , and Mn^{2+} , were observed (Table 3.2). This is attributable to the amino acid residues at the active site most likely to be aspartic acid and histidine. These metal ions resulted in a 6 – 26% decrease in enzyme activity, with Ca^{2+} showing the highest (26%) inhibition. The inhibition of PGase activity by Ca^{2+} is also reported by Schnitzhofer *et al.*, 2006. Other metal ions such as Fe^{2+} , Cu^{2+} and Ag^{2+} only slightly activated the enzyme activity. The addition of EDTA and 2-mercaptoethanol did not influence the enzyme activity, most likely due to the concentration limits of the assay. However, 2-

mercaptoethanol was expected to decrease the enzyme activity due to disruption of the disulfide bonds on the active site amino acids. These chemicals and metal ions were tested in an attempt to identify potential activators and inhibitors of the enzyme. From the results, it is clear that most of these compounds, with a few exceptions, did not influence the PGase activity (Table 3.2).

Table 3.2 Effect of metal ions and chemical reagents on PGase activity

Metal ions / Compound (5 mM)	Relative activity (%)
Control	100
KCl	93
MgCl ₂	101
ZnCl ₂	103
FeSO ₄	107
CuSO ₄	107
CaCl ₂	74
BaCl ₂	94
LiCl ₂	99
EDTA	103
2-Mercaptoethanol	95
Urea	96
AgNO ₃	107
MnSO ₄	94

The purified enzyme was also characterized with respect to its biochemical and substrate specificity. Polygalacturonase was found to be optimally active at pH 6.0 (Fig. 3.4). The optimum temperature of the enzyme was at 40 °C (Fig. 3.5). Temperatures above 65 °C significantly lowered the activity of PGase. The enzyme was stable with a half-life ($t_{1/2}$) greater than 3 h at 40 °C (Figure 3.6).

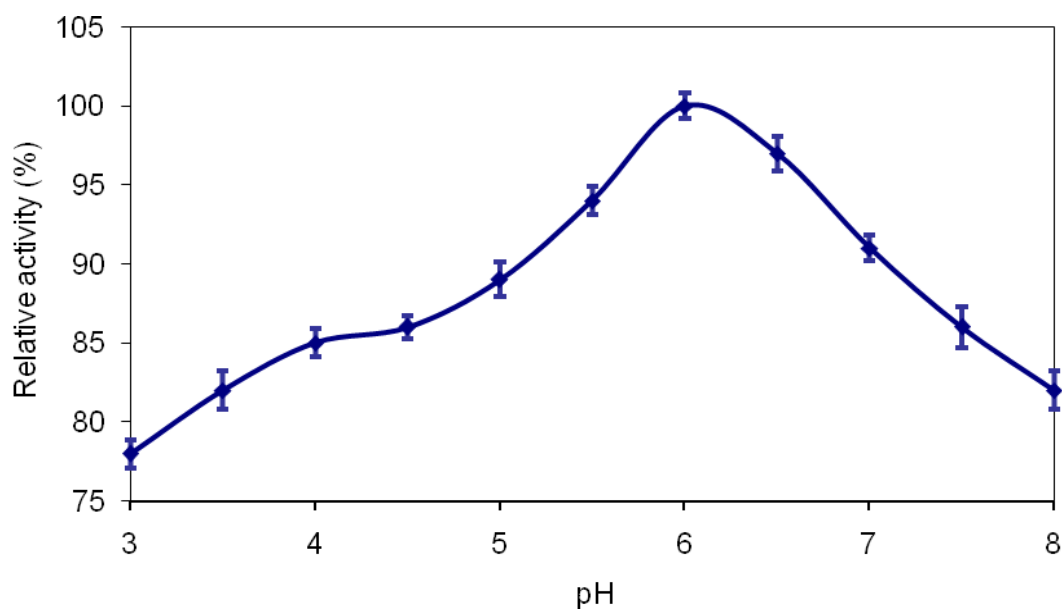


Figure 3.4 pH profile of PGase from *A. aculeatus*. Assay conditions described in 3.2.3. Error bars indicate the means ($\pm$ S.E.M) of three trials.

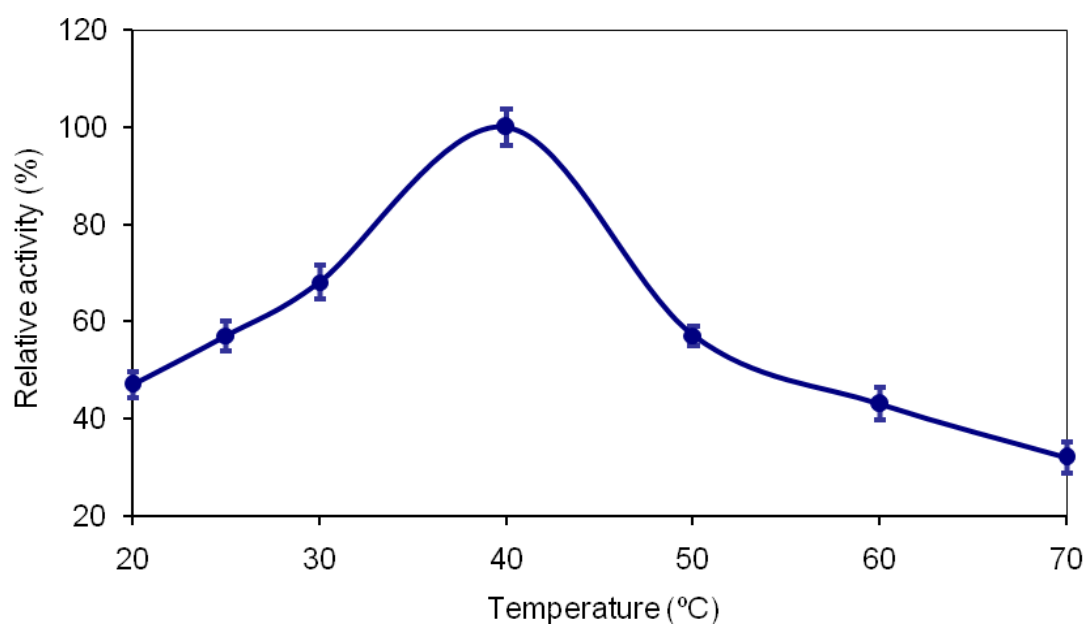


Figure 3.5 Temperature profile of PGase from *A. aculeatus*. Assay conditions described in 3.2.3. Error bars indicate the means ($\pm$ S.E.M) of three trials.

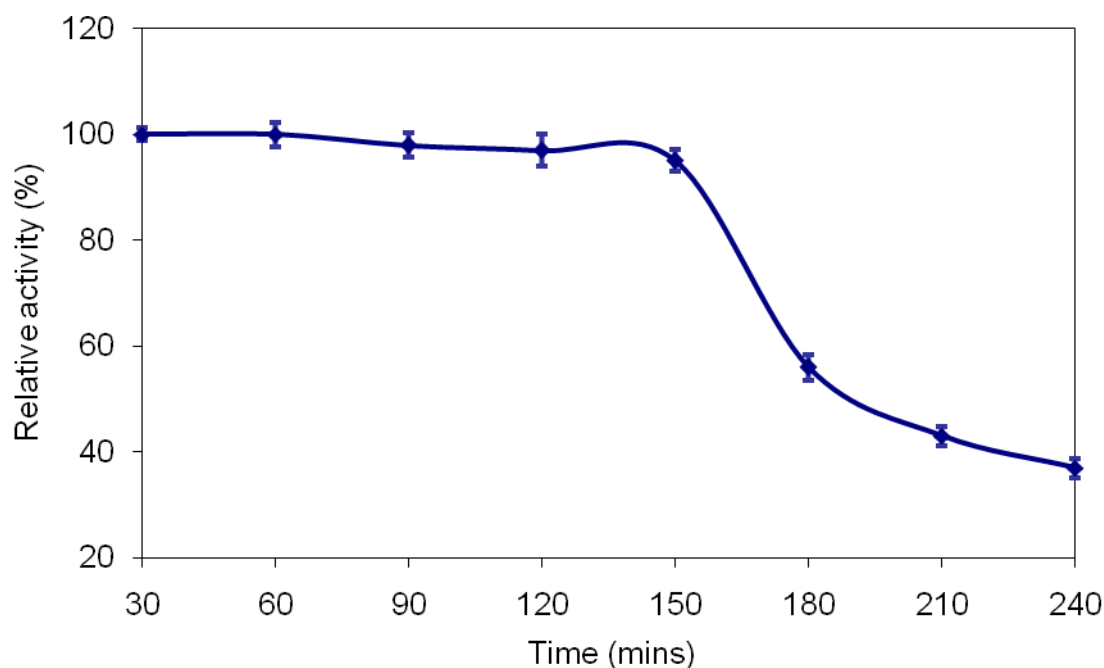


Figure 3.6 Thermal stability of PGase at 40°C. Assay conditions described in 3.2.3. Standard error bars indicate the means ($\pm$ S.E.M) of three trials.

3.3.3 Kinetic Parameters of PGase

The kinetic parameters of the PGase from *A. aculeatus* were determined using the Lineweaver-Burk plot. The $V_{\max}$ and K_m values were calculated to be $263 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ and $13.0 \text{ mg} \cdot \text{mL}^{-1}$, respectively, using pectin as a substrate (Fig. 3.7). The k_{cat} value, calculated using moles ($11.5 \mu\text{M}$) of the purified PGase, was found to be 1374 s^{-1} . The enzyme showed a catalytic efficiency (k_{cat} / K_m) of $106 \text{ s}^{-1} \cdot \text{mg} \cdot \text{mL}^{-1}$. Although the graph shows bundling of data points, which probably reflects saturation kinetics, these kinetic parameters were compared with those of other PGases reported in literature; however, there are slight differences depending on the source of the enzyme (Satyanarayana *et al.*, 2004; Tharanathan *et al.*, 2005; Dinischiotu *et al.*, 2007). Among the parameters investigated, the K_m value of the purified PGase showed a higher affinity for pectin compared to the PGase ($0.94 \text{ mg} \cdot \text{mL}^{-1}$) described by Dinischiotu *et al.* (2007).

Also, it is worth noting that the k_{cat} value for pectin hydrolysis (1374 s^{-1}) is in the high range compared to other reported PGases (Dinischiotu *et al.*, 2007), so is the $V_{\max}$ value ($263 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$) versus pectin concentration for other previously described

PGases (Tharanathan *et al.*, 2005; Dinischiotu *et al.*, 2007). Although the purified PGase showed comparatively lower catalytic efficiency ($k_{\text{cat}} / K_m = 106 \text{ s}^{-1} \cdot \text{mg} \cdot \text{mL}^{-1}$), the high substrate affinity ($K_m = 13.0 \text{ mg} \cdot \text{mL}^{-1}$) still demonstrates its greater specificity for pectin, which makes it a better candidate for pectin hydrolysis, hence greater inulin extraction from chicory roots.

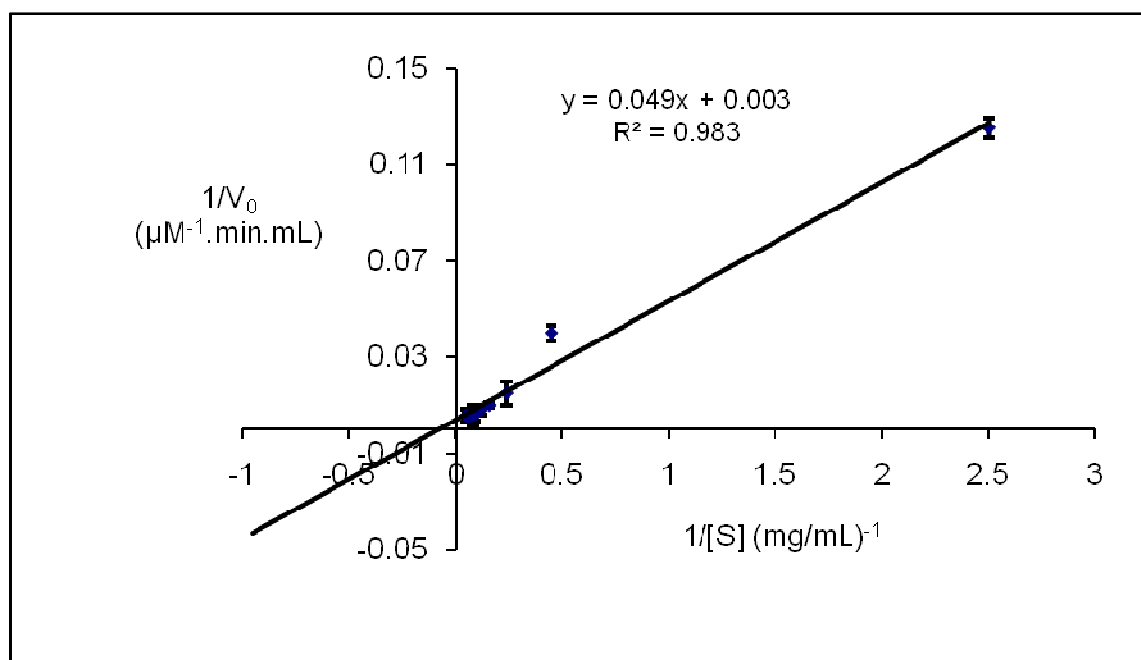


Figure 3.7 Lineweaver-Burk plot for the determination of V_{max} and K_m of the PGase from *A. aculeatus*.

3.4 Conclusion

Polygalacturonase was successfully purified from the commercial extract of *A. aculeatus* (Pectinex Ultra SP-L). A three-step purification procedure was employed to reach the aims of this chapter namely, $(\text{NH}_4)_2\text{SO}_4$ precipitation and successive anion exchange chromatography on DEAE-Sephacel and DEAE-Sephacel, respectively. The purified PGase had a molecular weight of 42 kDa as revealed by SDS-PAGE, with a final yield of 10% and 9.7 fold purification. The enzyme seemed to be very sensitive to Cu^{2+} , Ca^{2+} and Ag^{2+} heavy metal cations which usually inhibit enzymes by reacting with cysteine side chains. This led us to hypothesize about the presence of these amino acids in the catalytic site of the PGase. Analysis of the reaction products from inulin extraction suggested that PGase is an exo-acting as indicated by the elevated amount of reducing D-galacturonic acid residues in the reaction mixture.

The prolonged half-life at 40 °C, coupled with the higher substrate specificity, make this enzyme a potential candidate for the extraction of inulin from chicory roots. But for the extraction characteristics of inulin, the purified PGase would need to be compared with other pectinolytic enzymes in order to ascertain whether it is efficient or not. In the following chapter, inulin extraction from chicory roots was investigated using the purified PGase and a commercial pectinase.

