Enzymatic glucosylation:

sucrose glucosyltransferases and glucosidases in *O*- and *S*-glucoside synthesis

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G.H. Meulenbeld

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Contents

Heb der mar fiducie in,
want 't giet precies zoas 't giet.

(SKIK, *'t Giet zoas 't giet*)

Stellingen

Transglucosylering is meer dan alleen het toevoegen van een geactiveerde glucosyldonor.
(Dintinger et al., 1994 Biotechnol Lett 16: 689-692)

Teneinde een eigen theorie te verdedigen is het beter aanvullende experimenten te doen, dan tegenstrijdige literatuur te bekritiseren.

De benaming glucansucrase levert onevenredig veel verwarring op in vergelijking met de benaming dextransucrase.

De belangstelling voor myrosinases staat niet in verhouding met de brede verspreiding van andere thioglucoside hydrolases in de natuur.
(Fenwick et al., 1983 Crit Rev Food Science Nutr 18: 123-202)

"Wetenschappelijke nieuwsgierigheid" zou meer waardering moeten krijgen tegenover "nut en noodzaak" in discussies over onderzoek.

Aangezien een lift ook gebruikt wordt om omlaag te gaan, kan stijgen zeker ook als omgekeerd dalen worden omschreven.
(B.M. de Roode, 2001 Proefschrift Wageningen Universiteit)

Demonstranten tegen globalisering verliezen geloofwaardigheid door de hele wereld rond te reizen om zo hun bezwaren kenbaar te maken.

Verkeersveiligheid is ook gebaat bij het bekeuren van overtreders van de minimum-snelheid.

Kunst is de kunst van het verkopen.

De term "woon-werkverkeer" is meestal niet van toepassing op de avondspits.

Stellingen behorend bij het proefschrift "Enzymatic glucosylation:
sucrose glucosyltransferases and glucosidases in *O*- and *S*-glucoside synthesis."

Gerwin Meulenbeld, 30 oktober 2001.

1

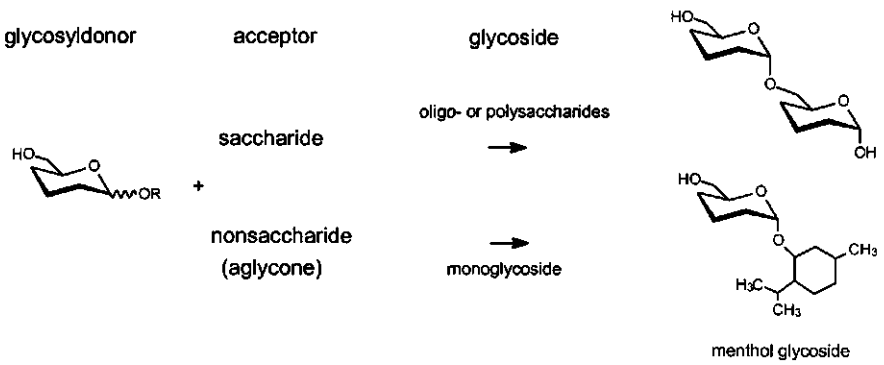
General Introduction

ENZYMATIC GLUCOSYLATION

Glycosylation is considered to be a relevant method for modifying physical properties like solubility and volatility of useful compounds. Areas of practical interest concern non-nutritive sweeteners, flavours and fragrances and site specific drug delivery control. For this reason there has been a growing interest in the past decade in the enzymatic synthesis of glycosides.

Glycosidic linkage

Glycosides are compounds composed of a saccharide and an acceptor molecule (containing a hydroxyl, amino or sulfhydryl group), which is covalently linked to the anomeric carbon group of the saccharide (glycosidic linkage). Basically two classes of acceptor molecules can be discriminated in glycosylation, saccharides and nonsaccharides (aglycones), which respectively result in the formation of oligo- or polysaccharides and monoglycosides (Scheme 1.1).



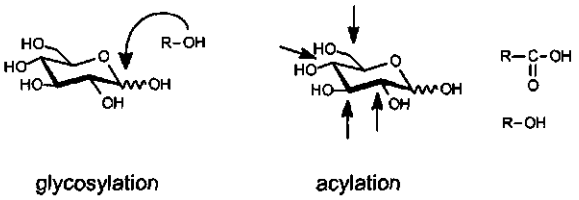
Scheme 1.1 Different acceptor molecules for glycosylation

The glycosidic linkage is one of the most abundant linkages. About 95% of the 200 billion tons of glucose formed annually is glycosidically linked to other glucose molecules in oligo- and polysaccharides (e.g. starch, cellulose) (Thiem, 1995). A small part of the glucose is glycosidically linked to other compounds than saccharides. Detailed analysis of fruit and vegetables has shown that a significant portion of the volatile flavour compounds occurs as nonvolatile precursors, most often as glycosides (Stahl-Biskup et al., 1993; Watanabe et al., 1993; Winterhalter and Schreier, 1994). In microorganisms the presence of glycosidically bound aglycones seems to be restricted to nucleosides.

The precise role of the glycosidically bound volatiles in plants is not always clear. It is believed that the glycosides play a role as fragrance precursor or that they may function as a protective mechanism in the plant defence system to prevent the lipophilic volatile phenols or alcohols from destroying membranes. (Fenwick et al., 1983; Vetter, 2000).

Acylation

Looking at the chemical differences between the hydroxyl groups of saccharides, two principally distinct coupling reactions between acceptor and saccharide molecules may occur, glycosylation and acylation (Scheme 1.2).



Scheme 1.2 Glycosylation and acylation

Saccharides can be acylated when reacting with (fatty) acids resulting in the formation of ester bonds (e.g. fatty acids in membrane glycolipids), or saccharides can form ether bonds (e.g. aminosaccharides). Synthetic sugar esters have been made chemically and enzymatically with lipases. The fatty acid sugar esters constitute a group of nonionic surfactants with potentially important applications as food emulsifiers or industrial (biodegradable) detergents (Ducret et al., 1995; Sarney and Vulfson, 1995; Gunstone, 1999).

In this thesis the focus is on enzymatic glycosylation.

IN VIVO GLYCOSIDE SYNTHESIS

In the biosynthetic machinery of living organisms, glycosyltransferases are used for the synthesis of glycosides. The glycosyltransferase reaction involves the highly specific transfer of a monosaccharide from an activated sugar donor to an acceptor molecule. The glycosyltransferases can be divided into Leloir enzymes and non-Leloir enzymes (Leloir, 1971). Glycosylhydrolase enzymes are generally used for the *in vitro* enzymatic glucosylation.

The Leloir enzymes require sugar nucleotides as activated glucosyl donors for glycosyl transfer. The biosynthesis of polysaccharides proceeds using sugar nucleotides and Leloir enzymes, e.g. the synthesis of starch, glycogen and cellulose using respectively ADP-glucose, UDP-glucose and GDP- (or UDP-)glucose. Also the synthesis of disaccharides (e.g. LacNAc using UDP-Gal and a galactosyltransferase, Scheme 1.3 reaction e) and the transfer of the glycosyl moiety to a protein, lipid or DNA is catalysed by Leloir enzymes (Toone et al., 1989; Ichikawa et al., 1992; Thiem, 1995).

Much of the research on glycoside synthesis concerns the synthesis of oligo- and polysaccharides using non-Leloir glycosyltransferases. Nowadays it is recognised that especially plants also have a large capability to glucosylate a wide range of different aglycones. Examples of characterised (plant) glucosyltransferases involved in the glucosylation of aglycones are, UDP-glucose: *p*-hydroxybenzoate glucosyltransferase (Bechthold et al., 1991), UDP-glucose: *p*-hydroxymandelonitrile glucosyltransferase

(Jones et al., 1999) and UDP-glucose: thiohydroximate glucosyltransferase (Reed et al., 1993).

Non-Leloir enzymes utilise other forms of activated glucose, e.g. sugar-1-phosphate and sucrose (Toone et al., 1989). Important examples of non-Leloir pathway enzymes are sucrose phosphorylase (EC 2.4.1.7), cyclodextrin glucanotransferase (EC 2.4.1.19) and sucrose glucosyltransferase (EC 2.4.1.5).

IN VITRO SYNTHESIS OF GLYCOSIDES

Acceptors

The *in vitro* synthesis of oligosaccharides has been extensively studied, mainly because of the importance of saccharides as components of glycoproteins and glycolipids. These oligosaccharides are involved in many types of molecular recognition (Varki, 1993). The complexity of these structures has hampered chemical or enzymatic synthesis, e.g. 3 different hexopyranose units can (theoretically) yield 720 different trisaccharides, while 3 different amino-acids only allow the synthesis of 6 different tripeptides. (Toone et al., 1989; Ichikawa et al., 1992; Palcic, 1999). The interest in oligosaccharide synthesis has also been stimulated because they have been proposed to stimulate the proliferation of bifidobacteria in the intestine, which is considered to be beneficial to the human health (Minami et al., 1983; Wijsman et al., 1989). In this respect, oligosaccharides can be designated as prebiotics (Monsan and Paul, 1995; Crittenden and Playne, 1996).

The research concerning enzymatic glycosylation of aglycones has mainly focussed on the synthesis of aliphatic alkyl glycosides. These glycosides have several specific properties allowing applications in detergency and emulsification and also have some antimicrobial effects (Panintrarux et al., 1995; Vulfson et al., 1990; Ljunger et al., 1994 and Vic et al., 1995). Much less has been published on the synthesis of non-alkyl aglycones. In Table 1.1 an overview is given of *O*-aglycones, varying from simple phenolic and non-alkyl aliphatic compounds to complex flavonoids that have been glycosylated using various enzymes.

Besides *O*-glycosides the occurrence of the *N*-glycosidic linkage is also widespread. The *N*-glycosidic linkage mainly occurs in nucleosides and their derivatives (Prasad et al., 1999) and glycosylated proteins. With respect to industrial applications (e.g. flavours) this type of glycosidic linkage is of minor interest. The formation of a third type of glycoside, the *S*-glycosidic linkage, has received very little attention in literature. This type of linkage is present in plants as glucosinolates, where they serve as a sink for nutrients like nitrogen and sulphur. The products of glucosinolate hydrolysis have a characteristic flavour and probably play an important role in the plant defence system (Mithen et al., 2000; Fenwick et al., 1983; Rask et al., 2000).

Table 1.1 Overview of different aliphatic (non-alkyl) and aromatic aglycones used in enzymatic glycosylation.

aglycone[1]	properties	enzyme (origin)	method[2] / donor	reference
•caffeic acid	antioxidant	α-amylase (*bacillus subtilis*)	TG/maltopentaose	Nishimura et al., 1995
•catechin	antioxidant (flavonoid)	sucrose phosphorylase (*Leuconostoc mesenteroides*)	TG/sucrose	Kitao et al., 1993
		sucrose glucosyltransferase (*Streptococcus sobrinus*)	TG/sucrose	Nakahara et al., 1995
		cyclodextrin glucanotransferase (*Bacillus macerans*)	TG/starch	Funayama et al., 1993
		GSase, amylase (*Bacillus subtilis*)	TG/starch, maltotriose	Funayama et al., 1994
		α-amylase (*Bacillus subtilis*)	TG/maltopentaose	Nishimura et al., 1994
		crude enzyme *Xanthomonas campestris*	TG/maltose	Sato et al., 2000
•chloro-amphenicol	antibiotic	β-galactosidase (*Aspergillus oryzae*)	TG/lactose	Schekerman et al., 1997
•furanone derivatives	flavour ingredient	sucrose phosphorylase (*Leuconostoc mesenteroides*)	TG/sucrose	Kitao et al., 2000
• (neo) hesperidin	antioxidant (flavonoid)	cyclodextrin glucanotransferase (*Bacillus* sp.)	TG/starch	Komentani et al., 1994
•hydroquinone		α-amylase (*bacillus subtilis*)	TG/maltopentaose	Nishimura et al., 1994
		sucrose phosphorylase (*Leuconostoc mesenteroides*	TG/sucrose	Kitao and Sekine, 1994
•kojic acid	anti bacterial reagent	cyclodextrin glucanotransferase (*Bacillus macerans*	TG/starch	Kitao and Sekine, 1994
		α-amylase (*bacillus subtilis*)	TG/maltopentaose	Nishimura et al., 1994
		β-galactosidase (*Bacillus circulans*)	TG/lactose	Hassan et al., 1995
•menthol	flavour ingredient	α-glucosidase (*Saccharomyces cerevisiae*)	TG/maltose	Nakagawa et al., 1996
•naringin	antioxidant (flavonoid)	matlogenic amylase (*Bacillus stearothermophilus*)	TG/maltotriose	Lee et al., 1999
		cyclodextrin glucanotransferase (*Bacillus* sp.)	TG/starch	Komentani et al., 1996
•serine and threonine		α-mannosidase (jack bean)	RH/mannose	Johansson et al., 1991
•vitamin E (derivative TM)	antioxidant	α-glucosidase (*Saccharomyces sp.*)	TG/maltose	Murase et al., 1997
•xylitol	flavour ingredient	sucrose phosphorylase (*Leuconostoc mesenteroides*)	TG/glucose-1-P	Kitao and Sekine, 1992

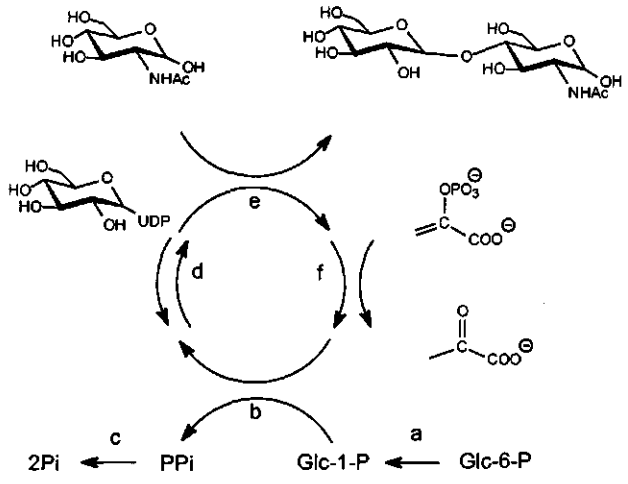
1) Trivial name 2) TG: transglycosylation, RH: reversed hydrolysis

The use of Leloir glycosyltransferases, the *in vivo* approach

In case of biocatalytic glycosylation there are some disadvantages associated with the use of (*in vivo*) Leloir glycosyltransferases. A serious problem related with large scale glycoside synthesis has been the preparation and availability of glycosyltransferases (Ichikawa et al., 1992). However, recent progress in genetic engineering has made several glycosyltransferases available in large quantities (Palcic, 1999; Endo and

Koizumi, 2000). Another problem using glycosyltransferases is the preparation of sugar nucleotides. Many chemical methods for the synthesis of sugar nucleotides have been reported, nevertheless the methods are laborious, expensive and difficult to apply on a large scale (Kretzschmar and Stahl, 1998). To overcome the difficulties associated with the preparation of sugar nucleotides and the inhibitory effects of the released nucleoside mono- or diphosphates, *in situ* regeneration (one-pot process) of sugar nucleotides was developed. Wong et al. (1982) demonstrated a large-scale synthesis of LacNAc using the β-1,4-galactosyltransferase reaction (Scheme 1.3), in which sugar nucleotides (UDP-Gal and UDP-Glc) were regenerated *in situ* using six different enzymes; phosphoglucomutase (EC 5.4.2.2), UDP-glucose pyrophosphorylase (EC 2.7.7.9), inorganic pyrophosphatase (EC 3.6.1.1), UDP-galactose 4-epimerase (EC 5.1.3.2), β-1,4-galactosyltransferase (EC 2.4.1.22) and a UTP pyruvate kinase (EC 2.7.1.40). Variations on the integrated cofactor-regeneration approach were also developed to glycosylate other acceptor molecules (Ichikawa et al., 1992).

In general, it has become clear that it is a laborious and a multi-step (expensive) endeavour to overcome the problems mentioned above. Therefore alternatives were developed for oligo- and polysaccharide synthesis and for the glycosylation of aglycones. Examples are the chemical, a chemoenzymological approach and the use of alternative (with respect to the *in vivo* mechanism) enzymes (Toone et al., 1989; Ichikawa et al., 1992; Thiem, 1995; Crout and Vic, 1998). However, it should be kept in mind that for the synthesis of specific oligosaccharides, the use of Leloir glucosyltransferases is often inevitable.



Scheme 1.3 Integrated cofactor-regenerated formation of LacNAc using phosphoglucomutase (a), UDP-glucose pyrophosphorylase (b), inorganic pyrophosphatase (c), UDP-galactose 4-epimerase (d), β-1,4-galactosyltransferase (e) and UTP-pyruvate kinase (f).

Chemical and chemoenzymological approach

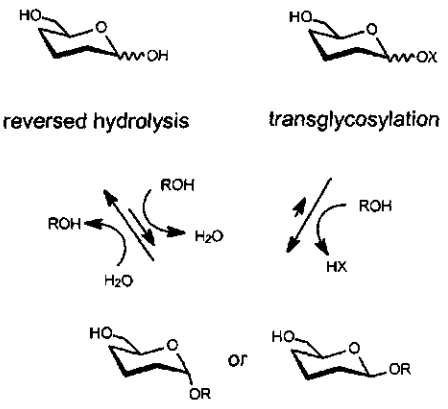
The chemical glycoside synthesis has been extensively developed. However, some features limit the application of existing synthetic methods for the practical preparation of glycosides (Toone et al., 1989).

As observed in oligosaccharide synthesis, the diversity in the amount of linkages exceeds that found in other areas of biological chemistry. In order to ensure regio- and stereoselectivity in both oligosaccharide synthesis and aglycone glycosylation, (complex) protection and deprotection steps are needed. Other problems are related to the activation of the glycosyl moiety, prior to transfer to the acceptor molecule and the necessity of using aqueous media. Many carbohydrates are only soluble in hydrophilic solvents (Dordick, 1989), while many organic chemical reactions are incompatible with aqueous systems.

Developments in the field of chemoenzymology sometimes (especially in oligosaccharide synthesis) provide solutions for problems faced when only carbohydrate chemistry or enzymology is applied (Dordick, 1992; Thiem, 1995). A field suitable for chemoenzymology is the enzymatic synthesis of oligosaccharide blocks, followed by a chemical regioselective coupling. This coupling may occur either with other oligosaccharides (Matsuo et al., 1998), simple chemicals (Matsuo et al., 1999) or even proteins (Gonzalez and Thiem, 1999). Another example of chemoenzymology was demonstrated for the production of simple aliphatic glucosides. Anomeric product mixtures of simple aliphatic alcohols obtained from acid-catalysed Fischer glycosylation were produced (Lee and Lee, 1974). In order to obtain control of glucoside configuration, the undesired glycosides can be enzymatically hydrolysed using α- or β- gluco- and galactosidases (Ruiz et al., 2001).

Enzymatic approach using glycosidases and non-Leloir glycosyltransferases

In the case of *in vitro* enzymatic aglycone glycosylation two strategies can be applied, the use of a thermodynamic (reversed hydrolysis) or a kinetic (transglycosylation) strategy (Scheme 1.4).



Scheme 1.4 Glycosylation mechanisms, reversed hydrolysis and transglucosylation.

Reversed hydrolysis glycosylation is a thermodynamic approach in which the reaction equilibrium, normally in favour of hydrolysis, is shifted towards synthesis. With this approach, final glycoside yields are generally low. Use of high substrate concentrations or heterogeneous catalysis in organic solvents with low water activity may result in a shift of the reaction equilibrium (Vic et al., 1995). Transglycosylation is a kinetic approach in which the (activated) glycosyl moiety of a substrate is transferred to acceptors other than water. For transglycosylation to occur a glycosidic linkage is hydrolysed prior to the transfer of the glycosyl moiety. Consequently accumulation of the product in high concentrations can be achieved.

Two classes of enzymes are available for the *in vitro* enzymatic glycosylation, glycosylhydrolases (EC 3.2.1) and glycosyltransferases (EC 2.4.1). Glycosylhydrolases (or glycosidases) normally cleave a glycosidic bond. However, they can be used to form glycosides by selecting conditions that favour synthesis, this is called reversed hydrolysis. Reversed hydrolysis is frequently applied e.g. in the synthesis of aliphatic alkylmonoglucosides. The glucosylation of aliphatic non-alkyl aglycones has been reported in the case of serine and threonine using α-mannosidase (Table 1.1). Glycosylhydrolases are also capable of transglycosylation. This is illustrated by the oligosaccharide formation using β-galactosidase and lactose (Nakanishi et al., 1983) and the list of nonsaccharide acceptors (aromatic and aliphatic alcohols) glycosylated by respectively α-amylase, β-galactosidase, α-glucosidase and α-mannosidase as presented in Table 1.1.

With respect to the *in vitro* synthesis of aglycones, the non-Leloir glycosyltransferases (especially sucrose phosphorylase, SPase) are of interest. SPase catalyses two types of transfer reactions. One is the transfer of a glucosyl moiety from glucose-1-phosphate. Using fructose as acceptor this reaction results in the synthesis of sucrose. The other reaction is the transfer of a glucosyl moiety from sucrose to an acceptor (e.g. inorganic phosphate). SPase has a broad acceptor specificity, besides the compounds mentioned in Table 1.1 also hydroxybenzenes, hydroxybenzoic acids, benzyl alcohol and hydroxybenzyl alcohols are glucosylated (Kitao and Sekine, 1994).

Another non-Leloir enzyme is cyclodextrin glucanotransferase (CGTase), which is predominantly capable of converting starch (and related substrates) into cyclodextrins. Reports on aglycone glucosylation by CGTase predominantly concern the glucosylation of a wide range of flavonoids like catechin and naringin and (neo)hesperidin (Table 1.1).

The requirement of expensive nucleotide donors is a disadvantage using Leloir glycosyltransferases. Therefore the use of glycosidases is more beneficial. Additionally, glycosidases are compared to the glycosyltransferases generally more available, less expensive, are generally tolerant to organic solvents and glycosides may be obtained in one-step reactions (Ichikawa et al., 1992; Vic et al., 1995; Palcic, 2000). The main disadvantage is that regioselectivity may not be observed in all cases and that generally low yields are obtained (Toone et al., 1989; Thiem, 1995; Ichikawa et al., 1992; Crout and Vic, 1998). However, there are enzymes that combine the merit of transglycosylation (high yields) with the ability to utilise inexpensive glycosyl donor

substrates as observed in the reversed hydrolysis mechanism. Examples are the frequently described SPase and the less familiar sucrose glucosyltransferase.

SUCROSE GLUCOSYLTRANSFERASES

Sucrose glucosyltransferases (or glucansucrases) are extracellular enzymes mainly produced by *Streptococcus* species present in the oral flora (commonly termed glucosyltransferase) or the soil bacterium *Leuconostoc mesenteroides* (commonly termed dextransucrase). The interest in glucansucrases can be attributed to their capacity to form glucose polymers, generically named glucans.

Glucan

Glucans are extracellular polysaccharides composed of a linear chain containing D-glucopyranosyl units, either principally linked through α(1-6) glucosidic bonds (dextran polymer), or through α(1-3) glucosidic bonds (mutan polymer) as well as linked alternately through α(1-6) and α(1-3) glucosidic bonds (alternan polymer). Glucans also differ in the type (α(1-2), α(1-3), α(1-4)) and amount of branch linkages (Robyt, 1995; Monchois et al., 1999). Water insoluble glucans (IG) predominantly contain α(1,3) linked glucose, while water soluble glucans (SG) are rich in α(1,6) linked glucose. Different kinds of glucans with different sizes and structures (type of glucosidic linkage), depending on the glucansucrase producing strain and the type of glucansucrase have been characterised.

The crucial role of *Streptococcus* species (especially *S. mutans*) together with sucrose in the development of human dental caries has been well documented (Hamada and Slade, 1980; Loesche, 1986). The ability of these organisms to convert sucrose into water (in)soluble glucans appears to play an important role in the attachment and colonisation of cariogenic bacteria. Some (*Streptococcus*) strains even express more than one glucansucrase and each glucansucrase has a specific role in the process leading to dental caries (Tsumori and Kuramitsu, 1997).

Despite the (dental) interest in glucansucrases from *Streptococcus* species, it is the dextran forming dextransucrase from *Leuconostoc mesenteroides* which is used in the industrial production of glucan and oligosaccharides (Monsan and Paul, 1995; Monchois et al., 1999).

Dextransucrases

Dextransucrases from *Leuconostoc mesenteroides* strains predominantly produce α(1,6) linkages (dextran), e.g. *L. mesenteroides* B-512F dextransucrase synthesises a linear water soluble dextran with 95% α(1,6) linkages in the main chains and 5% α(1,3) branch linkages (Jeanes and Seymour, 1979). An important exception is the dextran produced by *L. mesenteroides* NRRL B-1299 that contains the relatively rare α(1,2) glucosidic linkage. Both dextran types are produced on an industrial scale (Seymour et al., 1979a; Seymour et al., 1979c; Robyt, 1995; Remaud-Simeon et al., 2000). The occurrence of a high percentage of branch linkages is illustrated by *L. mesenteroides* B-

742. The B-742 dextran structure is essentially a B-512F structure containing some α(1,6) chains attached by an α(1,3) branch linkage, but having single glucose residues attached by α(1,3) branch linkage to every glucose residue in the α(1,6) chain (Seymour et al., 1979a and 1979b).

Glucosyltransferase

Streptococcal strains produce glucan, which is (generally) composed of high amounts of α(1,3) glucosidic linkages in comparison with dextrans. The diversity observed in glucan production is illustrated by *Streptococcus mutans* GS5. Three different glucansucrases are excreted; *gtf*B (1475 amino acids, Aoki et al., 1986; Shiroza et al., 1987), *gtf*C (1375 amino acids, Hanada and Kuramitsu, 1988; Ueda et al., 1988) and *gtf*D (1430 amino acids, Hanada and Kuramitsu, 1989; Honda et al., 1990), which produce different glucans with respectively 87% α(1,3) and 13% α(1,6), 85% α(1,3) and 15% α(1,6), 30% α(1,3) and 70% α(1,6) glucosidic linkages (Monchois et al., 1999; Remaud-Simeon et al., 2000). Other glucansucrase excreting streptococci are *S. mutans* LM7, *S. downei* Mfe28, *S. sobrinus* 6715, *S. sobrinus* OMZ176, *S. alivariius* ATCC 25975 and *S. gordonii* (Monchois et al., 1999; Remaud-Simeon et al., 2000).

One other notable difference between glucansucrases from *Leuconostoc* and *Streptococcus* is that *Leuconostoc* glucansucrases are inducible (requirement for sucrose in the culture medium) while *Streptococcus* species constitutively express glucansucrase (Robyt, 1995). Nowadays there are also *Leuconostoc* mutants available that constitutively produce glucansucrases (Kim and Robyt, 1994).

Other enzymes

Besides the glucansucrases mentioned above, there is also a third type of sucrose glucosyltransferase, amylosucrase (EC 2.4.1.4). This enzyme, excreted by *Neisseria perflava* and *N. polysaccharea*, uses sucrose to produce a linear polymer with α(1,4) linked glucosyl residues (Potocki de Montalk et al., 1999; Remaud-Simeon et al., 2000).

The transfer of fructose from sucrose (transfructosylation) is observed using levansucrase (EC 2.4.1.10) resulting in the formation of β(2,6) linked fructan. Levansucrase is produced by many different bacteria and the interest in this enzyme comes from the fructosyl transfer to a variety of saccharides, resulting in hetero oligosaccharide formation (Perez Oseguera et al., 1996 and references cited herein; Van Geel-Schutten et al., 1999).

MECHANISM OF ACTION OF GLUCANSUCRASES

Glucansucrases are able to transfer the glucosyl moiety of the donor substrate to the acceptor in a transglucosylation type of reaction with retention of (α-)configuration. Sucrose is the only natural donor substrate for glucansucrases and the energy necessary to catalyse all the (transfer) reactions comes from the cleavage of the glucosidic bond of sucrose. With sucrose, glucansucrases can catalyse four different reactions: (1)

synthesis of glucan by repeated transfer of glucose molecules to a growing glucose polysaccharide; (2) hydrolysis of sucrose by transferring the glucose moiety to water; (3) synthesis of oligosaccharides by the transfer of glucose molecules to other saccharide acceptors; and (4) synthesis of sucrose by fructose exchange.

The precise mechanism of glucan chain elongation by glucansucrases is still not fully understood and probably remains unsolved until the 3-dimensional structure is elucidated (Monchois et al., 1999). Interestingly, two mechanisms of glucan chain elongation have been proposed. The first involves elongation at the non-reducing end of the glucan chain (Kobayashi et al., 1986; Mooser et al., 1985), while the second assumes elongation to occur at the reducing of the glucan chain (Ebert and Schenk, 1968; Robyt et al., 1974). In glucan synthesis two steps can be distinguished, initiation and elongation of the glucan chain.

Initiation of glucan synthesis

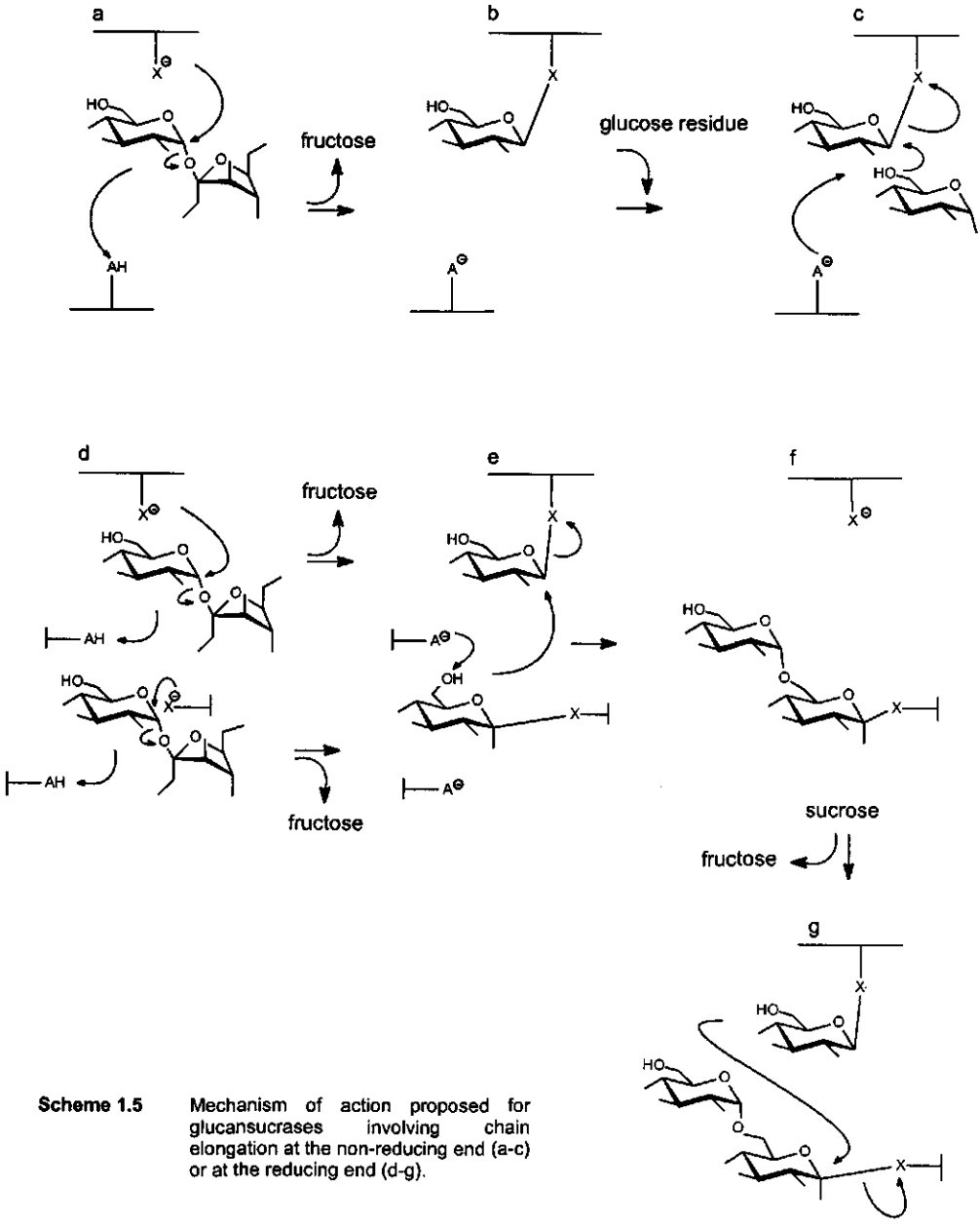
In the early years, the requirement of a primer to initiate the glucose polymerisation has often been discussed (Hehre, 1951; Koepsell et al., 1953; Germaine et al., 1974). This primer necessity was accepted in relation to studies on the mechanism of glycogen and starch synthesis mechanism carried out in this period. However, two observations illustrated the problem concerning the hypothesis of a primer-dependent glucan synthesis mechanism. The first is that glucansucrases are active in the absence of any exogenous primer (Kobayashi and Matsuda, 1980; Kobayashi and Matsuda, 1986; Robyt, 1995). Secondly, a dextran modified by a blocking group on the C-6 position of the glucosyl residue at the non-reducing, increased the rate of glucan synthesis equally as well as unmodified glucan (Robyt and Corrigan, 1977). This increase was explained by allosteric activation or the formation of α(1,3) branched dextran (Robyt and Taniguchi, 1976; Robyt, 1995).

In general, for synthesis of a polysaccharide to occur by a primer-dependent mechanism, the non-reducing ends of the primer are required for the addition of new monomer units (Kobayashi et al., 1986; Mooser et al., 1985). In order to explain chain elongation without the requirement of a primer (Robyt and Corrigan, 1977; Robyt, 1995), the two-site insertion mechanism with elongation at the reducing end was proposed (Ebert and Schenk, 1968; Robyt et al., 1974).

Elongation at the reducing end

The two-site insertion mechanism (Scheme 1.5 d-g) suggests that two identical nucleophilic sites are involved (Su and Robyt, 1994). At first two covalent glucosyl-enzyme complexes are formed in a process, which might be identical to the mechanism of glycosidases (Monchois et al., 1999). Subsequently the glucan chain is elongated at the reducing end by the nucleophilic attack of a C6 hydroxyl group on the C1 of the other glucosyl residue (implying a great flexibility in the glucosyl-enzyme complex) and a new sucrose molecule may bind to the nucleophilic site. During synthesis, the growing glucan chain is transferred from one site to the other in a continuous process. The two-site insertion mechanism may also explain the synthesis branched α(1,3)

glucans (Robyt and Taniguchi, 1976; Robyt, 1995). A C3 hydroxylgroup of an interior glucose residue on an acceptor glucan makes a nucleophilic attack at C1 of either the glucosyl-enzyme complex or the C1 of the glucanosyl-enzyme complex, thereby forming an α(1,3) linkage. The branching mechanisms of more complex and highly branched linkages other than α(1,3), such as α(1,4) and α(1,2) have not been studied (Kim and Robyt, 1995; Monchois et al., 1999).