CHAPTER 4

EXTRACTION OF INULIN FROM CHICORY
ROOTS

4 EXTRACTION OF INULIN FROM CHICORY ROOTS

4.1 Introduction

Inulin is used in various applications due to its health-promoting and technological properties (Gibson and Roberfroid, 1995; Ninness, 1999). It can be used as an alternative ingredient to substitute fats or natural sweeteners (fructose and sucrose) because it provides the same pleasant taste and texture, and is low in calories. Its functional use in products with low calories and reduced fat levels is already widespread in many developed countries (Figueira *et al.*, 2004).

With regards to inulin composition, Jerusalem artichoke and chicory contain the highest amounts (Waterhouse and Chatterton, 1993; Jianhua *et al.*, 2007). However, chicory is a valuable source of inulin due to the influence of harvest time and easy availability at low cost. In addition, chicory produces much higher yields of inulin per hectare as compared to Jerusalem artichoke. Another advantage is that, chicory possesses a relatively long-chain inulin as compared to other sources (Meijer and Mathijssen, 1992; Monti *et al.*, 2005). Chicory inulin also contains high fructose content for the production high fructose syrups (HFS) than any other raw material (Januário *et al.*, 1996).

Inulin is extracted from chicory roots on a commercial basis using various methods namely, hot-water extraction, alcohol precipitation, acid hydrolysis and ultrasound-assisted extraction (Roberfroid, 2000, Jianhua *et al.*, 2007). Although these methods are very common, they are deemed uneconomical and unsuitable for industrial use due to coloring of inulin products and rigorous downstream processing (Vandamme and Derycke, 1983; Singh *et al.*, 2006). Adding to these drawbacks is the fact that after harvest, fresh chicory roots must be processed or dried quickly, often within a 48-hour period, to avoid loss of yields due to spoilage, fermentation and/or desiccation (Dhellemmes, 1987). However, even the yields from the dried chicory roots are not satisfactory due to these inefficient extraction methods.

Enzymes are biological catalysts involved in reactions of synthesis and degradation, and have specific properties for their functioning and conditions that define their

catalytic efficiency (Palmer, 1995; Tharanathan *et al.*, 2005). Thus, the extraction limitations inherent from storage conditions and conventional extraction methods can be overcome by mild treatment with pectinolytic enzymes. One such example of pectinolytic enzymes application is in the fruit and vegetable processing industries, to increase the extraction of juice (Kashyap *et al.*, 2001; Crook and Corredig, 2006). By using the synergistic action of pectinolytic enzymes, complete breakdown of pectin can be achieved (Dinischiotu *et al.*, 2007; Martin *et al.*, 2007). Typical pectin-depolymerizing enzymes are the polygalacturonases (PGases) that cleave the backbone of pectin at the reducing end, thus releasing the D-galacturonic acid residues.

This chapter presents a novel method for the extraction of inulin through the complete depolymerization of pectins found in chicory roots using a purified PGase from *A. aculeatus*. A commercially available pectinase was also used to compare the extraction efficiency with the purified PGase. The inulin extract was further hydrolyzed using a crude endo-inulinase and its products were analyzed by HPLC, ESI-MS and NMR.

4.2 Materials and Methods

4.2.1 Reagents

Inulin and HPLC grade acetonitrile were purchased from Sigma-Aldrich (South Africa). Indole-3-acetic acid and HCl were obtained from Merck (South Africa). Novozyme 960 (crude endo-inulinase) was a gift from Novozymes (South Africa). FOS standards were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade and were obtained from readily available sources.

4.2.2 Inulin Assay

The inulin extract (100 μ L aliquot) was assayed by the addition of indole-3-acetic acid (50 mM, 150 μ L) and HCl (3 M, 3 mL). The mixture was briefly vortex-mixed and incubated at 60°C for 20 min. After cooling to room temperature for 30 min, the mixture was diluted with deionized water (3 mL) and briefly vortex-mixed. An

aliquot of 200 μL was transferred into a 96-well microplate and the absorbance was measured at 490 nm using a PowerWave™ plate reader. A calibration curve was constructed with inulin standard concentrations ranging from 0.1 – 1.0 mg/mL (Appendix F).

4.2.3 Extraction of Inulin from Chicory Roots

Dried and freshly-harvested chicory roots were obtained from Chicory SA (Alexandria, Eastern Cape). The roots were harvested at full maturity – 42 weeks. For inulin extraction, both dried and fresh chicory roots (1.0 g), were appropriately mixed with citrate buffer (33 mM, pH 6.0, 5 mL). To initiate the reaction, 100 μL of PGase enzyme with a specific activity of 28.3 U/mg were added into the reaction tube. The mixture was incubated at 40 °C for 90 minutes. The total amount of inulin extracted was determined by referring to the inulin standard curve.

4.2.4 Hydrolysis of Inulin Extracts

The extract was further hydrolyzed using a dialyzed crude endo-inulinase (Endo-I) (Mutanda and Whiteley, *pers. comm.*) to obtain inulo-oligosaccharides of varying chain length. The reaction mixture contained 3 mg/mL of inulin extract (450 μL) and the crude Endo-I (20 U/mL) in citrate buffer (50 mM, pH 6.0, 450 μL) in total volume of 1.0 mL and was incubated at 60 °C for 2 hours. For comparison purposes, commercial inulin (3 mg/mL) was similarly incubated under the same conditions with the crude Endo-I.

4.3 Analysis of Inulin Hydrolysates

4.3.1 HPLC Analysis

The hydrolysis products obtained from the inulin extract were analyzed by high performance liquid chromatography (HPLC) using a Beckman (4.5 × 120 mm) column system, coupled with a 2301 refractive index detector system maintained at $\pm 28^\circ\text{C}$. Sample volumes (50 μL) were filtered on Millex®-HV (0.45 μm) filter units (Millipore, USA) before injection. The mobile phase, acetonitrile – water (73:27) was degassed by vacuum filtration through a Millivac® pump (Millipore, USA). The samples were analyzed using an isocratic mode at a flow rate of 0.5 mL.min⁻¹ and the

column temperature was kept at ± 40 °C. A guard column was also used to prevent damage to the separation column. Oligosaccharides were identified by comparison of their retention time with the authentic (mono, di- and oligo-saccharide) standards (Appendix G), and their identity were further confirmed by nuclear magnetic resonance spectroscopy (NMR) and electrospray ionization mass spectrometry (ESI-MS).

4.3.2 Electro-Spray Mass Spectrometry (ESI-MS) Analysis

In this study, a reproducible platform for carbohydrate analysis has been developed using mass spectrometry coupled with an electrospray ion source, through direct injection. A single quadrupole MS system (Dionex, Sunnyvale, USA) was used. The MS system consisted of an ED50A electrochemical detector, an AS50 auto-sampler with sample cooling and thermal compartment for stabilizing the column, an amperometric cell and a 50 μ L injection loop. For the successful MS analysis of carbohydrates, some prior preparation of these compounds is required. Thus, LiCl (0.5 mM) was added to samples to improve the detection of the neutral carbohydrates. Lithium chloride forms charged complexes (Li-adducts) with carbohydrates. All carbohydrate samples were prepared at 0.5 mg/mL and were dissolved in deionized water prior to ionization with LiCl. The sample mixtures were filtered through a Millex[®]-HV membrane filter (0.45 μ m) before injection. These ionized carbohydrates were detected in the positive ion mode of the MS after formation of quasi-molecular ions with the lithium ions. The ESI-MS operation conditions were as follows: a probe temperature of 400 °C, 0.5 MPa nitrogen pressure and 75 V cone voltage.

4.3.3 NMR Analysis

The inulin extract mixtures analyzed by HPLC and ESI-MS were also characterized by ¹H NMR using an AMX 400Hz Bruker spectrometer (Bruker, Wissembourg, France). Inulin hydrolysates were prepared by freeze-drying to yield a total mass of 60 mg prior to analysis. The weighed samples were dissolved in D₂O (99.98%, 500 μ L). The proton NMR spectra were recorded at 1-min time interval during the NMR run and the resultant ¹H signals were compared with the predicted spectra of standard compounds (Appendix H). For NMR predictions, Isis Draw from MDL was used to prepare structure drawings and create 'mol' format files. NMR Predict from

Modgraph Consultants was used to prepare proton NMR predicted spectra. NMR Predict used the “Charge” algorithm developed by Professor Roy Abrahams at Liverpool University; and Mestre Nova from Mestrelab Research produced predicted spectra which can be processed by the NMR presentation and processing package, MestRe-C, also from Mestrelab Research.

The NMR signals were expressed in ppm relative to 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt (TTPA) used as an internal chemical shift reference at 0 ppm. The ^1H NMR signals were assigned by means of COSY experiments.

4.4 Results and Discussion

4.4.1 Extraction of Inulin from Chicory Roots

The extraction of inulin was performed enzymatically using two different pectinolytic enzymes; a PGase purified from *A. aculeatus* and a commercially obtained pectinase from Sigma-Aldrich. Both enzymes were incubated with chicory extracts under the same conditions (described in 4.2.3). Interestingly, both enzymes were used under the same conditions and the complete extraction of inulins was observed after 90 min with only the purified PGase (Fig. 4.1). After 90 minutes no further extraction of inulin was observed with both enzymes. In addition, the PGase showed a higher extraction rate compared to the commercial pectinase. This could be attributed to the fact that the purified PGase appears to have some degree of affinity for PGA ($K_m = 13.0 \text{ mg.mL}^{-1}$), although this is not significantly higher compared to other PGases (Dinischiotu *et al.*, 2007; Saad *et al.*, 2007).

Also, despite the fact that most naturally occurring pectins contain a high degree of esterification, and that PGases preferentially attack pectate over pectin; the activity of the purified PGase was not influenced by the degree of pectin methylation found in chicory roots. Thus, this feature could be useful in the food and beverage industry, particularly in fruit juice extraction and wine processing.

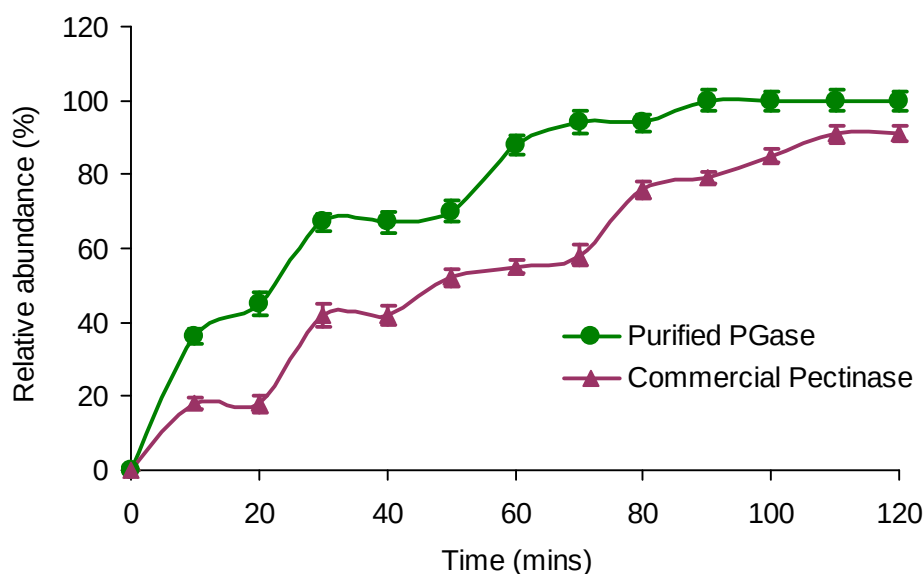


Figure 4.1 Inulin extractions from dried chicory roots at 40°C, 28.3 U/mg enzyme dosage, (100% extraction = 3.3 mg/mL).

Extraction yields from the freshly harvested chicory root (70 – 75% water content) and the dried chicory root were compared using the purified PGase (Fig. 4.2). The dried chicory root yielded the highest amount of inulin on a mass / mass basis (Table 4.1).

Table 4.1 Total amount of inulin per gram of both fresh and dried chicory root

Inulin extraction method	Fresh root (mg/mL)	Dried root (mg/mL)
PGase	1.1	3.3
HCl hydrolysis	1.3	3.1
Hot H ₂ O extraction	1.2	3.0

It is worth noting that the PGase also produced the same relative amount of inulin from both roots under economical experimental conditions; as opposed to other conventional methods that often require much energy input and expensive chemicals. Thus, the results obtained using the purified PGase from *A. aculeatus* could be applied as a new technology for the extraction of inulin in the chicory as well as other industries.

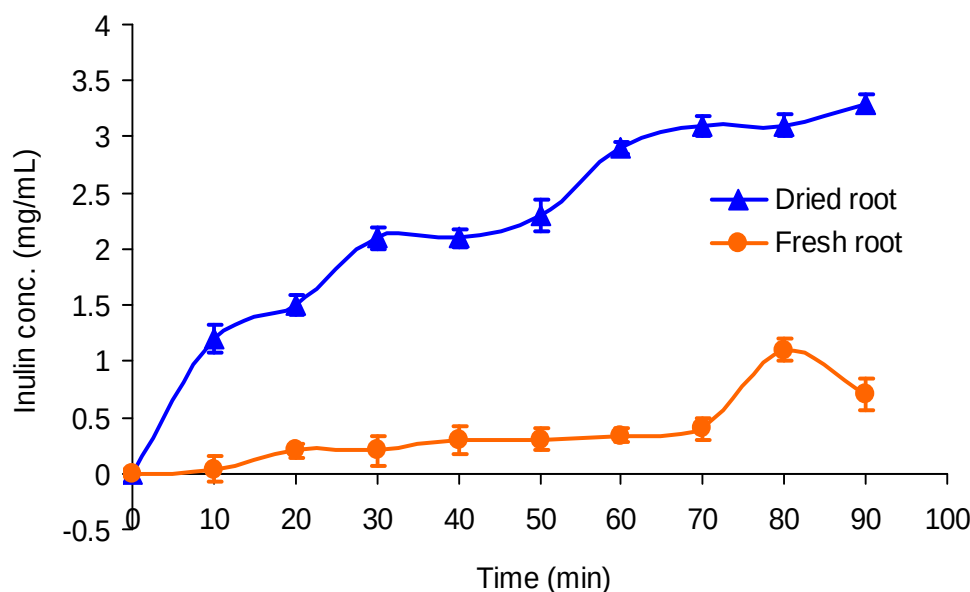


Figure 4.2 Inulin extraction yields from both fresh and dried chicory roots using PGase.

Although the purified PGase hydrolyzed the pectin within the cell wall of the chicory root to release the inulins, its mechanism of hydrolysis had to be determined. It was found that the pattern of hydrolysis of the pectin substrate was an exo mode of action based on the amount of liberated reducing sugars (D-galacturonic acid). To be classified as an exo enzyme, the relative concentration of reducing sugars typically has to be above 10% of the total sugars in the mixture with a slow change in viscosity (Shanley *et al.*, 1993).