Scheme 1.5 Mechanism of action proposed for glucansucrases involving chain elongation at the non-reducing end (a-c) or at the reducing end (d-g).

Elongation at the non-reducing end

The continuous process of chain elongation according to the two-site insertion mechanism is in clear contrast with the chain elongation at the non-reducing end (Mooser et al., 1985; Kobayashi et al., 1986). According to this mechanism the active site is composed of a sucrose and a (primer) glucan binding site. The C1 of a (covalent) glucosyl-enzyme complex is attacked by the C6 hydroxyl group of the non-reducing end of the growing glucan (or primer) chain (Scheme 1.5 a-c). For synthesis to continue, the elongated glucan chain must dissociate from the binding site, consequently the glucan synthesis proceeds in a discontinuous process.

Speculations about the nature of the primer (which is required in this mechanism) stated that sucrose itself was acting as a primer. However, no real evidence for this explanation is presented and the first oligosaccharide product of a sucrose primed reaction, theanderose, even inhibited glucan synthesis (Robyt, 1995).

Up to now, only one site capable of making a covalent bond with the glucose residue has been identified and sequenced, an active-site peptide containing an aspartic acid (Mooser et al., 1991).

ACCEPTOR REACTIONS USING GLUCANSUCRASES

Besides the synthesis of glucan, glucansucrases are also capable of transferring glucose from sucrose to other carbohydrates (acceptors) (Robyt, 1995). When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor products is produced. Acceptors may be classified as strong acceptors (e.g. maltose) or weak acceptors (e.g. fructose) according to their ability to compete with glucan synthesis (Monchois et al., 1999). Acceptors may also be divided in those that give homologous series of oligosaccharides and those that form just a single acceptor product containing one glucose residue more than the acceptor (Robyt, 1995). Besides saccharides, only the glucosylation of catechin by glucansucrase has been reported (Table 1.1).

With respect to the two-site insertion mechanism it is proposed that the acceptor molecule is bound at a separate acceptor binding site and not at the sucrose binding site (Tanriseven and Robyt, 1992; Su and Robyt, 1994). From a mechanistic point of view it is proposed that the C6 hydroxyl group at the non-reducing end of the acceptor attacks the C1 of the covalently bound glucosyl-enzyme complex (Robyt and Walseth, 1978). Therefore the mechanism of action of the acceptor is considered to be one of terminating glucan synthesis (Robyt and Walseth, 1978; Robyt, 1995) rather than one of priming glucan synthesis (Koepsell et al., 1953).

In contrast with the proposed separate acceptor binding site is the site-directed mutagenesis work by Monchois et al. (1997). A mutation of Asp-551 of the *L. mesenteroides* NRRL B-512F *dsr*S gene (which is homologous to the Asp residues identified by Mooser et al., 1991 and Kato et al., 1992) completely inhibited the synthesis of both glucan and oligosaccharides. Suggesting that one of the sucrose binding sites could also be an acceptor binding site (Monchois et al., 1999a).

STRUCTURAL ORGANIZATION OF GLUCANSUCRASES

Nowadays detailed information about the molecular basis of the genes coding for glucansucrases is becoming available (Monchois et al., 1999; Remaud-Simeon et al., 2000). Particularly *Streptococcus* glucosyltransferases have been studied, showing a close relationship between sequences of *Streptococcus* glucosyltransferases and *Leuconostoc* dextransucrases and a remarkable high degree of similarity based on secondary structure predictions (Monchois et al., 1999d).

Glucansucrases generally have a high molecular mass, around 160 kD, with the exception of dextransucrase-A of *L. mesenteroides* B1299 (Monchois et al., 1996). They are composed of four different structural domains. The N-terminal end begins with a short signal peptide of 35 to 38 amino acids with more than 50% identical or functionally equivalent residues (Remaud-Simeon et al., 2000). The signal peptide region is followed by a stretch of 140 to 260 amino acids which is highly variable. The variable region is followed by a highly conserved core region of about 900 amino acids including the catalytic domain, the N-terminal catalytic domain. The C-terminal region of the enzyme contains a glucan-binding domain covering about 400 amino acids (Ferretti et al., 1987; Abo et al., 1991; Giffard et al., 1993; Simpson et al., 1995).

The N-terminal catalytic domain

Sequence alignment and secondary structure prediction showed that the glucansucrase catalytic domain can be related to the α-amylase family, which contain a catalytic $(\beta/\alpha)_8$ barrel domain (MacGregor et al., 1996; Devulapalle et al., 1997). The glucansucrases are predicted to contain alternating β sheets and α helices, though the homologous elements appear to be circularly permuted with respect to those in amylases (MacGregor et al., 1996). Site directed mutagenesis experiments have allowed the location of catalytic amino acids in this region (Kato et al., 1992; Devulapalle et al., 1997; Monchois et al., 1997; Tsumori et al., 1997; Remaud-Simeon et al., 2000). Sequential N-terminal truncation experiments showed that key amino acids are also present in the first one-third of the core region (Monchois et al., 1999c). The secondary structure predictions performed concern the last two-thirds of the central core region.

The C-terminal glucan-binding domain

The C-terminal region is composed of a series of homologous direct repeats, designated A, B, C, and D repeats. Note that recently the presence of repeat motifs has also been observed in the N-terminal region (Janeček et al., 2000). The number of required units and the distribution of these repeats seems to be specific for each enzyme (Ferretti et al., 1987; Abo et al., 1991; Giffard et al., 1991). No real correlation between these repeats and enzyme function has been found (Ferretti et al., 1987; Monchois et al., 1999a). However, the synthesis of soluble glucan seems to be more sensitive towards deletions of the C-terminal domain compared to the synthesis of insoluble glucan (Lis et al., 1995; Monchois et al., 1998; Monchois et al., 1999a). Secondary structure predictions suggest that these repeat units might possess the structure of a functional

binding pocket (Eichel-Streiber et al., 1992).
The glucan-binding region is not directly involved in the catalytic process in glucan or oligosaccharide synthesis (Kato and Kuramitsu, 1990; Abo et al., 1991; Kato et al., 1992; Lis et al., 1995; Monchois et al., 1999b). It seems that its role may be to facilitate chain growth (of glucans and oligosaccharides) by making the release of products from the catalytic site easier (Monchois et al., 1998). Moreover, the presence of the C-terminal binding domain seems to be necessary to keep an active enzyme (Monchois et al., 1999a).

OUTLINE OF THIS THESIS

The aim of the research described in this thesis was the enzymatic synthesis of glucosides using nonsaccharides as acceptor molecules (aglycones). The initial focus was on the glucosylation of aromatic alcohols using non-Leloir glucosyltransferases, which are able to utilise (by transglucosylation) economically feasible glucosyl donor substrates. In the second part of the thesis the attention was shifted towards glucosidase type of enzymes with respect to hydrolysis and formation of thioglucosides.
In Chapter 2, the transglucosylation of the model aglycone (catechin, a flavonoid) is characterised using sucrose glucosyltransferases (glucansucrases) from *Streptococcus mutans*, generically named glucosyltransferases, and sucrose as glucosyl donor. Optimisation of reaction conditions is performed by direct consumption of inhibitory fructose by yeasts incapable of utilising sucrose. The acceptor specificity of *S. mutans* glucosyltransferase-D (GTF-D) and the use of organic solvents to engineer GTF-D transglucosylation activity is described in Chapter 3. As an outcome of this study bis-2-methoxyethyl ether is shown to affect the formation of high molecular weight GTF-D glucan and maltooligosaccharides. Also a change in the type of glucosidic linkage is observed upon bis-2-methoxyethyl ether addition (Chapter 4).
Chapters 5 and 6 focus on degradation and formation of thioglucosides using glycosylhydrolase type of enzymes. In Chapter 5 the isolation of the octylthioglucoside degrading *Sphingobacterium* sp. strain OTG1 is described. Chapter 6 finally, describes the use of almond β-glucosidase for the synthesis of thioglucosides. Under apparent reversed hydrolysis conditions the glucosylation of aliphatic thiols, in high yields is demonstrated.

2

Enhanced catechin transglucosylating activity of *Streptococcus mutans* glucosyltransferase-D due to fructose removal

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ABSTRACT

The catechin transglucosylating activities of several glucosyltransferases (GTFs) from the genus *Streptococcus* were compared. For this purpose, a mixture of 4 GTFs from *Streptococcus sobrinus* SL-1 and recombinant GTF-B and GTF-D from *Streptococcus mutans* GS-5 expressed in *Escherichia coli* were studied. It was shown that after removal of α-glucosidase activity, GTF-D transglucosylated catechin with the highest efficiency. A maximum yield (expressed as the ratio of moles of glucoside formed to moles of catechin initially added) of 90% was observed, using 10 mM catechin and 100 mM sucrose (K_m 13 mM) in 125 mM potassium phosphate, pH 6.0 at 37°C. ^1H and ^{13}C NMR spectroscopy revealed the structures of two catechin glucosides, respectively catechin-4'-*O*-α-D-glucopyranoside and catechin-4',7-*O*-α-di-D-glucopyranoside. Fructose accumulation during glucosyl transfer from sucrose to the acceptor competitively inhibited catechin transglucosylation (K_i 9.3 mM), whereas glucose did not inhibit catechin transglucosylation. The addition of yeasts was studied in order to minimise fructose inhibition by means of fructose removal. For this purpose, the yeasts *Pichia pastoris* and the mutant *Saccharomyces cerevisiae* T2-3D were selected because of their inabilities to utilise sucrose. Addition of *P. pastoris* or *S. cerevisiae* T2-3D to the standard reaction mixture resulted in a 2-fold increase in the duration of the maximum GTF-D transglucosylation rate. The addition of the yeasts also stimulated sucrose utilisation by GTF-D.

INTRODUCTION

Many compounds with interesting physiological or organoleptic properties occur in nature as glycosides. Therefore, methods of glycosylating compounds that would otherwise be too volatile or have a low solubility in aqueous systems are of interest to the pharmaceutical, cosmetics and food industries.

There are two distinct methods of enzymatic glycosylation, reversed hydrolysis and transglycosylation. Because of the hydrolytic activity of the glycosidases catalysing reversed hydrolysis, final glycoside yields are generally low. Use of high substrate concentrations or heterogeneous catalysis in organic solvents with low water activity may result in a reaction equilibrium shift towards reasonable glycoside synthesis (Vic et al., 1995). These complex reaction conditions can be avoided by using enzymes with transglycosylating activity and which cannot hydrolyse the glycosides formed.

Here we describe in detail the transglucosylating activity of glucosyltransferase-D (GTF-D) (EC 2.4.1.5) from *Streptococcus mutans* GS-5 towards the model acceptor compound catechin. Catechin is a polyphenol with a broad range of functions in medicinal and food applications (Kitao et al., 1993). Most studies on transglycosylation have focused on saccharides as acceptor molecules, resulting in the formation of oligosaccharides (Robyt, 1995). However, there have also been reports of various enzymes capable of transglycosylating (phenolic) alcohols: *Bacillus subtilis* X-23 α-amylase (EC 3.2.1.1) (Nishimura et al., 1994a/b; Nishimura et al., 1995), *Bacillus macerans* cyclodextrin glucanotransferase (EC 2.4.1.19) (Funayama et al., 1993) and *Leuconostoc mesenteroides* sucrose phosphorylase (EC 2.4.1.7) (Kitao and Sekine, 1992; Kitao et al., 1993; Kitao and Sekine, 1994a/b).

GTFs from the cariogenic streptococci are believed to play an important role in the formation of dental caries due to the production of glucans from dietary sucrose (Hamada and Slade, 1980; Loesche, 1986). The extracellular, constitutively produced GTFs are classified according to primer requirement to start glucan synthesis and to the type of glucan formed. The two main categories of glucans are the water insoluble glucans, predominantly containing α-1,3 linked glucose and the soluble glucans, rich in α-1,6 linked glucose (Robyt, 1995). Extracellular streptococcal fluids regularly contain more than one enzymatic activity, often occurring in high-molecular-weight aggregates. Genes encoding various GTFs have been isolated. *S. mutans* expresses four different genes, *gtf*A, *gtf*B, *gtf*C and *gtf*D (Robeson et al., 1983; Aoki et al., 1986; Hanada and Kuramitsu, 1988; Hanada and Kuramitsu, 1989). The latter gene encodes for GTF-D, which produces water soluble glucan in a primer stimulated manner (Hanada and Kuramitsu, 1989). Other *Streptococcus* species like *S. sobrinus* and *S. downei*, also contain multiple *gtf* genes encoding distinct GTFs (Yamashita et al., 1989; Giffard et al., 1993).

For GTFs closely related to *Leuconostoc mesenteroides* dextransucrase, synthesis of glucan is proposed to proceed according to a two-site insertion mechanism, resulting in addition of glucose to the reducing end of the glucan (Robyt and Walseth, 1978; Su and Robyt, 1994; Robyt, 1995). Leucrose (5-*O*-α-D-glucopyranosyl-D-fructose) and

glucose (arising from an acceptor reaction with water) are formed as minor products. Besides the transfer of glucose from sucrose to glucan, other carbohydrates such as monosaccharides (D-mannose, D-galactose), disaccharides (maltose, lactose) or oligosaccharides (maltotriose), can act as acceptors for transglucosylation by dextransucrase, resulting in a homologous series of oligosaccharide products (Robyt and Eklund 1983; Robyt, 1995).

In this study we compared the transglucosylating activities of several *S. mutans* and *S. sobrinus* GTFs towards catechin as a model acceptor. The highly efficient catechin transglucosylation by GTF-D and especially the influence of fructose on GTF-D transglucosylating activity are described in more detail.

MATERIALS AND METHODS

Growth conditions of *Streptococcus sobrinus* SL-1 and partial purification of GTF-T

Streptococcus sobrinus SL-1 was kindly provided by R.R.B. Russell (Department of Oral Biology, University of Newcastle) and stored in glycerol (45% v/v) at -80°C. A single colony of *S. sobrinus* SL-1 grown on a MRS plate at 37°C was used to inoculate 25 mL of MRS medium supplemented with cysteine (0.5 g/L) and was grown statically at 37°C. Overnight cultures (1 mL) were used to inoculate 250 mL of modified Terleckyj (Terleckyj et al., 1975) medium and were incubated statically at 37°C. After reaching the stationary phase at an optical density at 600 nm of 1.5 to 2.0, the culture supernatant was obtained by centrifugation (16,300 × *g*, 15 min, 4°C). The mixture of the 4 glucosyltransferases (GTF-T) was partially purified and concentrated by ammonium sulphate precipitation (Fukui, 1982). The supernatant was brought to 55% (326 g/L) saturation with ammonium sulphate. The precipitate was collected by centrifugation (39,100 × *g*, 15 min, 4°C), dissolved in potassium phosphate buffer (125 mM, pH 6.0) and dialysed against the same buffer.

Cultivation of *Escherichia coli* expressing the recombinant GTF-B and GTF-D and preparation of cell free extracts

Escherichia coli containing *Streptococcus mutans* GS-5 *gtf*B (pYNB13) (Tsumori et al., 1997) and *E. coli* containing *S. mutans* GS-5 *gtf*D (pYND72) (Shimamura et al., 1994) were kindly provided by H.K. Kuramitsu (Department of Oral Biology, State University of New York at Buffalo). The *E. coli* strains were stored in glycerol (14% v/v) at -80°C. Both strains were grown aerobically at 30°C in TY medium (1% NaCl, 3.2% tryptone and 2% yeast extract) with 0.2 mM isopropyl β-D-thiogalactoside. pYNB13 was maintained in medium supplemented with 50 μg/mL ampicillin. pYND72 was maintained in medium supplemented with 68 μg/mL chloramphenicol. Overnight cultures were harvested by centrifugation (16,300 × *g*, 15 min, 4°C). The cells were washed with Tris-HCl buffer (20 mM, pH 7.5) and resuspended in the same buffer at a concentration of 0.2 ± 0.1 g of wet biomass/mL. After sonication (Sonifier 250, Branson: duty cycle 30% output control 3) 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mg of DNase were added to the lysate. After centrifugation (39,100 × *g*, 15 min,

4°C), the supernatant was dialysed against Tris-HCl buffer (20 mM, pH 7.5) and used as crude enzyme solution.

Partial purification of GTF-B

GTF-B produced with *E. coli* was partially purified by ammonium sulphate precipitation. The crude enzyme solution was brought to 10% (50 g/L) saturation with ammonium sulphate. The supernatant with GTF activity obtained after centrifugation (39,100 × *g*, 15 min, 4°C) was brought to 40% (169 g/L) saturation and allowed to stand for 30 min. After centrifugation (39,100 × *g*, 15 min, 4°C) the precipitate was dissolved in potassium phosphate buffer (125 mM, pH 6.0) and dialysed against the same buffer.

Partial purification of GTF-D

GTF-D was initially partially purified by applying the cell free extract on a DEAE Sepharose CL-6B (Pharmacia) column (2.5 × 30 cm), previously equilibrated with Tris-HCl buffer (20 mM, pH 7.5). Proteins were eluted using a linear gradient of 0 to 0.7 M NaCl in the same buffer. Fractions containing GTF activity were concentrated by ultrafiltration (YM30, Amicon). The concentrated solution was dialysed against potassium phosphate buffer (125 mM, pH 6.0). Under the same experimental conditions GTF-D was also be purified using a DE-52 cellulose anion exchange column (Whatman).