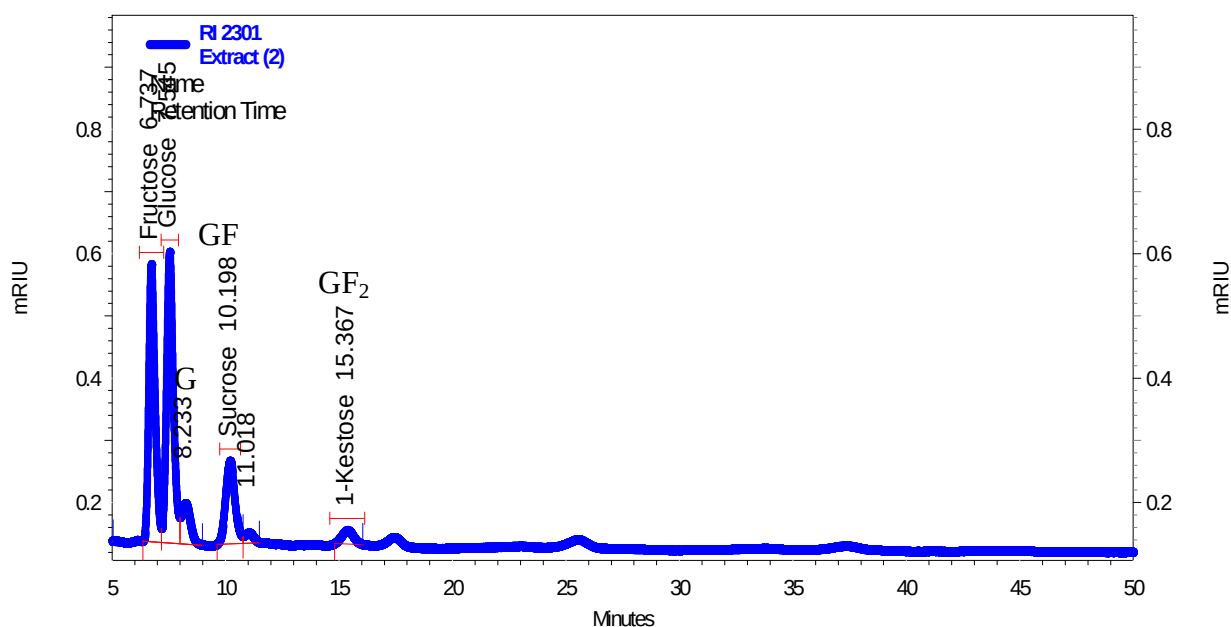
4.4.2 HPLC Analysis of Inulin Hydrolysates

In order to identify the different inulo-oligosaccharides produced by the action of Endo-I, a set of commercially available oligosaccharide standards was prepared and analyzed by HPLC (Appendix G). From both the extracted and commercially obtained inulin, the inulin hydrolysates (obtained by crude Endo-I hydrolysis) were also analyzed by HPLC. Table 4.2 shows the relative oligosaccharide quantification obtained from intensities of the HPLC traces. The hydrolysis of inulin extract yielded a mixture of GF₂, GF₃ and various simple sugars as illustrated in Figure 4.3. When using commercially obtained inulin as substrate, hydrolysates ranging from GF₂ to GF₄ and a high amount of fructose were obtained (Fig. 4.4).

Table 4.2 Oligosaccharides composition from both extracted and commercial inulin hydrolysis with endo-inulinase determined by HPLC.

Sugar/oligosaccharide	Inulin extract (%)**	Commercial inulin (%)**
Fructose	59	93
Glucose	14	14
Sucrose	28	71
DP 2	13	34
DP 3	ND*	12
DP 4	ND*	ND*
DP 5	ND*	ND*

ND*: Not detected; (%)**: Percentages are relative to the units not the actual composition of the mixture.

**Figure 4.3** HPLC analysis of extracted inulin hydrolysates after treatment with endo-inulinase.

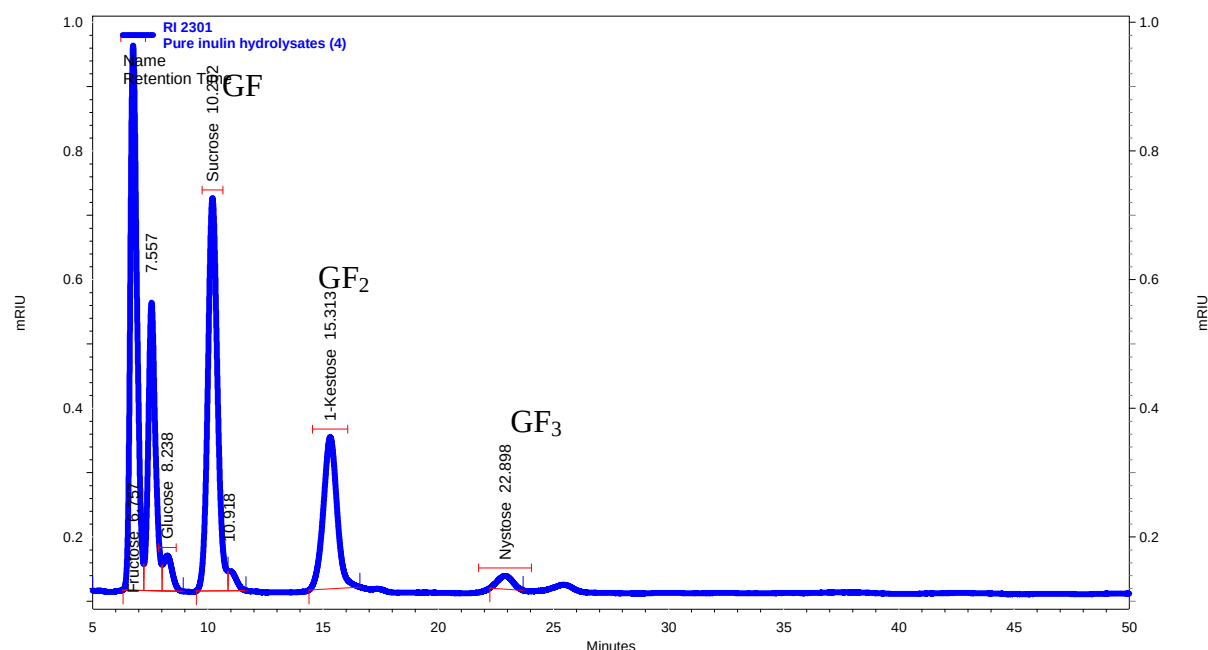


Figure 4.4 HPLC analysis of commercial inulin hydrolysates after treatment with Endo-I.

Figure 4.4, shows a large peak area with a high relative abundance of different sugars. However, in this figure, the mixture also shows the presence of two less abundant peaks at 17.5 and 25.5 mins. Since an Endo-I was used for inulin hydrolysis; it is probable that these smaller peaks could correspond to inulotrioses (F_3), tetroses (F_4) and so on. However, there were no immediate F_n -type oligosaccharide standards available to confirm this.

To date, there is still no single method for the determination of all carbohydrate compounds that exist in nature. Moreover, analytical methods such as HPLC have considerable limitations, such as lacking sensitivity, on the analysis of certain compounds especially structurally complex carbohydrates (Townsend, 1995; Wei and Ding, 2000). Given all these factors, different analytical tools may be useful for the analysis of different types of sugars. In this case, mass spectrometry with electrospray ionization (ESI-MS) was used to ascertain if there could be other higher DP oligosaccharides in the inulin hydrolysis mixture.

4.4.3 ESI-Mass Spectrometry Analysis of Hydrolysates

Ionization of neutral oligosaccharides with LiCl was used to improve their signals on mass spectrometric analysis. In order to confirm the signal response from both the extracted and the commercially obtained inulin hydrolysates, a mass calibration was performed using known oligosaccharide standards (Fig. 4.5). The signal strength was over 5 orders of magnitude using nystose (GF₃) for the detection limit. The calibration signals of the Li-adducts of the different sugars are indicated in Table 4.3.

Table 4.3 The calibration signals of different sugars on a mass spectrum

Sugar compound	Fragment ions (<i>m/z</i>)	Minimum detection limit (MDL)	Relative abundance (%)
Fructose	187	0.2 mg/mL	25
Glucose	187	0.2 mg/mL	25
Sucrose	349	0.2 mg/mL	99
1-Kestose	512	0.2 mg/mL	38
Nystose	674	0.2 mg/mL	13
¹ F-Fructosylnystose	837	0.2 mg/mL	81

Under the ESI-MS operational conditions employed, fragment ions from glycosidic cleavages and water adducts were also observed. According to the numbering system proposed by Domon and Costello (1988), the mass loss of 162 (fragment ion at *m/z* 187) is indicative of a hexose. The quasi-molecular ion at the mass to charge ratio (*m/z*) of 350 clearly corresponds to the base peak of sucrose, whilst the fragment ion at *m/z* 205 is the water adduct. Such water adducts are easily detected in the ESI mode of the MSQ mass spectrometer (Bruggink *et al.*, 2005). The quasi-molecular ion at *m/z* 169 indicates a glycosidic cleavage on the oxygen atom from the glucose side of the linkage.

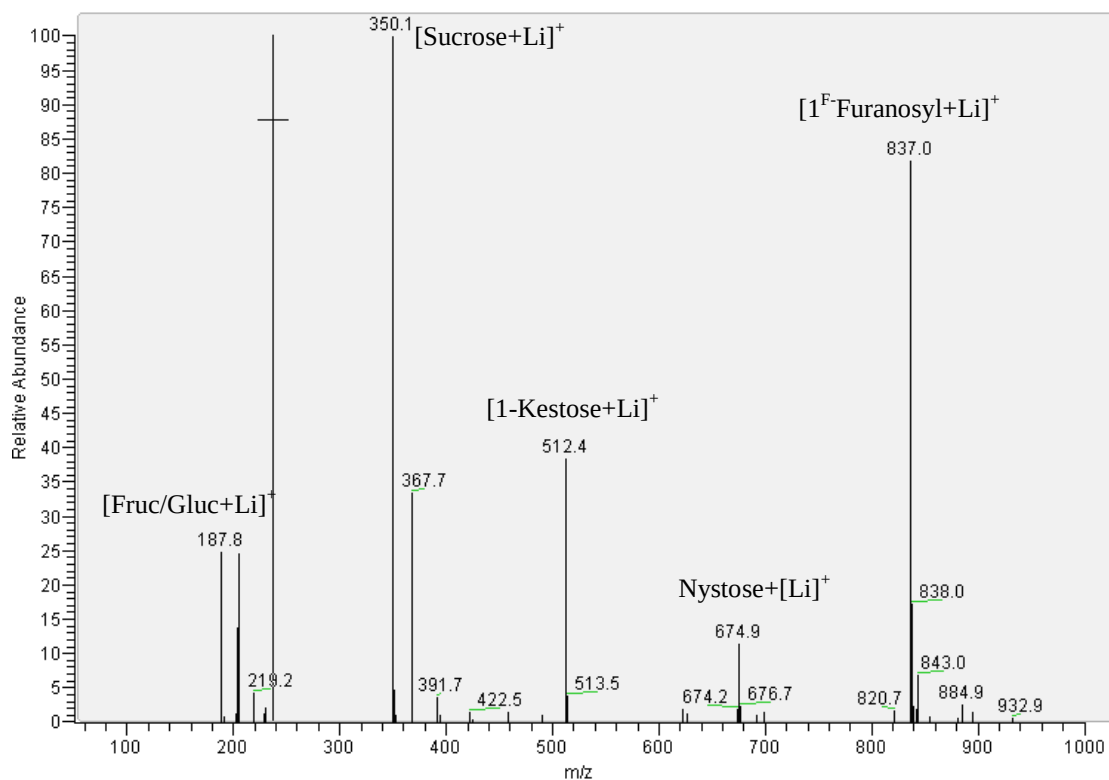


Figure 4.5 Mass spectrum of sugar and oligosaccharide standards.

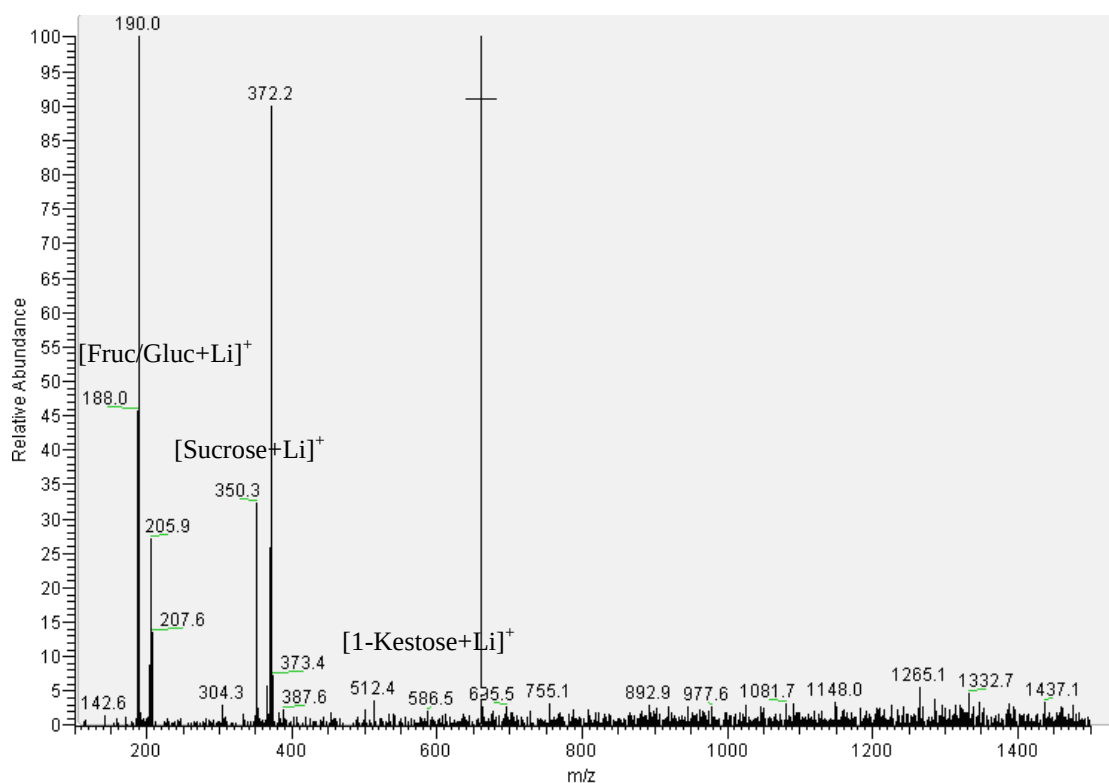


Figure 4.6 Mass spectrum of inulin extract hydrolysates at 0.5 mg/mL.

In Figure 4.6, the overall oligosaccharides detected relative to the MS spectrum of the standards were predominantly low DP sugars: 1-kestose and below. For this MS spectrum, the oligosaccharide mixture was prepared with the inulin extracted from chicory roots (3.3 mg/mL). But when the mixture was prepared from the commercial inulin (3 mg/mL), the resulting ESI-MS spectrum revealed higher ionization states of some of the oligosaccharides in the mixture (Fig. 4.7). For example, the MS spectrum showed two Li-sugar adducts at m/z values of 674.8 and 837, corresponding to nystose and 1-^FFuranosylnystose, respectively. This observation may be ascribed to the fact that the source of inulin from the chicory root came from one harvest and one batch in one area while the commercial source is likely to have come from several batches over a wide range of source material, which explains the detection of nystose and furanosyl-nystose in it.

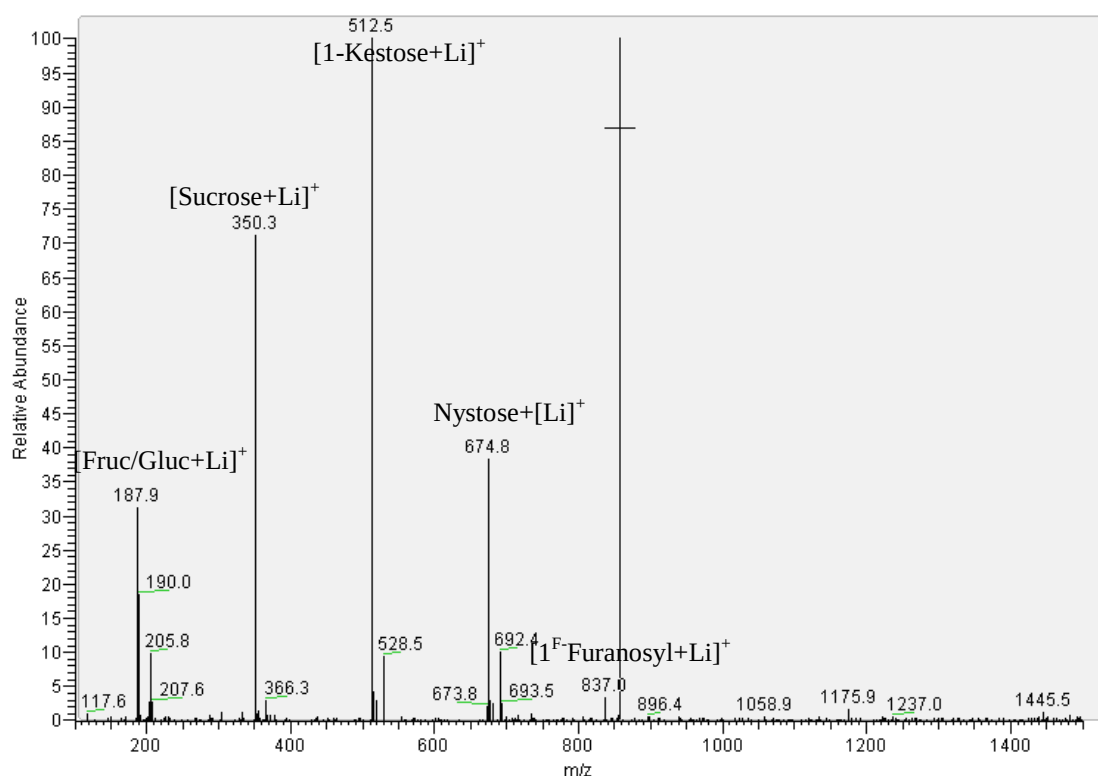


Figure 4.7 Mass spectrum of commercial inulin hydrolysates at 0.5 mg/mL.

These results indicate that the ESI-MS technique developed in this work is capable of analyzing various oligosaccharides present in mixtures. However, there are still some limitations on the use of MS for the quantitative analysis of oligosaccharides due to differences in ionization efficiencies of the various components present in the mixture

(Zaia, 2004). Thus, the use of other high resolution and sensitive techniques (such as NMR) offers promise for the detection and characterization of changes relative to the abundance of specific oligosaccharides present in complex mixtures.

4.4.4 ^1H NMR Analysis of Inulin Hydrolysates

The high-resolution proton (^1H NMR) spectrum was used for oligosaccharide analysis by NMR. The proton spectrum from the extract (Fig. 4.8) showed the presence in the anomeric region of some well-resolved resonances corresponding to (H1) and (H3). However, the other remote skeletal protons resonated in the region 3.62 – 3.98 ppm, giving rise to a cluster of poorly resolved resonances. The ^1H NMR spectrum of the commercial inulin hydrolysates revealed the presence of intense signals in the region between 3.50 and 4.20 ppm (Fig. 4.9). This observation was confirmed by the comparison of chemical shifts of the protons with the predicted ^1H NMR spectra of 1-kestose and nystose (Appendix H). This also allowed the assignment of the ^1H NMR signal at 5.28 to H-1 proton of the α -glucose residue of sucrose. It may also be seen that the significant signals for both spectra lie between 3 and 5.5 ppm. The large signal at 4.82 ppm is from residual water in the D_2O solvent.

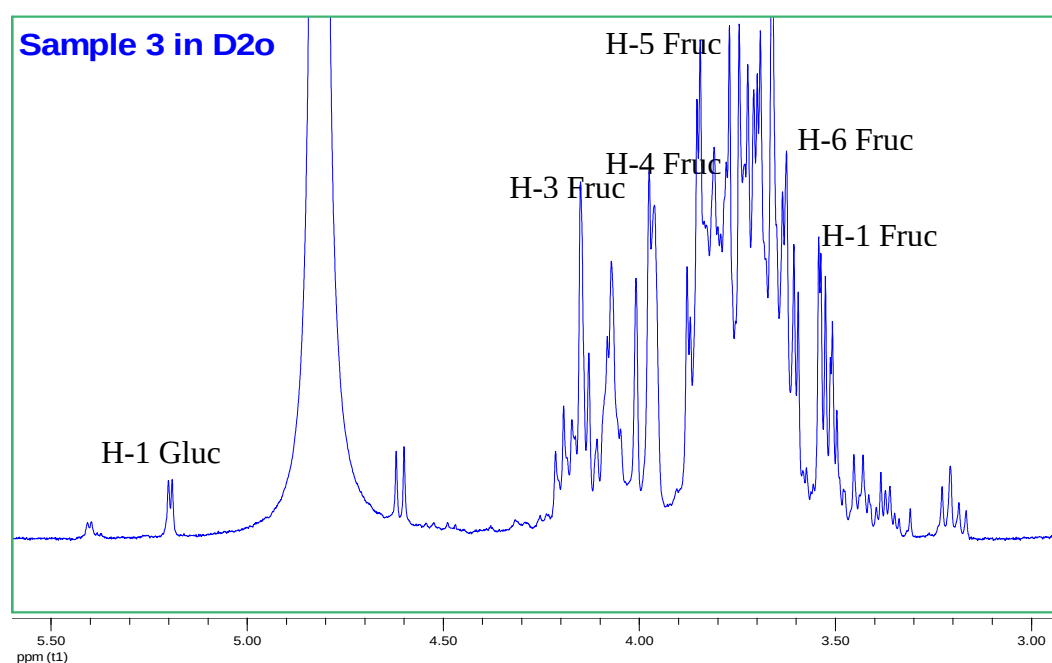


Figure 4.8 ^1H NMR spectrum of the extract hydrolysis. The hydrolysis reaction was catalyzed by endo-inulinase.

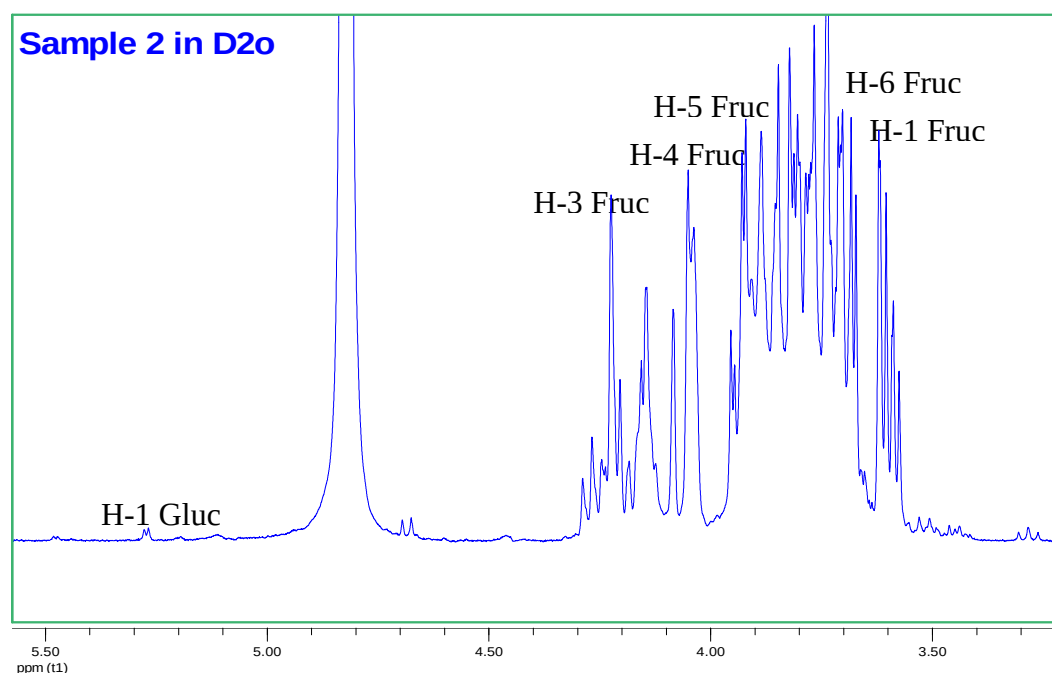


Figure 4.9 ^1H NMR spectrum of the commercial inulin hydrolysis. The hydrolysis reaction was catalyzed by endo-inulinase.

However, it is difficult at this stage to confirm whether kestose or nystose are present due to the lack of down-field resonances in the experimental spectra when compared to the predicted spectra of the two compounds. The singlet signal at about 5 ppm in the nystose spectrum (Appendix H) is a characteristic signal to differentiate between these two compounds when only the up-field sections are shown.

4.5 Conclusion

A reproducible and efficient inulin extraction method has been developed using the purified PGase. The applicability of this method was shown by comparison with other conventional methods such as strong acids and hot-water extraction. Most of the pectinolytic enzymes reported in literature have only been applied in the depolymerization of other carbohydrates such as chitin, as well as bioscouring of cotton, whereas this investigation presents a new dimension which offers the potential for inulin extraction for the food and pharmaceutical industries. Furthermore, the fact that there was no further inulin extracted after 90 minutes could be an indication of complete extraction of inulin from the chicory root. This observation was confirmed when the same amount of chicory root was extracted using acid (HCl) hydrolysis or

hot water extraction. The purified PGase was free of pectate lyase and pectin methyl esterase activities and yet it hydrolyzed 90 – 97% esterified pectins with a relatively high degree of methylation. These results indicate that *A. aculeatus* PGase efficiently degrades esterified pectin in the absence of methyl esterases.

The method described here demonstrates the successful application of biocatalysis in combination with sensitive analytical tools such as mass spectrometric detection and NMR. Both ESI-MS and NMR analyses have been very useful in this study, and the advantages can be applied in the analysis of FOS with detailed structural representation. The resulting mass difference determined by ESI-MS provides a tool to distinguish isotopic clusters from each of the compounds in the mixture. Above all, the possibility of comparing the ^1H -NMR experimental values with the predicted ones constitutes a powerful tool when compounds with simple structural representation are analyzed; however, predictions have limitations when analyzing a mixture of compounds, which often have a large number of chemically-shifted nuclei, as observed above.

CHAPTER 5

PHARMACEUTICAL PROPERTIES OF SC-FOS

AGAINST CARCINOGENIC ENZYME

ACTIVITIES

5 PHARMACEUTICAL PROPERTIES OF SC-FOS AGAINST CARCINOGENIC ENZYME ACTIVITIES

5.1 Introduction

It has been reported that the human gastrointestinal tract is residence to a highly complex and diverse bacterial population (Conway, 1995; Hao and Lee, 2001). Although little is known about the specific strains and strain characteristics of the intestinal microecology, research suggests that intestinal microflora are divided into two categories; health-promoting species (that provide good digestion and increased resistance to infection) and pathogenic species that produce toxic by-products contributing to colon carcinogenesis (Salminen *et al.*, 1998; Burns and Rowland, 2000). In particular, bacterial species such as *Clostridia*, *Fusobacteria* and *Bacteriodes* have been implicated in colon carcinogenesis through their carcinogenic enzyme activities (Pool-Zobel *et al.*, 2001; Bosscher *et al.*, 2006). *Bifidobacteria* and *Lactobacilli*, on the other hand, have been shown to have potent anti-carcinogenic activities (Hosoda *et al.*, 1996). For example, *Lactobacilli* have been shown to break down carcinogens such as N-nitrosamines (Rowland and Grasso, 1975). The link between these beneficial bacteria and cancer suppression may be explained, in part, by the sc-FOS fermentation in the large intestine, which results in the production of anti-tumorigenic compounds (SCFA and bacteriocins) and the alteration of physicochemical conditions in the colon (Fujisawa *et al.*, 2001; Topping *et al.*, 2003).

Under normal physiological conditions, many toxic compounds are detoxified in the liver to form glucuronides before entering the intestine via the bile. However, in the colon, the bacterial enzyme β -glucuronidase from *C. perfringens* has the ability to de-conjugate glucuronides due to its broad substrate specificity (Hirayama and Rafter, 1999; Pool-Zobel *et al.*, 2001). De-conjugation of these compounds in the colon releases the parent carcinogenic compound, thereby initiating tumour formation. Fujisawa *et al.* (2001) and Priebe *et al.* (2002) have shown that a decrease in pH significantly decreases β -glucuronidase and other carcinogenic enzyme activities.

Another such enzyme implicated in colon carcinogenesis and reported to be inhibited by SCFA is urease. In the colon, urease is mainly produced by anaerobes such as *Helicobacter pylori* and it catalyzes the breakdown of urea into ammonia and CO₂. Ammonia neutralizes the gastrointestinal environment and thus enables *H. pylori* to survive and damage the mucosal tissue of the intestine, thereby promoting tumour initiation (Mobley *et al.*, 1991). Detoxifying enzymes such as glutathione S-transferase and β -glucosidase inactivate the procarcinogens formed by pathogenic bacteria. Unfortunately β -glucosidase is reported to be involved in both detoxification and activation of procarcinogens; this apparently depends on the localization of the enzyme (Pool-Zobel *et al.*, 2001).