α-Glucosidase activity

Enzyme solutions were incubated with *p*-nitrophenyl α-D-glucopyranoside (10 mM) and potassium phosphate buffer (125 mM, pH 6.0) in a volume of 1 mL. α-Glucosidase activity present in the crude enzyme solution was measured by monitoring the increase of *p*-nitrophenolate at 405 nm at 30°C. The molar extinction coefficient for *p*-nitrophenolate at pH 6.0 was 2300 $M^{-1} \times cm^{-1}$.

Glucosyltransferase transglucosylating activity

GTF transglucosylating activity was quantified by two different methods, measurement of the formation of reducing sugars (RS activity) and of catechin glucosides (CG activity).

RS activity was measured by the dinitrosalicylic acid (DNS) method (Miller, 1959) or by high performance liquid chromatography (HPLC-RI analysis). With the DNS method, samples of the reaction mixture were centrifuged (13,000 × *g*, 5 min) and 100 µL of the supernatant was mixed with 100 µL of DNS solution. The mixture was heated at 100°C for 5 min. After cooling, 1 mL of water was added and the absorption at 575 nm was measured spectrophotometrically. Glucose was used as a standard. Quantification of the amount of reducing sugars by HPLC-RI analysis was achieved using a Rezex RCM-Monosaccharide column (300 × 7.8 mm) (Phenomenex) with refractive index (RI) detection. Ultrapure water was used as mobile phase at a constant flow rate of 1 mL/min at 65°C.

One unit of RS activity was defined as the amount of enzyme which caused the release of 1 μmol of reducing sugar (expressed as glucose equivalent) per minute in 125 mM potassium phosphate buffer pH 6.0, with 60 mM sucrose at 37°C.

CG activity was measured using HPLC-UV. Catechin and catechin glucoside concentrations were measured on a C_{18} reversed phase column (200 × 3 mm) (ChromPack). As mobile phase a 30:70% (v/v) mixture of methanol and water (adjusted to pH 2.5 with ortho phosphoric acid) was used at a constant flow rate of 0.5 mL/min at 25°C. Catechin and catechin glucosides were detected spectrophotometrically at 260 nm.

One unit of CG activity was defined as the amount of enzyme which catalyses the formation of 1 μmol of catechin glucoside per minute in 125 mM potassium phosphate buffer pH 6.0, with 60 mM sucrose and 10 mM catechin at 37°C.

Catechin transglucosylation conditions

Catechin transglucosylation studies were performed with a reaction solution containing potassium phosphate buffer (125 mM, pH 6.0), sucrose, (+)-catechin (Sigma) and water. After addition of GTF the reaction mixture was incubated statically at 37°C.

Samples for HPLC analysis were prepared by mixing 50 μL of reaction aliquots with 450 mL of water (adjusted to pH 2 with HCl). After centrifugation (13,000 × g, 5 min), 400 μL of supernatant was used for analysis.

Separation of catechin transfer products

GTF-D catechin transfer products for structure elucidation were separated using a 2-step approach. First, 200 μL of reaction mixture was put on a silica column (10 × 1.5 cm; Silica gel 60 Merck, particle size 0.063 - 0.200 mm). Two fractions (A and B) were collected by eluting the silica column with 23 mL 50% ethanol:H_2O (v/v) and 18 mL 50% ethanol:H_2O (v/v) with 5 % acetic acid (v/v). Both fractions were concentrated by vacuum distillation and dissolved in 125 mM potassium phosphate, pH 6.0. Further purification of catechin transfer products was done by collecting the individual peaks using the HPLC-UV method described earlier.

NMR spectroscopic analysis

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a 200 MHz AC200 spectrometer at room temperature. All Nuclear Overhauser Effect (NOE)-difference and 2-dimensional NMR spectra measured were collected with a 400 MHz Bruker DPX 400 spectrometer.

CO_2 measurements

CO_2 concentrations were determined by analysing 100 μL gas phase samples on a Hewlett Packard HP 6890 gas chromatography system containing a Chrompack Poraplot Q column.

Growth conditions of *Saccharomyces cerevisiae* T2-3D and *Pichia pastoris*

The mutant yeast *Saccharomyces cerevisiae* T2-3D (Wenzel et al., 1992) was kindly provided by J.T. Pronk (Department of Microbiology, University of Delft). *S. cerevisiae* T2-3D was stored in glycerol (20% w/v) at -80°C. *Pichia pastoris* (CBS 704) was stored in glycerol (50% v/v) at -80°C. Both yeasts were grown aerobically in 1 liter of mineral salts medium with glucose (2% w/v) and yeast extract (0.2% w/v, Difco). *P. pastoris* and *S. cerevisiae* were harvested by centrifugation (39,100 × *g*, 15 min, 4°C). After washing the cells, *P. pastoris* and *S. cerevisiae* were stored (-20°C) at concentrations of respectively 66 and 70 mg (dry weight) mL^{-1}.

RESULTS

Partial purification of GTFs

The culture supernatant from *Streptococcus sobrinus* SL-1 was harvested during the stationary phase. The initial RS activity of the mixture of 4 glucosyltransferases (GTF-T), 0.16 U/mL (DNS method), which was comparable with other reported activities for *S. mutans* strains (Furuta et al., 1982; Furutani et al., 1988), was purified 13-fold by ammonium sulphate precipitation with 83% recovery (0.86 U/mg). After dialysis no α-glucosidase activity was detected. This GTF-T enzyme preparation was used in the initial experiments as shown in Table 2.1.

GTF-B and GTF-D, both expressed in *E. coli* containing the respective *S. mutans* GS-5 *gtf*B and *gtf*D genes (Shimamura et al., 1994; Tsumori et al., 1997), were partially purified with the objective of removing α-glucosidase activity present in the cell free extract. When the cell free extract containing GTF-B was applied to a DEAE Sepharose CL-6B column, GTF, assayed as RS activity (DNS method), could not be recovered using a NaCl gradient (Ma et al., 1996; Robyt, 1995). Subsequently, GTF-B was partially purified by ammonium sulphate precipitation to remove the α-glucosidase activity. The RS activity of GTF-B was purified 2-fold with 39% recovery. This partially purified enzyme preparation (5.4 U/mL and 0.5 U/mg RS activity, DNS method) was used in the initial experiments (Table 2.1).

Using cell free extract containing GTF-D, it was possible to elute RS activity (DNS method) from the DEAE Sepharose CL-6B column. The pooled fractions devoid of α-glucosidase activity were used in the initial experiments to compare the various GTF activities (Table 2.1). GTF-D was purified with 44% recovery, while the specific activity increased 9-fold (9.1 U/mg). The RS active fractions (DNS method) did not elute as a sharp peak but like a smear (0.05 M to 0.4 M NaCl). This could be overcome by using a DE-52 cellulose anion exchange column (Hanada and Kuramitsu, 1989). In this way GTF-D was purified 8-fold with 55% recovery (7.4 U/mL and 7.2 U/mg RS activity, HPLC-RI method). This GTF-D preparation was used to perform the kinetics experiments and to identify catechin transfer products.

Catechin transglucosylation with different GTFs

The partially purified GTF preparations (0.3 U/mL) were compared for their transglucosylating activities towards 5 mM catechin as model acceptor. Both the RS activity (expressed as reducing sugars released in the presence of catechin, DNS method) and the CG activity (expressed as glucose incorporated into catechin, HPLC-UV method) were determined (Table 2.1).

With all enzyme preparations, the addition of catechin resulted in approximately a 2-fold increase of the RS activity. A comparison between the CG and RS activities revealed that GTF-D had the highest efficiency for catechin transfer product formation at 5 mM catechin. Therefore catechin transglucosylation was further studied using this enzyme.

Table 2.1 Comparison between transglucosylation activity of *S. sobrinus* GTF-T, *S. mutans* GTF-B and *S. mutans* GTF-D.

enzyme	RS activity		CG activity
	- catechin (U/mL)[1]	+ catechin (U/mL)[1,2]	(U/mL)[2]
GTF-T	0.3	0.62	0.004
GTF-B	0.3	0.66	0.012
GTF-D	0.3	0.60	0.035

1 Reaction mixtures containing 125 mM potassium phosphate buffer (pH 6.0), 60 mM sucrose and enzyme solution (0.3 U/mL). The reaction mixtures were incubated for 30 min at 37°C. Analysis using DNS method.

2 Addition of 5 mM catechin.

Characteristics of GTF-D transfer products

HPLC analysis showed that during the incubation of GTF-D with catechin and sucrose, three distinguishable peaks, hereafter labelled peak 1 (t_r 7.63), 2 (t_r 7.85) and 3 (t_r 8.37) in order of increasing HPLC retention times (t_r), were detected (Figure 2.1A).

While the total area of peaks 1, 2 and 3 increased, the catechin peak area decreased (peak 4, t_r 8.80, Figure 2.1A and B). Catechin polymerisation was not observed under these conditions. Incubation of the reaction solution with *Aspergillus niger* amyloglucosidase (Sigma) (EC 3.2.1.3) resulted in a decrease in the three product peaks and an increase in catechin (data not shown).

Comparison of the UV spectra between 200 and 400 nm of catechin and of the catechin transfer products revealed no significant differences. Besides absorption maxima at 220 nm and 277 nm, an absorption minimum at 251 nm and a shoulder at 231 were detected. Therefore, the molar extinction coefficients of catechin and the catechin transfer products were assumed to be the same.

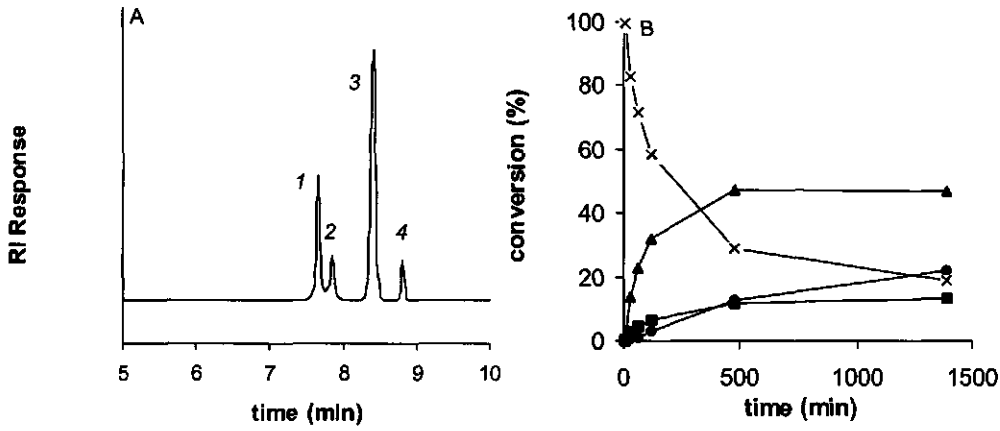


Figure 2.1 Formation of catechin transfer products by *S. mutans* GTF-D. A: HPLC chromatogram, showing the three catechin transfer products (*1*, *2* and *3*) and catechin (*4*) after 24 hours of incubation using 10 mM of catechin, 60 mM sucrose and 37 mU/ml GTF-D at 37°C. B: The formation of the three catechin transfer products (*1* (●), *2* (■), *3* (▲)) and catechin (×), using 10 mM of catechin, 60 mM sucrose and 37 mU/ml GTF-D at 37°C.

Identification of catechin transfer products

The exact identity of the GTF-D catechin transfer products was established by a comprehensive NMR spectroscopic study. To obtain the purified catechin transfer products, the mixture of GTF-D catechin transfer products was separated in 2 steps as described in the materials and methods section. After the first step (silica column chromatography) 2 fractions, labelled as A and B, were collected. Fraction A contained peak 1 and 3, hereafter labelled as products *1* and *3*. Fraction B contained peak 2, hereafter labelled as product *2* and catechin. After the purification we obtained 6.7, 3.2 and 16.1 μmol of products *1*, *2* and *3* respectively, from the initial 250 μmol of catechin (25 mL reaction volume containing 60 mM sucrose). Product *2* was not chemically stable upon prolonged storage, so no further attempts were made to elucidate its structure.

The spectroscopic structure elucidation of products *1* and *3* started with the assignment of catechin protons and carbon atoms (Scheme 2.2, Table 2.2). This assignment was based on COLOC measurements in dimethyl sulfoxide-d6 (DMSO) in combination with ^{1}H and ^{13}C (broad-band proton-decoupled and DEPT) NMR. The assignment of catechin is in line with a recent analysis (Davis et al., 1996) in which the assignment was based on HMQC, HMBC and NOE-difference spectroscopy.

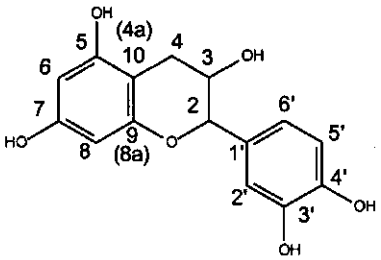
After the assignment of catechin, the spectra of products *1* and *3* were analysed. These spectra were obtained using a combination of ^{1}H, ^{13}C, H,C-correlated HETCOR + COLOC spectroscopy and NOE-difference spectroscopy. The ^{1}H-NMR spectra showed peaks in the range of 3.14-3.91 ppm, characteristic of sugar protons. Critical in the assignment of products *1* and *3* was the analysis of H-1'' (proton at the anomeric sugar carbon) and H-2 peaks (Table 2.2). Both proton peaks were assigned unambiguously

and were clearly separated from any other peaks in the spectrum. Based on the integral ratio of the peaks for H-1" and H-2 with respect to the peaks at 3.14-3.91 ppm, it was concluded that product *1* and *3* are sugar derivatives of catechin. For product *3*, the integral ratio of H-1" to H-2 to the 3.14 to 3.91 ppm peaks was 1.00:0.80:10.91, which is indicative for a mono-glucosylated catechin. For product *1*, this ratio was 2.00:0.92:21.90, which is indicative for a diglucose substituted catechin.
Based on the coupling constant between the glucose H-1'' and H-2'' (J=3.6 Hz, data not shown) it was concluded that both products *1* and *3* were α-glucosylated catechin molecules. In the case of a β-glucosylated catechin molecule, the coupling constant between H-1'' and H-2'' should be about 7 Hz.

Table 2.2 Assigned NMR data for catechin and product *1* and *3*.

Catechin:	Product *1*:		Product *3*:	
^{1}H NMR spectrum:				
δ ppm: 2.45 (dd, 1H, H-4a)	2.40 (H-4a)		2.35 (H-4a)	[- 0.10]
2.75 (dd, 1H, H-4b)	2.70 (H-4b)		2.70 (H-4b)	
3.92 (m, 1H, H-3)	3.89 (H-3)	[- 0.03]	3.85 (H-3)	[- 0.07]
4.58 (d, 1H, H-2)	4.60 (H-2)	[+0.02]	4.55 (H-2)	[- 0.03]
4.95 (d, 1H, OH)				
5.79 (d, 1H, H-8)	6.05 (H-8)	[+ 0.26]	5.70 (H-8)	[- 0.09]
5.98 (d, 1H, H-6)	6.16 (H-6)	[+ 0.18]	5.90 (H-6)	[- 0.08]
6.69 (dd, 1H, H-6')	6.73 (H-6')	[+ 0.04]	6.72 (H-6')	[+ 0.03]
6.78 (d, 1H, H-5')	7.15 (H-5')	[+ 0.37]	7.14 (H-5')	[+ 0.36]
6.82 (d, 1H, H-2')	6.80 (H-2')	[- 0.02]	6.79 (H-2')	[- 0.03]
	5.18, 5.22 (H-1" gluc)		5.17 (H-1" gluc)	
^{13}C NMR spectrum:				
δ ppm: 27.87 C-4	27.75		27.86	
	60.62, 60.75		60.77	
66.37 C-3	66.02		66.30	
	69.87-73.73 (8x)		70.01-73.75 (4x)	
81.04 C-2	80.77		80.70	
93.93 C-8	95.50*, 96.72*	[+1.57]	93.87	
95.19 C-6	97.97*	[+2.78]	95.26	
99.13 C-10(4a)	100.30*, 102.07*		99.03*, 100.28*	
114.57 C-2'	114.75		114.83	
115.16 C-5'	117.31	[+2.15]	117.25	[+2.09]
118.50 C-6'	118.21		118.30	
130.67 C-1'	134.51	[+3.84]	134.70	[+4.03]
144.89 C-3'	144.72		144.71	
144.89 C-4'	147.04	[+2.15]	147.01	[+2.12]
155.42 C-9(8a)	155.11*		155.24*	
156.23 C-5	156.14*		156.25*	
156.51 C-7	156.57*		156.54*	

δ Values versus tetramethyl silane in parts per million. Values in straight parentheses the differences (in ppm) with catechin.
* Assignments may be interchangeable.



Scheme 2.2 Structure and atom numbering of catechin.

The position of the glucose substituent of product *3* was assigned by comparison of the ^{1}H and ^{13}C NMR data (Table 2.2) with literature data. Our data were in line with data obtained (in CD_3OD) for substitution at the 4'-position (Foo and Karchesy, 1989), as the δ-value for H-5' has shifted 0.36 ppm downfield with respect to its position in catechin. This was at variance with data obtained for substitution at the 3'-position (Funayama et al., 1993). Subsequently, NOE-difference ^{1}H spectroscopy, which showed no cross-coupling on irradiation at H-2 and H-8, was performed. This indicated that no substitution at the 3- or 7-position had taken place. Clear cross-coupling with the H-1" peak (5.17 ppm) upon irradiation of H-5' was observed. This unambiguously supported the assignment for substitution at 4'. Therefore product *3* was identified as catechin-4'-α-*O*-glucoside.