Although sc-FOS have been associated with several health-promoting and anti-carcinogenic properties (Fuller and Gibson, 1997; Tanriseven and Gokmen, 1999; Gibson, 1999), their direct or indirect interaction with carcinogenic enzymes is not yet clearly understood. Even though there is ample research evidence describing the modulation and alteration of intestinal bacteria by sc-FOS (Bouhnik *et al.*, 1997; Campbell *et al.*, 1997), however, the direct effects of sc-FOS and their fermentation products (SCFA) have not been well elucidated. This is exacerbated by the fact that, studies on the *in vivo* (fecal) enzyme activities have proved to be extremely difficult to interpret (Harris and Ferguson, 1999). The aim of this study was to investigate the *in vitro* effects of sc-FOS and SCFA on various intestinal carcinogenic (β -glucuronidase, urease) and detoxifying (β -glucosidase) enzymes and to comparatively evaluate these compounds as potential intervention agents against colon carcinogenesis.

5.2 Materials and Methods

5.2.1 Reagents

The following commercial sc-FOS were used in the study: Raftilose Synergy, Raftiline HP, and Raftilose P95 (Orafti, Tienen, Belgium). 1-kestose, nystose and 1^F-fructofuranosylnystose were obtained from Wako Pure Chemicals (Osaka, Japan). SCFA

(acetic, *n*-butyric, L-lactic and propionic acid) and the enzymes (β -glucuronidase, β -glucosidase and urease) were obtained from Sigma-Aldrich (South Africa). All other chemicals were purchased from Merck (South Africa) unless otherwise indicated.

5.3 Enzyme Assays and Effect on Anti-carcinogenic enzymes

5.3.1 β -Glucuronidase Assay

The assay of β -glucuronidase activity was modified as previously described by Aich *et al.* (2001). The *p*-nitrophenyl- β -D-glucuronide (PNPG) substrate was first equilibrated in the water bath at 37°C for 10 min. The assay mixture contained 0.9 mL of phosphate buffer (20 mM, pH 6.8) and 0.1 mL of substrate. The reaction was initiated by adding 20 μ L of enzyme (3 U/mL). The mixture was slightly vortexed and incubated at 37 °C for 30 minutes. After incubation, the reaction was stopped by the addition of sodium carbonate (1 M, 0.1 mL). The mixture was vortex-mixed and an aliquot (200 μ L) was transferred into a 96-well microplate and the absorbance was measured at 403 nm. One unit (IU) of β -glucuronidase activity was defined as the amount of enzyme capable of releasing 1 μ mol of *p*-nitrophenol under the assay conditions used. The amount of *p*-nitrophenol produced was determined by referring to the standard curve obtained using *p*-nitrophenol standards (0.1 – 0.4 mM, Appendix I).

5.3.2 β -Glucosidase Assay

The β -glucosidase activity was measured using a slightly modified assay by Hagental *et al.* (2001). The assay mixture contained 0.2 mL of sodium citrate buffer (50 mM, pH 4.8) and 0.3 mL of *p*-nitrophenyl- β -D-glucopyranoside (PNPG) (10 mM). After addition of the enzyme (20 U/ml, 25 μ L), the mixture was incubated at 37 °C for 15 min in a temperature-controlled water bath. After incubation, sodium carbonate (100 mM, 3 mL) was added to stop the reaction and for color development. The absorbance of the reaction mixture and control (with PNPG and citrate buffer) was measured at 400 nm in a 96-well microplate using a PowerWave™ plate reader. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of *p*-nitrophenol per minute

at 37°C from the substrate (PNPG). The amount of *p*-nitrophenol was determined from the standard curve obtained using *p*-nitrophenol standards.

5.3.3 Urease Assay

The assay for urease activity was determined by measuring the release of ammonia (from urea). The reaction mixture contained urea (100 mM) in 20 mM phosphate buffer (20 mM, pH 7.4) and a 100 μ L aliquot of the enzyme (0.5 U/mL) in a total volume of 1.0 ml. After incubation for 15 minutes at 37°C, the reaction was stopped by adding 2 ml of 0.5% phenol – 0.0025% sodium nitroprusside solution, followed by 2 ml of 0.25% sodium hydroxide – 0.25% sodium hypochlorite solution. The reaction mixture was further incubated at 55 °C for 8 min for color development, and the absorbance was measured at 625 nm. The amount of ammonia produced was determined by referring to a standard curve obtained with ammonium chloride standards (Appendix K). Urease activity was expressed as the amount in micromoles (μ mol) of urea hydrolyzed per min under the assay conditions used.

5.3.4 Effects of sc-FOS and SCFA

The inhibitory effect of sc-FOS and SCFA was evaluated by pre-incubating the enzymes with various mixtures of sc-FOS (0.5%, v/v) and SCFA (0.3%, v/v) under the described assay conditions. After the termination of each assay, the effects of sc-FOS and SCFA were immediately analyzed by comparing the reaction mixtures with the non-treated mixtures (controls). Controls were taken as 100% activity.

5.3.5 Statistical Analyses

All the experimental results were analyzed by two-factor ANOVA (Microsoft Excel 2003). The differences with *P*-values ≤ 0.05 were considered significant. Results are expressed as means $\pm$ SEM (n = 3).

5.4 Results and Discussion

5.4.1 Effects of sc-FOS and SCFA on β -glucuronidase Activity

The results of the effects of sc-FOS and SCFA on the gut microbial enzymes are presented as average values of three experimental runs in the figures. To determine the influence of SCFA on the reaction mixtures, the pH of the reaction mixtures was monitored before and after treatment with SCFA. The results indicate that in the absence of significant pH changes, the effects of sc-FOS and SCFA on various carcinogenic and therapeutic enzymes varied markedly. The activity of β -glucuronidase was significantly ($P < 0.05$) decreased by the presence of various sc-FOS (0.5%, v/v) supplements in the reaction mixture (Fig. 5.1). Among the sc-FOS added in the reaction mixture, the most significant decrease (79%) on β -glucuronidase activity was observed with nystose (GF₃), whereas the least, but significant, effect (75%) was obtained with the probiotic supplement (Culturelle™) relative to the control. The commercial names of the sc-FOS and the probiotic supplement used in the experiments are tabled in Appendix J.

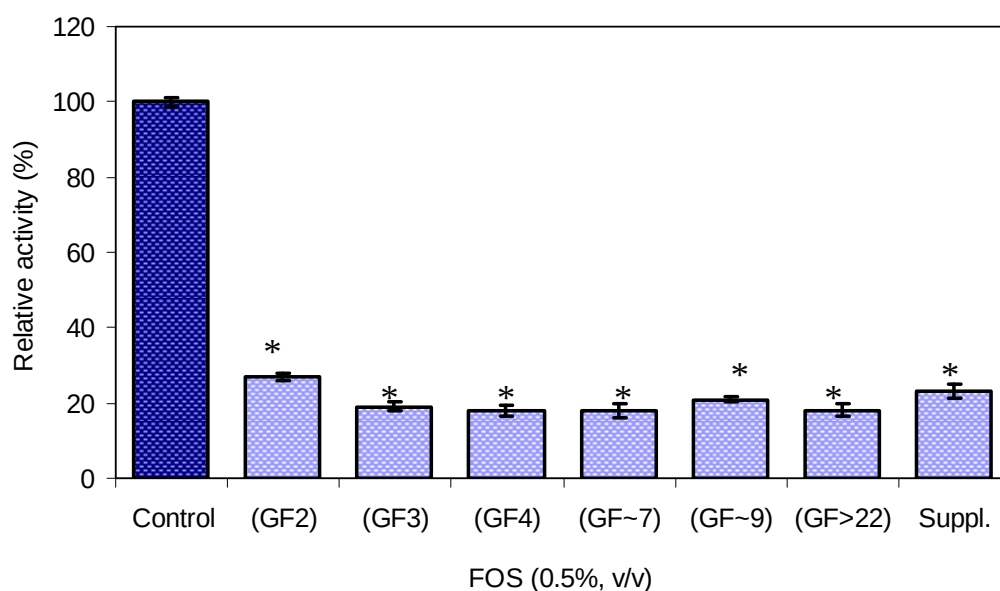


Figure 5.1 Effect of sc-FOS (0.5%, v/v) on β -glucuronidase activity. Values are represented as the means ($\pm$ SEM) of three trials. (*Significantly different from control, $P < 0.05$), (GF_n: G=glucose; F=fructose).

The effect of SCFA on β -glucuronidase activity was more pronounced compared to that of sc-FOS. The enzyme activity was significantly lower ($P < 0.05$) in the presence of acetate, butyrate and lactate (94, 91, and 93% decrease, respectively) relative to the control. A similar significant decrease (87%) was observed with propionate (Fig. 5.2). Overall, the activity of β -glucuronidase was markedly lowered by all the SCFA. These results strongly support the evidence that the risk of colon cancer caused by carcinogenic enzyme activities may be significantly lowered by SCFA produced by beneficial bacteria (*Lactobacilli* and *Bifidobacteria*) after FOS fermentation. More accurately, this SCFA inhibitory effect on bacterial β -glucuronidase activity is a result of the pH decrease and therefore indicates that the acidification of the colonic environment may reduce the incidence of colon carcinogenesis.

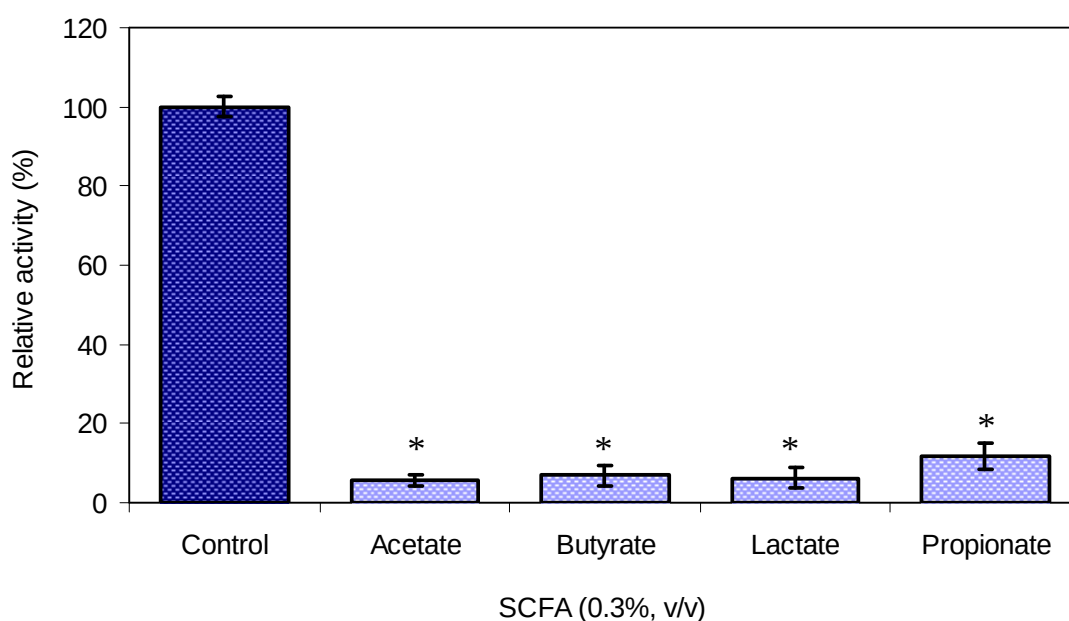


Figure 5.2 Effect of SCFA (0.3%, v/v) on β -glucuronidase activity. Values are represented as the means ($\pm$ SEM) of three trials. (*Significantly different from control, $P < 0.05$).

5.4.2 Effects of sc-FOS and SCFA on Urease Activity

For the urease enzyme, most of the sc-FOS did not exert any significant inhibitory effect when compared to the control treatment (Fig. 5.3). However, GF₄ reduced the activity of urease by 28%, although the effect was not as pronounced when compared to that of SCFA. It would have been expected that the longer chain oligosaccharides would decrease the enzyme activity compared to the control. However, the explanation for the observed trend could be drawn from the fact that most of the long-chain oligosaccharides are less soluble; indicating that solubility of fiber influenced the activity of this enzyme.

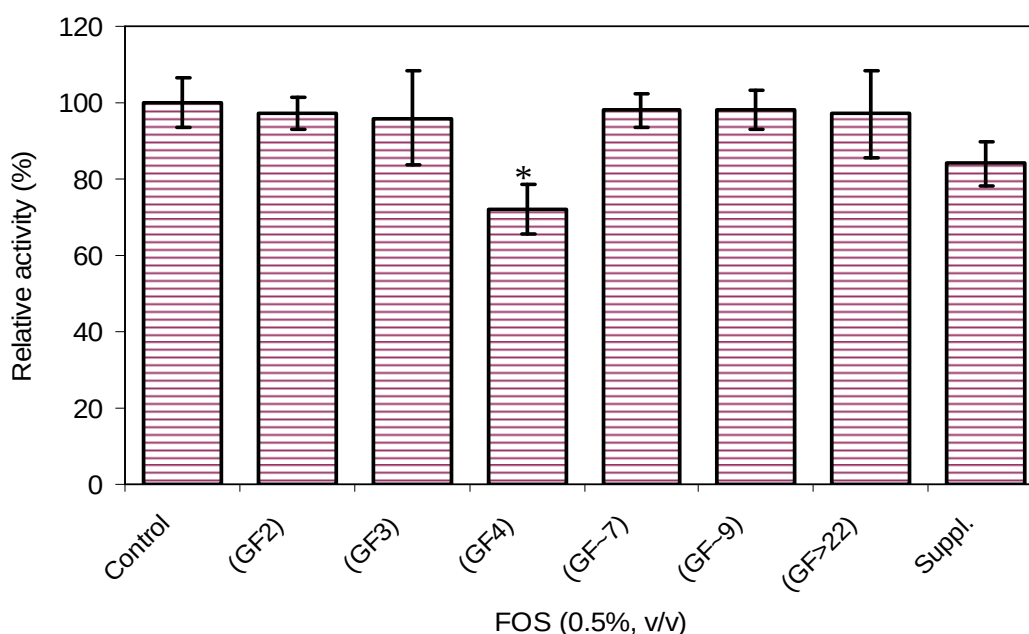


Figure 5.3 Effect of sc-FOS on urease activity. (*Significantly different from control, $P < 0.05$).

After analyzing the effect of SCFA on urease, it was found that the effect was varied. Two of the SCFA (butyrate and propionate) showed significantly lower levels (98 and 96%, respectively) of urease activity relative to the control. In contrast, lactate had a less significant effect (60% decrease), while acetate had a moderate (44%) inhibitory effect on the enzyme (Fig. 5.4).