Based on the assignment of the mono-glucosylated product *3*, the spectra of the diglucosylated product *1* were interpreted. The H-5' signal of product *1* has a very similar downfield shift, as was observed for this proton in product *3* (Table 2.2), indicative of the substitution of one glucose molecule at the 4'-position of catechin. Observation of the substantial difference of the ^{1}H signals for H-6 and H-8 suggests that substitution of the second glucose molecule has taken place at the 5- or 7-position, rather than e.g. at the 3- or 3'-position, or that a second substitution on the glucose at the 4'-position has occured. In combination with literature data for substitution at the 5-position (Cui et al., 1992) or 7-position (Foo and Karchesy, 1989), it became clear that simple 1-dimensional ^{1}H or ^{13}C NMR spectroscopy could not answer this question. However, with NOE-difference ^{1}H spectroscopy the structure of product *1* could be safely assigned. Irradiation at H-8 gave a cross-peak with the H-1" of one glucose molecule, while irradiation at H-5' gave cross-coupling with the H-1" of the second glucose. Consequently, product *1* was identified as catechin-4',7-*O*-α-di-D-glucoside.

Hydrolysis of catechin glucosides by amyloglucosidase

The identification of product *3* as catechin-4'-*O*-α-glucoside and product *1* as catechin-4',7-*O*-α-di-D-glucoside made it tempting to assume that product *2* was catechin-7-*O*-α-glucoside. Hydrolysis of the identified catechin glucosides by *Aspergillus niger* amyloglucosidase was used in an attempt to confirm this assumption.

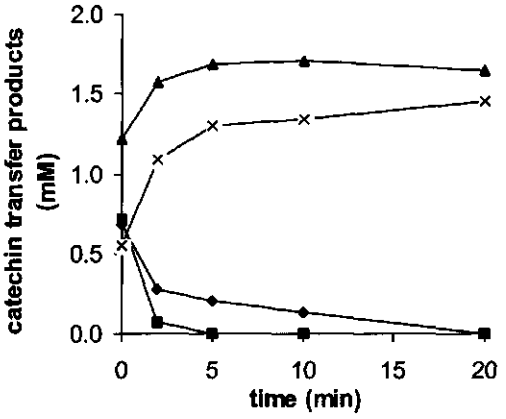


Figure 2.3 Hydrolysis of the three catechin transfer products (*1* (◆), *2* (■), *3* (▲)) and the formation of catechin (×), using 140 U/ml *Aspergillus niger* amyloglucosidase at 55°C.

Incubation of product *1* with amyloglucosidase resulted in hydrolysis of product *1* and simultaneous accumulation of product *3*, while no accumulation of product *2* was observed. Prolonged incubation with *A. niger* amyloglucosidase resulted in complete hydrolysis of product *3* with quantitative recovery of catechin (data not shown). Based on this experiment, the identity of product *2* as catechin-7-*O*-α-glucoside could not be confirmed. More insight was gained hydrolysing a mixture of the three different catechin glucosides by *A. niger* amyloglucosidase (Figure 2.3). Here the direct formation of catechin due to hydrolysis of product *2* and the formation of product *3* due to hydrolysis of product *1* can be observed.

Kinetics of GTF-D catechin transglucosylation

Catechin transglucosylation assays were performed using 125 mM potassium phosphate buffer at pH 6.0. It appeared that under these reaction conditions the reaction rate was more or less constant during the first 60 min (Figure 2.1B). Subsequently initial CG activities were determined based on the amount of catechin glucoside formed after 60 min of incubation. Variations in the potassium phosphate concentration (25-250 mM) and pH (5.0-7.5) had very little effect on the initial CG activity (using 60 mM sucrose and 10 mM catechin).

The effect of the catechin concentration on initial catechin transglucosylation rates was studied at catechin concentrations varying between 1 and 20 mM with 60 mM sucrose. The reaction appeared to be first order with respect to catechin concentrations below 15 mM (60 mU/mL, CG activity). The yield of the catechin transglucosylation reaction (expressed as the ratio of moles catechin glucoside formed after 24 h compared to the amount of catechin initially added) varied from 35% at 1 mM initial catechin to 75% at an initial catechin concentration of 15 mM. Due to the low water solubility of catechin (25 mM) the K_m could not be determined.

Using an initial concentration of 10 mM catechin, the effect of the initial sucrose

concentration on the glucoside yield was studied. The yield was 55% at a 10 mM initial sucrose concentration and increased to 90% at sucrose concentrations above 100 mM. The K_m for sucrose was estimated to be 13 mM (24 mU/mL, CG activity) by plotting the experimental data in a Lineweaver-Burk plot.

Fructose inhibition of GTF-D activity

The total catechin glucoside formation rate, as shown in Figure 2.1B, gradually decreased as the reaction proceeded. The decrease in catechin concentration during the reaction was simulated, assuming first order kinetics with respect to the catechin concentration and zero-order kinetics with respect to the sucrose concentration. Comparison of the experimentally determined data (Figure 2.1B) with the simulated curve indicated (data not shown) that the decrease in reaction rate was stronger than would be expected based solely on the decrease in catechin concentration. In other studies with glucosyltransferases, the accumulation of fructose was shown to competitively inhibit GTF enzyme activity (Devulapalle and Mooser, 1994). We therefore studied the inhibitory effect of fructose on catechin transglucosylation by GTF-D.

Reaction mixtures containing 37 mU/mL GTF-D, 10-150 mM sucrose and 0-200 mM fructose indicated the competitive inhibition of catechin transglucosylation by fructose. Using Lineweaver-Burk plots (data not shown), the K_i for fructose was calculated to be about 9.3 mM ± 1.0 mM. Under the same experimental conditions used to determine fructose inhibition, glucose (10-150 mM) showed no significant inhibition of catechin transglucosylation.

Monosaccharide consumption by *Pichia pastoris* and *Saccharomyces cerevisiae* T2-3D

To overcome the inhibitory influence of fructose accumulation on catechin transglucosylation, we studied the possibility of using yeasts to remove fructose. Two yeasts, the methylotrophic yeast *Pichia pastoris* and a mutant *Saccharomyces cerevisiae* T2-3D, were selected. Both strains are incapable of fermenting sucrose. This was confirmed by measuring saccharide dependent carbon dioxide formation. No additional formation of CO_2 was observed after addition of sucrose in contrast to addition of either glucose or fructose. Furthermore, the sucrose concentration, monitored by HPLC-RI, did not change during incubation of *P. pastoris* or *S. cerevisiae* with sucrose.

The potential toxic and/or inhibitory influence of the polyphenol catechin on *P. pastoris* and *S. cerevisiae* was also examined. Both yeasts were incubated for 60 min with different concentrations of catechin (1-10 mM). The maximal CO_2 formation rate was determined after addition of a pulse of fructose (20 mM). With *P. pastoris* there appeared to be no effect on fructose fermentation, whereas *S. cerevisiae* appeared to be slightly inhibited by higher catechin concentrations. Based on these experiments *P. pastoris* appeared to be the most suitable strain for the selective removal of fructose during catechin transglucosylation. However, both yeasts were tested.

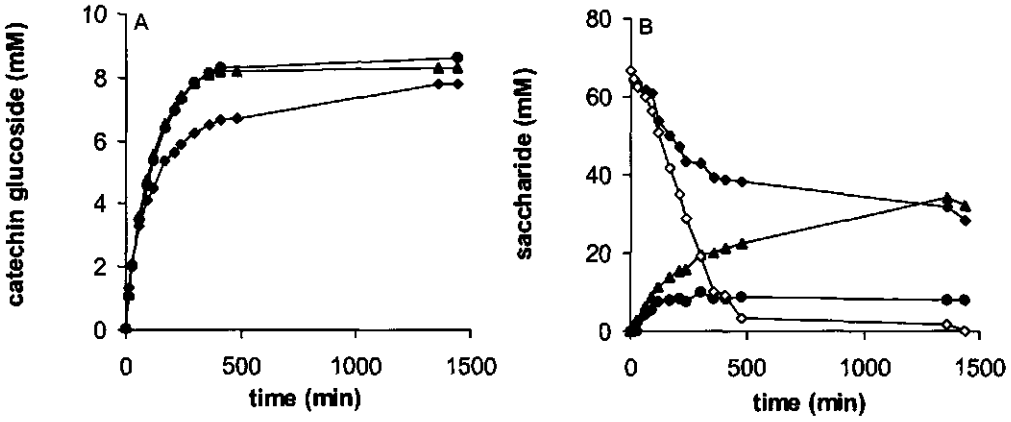


Figure 2.4 A: Catechin glucoside formation by *S. mutans* GTF-D in the absence (◆) or presence of *Pichia pastoris* (●, 66 mg dry weight/ml) or *Saccharomyces cerevisiae* T2-3D (▲, 70 mg dry weight/ml). B: Saccharide concentrations (◆sucrose, ▲fructose, ●glucose) in the absence (closed symbol) and in the presence (open symbol) of *P. pastoris*. The saccharide profile with *S. cerevisiae* T2-3D was very similar to the saccharide profile with *P. pastoris*. The reaction solutions (5 ml) contained 125 mM potassium phosphate buffer pH 6.0, 60 mM sucrose, 10 mM catechin and 37 mU/ml GTF-D.

During a 24 h incubation period in the absence or presence of yeast cells (*P. pastoris* or *S. cerevisiae*) both catechin glucoside and saccharide concentrations (fructose, glucose and sucrose) were monitored (Figure 2.4A and B). In the presence of *P. pastoris* or *S. cerevisiae* no accumulation of significant amounts glucose or fructose was observed. Incubation without added yeasts showed accumulation of 29 mM fructose and 8 mM glucose after 24 hours of incubation. Fructose and glucose consumption by *P. pastoris* (Figure 2.4A and B) or *S. cerevisiae* resulted in a prolongation of the maximum CG activity and an increase in the final catechin glucoside yield.
Interestingly, monosaccharide removal also affected sucrose consumption by GTF-D. After 500 min the sucrose was almost completely consumed in the presence of the yeasts, whereas without yeast addition more than half of the sucrose was still present.

DISCUSSION

In this study we have compared the transglucosylating activity towards catechin of several glucosyltransferases (GTFs) from the genus *Streptococcus*. Transglucosylation characteristics of *Streptococcus mutans* GS-5 glucosyltransferase-D were studied in more detail. Although catechin has a complex structure, containing five hydroxyl groups, this compound was used as a model acceptor to allow comparison with literature data on other catechin transglucosylating enzymes (Funayama et al., 1993; Kitao et al., 1993; Nakahara et al., 1995).
The different GTF preparations tested were a mixture of the four GTFs from *Streptococcus sobrinus* SL-1 (GTF-T) and the heterologously produced GTF-B and

GTF-D from *S. mutans* GS-5. Note that the three GTF preparations were not purified to homogeneity.

All GTF preparations were capable of catechin transglucosylation (Table 2.1), although at different rates. The catechin glucosylation (CG) activity of GTF-D was almost 3 times higher than the CG activity of GTF-B and 8 times higher than the CG activity of GTF-T. Considering the ratio between the RS and CG activity of the three GTF preparations, we decided to study the transglucosylation of catechin in more detail using the GTF-D from *S. mutans* GS-5.

Besides catechin transglucosylating activity, all three GTF preparations showed an increase in the formation rate of reducing sugars in the presence of catechin. Especially in the case of GTF-T. This increase can hardly be explained by assuming that catechin is a better glucosyl acceptor than dextran, because almost no catechin glucoside formation was observed. An explanation could be that upon dextran chain elongation the glucosyl transfer rate decreases. If we assume that the presence of catechin results in the premature displacement of the growing dextran chain from the active site, catechin addition would result in an enhanced rate of (short chain) dextran formation and hence also in an increase in the reducing sugar formation.

Based on HPLC-analysis, the glucosylation of catechin resulted in the formation of (at least) 3 catechin transfer products. Two catechin transfer products were characterised in more detail. Although the catechin glucoside separation was not optimised, sufficient catechin glucoside was isolated to perform ^{1}H and ^{13}C NMR spectroscopy. The structures of two catechin transfer products were identified as catechin-4'-*O*-α-D-glucoside and catechin-4',7-*O*-α-di-D-glucoside. In literature the characterisation of a 4'-*O*-α (Nakahara et al., 1995) and 3'-*O*-α (Funayama et al., 1993; Kitao et al., 1993) glucosylated catechin have been described. The catechin diglucoside characterised in this study is to our knowledge the first catechin diglucoside described. The structure of the third catechin transfer product (labeled as product *2*) could not be spectroscopically elucidated. However, the deglucosylation study with *Aspergillus niger* amyloglucosidase suggests that this compound could be 7-*O*-α mono-glucosylated catechin. Note that the *A. niger* amyloglucosidase, preferentially deglucosylates the 7-*O*-α glucose of the diglucoside. The fast hydrolysis of product *1* into product *3* and the fast hydrolysis of product *2* into catechin are therefore indicative for glucosylation of C-7 rather than C-5, C-3 or C-3' in product *2*.

In comparison with literature data concerning catechin transglucosylation (Kitao et al., 1993; Nakahara et al., 1995), the catechin transglucosylation efficiency of GTF-D appears to be very high. Based on the data in Table 2.1, a minimal glucosyltransfer efficiency of 58% can be calculated assuming all the reducing sugars formed are fructose. In a number of studies the transfer ratio (or yield) has been used to quantify the transglucosylating potential of enzyme preparations (Kitao et al., 1993; Nakahara et al., 1995). The transfer ratio is expressed as the ratio between the moles of catechin glucoside formed and moles of catechin initially added. Depending on the sucrose concentration the transfer ratio for catechin glucosylation with GTF-D varied between 55 and 90%. Sucrose phosphorylase (Kitao et al., 1993) was reported to have a similar

transfer ratio, but a much higher sucrose concentration (30% w/v) was used. The mixture of GTFs from *S. sobrinus* (Nakahara et al., 1995) also transglucosylates catechin, but the reported transfer ratio is less than the transfer ratio of the partially purified GTF-D that we studied. The data reported for catechin transglucosylation with cyclodextrin glucanotransferase are difficult to compare because of the use of soluble starch as glucosyl donor (Funayama et al., 1993).

Detailed analysis of the catechin glucosylation kinetics made it clear that the decrease in the catechin concentration during the reaction could not solely explain the observed decrease in GTF-D activity. We showed that fructose, which can be regarded as a side product of the transglucosylation reaction, competitively inhibits GTF-D. The observed decrease in GTF-D activity as the reaction proceeds, is therefore most likely the result of a combination of the decrease in catechin concentration and fructose inhibition, although product inhibition by catechin glucosides cannot be ruled out.

To demonstrate and to minimise the influence of fructose on catechin transglucosylation, the effect of fructose removal from the reaction mixture was studied. To achieve this, two yeasts *Pichia pastoris* and *Saccharomyces cerevisiae* T2-3D, incapable of sucrose consumption, were used. Addition of *P. pastoris* or *S. cerevisiae* to the catechin transglucosylation mixture resulted in a 2-fold increase of the duration of the maximum GTF-D activity, an increase of catechin glucoside production (24%) and complete sucrose utilisation (Figure 2.4A and B). The decrease in catechin glucoside formation rate after 500 minutes of incubation was most likely due to sucrose depletion and the very low catechin concentrations. Interestingly, the removal of fructose and hence fructose inhibition by fermentation, also resulted in complete sucrose utilisation. As sucrose consumption by *P. pastoris* and *S. cerevisiae* was ruled out, the complete sucrose utilisation by GTF-D is due to the removal of fructose.

The transglucosylation efficiency based on sucrose utilisation by GTF-D can be very high. However, the use of the yeasts to enhance the GTF-D transglucosylating rate, resulted in a drastically decreased efficiency of sucrose utilisation. Therefore, depending on the optimisation objective, yeasts should be added or omitted. It would be interesting to study whether formation of glucan can also be stimulated by fructose removal as has been shown here for catechin transglucosylation.

3

Transglycosylation by *Streptococcus mutans* glucosyltransferase-D: acceptor specificity and engineering of reaction conditions

G.H. Meulenbeld
S. Hartmans

ABSTRACT

The acceptor specificity of *Streptococcus mutans* GS-5 glucosyltransferase-D (GTF-D) was studied, in particular towards nonsaccharide compounds (aglycones). Dihydroxy aromatic compounds like catechol, 4-methylcatechol and 3-methoxycatechol were glycosylated by GTF-D with a high efficiency. Transglycosylation yields were respectively 65, 50 and 75% using 40 mM of acceptor and 200 mM of sucrose as glucosyl donor. 3-Methylcatechol was also glycosylated though at a significantly lower rate. A number of other aromatic compounds such as phenol, 2-hydroxybenzaldehyde, 1,3-dihydroxybenzene and 1-phenyl-1,2-ethanediol were not glycosylated by GTF-D. Consequently GTF-D aromatic acceptors appear to require two adjacent aromatic hydroxyl groups. In order to facilitate the transglycosylation of less water soluble acceptors the use of various water miscible organic solvents (cosolvents) was studied. The flavonoid catechin was used as a model acceptor. Bis-2-methoxyethyl ether (MEE) was selected as an useful cosolvent. In the presence of 15% (v/v) MEE, the specific catechin transglucosylation activity was increased 4-fold due to a 12-fold increase in catechin solubility. MEE (10-30% v/v) could also be used to allow the transglycosylation of catechol, 4-methylcatechol and 3-methoxycatechol at concentrations (200 mM) otherwise inhibiting GTF-D transglycosylation activity.