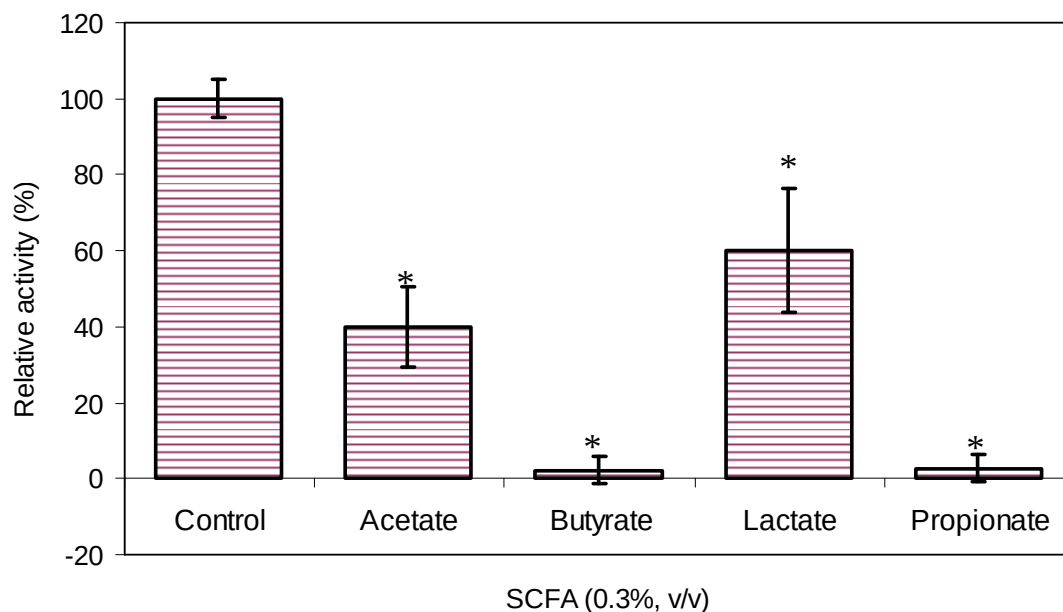


Figure 5.4 Effect of SCFA on urease activity (*Significantly different from control, $P < 0.05$).

5.4.3 Effects of sc-FOS and SCFA on β -glucosidase

There was no significant ($P > 0.05$) effect on the activity of β -glucosidase upon the addition of various sc-FOS in the reaction mixtures (Fig. 5.5). On the contrary, some of the sc-FOS (GF₄ and GF>22) even elevated the enzyme activity. This observation is similar to the findings in literature (Marteau *et al.*, 1990; Pool-Zobel *et al.*, 2001) citing the induction of β -glucosidase upon the addition of FOS. There were no differences in the effect of FOS between the control and the GF₄/GF>22 treated mixtures.

With regards to the effect of SCFA on β -glucosidase, only lactate showed a very good inhibitory effect (86%), whereas butyrate and propionate only had a moderate effect (58 and 59% decrease, respectively). In the mixture treated with acetate, the inhibitory effect was slightly lower (42%) as compared to the other SCFA, but still significantly different ($P < 0.05$) from the control (Fig. 5.6).

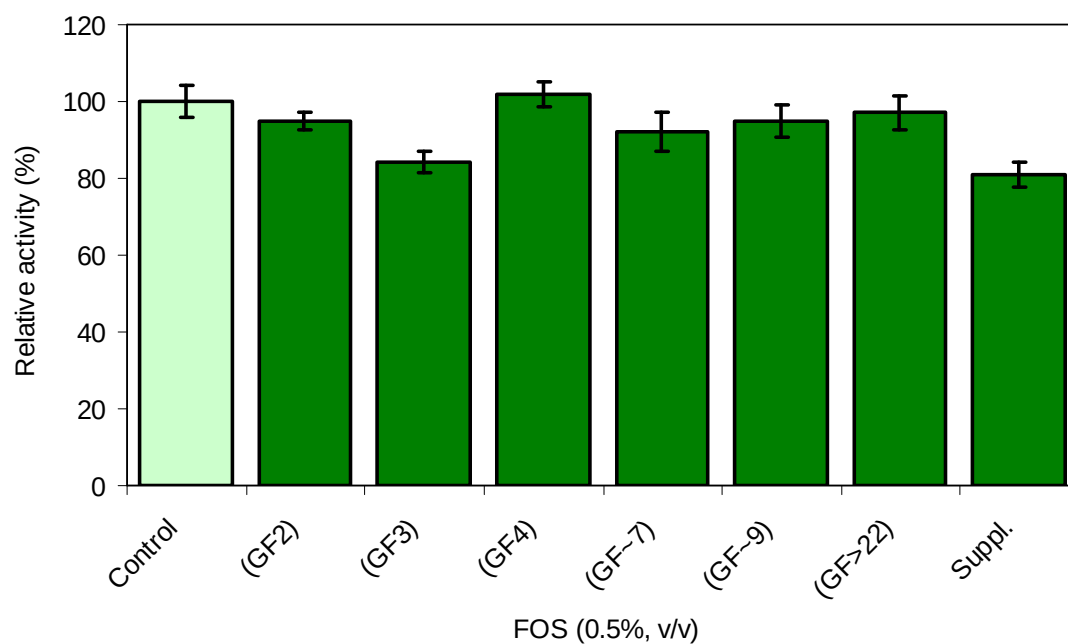


Figure 5.5 Effect of sc-FOS on β -glucosidase activity.

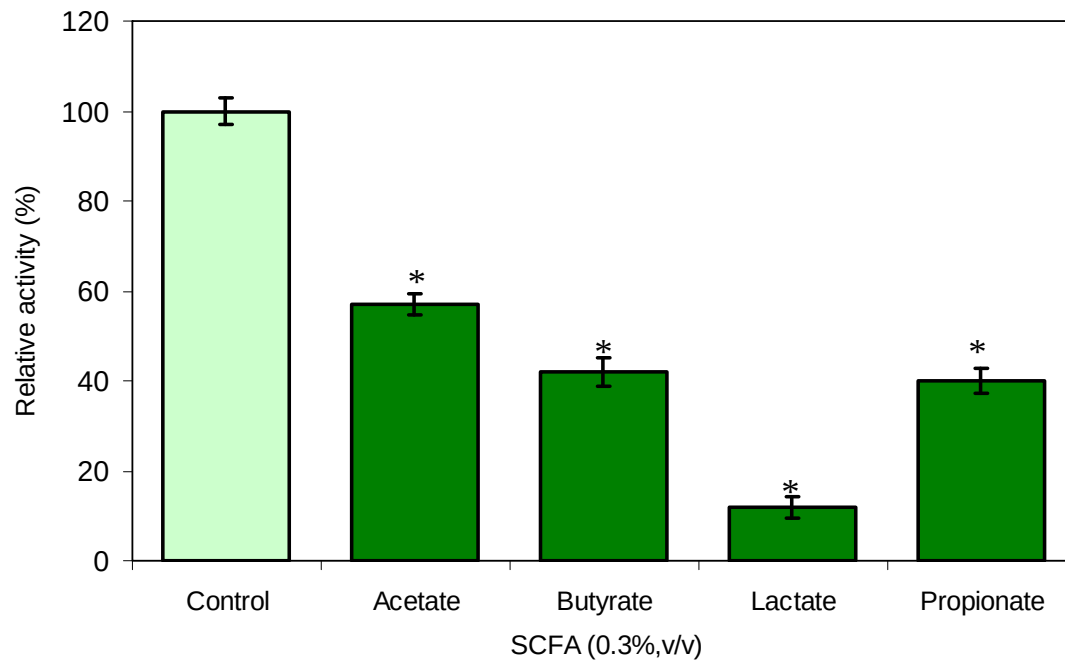


Figure 5.6 Effect of SCFA on β -glucosidase activity (*Significantly different from control, $P < 0.05$).

5.5 Conclusion

Short-chain fructo-oligosaccharides do possess anti-carcinogenic properties. However, one of the most likely possible mechanisms by which FOS offer protection against tumour development in the colon is through fermentation by colonic bacteria. As observed, treatment with sc-FOS did not significantly decrease most of the enzyme activities, except β -glucuronidase. Treatment with most of the SCFA significantly lowered all bacterial carcinogenic enzymes as observed by several statistically significant trends.

Although this is an *in vitro* approach which gives only a rough picture regarding the exact mechanism of intestinal modulation, but when one considers the observed enzyme inhibition, it can be concluded that the pH of the colonic environment plays a pivotal role in intestinal modulation. This points out that the fermentation of sc-FOS in the colon and subsequent production of SCFA may be the only mitigating factor against colon carcinogenesis. The observed SCFA effects on these enzymes and in particular β -glucuronidase, justify this conclusion. Among the SCFA analyzed, butyrate, lactate and propionate showed the most significant inhibitory effect on the enzymes. Since the reactions were carried out in strong buffered solutions and the pH monitored before and after the addition of SCFA; an insignificant (± 0.8) pH difference in the reaction mixtures was observed. This is an acceptable shift for any functional enzyme activity due to the fact that the variation of activity with pH, within a range of 2-3 units each side of the pI, is normally a reversible process (Sugunan, *et al.*, 2006). The observed inhibition is therefore most probably not attributed to the denaturing of enzymes or any disruption of optimal enzyme performance due to pH effects.

The probiotic supplement (Culturelle™) used in this study was coated with an oligosaccharide fraction from inulin. Clearly from the results, this combination does not exert any effect on either enzyme. In conclusion, the fermentation of sc-FOS by colonic microflora and the subsequent production of SCFA may provide a protective effect against carcinogenic enzymes in the colon.

CHAPTER 6

FINAL CONCLUSIONS AND FUTURE
PERSPECTIVES

6 FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 General Discussion

6.1.1 Purification and Characterization of FTase

Given the important functions and applications of FOS, novel enzymes with superior biosynthetic properties are of great importance. In this project, a fructosyltransferase from *A. aculeatus* was purified and biochemically characterized.

For the characterization and application of the enzyme, the purification was achieved through three main purification steps, namely PEG precipitation, anion exchange chromatography and size-exclusion chromatography.

The purified FTase is shown to be a single-band protein (MW $\pm$ 85kDa), and possibly a monomeric glycoprotein as determined by SDS-PAGE. The optimum pH and temperature of FTase from *A. aculeatus* were determined to be 6.0 and 60 °C, respectively. The enzyme was found to be highly stable at 60 °C, with a half life of 8 hours. The lyophilized enzyme was also stable at -20 °C for 8 months.

The turn-over number (k_{cat}) for the FTase was calculated to be 602.5 min⁻¹ and its catalytic efficiency (k_{cat}/K_m) was 0.8 min⁻¹.mM⁻¹. Although its catalytic efficiency was shown to be lower compared to other FTases reported in literature, its estimated affinity for sucrose was higher (K_m = 752.3 mM). This purified FTase was used in another independent study in our laboratory to synthesize various chain-lengths FOS. Typically, the amount of free fructose quantified by HPLC is used as a direct measurement of the hydrolytic activity, whereas the amount of glucose minus the amount of free fructose corresponds to the transferase activity (Van Hijum *et al.*, 2004). These parameters were also determined in a separate study in our laboratory. Fructo-oligosaccharides production with the purified FTase yielded various sc-FOS, however, no production of FOS higher than GF₄ (nystose) was detected.

6.1.2 Purification and Characterization of PGase

Similarly, a PGase from *A. aculeatus* was purified to apparent homogeneity in a three-step procedure: ammonium sulphate precipitation, anion exchange chromatography on DEAE-Sephadex and DEAE-Sephacel, respectively. Various catalytic and physicochemical properties of PGase were determined. The temperature effect showed the optimum PGase activity at 40 °C; while the effect of pH showed that this enzyme is optimally active at pH 6.0. The enzyme was relatively stable even after 2 hours of incubation at 40 °C ($t_{1/2} > 3$ h). Further stability of PGase was also shown after 6 months of storage at -20 °C and upon the addition of various chemical reagents. The PGase hydrolyzed pectin with a K_m of 13.0 mg.mL⁻¹ and k_{cat} of 1374 s⁻¹. The catalytic efficiency of the PGase action was determined to be 105.9 s⁻¹.mg.mL⁻¹.

6.1.3 Extraction of Inulin from Chicory Roots

The purified PGase was successfully used, in comparison with a commercially obtained pectinase, to break the cell wall (pectins) of chicory roots to release the inulins within. The extraction was carried out in citrate buffer (50 mM, pH 6.0) as well as in distilled water, and was investigated using both fresh and dried chicory roots. Complete inulin extraction was achieved with the purified PGase for each root sample analyzed. The dried chicory tuber was found to be the best and most suitable source for efficient inulin extraction.

Of the total inulins in each root sample, 98% extraction was achieved using the purified PGase with a specific activity of 28.3 U/mg. Furthermore, inulin extraction using PGase required only 40 minutes for optimal inulin release as compared to the time-consuming and high energy input conventional extraction methods. To our knowledge, there are no studies in literature showing the pectinolytic activity of the PGase which produced more than 98% yields of inulin from any source.

To identify the various oligosaccharides present in the mixtures, different analytical methods were used, namely HPLC, ESI-MS and ¹H NMR. Using both HPLC and ESI-

MS, the major products identified were GF₂, GF₃, and GF₄, with a high abundance of low-molecular weight sugars such as fructose, glucose and sucrose. The analytical techniques employed allowed for the confirmation of the hydrolysis products from both the extracted and the commercially obtained inulin. The sensitivity of ESI-MS analysis gives it an unmatched advantage over HPLC. An example is the identification of the inulo-oligosaccharide [GF₄+Li]⁺ peak at m/z 837. From the mass spectra data obtained, it is possible that higher DP oligosaccharides could have been detected if the mass to charge detection was increased to 1500 m/z . A singly charged GF₆ at m/z 1159 was also detected in one of the spectra generated. The ESI-MS data revealed that techniques such as HPLC are prone to limitations with regard to complex carbohydrate analysis.

However, there are also limitations on the use of ESI-MS for the quantitative analysis of various compounds present in mixtures. Another drawback of this technique is the derivatization procedure which is time-consuming and often requires the availability of standards for every oligosaccharide to be identified. Thus, high resolution NMR techniques can be very useful in identifying higher DP oligosaccharides in complex mixtures. This capability arises from the inherent property of high-resolution ¹H NMR spectra of oligosaccharides, allowing correlations to be made at low field and well-resolved regions of the spectra.