INTRODUCTION

Glycosylation can be a useful method for improving the chemical properties of compounds with interesting physiological or organoleptic properties. The benefits of glycosylation may be an increased solubility or a decreased volatility in comparison with non glycosylated molecules.

Most of the research on enzymatic synthesis of glycosidic linkages has focused on the synthesis of oligosaccharides. For enzymatic oligosaccharide synthesis three strategies can be applied (Ichikawa et al., 1992). The first is the use of glycosyltransferases of the Leloir pathway, which require (expensive) sugar nucleotides as glycosyl donors. The second is the use of non-Leloir pathway enzymes, which e.g. require sugar-1-phosphate as glycosyl donor. The third is the use of the glycosidase type of enzymes, which in general use inexpensive mono- or disaccharides as glycosyl donors.

Glycosylation with glycosidases can be performed either by reversed hydrolysis or by transglycosylation. The hydrolytic activity of the glycosidases catalysing reversed hydrolysis glycosylation, usually results in low glycoside yields. However, glycoside yields can be increased by lowering the water activity, increasing the substrate concentrations or by extracting the glycosides from the reaction mixture. The disadvantages of these complicated reaction conditions using the reversed hydrolysis mechanism are obvious. Enzymatic transglycosylation using glucansucrases (EC 4.2.1.5) from *Streptococcus* species (glucosyltransferases) or *Leuconostoc mesenteroides* (dextransucrases) is more efficient. With these enzymes only sucrose can be used as an inexpensive glucosyl donor and relatively uncomplicated reaction conditions can be applied, because the glucansucrases do not (significantly) hydrolyse the glycosides formed.

Using sucrose as substrate, glucansucrases can catalyse four different reactions: (i) synthesis of glucan by repeated transfer of glucose molecules to a growing glucose polysaccharide, (ii) hydrolysis of sucrose by transferring the glucose moiety to water, (iii) synthesis of oligosaccharides by the transfer of glucose molecules to other saccharide acceptors, (iv) isotopic exchange by the reverse reaction of glucosyl-enzyme complex formation leading to the formation of sucrose and leucrose. The precise mechanism of glucansucrases is still not fully understood (Robyt, 1995; Monchois et al., 1999). In literature elongation of the glucan chains has been proposed to occur at the non-reducing end (Mooser et al., 1985; Kobayashi et al., 1986) or at the reducing end (Ebert and Schenk, 1968, Robyt et al., 1974).

Most studies on transglycosylation (with glucansucrases) have focused on saccharides as acceptor molecules, primarily resulting in the formation of oligosaccharides (e.g. Robyt, 1995). There are some exceptions. In some of these studies the flavonoid catechin was used as a model acceptor. The transfer of glucose molecules to catechin was described for a mixture of several glucosyltransferases from *Streptococcus sobrinus* (Nakahara et al., 1995) and for *S. mutans* GS-5 glucosyltransferase-D (GTF-D) (Meulenbeld et al., 1999). Catechin can also be transglucosylated by *Bacillus macerans* cyclodextrin glucanotransferase (EC 2.4.1.19) using starch as glucosyl donor

(Funayama et al., 1993). *Leuconostoc mesenteroides* sucrose phosphorylase (EC 2.4.1.7) is capable of transglucosylating catechin as well as transglucosylating various (substituted) hydroxyaromatic derivatives (Kitao and Sekine, 1993; Kitao and Sekine, 1994).

For *S. mutans* GS-5 GTF-D we previously showed that it forms three different catechin glucosides: catechin-4'-*O*-α-D-glucopyranoside, catechin-4',7-*O*-α-di-D-glucopyranoside and catechin-7-*O*-α-D-glucopyranoside (the latter one was not spectroscopically elucidated). This range of glucosylated catechins prompted us to study the acceptor specificity of GTF-D in more detail. Besides testing a range of hydroxylated aromatic acceptors, we have also studied the use of water miscible organic solvents (cosolvents) in transglycosylation reactions (Girard and Legoy, 1999). These cosolvents were used to engineer the GTF-D acceptor solubility and to improve the yield of glycosylated products.

MATERIALS AND METHODS

Glucosyltransferase-D

Escherichia coli containing the *Streptococcus mutans* GS-5 *gtf*D gene (pYND72) (Shimamura et al., 1994) was kindly provided by H.K. Kuramitsu (Department of Oral Biology, State University of New York at Buffalo). Glucosyltransferase-D (GTF-D) from *E. coli* was purified as described earlier (Chapter 2, Meulenbeld et al., 1999).

Quantification of GTF-D transglycosylation products

GTF-D transglycosylation products were quantified by three different methods: formation of reducing sugars, formation of glucan and the formation of glycosides.

Reducing sugars

The formation of reducing sugars (RS) was measured using the dinitrosalicylic (DNS) method (Miller, 1959) or by high performance liquid chromatography with refractive index detection (HPLC-RI). With the DNS method, samples of the reaction mixture were diluted 10-fold and centrifuged (13,000 × *g*, 5 min). A 100 μL amount of the supernatant was mixed with 100 μL of the DNS solution and heated at 100°C for 5 min. After cooling, 1 mL of water was added and the absorption at 575 nm was measured spectrophotometrically. Glucose was used as the standard. The DNS solution (100 mL) contained 1.6 g NaOH, 1.0 g 3,5-dinitrosalicylic acid, 30.0 g KNa-tartrate 0.2 g phenol and 50 mg of Na_2CO_2.

Quantification of the amount of reducing sugars by HPLC-RI analysis was performed using a Nucleogel SUGAR Pb column (300 × 7.7 mm) (Macherey-Nagel) with RI-detection. Ultrapure water was used as mobile phase at a constant flow rate of 0.4 mL/min at 75°C.

Samples for analysis by HPLC-RI were prepared by mixing 50 μL of reaction aliquots with 450 μL of water (adjusted to pH 2 with HCl). Before analysis the samples were centrifuged (13,000 × *g*, 5 min).

Glucan

Glucan was determined according to the method described by Dubois et al. (1956). A 1,0 mL amount of ethanol (75% v/v, -20°C) was added to 100 µL of reaction mixture and centrifuged (13,000 × *g*, 5 min). The precipitate was washed with ethanol (75% v/v, -20°C) and dissolved in NaOH (0.5 M). This glucan solution was diluted to a concentration of 0.1-2 mM glucose (final volume of 2.0 mL). Within 10 sec after the addition of 50 µL phenol (80% w/v) to the 2 mL diluted glucan solution, 5 mL of concentrated sulphuric acid was added. After mixing, this solution was allowed to stand for 10 min at room temperature followed by 15 min incubation at 37°C. The absorption at 490 nm was recorded spectrophotometrically. Glucose was used as a standard and the outcomes are expressed as mM glucose.

Acceptors and corresponding glycosides

The concentration of acceptors and the corresponding glycosides were measured using high performance liquid chromatography with spectrophotometric detection (HPLC-UV). Analysis were performed using a C_{18} reversed phase column (200 × 3 mm) (ChromPack). A 40:60% (v/v) mixture of methanol and water (adjusted to pH 2.5 with ortho-phosphoric acid) was used as mobile phase at a constant flow rate of 0.35 mL/min at 25°C. Catechin and catechin glucosides were analysed using the same column but with a 30:70% (v/v) mixture of the mobile phase mentioned above and a constant flow rate of 0.5 mL/min at 25°C. Acceptors and corresponding glycosides were routinely detected spectrophotometrically at 260 nm. Samples for analysis were prepared the same way as described for the HPLC-RI method.

GTF-D transglycosylation activity

GTF-D activity was quantified by two different methods: formation of reducing sugars (RS-DNS) and formation of catechin glucosides (HPLC-UV).

One unit (U) of RS activity is defined as the amount of enzyme that causes the release of 1 µmol of reducing sugars per minute (quantified using the DNS-method) in 125 mM potassium phosphate buffer (pH 6.0) with 60 mM sucrose at 37°C. One unit of catechin glucoside (CG) activity is defined as the amount of enzyme which catalyses the formation of 1 µmol of catechin glucoside (HPLC-UV method) per minute.

GTF-D transglycosylation conditions

A typical GTF-D transglycosylation reaction was carried out in a reaction solution containing potassium phosphate buffer (20 mM, pH 6.0), sucrose (200 mM) and water in a total volume of 1 mL. Depending on the type of experiment, acceptors and organic solvents were added. After addition of GTF-D and mixing, the reaction mixtures were incubated statically at 37°C.

RESULTS AND DISCUSSION

Acceptor specificity of *Streptococcus mutans* GS-5 glucosyltransferase-D

Previously we showed that *Streptococcus mutans* GS-5 glucosyltransferase-D (GTF-D) glucosylated the C-4' and C-7 hydroxyl groups of catechin (Scheme 2.2) (Meulenbeld et al., 1999), resulting in the formation of catechin-4'-*O*-α-D- and catechin-4',7-*O*-α-di-D-glucopyranoside and most likely catechin-7-*O*-α-D-glucopyranoside. Based on these results we anticipated that GTF-D could be used to glucosylate a whole range of hydroxy aromatic compounds.

To study the GTF-D acceptor specificity, selected hydroxy aromatic compounds were incubated at two concentrations (40 and 200 mM) together with GTF-D and sucrose. Initially only the formation of the corresponding glycosides was determined (Table 3.1). The data presented in Table 3.1 show that catechol and the substituted catechols 3-methylcatechol, 4-methylcatechol and 3-methoxycatechol are all transglucosylated by GTF-D. In each case only one product peak was observed, suggesting that only one hydroxyl group was glycosylated. Confirmation of the identity of the reaction products (glycosides) was obtained by the specific hydrolysis of these reaction products using the *Aspergillus niger* amyloglucosidase (EC 3.2.1.3). When amyloglucosidase (2 mg/mL, 60 min at 55°C) was used, the area (HPLC chromatograms) of the catechol glycoside peak decreased, while the area of the catechol peak increased correspondingly (data not shown). The same was observed with the substituted catechols. Comparison of the UV spectra between 200 and 400 nm of the acceptors and the corresponding glucosides, revealed no significant differences. Consequently the molar extinction coefficients of acceptors and glycosides were assumed to be the same.

The monohydroxy aromatic compounds in Table 3.1, as well as several other aromatic compounds like 4-methylphenol, 3-methylphenol and 4-hydroxyphenol (40 and 100 mM), 3,5-dihydroxybenzylalcohol (40 and 200 mM), 2-methoxy-4-methylphenol (40 and 100 mM) 2-methoxybenzylalcohol and 3-methoxybenzylalcohol (40 and 100 mM) were not glycosylated.

We have previously reported the glucosylation of the catechin C-7 hydroxyl group (Meulenbeld et al., 1999). Because of the similarity between 3-hydroxyphenol and the 1,3-dihydroxy structure of catechin at C-5 and C-7, we expected that 3-hydroxyphenol would also be glycosylated by GTF-D. Nevertheless, 3-hydroxyphenol was not glycosylated by GTF-D. This observation and the data from Table 3.1 therefore strongly indicate that GTF-D requires adjacent hydroxyl groups on the aromatic ring for glycosylation. Possibly one hydroxyl group is required to (covalently) interact with the enzyme to ensure a proper acceptor orientation in the active site necessary for the second hydroxyl group to react with the C1-atom of glucose. Molecules with only one hydroxyl group interacting with the enzyme would not have a second hydroxyl group available for glycosylation. The fact that 3-methylcatechol is more difficult to glucosylate compared to catechol, 4-methylcatechol and 3-methoxycatechol may indicate that GTF-D is inhibited at low concentrations of this compound or that the proper orientation in the active site necessary for glucosyltransfer is difficult to obtain.

Table 3.1 *S. mutans* GTF-D acceptor specificity.

	concentration[1] (mM)	glycoside (mM)	glucan (mM)
water	-	-	87
catechol	200	nd [2]	6
	40	26	100
3-methoxycatechol	200	32	3
	40	30	72
3-methylcatechol	200	nd	2
	25 [3]	3	44
4-methylcatechol	200	nd	1
	40	20	44
phenol	200	nd	< 1
	40	nd	71
3-hydroxyphenol	200	nd	< 1
	40	nd	67
benzylalcohol	200	nd	25
	40	nd	82
2-hydroxybenzylalcohol	200	nd	29
	40	nd	77
2-ethoxybenzylalcohol	200	nd	37
	40	nd	86
1-phenyl-1,2-ethanediol	200	nd	63
	40	nd	79

1 Reaction solution containing, 20 mM potassium phosphate (pH 6.0), 200 mM sucrose, 300 mU/mL GTF-D at 37°C. Samples were taken after 310 min of incubation.
2 Not detected.
3 Incubation (24 h) with 600 mU/mL GTF-D at 37°C.

Inhibition of GTF-D transglycosylation.

As mentioned above, all the potential acceptor molecules were initially incubated at two concentrations (Table 3.1). With catechol and 4-methylcatechol no glucoside formation was observed at an initial acceptor concentration of 200 mM. However, in both cases glycosylation was observed at an initial acceptor concentration of 40 mM. This suggests that catechol and 4-methylcatechol are inhibitory at concentrations of 200 mM. To verify whether GTF-D is inhibited (or inactivated) by high concentrations (200 mM) of catechol and substituted catechols, an alternative method to measure GTF-D activity was used.

In addition to the capacity to glucosylate non saccharide acceptor molecules, GTF-D forms glucan even in the presence of catechin (data not shown). The amount of glucan formed can be used as a measure of GTF-D activity. As can be seen in Table 3.1, the amount of glucan formed is strongly reduced in the presence of 200 mM catechol, substituted catechols and monohydroxy aromatic compounds. However, at acceptor

concentrations of 40 mM glucan formation similar to the levels in the absence of acceptor was observed with all the substrates tested, showing that GTF-D was active under these conditions. Interestingly the non-phenolic aromatic compounds with hydroxylated aliphatic side-chains were much less inhibitory than the compounds with aromatic hydroxyl groups.

From the data presented in Table 3.1 it was not possible to conclude what type of inhibition the phenolic compounds exerted on GTF-D. To discriminate between inhibition and inactivation, the GTF-D transglycosylation activity in the presence of various concentrations of phenol was studied in more detail (Table 3.2). After 310 min of incubation the amount of glucan formed was determined. This was followed by a 5-fold dilution of the reaction mixtures, resulting in a 5-fold decrease in the phenol concentration. 24 Hours after the start of the experiment, the amount of glucan (and reducing sugars, data not shown) was determined in the diluted incubation mixtures. Table 3.2 shows that 200 mM of phenol prevents the formation of glucan and that the amount of glucan formed is reduced in the presence of 100 mM of phenol. A 5-fold dilution of the incubation mixture with 200 mM of phenol does not result in the recovery of glucan forming activity, suggesting that GTF-D is irreversibly inactivated by 200 mM of phenol. At lower concentrations phenol also appears to inactivate GTF-D upon prolonged incubation.

Table 3.2 Effect of phenol on glucan formation by *S. mutans* GTF-D.

phenol (mM)	glucan (mM)	
	310 min[1]	1440 min[2]
0	88	74
40	81	50
100	48	28
200	0	0

1 Reaction solution containing 20 mM potassium phosphate (pH 6.0), 200 mM sucrose and 300 mU/mL GTF-D at 37°C.
2 Reaction solution diluted 5-fold in 200 mM sucrose and 20 mM potassium phosphate (pH 6.0).

The use of organic solvents in engineering GTF-D transglycosylation activity

In our previous study concerning the transglucosylation of catechin, high concentrations of catechin glucosides could not be obtained because of the limited water solubility of catechin. To increase the product concentration a higher initial substrate concentration would be required, or alternatively the acceptor might be added in portions. To increase the catechin solubility, water miscible solvents could be applied. We therefore studied the effect of water miscible organic solvents (cosolvents) on catechin transglucosylation. The choice of water miscible solvents (monophasic system) was further motivated by the fact that sugars are only soluble in hydrophylic and thus water miscible solvents (Dordick, 1989).

Choice of organic solvents

To determine the effect of the selected cosolvents on GTF-D transglycosylation activity, the formation of catechin glucosides, glucan and reducing sugars was determined in the presence of 30% (v/v) cosolvent (Table 3.3). Addition of the cosolvents methanol, ethanol, 1-propanol, 2-propanol, acetonitrile, acetone and N,N-dimethylformamide apparently inactivated GTF-D, because the formation of reducing sugars was not observed (data not shown). To select an appropriate cosolvent, the ratio between glucan and reducing sugars formation and the absolute amount of catechin glucosides formed were considered (Table 3.3). A reducing sugars/glucan ratio similar to the situation without cosolvent, suggests that the transfer of glucose to glucan was not influenced by addition of the cosolvent. This situation was observed with bis-2-methoxyethyl-ether.

Table 3.3 The influence of organic solvents on *S. mutans* GTF-D transglycosylating activity.

solvent	reducing sugars (mM)[1,2]	glucan (mM)[1]	ratio (-)[3]	catechin glucosides (mM)[4]
none	135	87	1.6	7.5
1,2-ethanediol	159	10	15.9	1.7
1,2-propanediol	94	17	5.5	2.6
1-methoxy-2-propanol	3	2	1.5	0.8
1,3-butanediol	82	15	5.5	1.5
1,4-butanediol	20	3	6.7	1.2
bis-2-hydroxyethyl ether	147	3	49.0	0.9
bis-2-methoxyethyl ether	127	79	1.6	2.2
bis-2-ethoxyethyl ether	11	-	-	-
dimethylsulfoxide	32	9	3.6	0.8

1 Reaction mixtures containing 20 mM potassium phosphate buffer (pH 6.0), 200 mM sucrose, solvent (30 % v/v) and 150 mU/mL GTF-D. Samples were taken after 24 h.
2 The concentration of reducing sugars determined using the DNS method (Miller, 1959).
3 Reducing sugars/glucan.
4 Reaction mixtures contained 20 mM potassium phosphate buffer (pH 6.0), sucrose (60 mM), solvent (15 % v/v), catechin (10 mM) and 150 mU/mL GTF-D. Catechin glucoside formation after 24 h of incubation was determined using HPLC-UV.

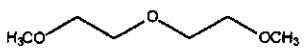
In the presence of bis-2-hydroxyethyl ether (diethylene glycol, DEG) the reducing sugars/glucan ratio was 30-fold higher than in the absence of the cosolvent. Almost no glucan could be detected while the concentration of reducing sugars formed was comparable to the control without solvent. This means that the glucose moiety is transferred to DEG or H_2O (sucrose hydrolysis). In the latter case glucose and fructose would be formed in equimolar amounts. Therefore the individual contribution of fructose and glucose to the total amount of reducing sugars was determined using HPLC-RI. GTF-D was incubated with various DEG concentrations (0-30% v/v) and

samples were taken and analysed as described (data not shown). The glucose concentration was not significantly higher in the presence of DEG. Consequently DEG induced sucrose hydrolysis was ruled out, suggesting glucose was transferred to DEG resulting in the formation of DEG glucosides.

Although DEG glucoside formation appears logical, the formation of undetectable glucan can not be excluded. DEG may induce the formation of glucan with modified molecular weight, which is not precipitated using 75% ethanol (Dubois et al., 1956). Analysis of the molecular weight distribution of the glucan formed, could provide conclusive answers.

Based on the increased reducing sugars/glucan ratio, other alcohols mentioned in Table 3.3, perhaps with the exception of 1-methoxy-2-propanol, are most likely all transglucosylated by GTF-D.

With bis-2-methoxyethyl ether (MEE, Scheme 3.1) the transfer ratio indicates a negligible influence of the cosolvent on GTF-D transglycosylation activity compared to the control without cosolvent. Combined with the absolute amount of catechin glucosides formed, the ether MEE was considered as a suitable cosolvent for further experiments.



Scheme 3.1 Structure of bis-2-methoxyethyl ether (MEE).

Improved catechin glucosylation with bis-2-methoxyethyl ether

As our initial objective was to increase the solubility of catechin to increase the concentration of catechin glucosides, we studied the effect of the catechin concentration on the GTF-D transglucosylation activity in the presence of MEE as cosolvent. By adding 15% (v/v) of MEE the catechin solubility was increased to 300 mM compared to 25 mM in water. The kinetics of catechin glucoside formation in water were first order with respect to the catechin concentration up to a catechin concentration of about 15 mM (Figure 3.2A). This suggested that higher specific catechin transglucosylation activities could be obtained if higher catechin concentrations could be employed by adding the cosolvent. This increase in the specific catechin transglucosylation activity is demonstrated in Figure 3.2B.

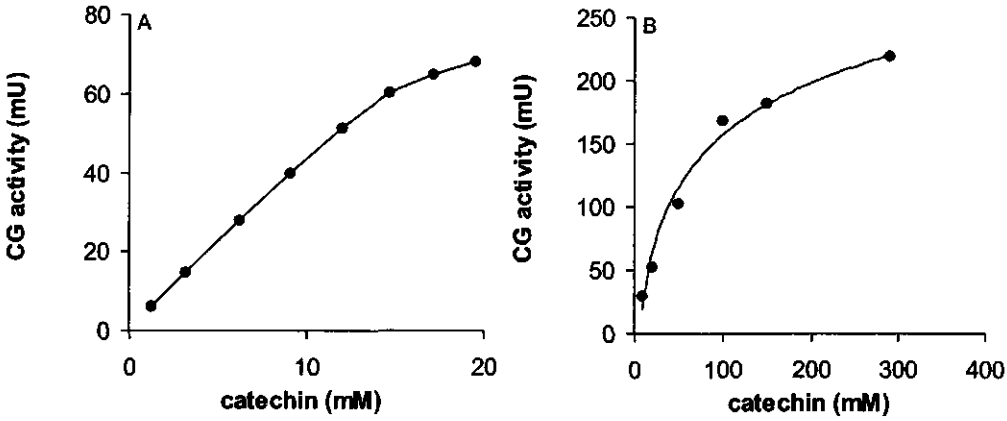


Figure 3.2 A: Initial (0-60 min) catechin transglucosylation by *S. mutans* GTF-D in absence of bis-2-methoxylethyl ether. Using 150 mU/ml GTF-D, 200 mM sucrose and 20 mM potassium phosphate (pH 6.0) at 37°C the catechin transglucosylation activity was determined. B: The initial (0-60 min) catechin transglucosylation in the presence of 15% (v/v) bis-2-methoxylethyl ether.

Comparison of the data in Figure 3.2A and B shows that the catechin glucosylation activity in the presence of the cosolvent was inhibited to some extent compared to catechin glucosylation without cosolvent added. Therefore we examined the influence of MEE on GTF-D transglycosylation in more detail at a catechin concentration of 10 mM (Figure 3.3).

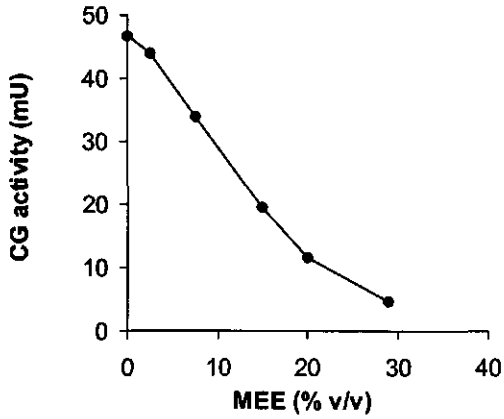


Figure 3.3 Bis-2-methoxyethyl ether (MEE) influencing catechin transglucosylation by *S. mutans* GTF-D. Using catechin (10 mM), sucrose (60 mM), 20 mM potassium phosphate (pH 6.0) and 150 mU/ml GTF-D the initial (0-60 min) catechin transglucosylation activity was determined at 37°C.

Interestingly the formation of glucan was not significantly inhibited in the presence of 30% (v/v) MEE (Table 3.3). Competition between MEE and GTF-D acceptors (catechin, glucan and fructose) for the acceptor site could be ruled out because the formation of glucan was not inhibited by the addition of MEE in contrast with the catechin glucosylation.

To explain the inhibition of catechin glucosylation due to the presence of MEE, we assume that by introducing MEE into the reaction solution the partitioning of catechin between GTF-D and the solvent changes. Due to the increased solubility of catechin, the apparent catechin concentration at the interface of the enzyme is lowered. This lower apparent catechin concentration results in a decreased catechin transglucosylation activity. The concept of lowering the apparent acceptor concentration by addition of MEE was further supported by incubating the irreversible inhibitor phenol (200 mM) in the presence of MEE (30% v/v). In this case GTF-D activity could be observed (formation of reducing sugars, data not shown), whereas in the absence of MEE 200 mM of phenol completely inhibited GTF-D.

This concept of a lowered apparent acceptor concentration due to the addition of a cosolvent could perhaps also be used to glycosylate other acceptors at concentrations which otherwise inactivate GTF-D. To demonstrate this we incubated GTF-D with 200 mM of catechol, 4-methylcatechol or 3-methoxycatechol and with various MEE concentrations and observed the formation of the corresponding glycosides after 60 min of incubation (Table 3.4). The addition of MEE resulted in glycosylation of all three acceptors, at acceptor concentrations that did not allow transglycosylation by GTF-D in the absence of MEE. The optimal MEE concentration will vary depending on concentration and the properties of the acceptor used.

Table 3.4 Engineering of *S. mutans* GTF-D transglycosylating activity.

MEE (%)[1]	catechol (mM)[2]	4-methylcatechol (mM)[2]	3-methoxycatechol (mM)[2]
0	4.3	0.9	23.7
10	14.5	10.6	36.3
20	24.4	35.7	45.1
30	24.3	24.7	25.6

1 Reaction solution containing 20 mM potassium phosphate (pH 6.0), 200 mM sucrose, 200 mM of acceptor and 300 mU/mL GTF-D at 37°C. Samples were analysed after 60 min of incubation.
2 Concentration of the corresponding glycoside.

Although the MEE/GTF-D system was initially studied to glucosylate catechin at higher concentrations, we show here that it can be used as a tool to engineer GTF-D transglycosylating conditions, allowing higher initial acceptor concentrations and hence higher product concentrations.

4

Modifying the formation of glucan and maltooligosaccharides by *Streptococcus mutans* glucosyltransferase-D

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ABSTRACT

The organic cosolvent bis-2-methoxyethyl ether (MEE) affects the formation of glucan by a sucrose glucosyltransferase-D (GTF-D) from *Streptococcus mutans* GS-5. A 20% increase in the formation rate of reducing sugars (fructose and glucose) was observed upon MEE (10-20% v/v) addition. The increase in GTF-D activity could be partially attributed to an increased formation of high molecular weight glucans (10^2-10^3 kDa). Also the type of glucosidic linkage changed upon MEE addition. An increase in the amount of $\alpha(1,3)$ glucosidic linkages was observed. Bis-2-methoxyethyl ether also influenced the formation of malto acceptor products. In the presence of the cosolvent the number of different malto acceptor products decreased, resulting in the formation of only short maltooligosaccharides.

INTRODUCTION

Carbohydrates display many different physical and physiological characteristics, which can also be attributed to the type and amount of glycosidic linkages. The carbohydrate dextran is of interest to the food (oligodextran as prebiotic) and pharmaceutical (polydextran as blood volume expander) industries (Monsan and Paul, 1995; Barker et al., 1993). The molecular weight of dextran may vary from oligosaccharides to high molecular weight polymers (10^3-10^5 kDa) (Monsan and Paul, 1995; Robyt, 1995). For clinical-grade dextrans, the molecular weight may vary between 12 and 98 kDa, with an average of 40 kDa (Barker et al., 1993).

The predominantly α(1,6) linked glucose polymer dextran belongs to a class of polysaccharides generically named glucans. Glucose polymer members of this class might also be linked through α(1,3) (mutan polymer) or alternately linked through α(1,6) and α(1,3) glucosidic bonds (alternan polymer) in the main chain (Côté and Robyt, 1982; Robyt, 1995). These glucans are synthesised by sucrose glucosyltransferase (or glucansucrase) using sucrose as glucosyl donor. The glucansucrase from the soil bacterium *Leuconostoc mesenteroides* used to produce dextran is generically named dextransucrase (EC 2.4.1.5). In a transglucosylation type of reaction, glucose molecules from sucrose are successively α(1,6) linked in the main linear chain. Variation in the type of dextran (e.g. branch linkage) strongly depends on the dextransucrase producing strain (Monchois et al., 1999; Robyt, 1995). We focused our studies on glucansucrases from *Streptococcus* species (generically named glucosyltransferase, EC 2.4.1.5) and in particular *S. mutans* GS-5 glucosyltransferase-D (GTF-D).

The main interest in *Streptococcus* glucosyltransferases is related to the involvement of their glucan in the development of dental caries (Loesche, 1986). Glucosyltransferases from *Streptococcus* species produce glucans that are usually composed of a high percentage of α(1,3) glucosidic linkages. Great diversity is observed in the type of glucan that is formed. That is illustrated by the variety of glucansucrases from *S. mutans* GS-5. Three different glucansucrases are excreted by this microorganism and each glucansucrase produces a different glucan with varying percentages of α(1,3) and α(1,6) linkages (Robyt, 1995; Monchois et al., 1999; Remaud-Simeon, 2000). Besides the formation of glucan, glucansucrases also glucosylate aglycones like catechin and (substituted) catechols using sucrose in a transglucosylation type of reaction (Nakahara et al., 1995; Meulenbeld and Hartmans, 2000).

We previously observed that by adding an organic solvent (bis-2-methoxyethyl ether, MEE), glucosylation at otherwise inhibitory aglycone concentrations was possible (Meulenbeld and Hartmans, 2000). This organic solvent MEE was selected from a range of solvents because the transfer of glucose to glucan remained efficient even in the presence of 30% (v/v) of MEE. In this study we describe the impact of MEE on GTF-D in more detail with respect to the synthesis of glucan and maltooligosaccharides.

MATERIALS AND METHODS

Materials

Escherichia coli containing the *Streptococcus mutans* GS-5 *gtf*D gene (pYND72) (Shimamura et al., 1994) was kindly provided by H.K. Kuramitsu (Department of Oral Biology, State University of New York at Buffalo). Glucosyltransferase-D (GTF-D) from *E. coli* was purified (8.5 U/mg of protein, 70 U/mL) as described earlier (Meulenbeld et al., 1999).

GTF-D activity was quantified by the formation of reducing sugars (RS, using the DNS-method, see below). One unit (U) of RS activity is defined as the amount of enzyme which causes the release of one µmol of reducing sugars per minute in 25 mM potassium phosphate buffer (pH 6.0) with 200 mM sucrose at 37°C.

Bis-2-methoxyethyl ether (MEE) was obtained from Aldrich. All the other chemicals used were of analytical grade.

Typical GTF-D transglucosylation conditions

A typical GTF-D transglycosylation reaction was carried out in a reaction solution containing 25 mM potassium phosphate buffer pH 6.0, 200 mM sucrose and water in a total volume of 1 mL. Depending on the type of experiment MEE was added. Reactions were initiated by adding GTF-D (350 mU) followed by static incubation at 37°C.

Quantification of GTF-D transglucosylation products

GTF-D transglucosylation products were quantified by two different methods, respectively the formation of reducing sugars and the formation of glucan.

Reducing sugars

Reducing sugars (RS) are measured using the dinitrosalicylic (DNS) method (Miller, 1959). A 100 µL amount of diluted reaction sample (10-fold using water) was mixed with 100 µL of the DNS solution and heated for 5 min at 100°C. After cooling, a 1 mL amount of water was added and the absorption at 575 nm was measured spectrophotometrically. Glucose was used as standard and the outcomes are expressed as mM glucose. The DNS solution (100 mL) contained 1.6 g NaOH, 1.0 g 3,5-dinitrosalicylic acid, 30.0 g KNa-tartrate 0.2 g phenol and 50 mg of Na_2CO_2.

Glucan

Glucan was quantified according to the method described by Dubois et al. (1956) using ethanol to precipitate glucan chains. Different precipitation mixtures varying in the percentage of ethanol, were prepared by adding water to ethanol (100% v/v). The different ethanol mixtures (60, 75 and 100% v/v) were stored at -20°C. A 2 mL amount of ethanol mixture was added to 50 µL of reaction mixture, followed by thorough mixing and centrifugation (13,000 × g, 15 min, at room temperature). The precipitate was washed once with the same percentage of ethanol and dissolved in NaOH (0.5 M). This glucan solution was diluted to a concentration of 0.1-2 mM glucose (final volume

of 1 mL). Within 10 sec after the addition of 25 µL phenol (80% w/v), 2.5 mL of concentrated sulphuric acid was added. After mixing, this solution was allowed to stand for 10 min at room temperature and subsequently incubated for 15 min at 37°C. The absorption at 490 nm was recorded spectrophotometrically. Glucose was used as a standard and the outcomes are expressed as mM glucose.

Analysis of mono-, oligo- and polysaccharides

Mono- and oligosaccharides were characterised using high performance anion exchange chromatography (HPAEC). Polysaccharides were characterised using high performance size exclusion chromatography (HPSEC).

High performance anion exchange chromatography

High performance anion exchange chromatography (HPAEC) was performed using a Carbopac PA-1 (250 × 4 mm, Dionex) column, in combination with a Carbopac P-100 guard column (Dionex). The eluent (1 mL/min) was monitored using a Dionex ED40 detector in the pulsed amperometric detection mode (PAD mode).

The gradient to analyse the monomers (fructose and glucose) was as follows: 0-26 min 16 mM NaOH, 26-33 min to 100 mM NaOH, 33-45 min to 40 mM NaOAc in 100 mM NaOH, 45-78 min to 300 mM NaOAc in 100 mM NaOH, 78-83 min to 1000 mM NaOAc in 100 mM NaOH, 83-88 min 1000 mM NaOAc in 100 mM NaOH.

The gradient to analyse malto acceptor products (maltooligosaccharides) was as follows: 0-5 min from 0 to 70 mM NaOAc in 100 mM NaOH, 5-25 min to 200 mM NaOAc in 100 mM NaOH, 25-40 min 200 mM NaOAc in 100 mM NaOAc.

High performance size exclusion chromatography

The samples obtained after enzymatic treatment were freeze dried (1 mL) and dissolved in 200 µL of 0.5 M NaOH prior to high performance size exclusion chromatography (HPSEC).

HPSEC was performed using three TosoHaas TSK PW_{XL} 2500, 3000 and 4000 columns (each 300 × 7.5 mm) in combination with a TosoHaas TSK PW_{XL} guard column. Samples were eluted (0.8 mL/min) using 0.2 M $NaNO_3$ at 30°C. The eluate was monitored using a Shodex SE-71 refractive index detector. The system was calibrated with pullulan standards (Polymer laboratories) with molecular weights in the range of 1 to 500 kDa.