6.1.4 Pharmaceutical Properties of *sc*-FOS and SCFA.

Based on the results obtained in this study, no direct significant ($P > 0.05$) effect of the *sc*-FOS on either the carcinogenic or therapeutic enzymes (with the exception of β -glucuronidase) was observed. These results suggest that to be effective, FOS first require fermentation in the colonic environment. The SCFA which are the main products of FOS fermentation were found to significantly ($P < 0.05$) decrease both types of enzymes. Among the SCFA tested, butyrate, lactate and propionate were frequently observed to suppress the carcinogenic enzyme activities.

In particular, urease and β -glucuronidase activities were profoundly decreased by these SCFA; whereas a minimal increase in β -glucosidase activity was observed with FOS

treatment. Similar *in vivo* and *in vitro* inhibitory effects of SCFA have been reported by other investigators (Ogata *et al.*, 1999; Hughes and Rowland, 2000). These significant changes in microbial enzyme activities are therefore indicative of the anti-carcinogenic effect of FOS after fermentation in the intestinal environment. Although the effects of sc-FOS treatment in this study gives only a rough sketch, the results obtained with SCFA treatment, however, tell something of the modulatory effects of prebiotics on intestinal microflora. Additionally, if the findings with SCFA are stable and can be verified in any *in vivo* studies, this could have far-reaching, positive implications on the proposed anti-carcinogenic effects of prebiotics, also keeping in mind the other physiological effects such as immuno-modulation, cholesterol-lowering and therapeutic benefits.

6.2 Future Perspectives

The importance of FOS in various pharmaceutical and food industries, as well as their increasing bio-functional applications, seem to justify future investigations to obtain more effective enzymes for the synthesis of specific chain-lengths FOS preparations. This achievement will, in future, fill the current gap in the physicochemical and catalytic properties of fructosyl transferring enzymes.

More specifically, future work should elucidate the possible interactions between the active site of the enzyme and substrates, which will give more insight into the exact nature of the binding conformations. Also, an interesting question that should be addressed is what determines the specificity of β -(2 $\rightarrow$ 1) versus β -(2 $\rightarrow$ 6) linkages and the sizes of FOS produced. Thus far, no detailed mechanism of synthesis and structure/function characterization of FTases has been reported. This is complicated by the fact that during the transfer reaction, FTases generate new fructan molecules, which in turn can be used as acceptor molecules to elongate the FOS chain. By looking at detailed structure/function characterization of recombinant or purified FTase enzymes, more insight could be obtained regarding the mechanism of catalysis of FTase enzymes. Subsequently, these structure and mechanism investigations will provide the breakthrough synthesis of specific chain-lengths FOS. Ultimately, a new generation of FOS will emerge, that will offer better and more diverse bio-functional properties.

However, several questions remain, for example: what is the major contributing prebiotic property of a particular FOS (i.e. chain-length, solubility and dosage), and how the different FOS are fermented by various probiotic strains. These are questions to which we presently do not have the answers. It is possible that different probiotic strains target different FOS. All of these hypotheses have various degree of support, mainly originating from *in vitro* and animal experiments. Thus, more work needs to be done to identify other specific probiotic strains and FOS characteristics responsible for specific anti-tumorigenic effects. However, even with these reservations in mind and mindful of the limited human clinical studies available, the use of lactic bacteria for cancer suppression is certainly interesting and therefore deserves more scrutiny. The latter should involve carefully designed human clinical studies to corroborate the wealth of animal studies.

In terms of oligosaccharides analysis, large biomolecules present in complex mixtures usually require prior knowledge regarding the spectroscopic properties of the molecule, especially when using high resolution NMR. Therefore, in future, this would also require that sophisticated experimental techniques be applied, such as multidimensional spectroscopy or signal processing.

6.3 Conclusions

In the present study, a procedure was developed for the successful isolation and purification of two different enzymes, namely FTase and PGase, present in the commercial extract (Pectinex[®] Ultra SP-L) of *A. aculeatus*. It was shown that the FTase enzyme was capable of synthesizing various, short chain FOS. The kinetic and physicochemical characterization of this enzyme did not, however, allow us to speculate on the mechanism of increasing chain-length FOS synthesis; suggesting that further elucidation of substrate-binding regions and enzyme mechanism are of particular importance.

Similarly in this study, a reproducible and efficient method for the extraction of inulin from chicory roots was developed using the purified PGase at its optimal parameters.

Also in conclusion, this work presents another approach for oligosaccharide analysis using ESI-MS and ^1H NMR. Although different in their speed and sensitivity, the results presented demonstrate that both these techniques are capable of providing accurate, relative quantitative and structural data on the differences in the abundance of oligosaccharides present in mixtures. The variability of the analytical data obtained when using these techniques was small, as compared to HPLC. The resulting spectra showed relative uniformity and consistency between the theoretical (standards and predicted) and experimental samples.

Finally, we demonstrated with *in vitro* studies that SCFA, rather than FOS, may be responsible for inhibiting pro-carcinogenic enzymes (β -glucuronidase and urease) in the colon. This anti-carcinogenic activity is because the major SCFA produced are organic acids with pK_a values of approximately five. Their production may lead to the acidification of the intracolonic environment and resultant inhibition of sensitive enzymes. These results indicate that *in vitro* models can provide some insight into the modulation of metabolic activities in the colonic environment. However, the *in vitro* studies and techniques currently available should be further validated using well-designed, randomized *in vivo* clinical studies if the aim is to design prebiotics for specific targets (e.g. immuno-modulation, anti-carcinogenicity or other therapeutic properties). Furthermore, *in vivo* studies should also evaluate whether the large intestine is the right target or whether the clinical trials should focus on the small intestine or both.

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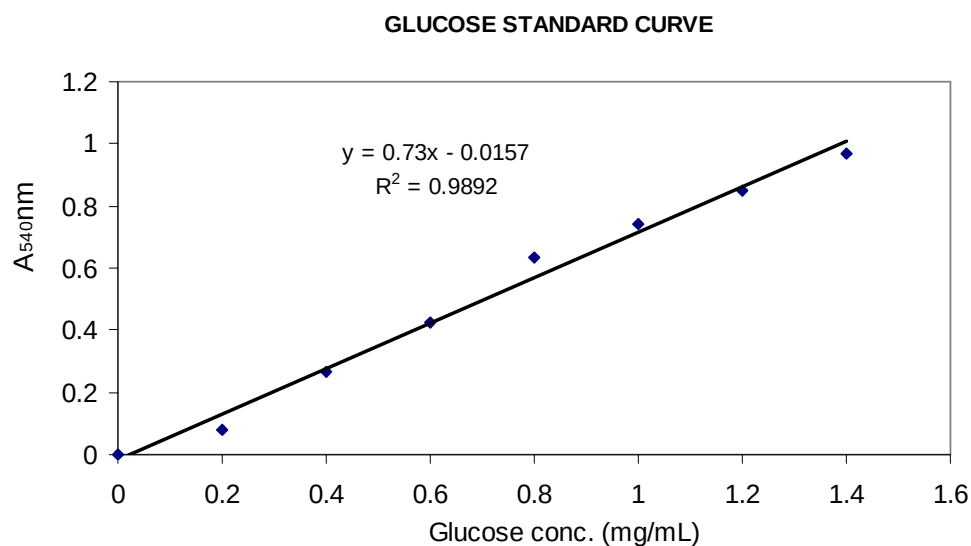
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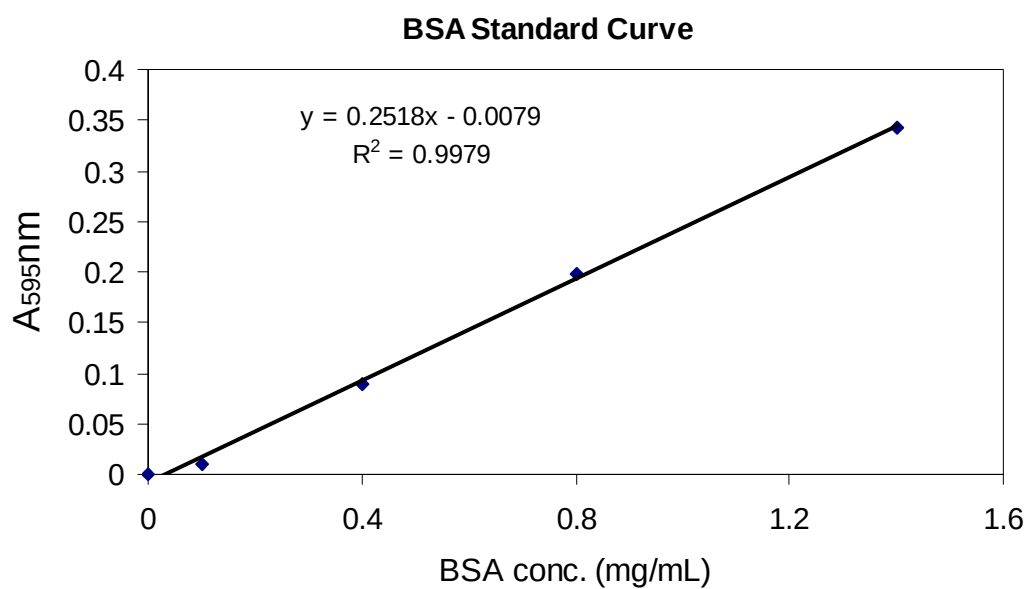
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APPENDICES



APPENDIX A Glucose standard curve



APPENDIX B Bovine serum albumin standard curve

APPENDIX C: Kinetic Constant Equations

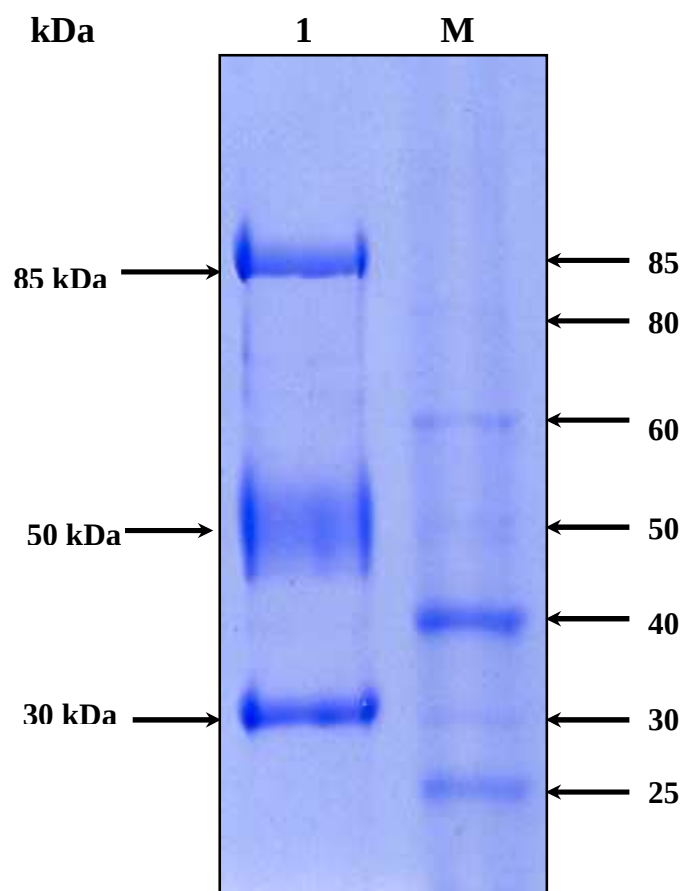
The turn-over number was calculated using the equation:

$$k_{\text{cat}} = V_{\text{max}}/[E_t],$$

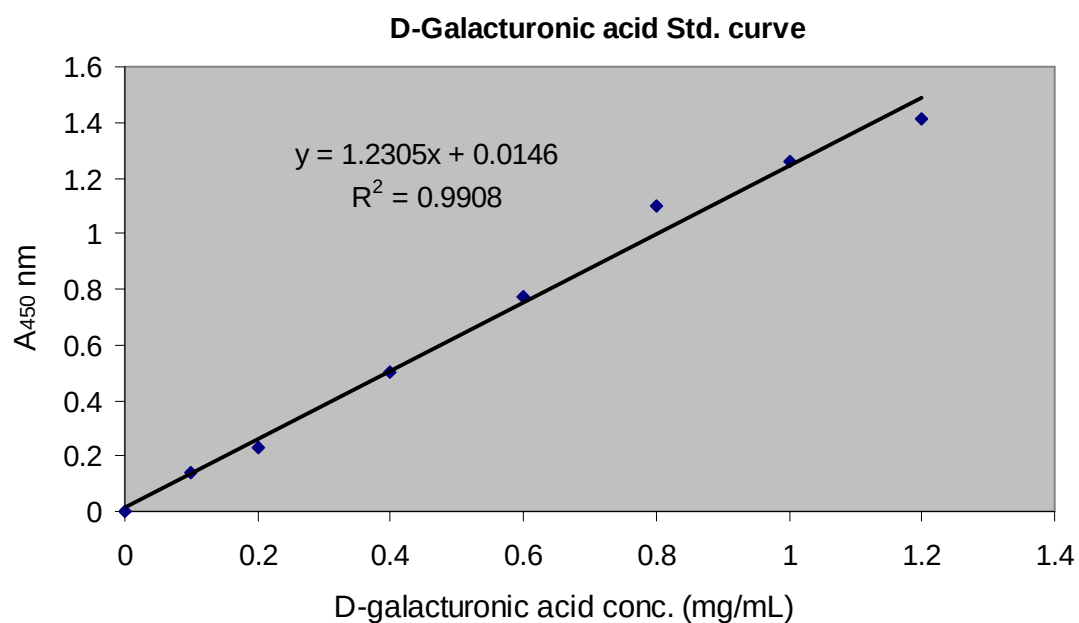
where V_{max} is the maximum velocity of the enzyme and $[E_t]$ is the total amount of enzyme.

The catalytic efficiency was determined using the equation:

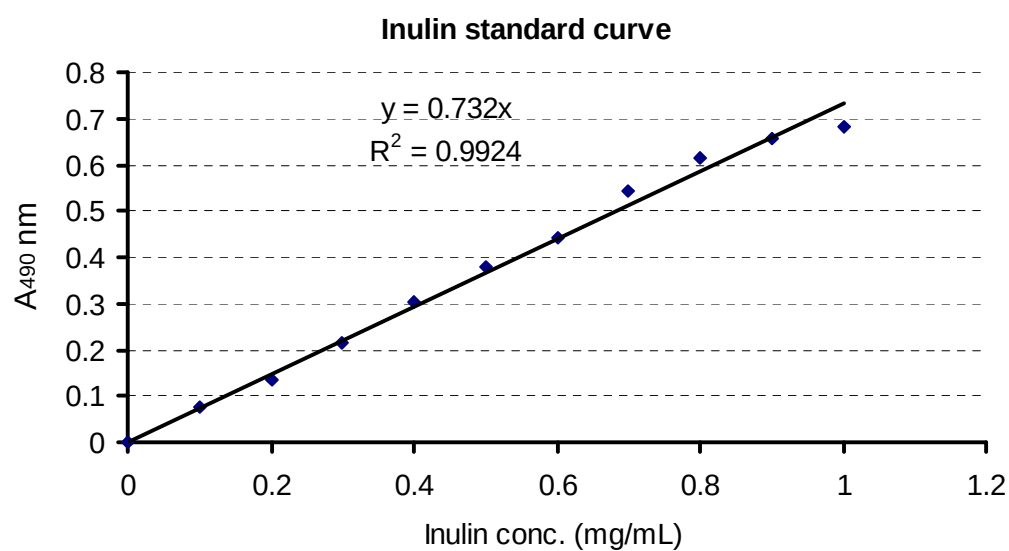
$$k_{\text{cat}} / K_m$$



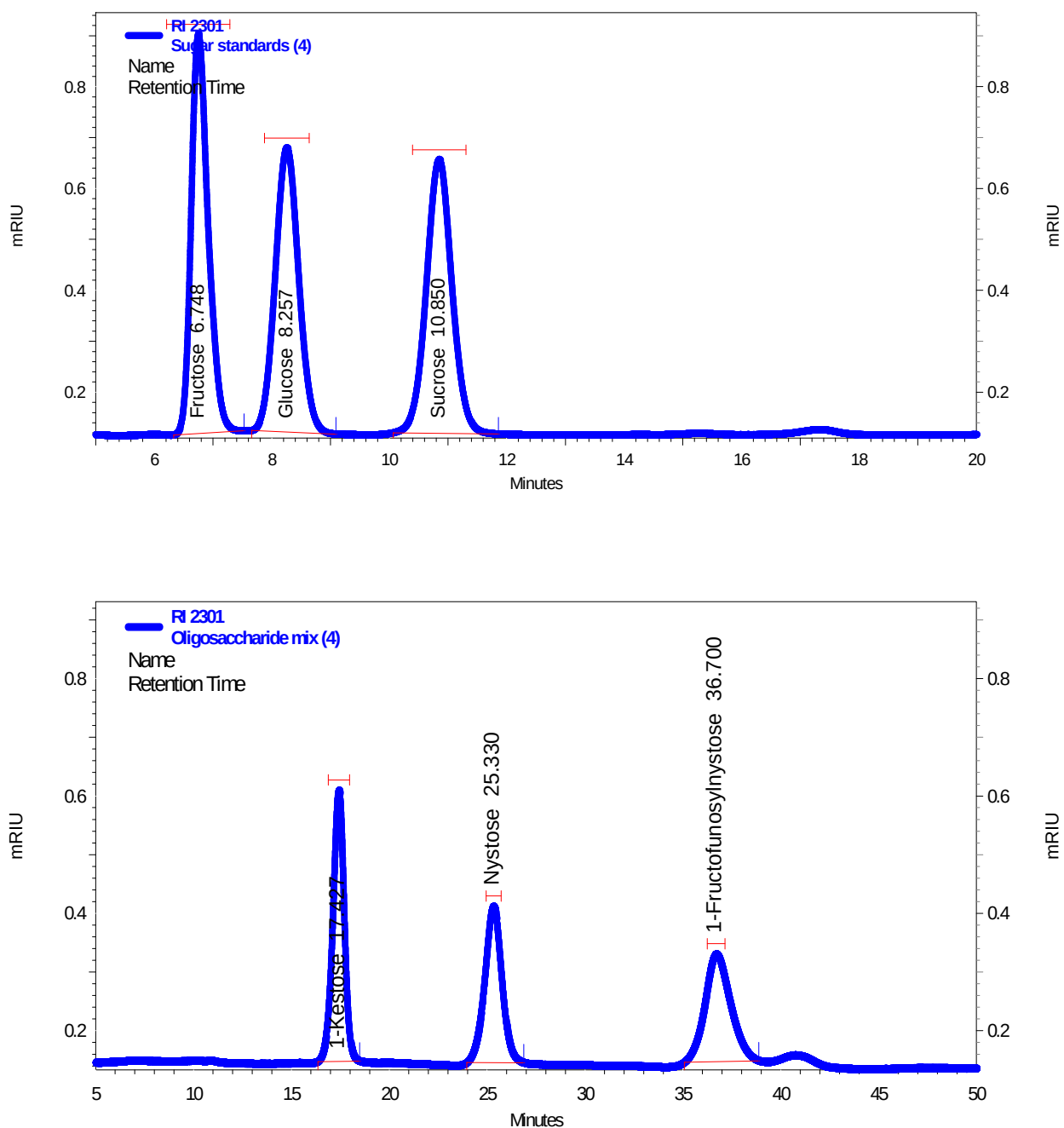
APPENDIX D Native PAGE analysis of the purified FTase. Lane 1, FTase protein showing three species with different charges; Lane M, molecular weight markers. The stacking gel concentration was 5% and the separating gel was 12%. The gel was stained with Coomassie Brilliant Blue R-250 and destained with acetic acid:methanol: water (1:1:8, v/v).



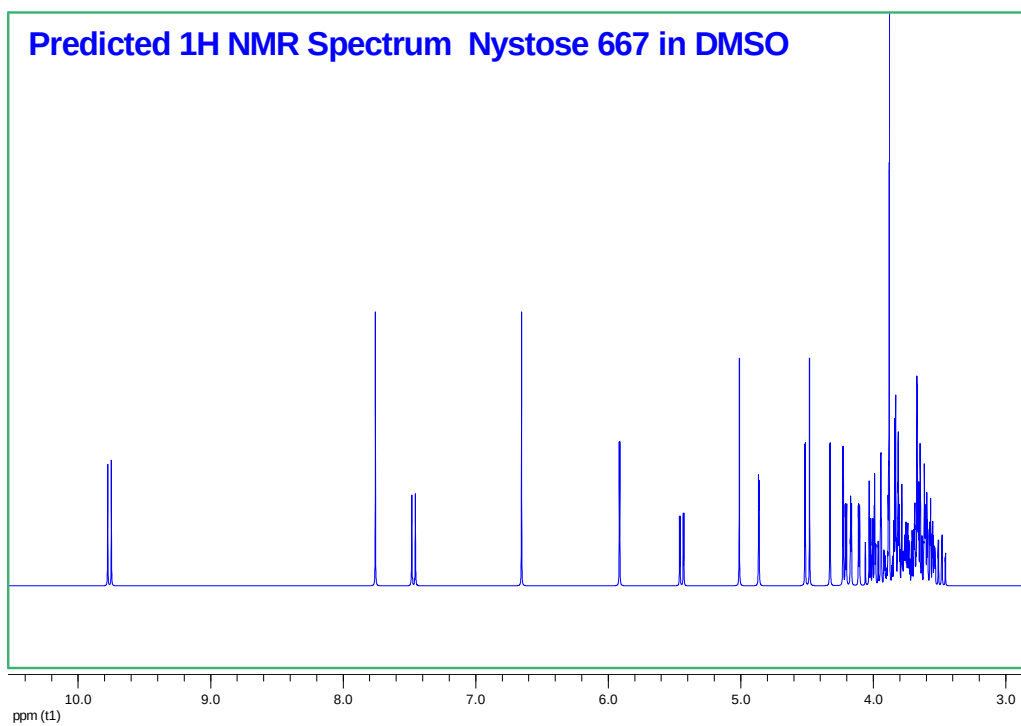
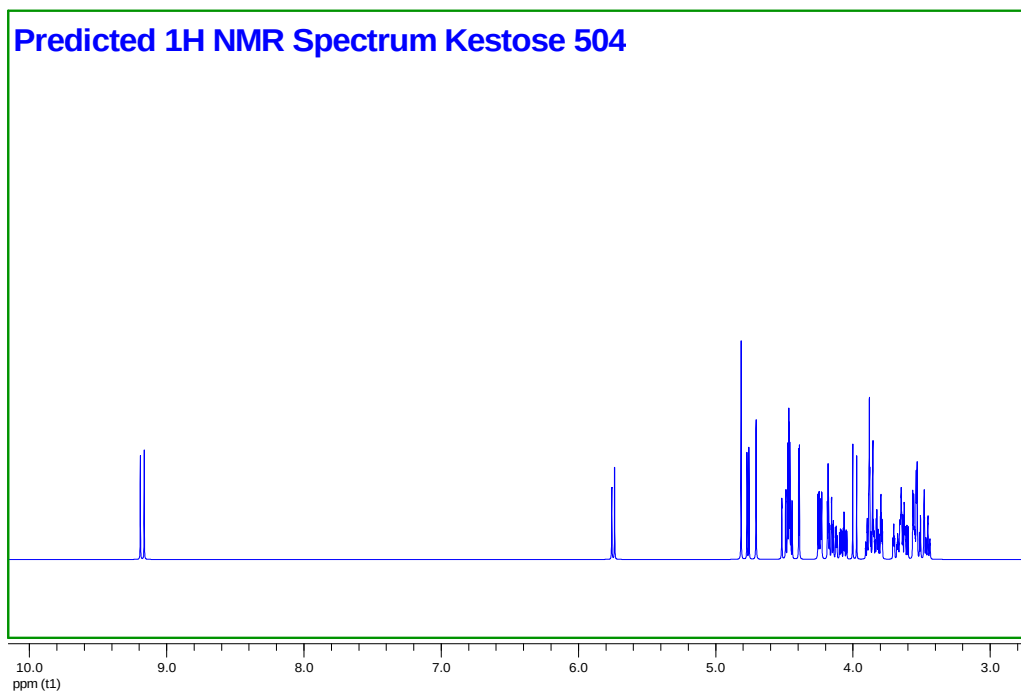
APPENDIX E D-galacturonic acid standard curve.



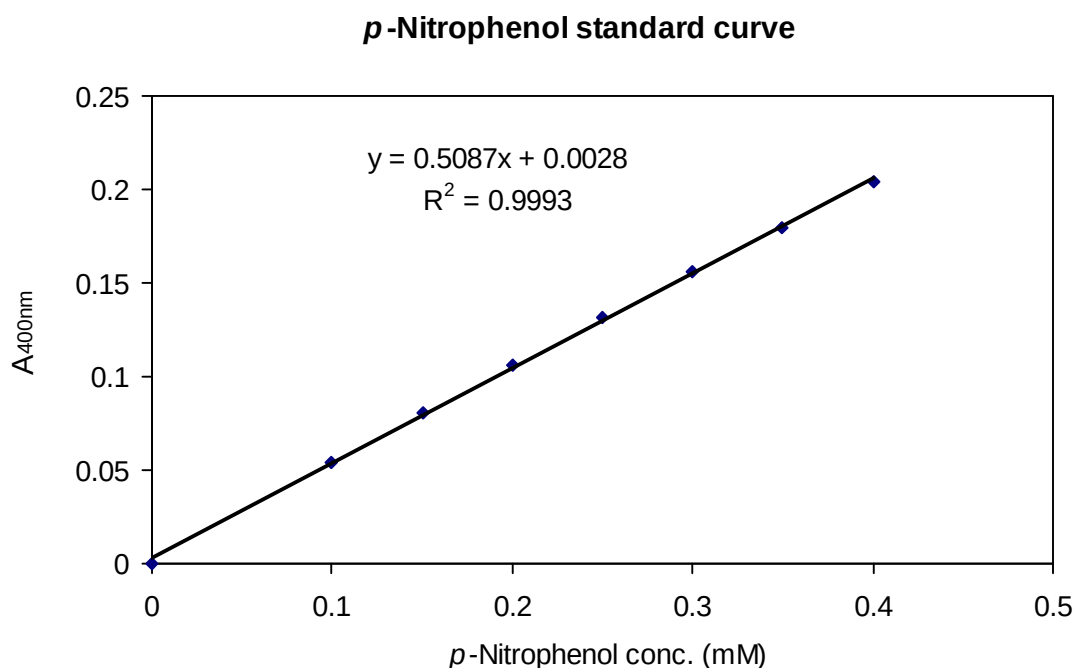
APPENDIX F Inulin standard curve



APPENDIX G Sugar and Oligosaccharide Standards



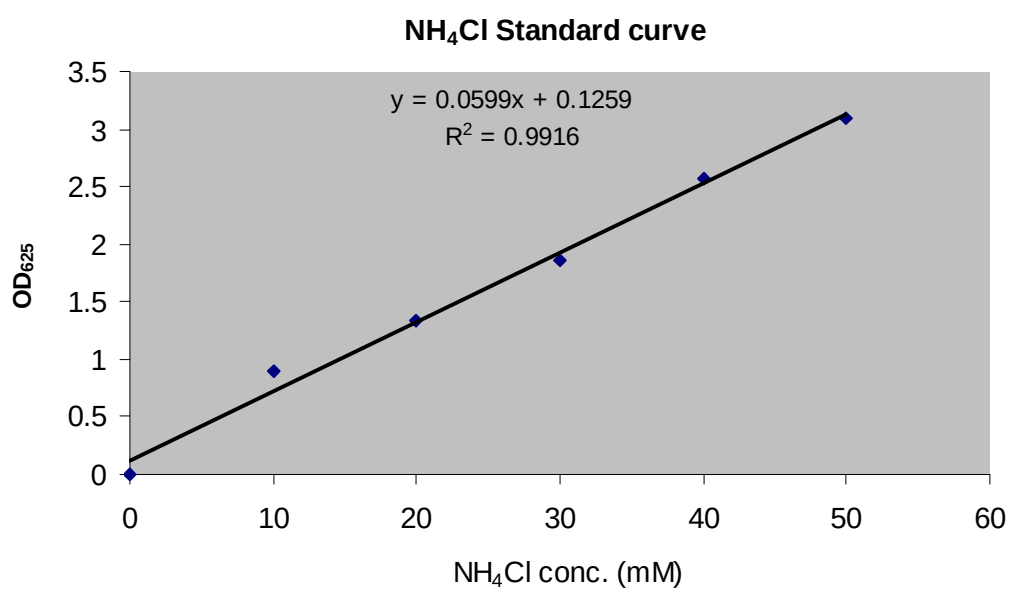
APPENDIX H ^1H -NMR Predictions of Kestose and Nystose



APPENDIX I *p*-Nitrophenol Standard Curve

GF2:	1-Kestose
GF3:	Nystose
GF4:	1-Fructofuranosyl-nystose
GF~7:	Raftiline [®] GR
GF~9:	Raftilose
GF>22:	Raftiline [®] HP
Probiotic Supplement:	Culturelle™ (Each capsule contains: <i>B. longum</i> ; <i>L. rhamnosus</i> 10×10 ⁷ ; glucose; oligosaccharides; potato starch; locus bean gum)

APPENDIX J Oligosaccharide Chemical Names



APPENDIX K

Ammonium Chloride Standard Curve