Linkage analysis

Linkage analysis was established by methylation, followed by hydrolysis with 2 M trifluoracetic acid and conversion of the products into partially methylated alditol acetates. The products of methylation analysis were identified and quantified by capillary CLC and GLC-MS (Schols et al., 1990; Verbruggen et al., 1995).

RESULTS AND DISCUSSION

Formation of reducing sugars and glucan in the presence of MEE

In a previous study we described the use of the water miscible organic (co)solvent bis-2-methoxyethyl ether (MEE) in the transglucosylation of (substituted) catechols. The addition of MEE resulted in the formation of glucosides at aglycone concentrations otherwise inhibiting *Streptococcus mutans* GS-5 glucosyltransferase-D (GTF-D) transglucosylation activity (Meulenbeld and Hartmans, 2000). The observation that GTF-D glucan formation activity remained efficient in the presence of MEE prompted us to study the impact of MEE on GTF-D in more detail.

In the initial screening to select a suitable cosolvent, a high concentration (30% v/v) of cosolvent was used (Meulenbeld and Hartmans, 2000). Despite the small decrease in the formation rate of reducing sugars (RS, fructose and glucose) and glucan, MEE was regarded as the most suitable cosolvent. Interestingly, by using 20% (v/v) of MEE an increase of 20% in RS formation rate was observed (Figure 4.1A), an increase which could be accurately reproduced. By varying the MEE concentration, the optimum in RS formation rate was determined (Figure 4.1B). The RS formation rate decreased at MEE concentrations above 20% (v/v). In the presence of 40% (v/v) MEE, GTF-D was completely
inactivated.

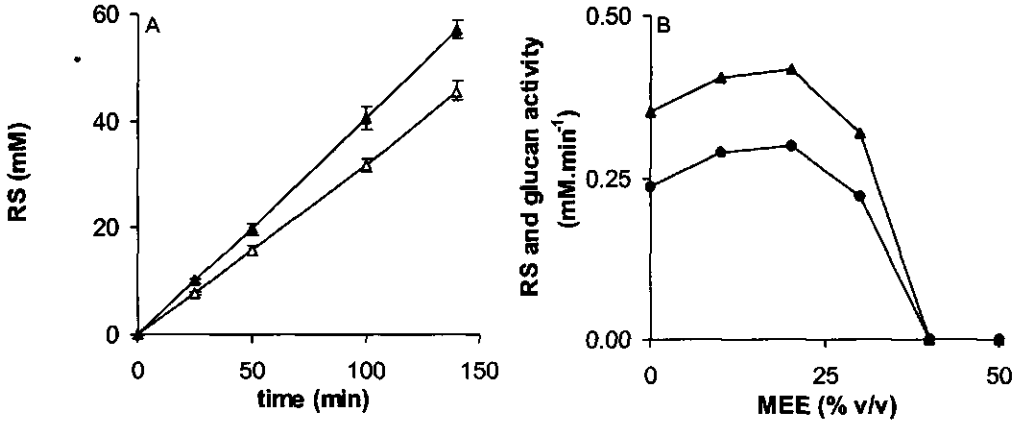

Figure 4.1 Transglucosylation activity of *S. mutans* GTF-D as a function of bis-2-methoxyethyl ether concentration. A: Formation of reducing sugars (RS) in presence (closed symbol) and absence (open symbol) of 20% (v/v) MEE. B: Formation of reducing sugars (RS activity, ▲) and glucan (glucan activity, ●), using 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C.

The DNS method used to monitor GTF-D activity, is only suitable to quantify the total amount of reducing sugars, glucose and fructose. To discriminate between the individual contribution of fructose and glucose to the total amount of RS, HPAEC was performed. Samples for analysis were obtained after 150 min of incubation using

standard conditions. The results obtained using HPAEC show (Table 4.1) that in the absence of MEE 6.5 mM glucose and 34.1 mM fructose were formed (total RS 40.6 mM), while in the presence of MEE 9.5 mM glucose and 39.8 mM fructose were formed (total RS 49.3 mM). These HPAEC results confirm the increase in the total amount of RS due to the addition of MEE as observed using the DNS method. The RS levels quantified using HPAEC are slightly (1.17-fold) lower compared to the DNS method for both reaction conditions. This difference might be caused by over-oxidation using the DNS method (Voragen et al., 1971).

Table 4.1 Formation of reducing sugars and glucan by *S. mutans* GTF-D.

	reducing sugars (mM)[1]	
	0% MEE	20% MEE
glucose (HPAEC)[2]	6.5	9.5
fructose (HPAEC)[2]	34.1	39.8
	glucan (mM)[1]	
	0% MEE	20% MEE
based on HPAEC[3]	27.6	30.3
precipitation (%)[4]		
100	30.7	37.0
75	22.8	29.4
60	12.7	18.8

1 Amount of reducing sugars or glucan after 150 min of incubation using 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C in 1 mL total volume.
2 Reducing sugars determined by HPAEC.
3 Calculated amount of glucan based on HPAEC analysis of fructose and glucose.
4 Percentage of ethanol used to precipitate glucan (Dubois et al., 1956).

Stimulation of glucan formation using MEE

Assuming that the increase in the amount of fructose (as determined by HPAEC) can be used to quantify the transfer of glucose to glucan, it is obvious that the increase in RS formation can not solely be attributed to MEE stimulated sucrose hydrolysis. In that case the increase (in absolute amounts) in glucose and fructose would be the same. Therefore, a part of the additionally formed glucosyl moiety is transferred to another acceptor than water, most likely glucan. The HPAEC data suggest that in the absence of MEE 27.6 mM of glucose ([fructose]–[glucose]) is transferred to form glucan while in the presence of MEE 30.3 mM of glucose is transferred to form glucan (Table 4.1).

In order to actually quantify the amount of glucan, precipitation of the glucan chains using ethanol was performed (Dubois et al., 1956). To ensure that all of the glucan was precipitated, including small glucan fragments (e.g. oligosaccharides), 100% (v/v) ethanol (97% v/v when corrected for dilution with the sample) was used. The data presented in Table 4.1 confirm that MEE addition stimulates glucan formation, although there is some discrepancy between the total amount of glucan formed based on

HPAEC calculations and determined using the precipitation method. The stimulation of glucan formation showed an optimum value using 20% (v/v) of MEE (Figure 4.1B).

Characterisation of glucan

The glucan precipitation behaviour depends on the percentage of ethanol used. By decreasing the ethanol percentage only longer glucan chains are precipitated. Therefore two other ethanol precipitation mixtures (besides 100% v/v) were used, respectively 75 and 60% (v/v) (73 and 59% when corrected for dilution with the sample). The data (Table 4.1) indicate that in the presence of MEE more long chain glucans (higher molecular weight) are formed. Using 60% (v/v) ethanol, 50% more glucan is precipitated when MEE was present during glucan synthesis.

To verify if MEE induces GTF-D to produce more long chain glucans, the molecular weight distribution of glucan was determined by HPSEC (Figure 4.2). The samples used for HPAEC were also used for HPSEC analysis. It is obvious that the addition of MEE results in increased amounts of high molecular weight (HMW) glucan (500-10^3 kDa). Detailed analysis (using HPAEC) revealed that MEE had no effect on oligosaccharide formation (data not shown).

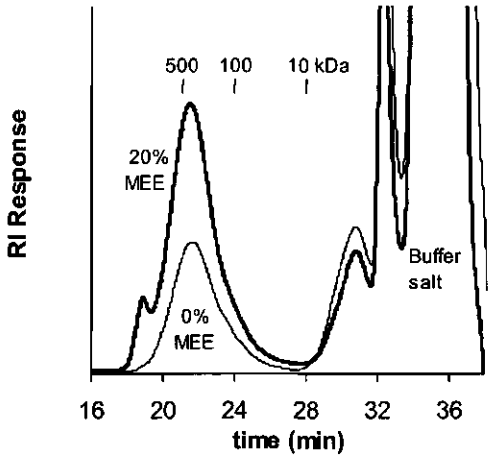

Figure 4.2 High performance size exclusion chromatography pattern of glucan formed by *S. mutans* GTF-D. Formation of glucan was in the absence or presence of 20% (v/v) MEE, 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C during 150 min.

GTF-D glucan was also characterised by looking at the type of glucosidic linkages formed in the absence or presence of MEE using linkage analysis. The analysis (Table 4.2) shows that a significant change in the type of glucosidic linkage occurs due to MEE addition. The percentage of α(1,3) linkages increases significantly, while the percentage of α(1,6) linkages decreases. Therefore, besides affecting the amount of glucan synthesised, MEE also induces the formation of a different type of glucan because of the change in type of glucosidic linkages.

Table 4.2 Analysis of glucosidic linkages in GTF-D glucan.

condition	ratio α(1,3) / α(1,6)[1]
0% (v/v) MEE	0.20
20% (v/v) MEE	0.37

1 Ratio of glucosidic linkages.

Glucansucrases contain a N-terminal catalytic domain and a C-terminal glucan binding domain. This domain is responsible for the binding of glucan and is not directly involved in the catalytic process (Monchois et al., 1999). Previous site directed mutagenesis studies showed that amino acid modifications, especially mutations of an aspartate, influenced the molecular weight distribution of the glucan and the size of the oligosaccharide produced (Shimamura et al., 1994; Monchois et al., 2000). Changing the *Streptococcus downei* glucosyltransferase aspartate-569 into aliphatic amino acids, increased the yield of shorter oligosaccharides. In case of glucan stimulation by MEE, the glucan binding domain is changed, perhaps by a change in enzyme conformation (Robyt and Corrigan, 1977; Robyt, 1995). This change eventually results in an increased formation of high molecular weight glucan.
The characteristic response of GTF-D towards an external factor like MEE prompted us to study the role of the pH in more detail, as the pH plays an important role in enzyme conformation.

Role of pH in glucan formation

The influence of pH on the formation of reducing sugars in the absence or presence of MEE was determined. Different buffers were used (sodium acetate pH 4-6, potassium phosphate pH 6-7, glycine/NaOH pH 8-11) and the initial formation rate of reducing sugars (RS activity) was determined in the absence or presence of MEE (Figure 4.3). Interestingly, using a high pH value (pH 9), GTF-D stimulation was increased 6-fold by MEE, compared to 1.2-fold stimulation by MEE at lower pH values. This increase was also observed using Tris/HCl buffer (pH 7-9, data not shown). However, Tris appeared to inhibit GTF-D activity to some extent. This was demonstrated by comparing the RS activities using various buffers at pH 7. Using MES, MOPS, Imidazole and HEPES buffers, the RS activity and the stimulatory influence of MEE was the same as with phosphate buffer. However, using Tris/HCl or Tris/Maleate buffers the RS activity decreased significantly to respectively 0.15 and 0.20 $mM.min^{-1}$. A stimulation of RS formation by MEE could still be observed.
From these results it is obvious that the presence of the organic cosolvent (MEE) induces GTF-D to withstand higher pH values, possibly by a change in enzyme conformation. It should be kept in mind that in aqueous environments the pH is one of the most influential parameters affecting enzymatic activity, but that it has very little effect in organic solvents or low water environments (Klibanov, 2001; Dordick, 1989).

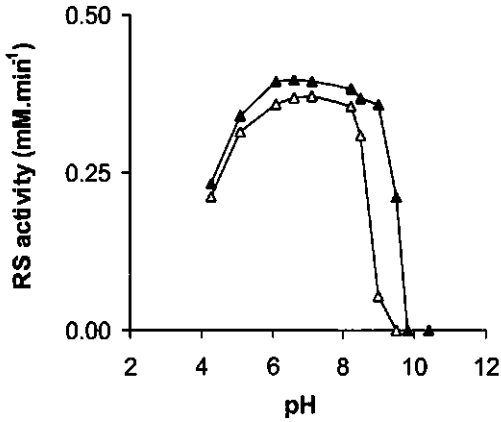

Figure 4.3 Role of pH on transglucosylation activity of *Streptococcus mutans* GTF-D. RS activity in the presence (▲, closed symbol) or absence (open symbol) of 20 % (v/v) MEE, using 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C.

Formation of malto acceptor products

In literature the stimulation of glucansucrase activity by the addition of maltose has been described, resulting in decreased glucan concentrations and relatively high malto acceptor product concentrations (Su and Robyt, 1993; Kitaoka and Robyt 1999). Because maltose itself acts as a reducing sugar, the DNS method could not be applied with sufficient accuracy. In stead, glucan and the malto acceptor products (hereafter also designated as glucan) were determined by ethanol precipitation (100% v/v). Note that this ethanol precipitation does not provide insight in the type of transfer products formed, but does give an indication of the overall (glucosyl) transfer activity.

The long established fact that maltose acts as very good acceptor is confirmed by the data presented in Figure 4.4A. Addition of a small amount of maltose results in a 7-fold increase in the glucan formation rate. In the presence of MEE (20% v/v) only a 4-fold increase in glucan formation, compared to the control without MEE, was observed upon maltose addition. Therefore the influence of other MEE concentrations was also examined resulting in the pattern as presented in Figure 4.4A. In the absence of maltose the same results as in Figure 4.1B were obtained.

Variations in the sucrose/maltose ratio (0.2-10, using a fixed amount of sucrose) using 0 or 20% (v/v) MEE showed that an increase in the amount of maltose resulted in an increase in glucan formation. In all cases, the addition of MEE (20% v/v) resulted in a decrease in glucan formation (Figure 4.4B).

The specific pattern of MEE influencing glucan formation in the presence of maltose (Figure 4.4A) can be explained by discriminating between two effects. The first is the effect as observed in Figure 4.1B, the stimulation of glucan formation by MEE. The second effect is the influence of MEE on the glucosyl transfer to maltose. Apparently this transfer in the presence of MEE is altered, resulting in a decreased glucan (or malto acceptor product) formation rate. Although this rate is still high compared to the

situation where no maltose is added. By combining the stimulatory influence of MEE on GTF-D activity and its inhibitory influence in the presence of maltose, curves as presented in Figure 4.4 could be obtained.

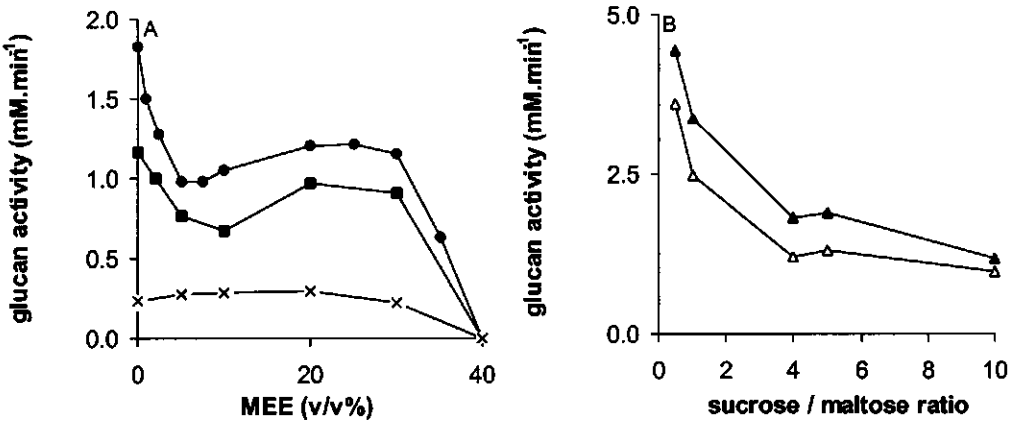

Figure 4.4 Role of maltose on transglucosylation activity of *Streptococcus mutans* GTF-D. A: Glucan activity using 40 (●), 20 (■) or 0 (×) mM maltose, variable MEE, 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C. B: Glucan activity, varying the sucrose/maltose ratio in the absence (▲) or presence (open symbol) of 20% MEE (v/v), 350 mU/mL GTF-D, 200 mM sucrose in 25 mM potassium phosphate buffer pH 6.0 at 37°C.

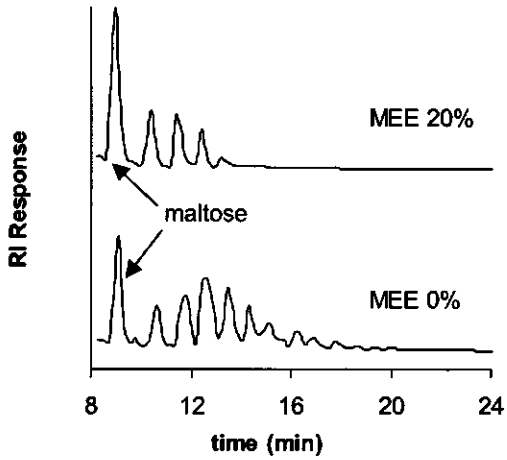

Figure 4.5 Molecular weight distribution of maltooligosaccharides using high performance size exclusion chromatography. Formation of maltooligosaccharides in the absence or presence of 20% (v/v) MEE, 350 mU/mL GTF-D, 200 mM sucrose and 40 mM maltose in 25 mM potassium phosphate buffer pH 6.0 at 37°C during 60 min.

To determine the influence of MEE on the type of malto acceptor products formed, their molecular weight distribution was analysed. Using a sucrose/maltose ratio of 5/1 in the presence or absence of 20% (v/v) MEE the malto acceptor products (maltooligosaccharides) were analysed using HPAEC (Figure 4.5). The HPAEC data indicate that MEE influences the formation of maltooligosaccharides. By adding the aliphatic cosolvent MEE, the synthesis of long chain maltooligosaccharides is no longer observed. The differences in retention times observed in the analysis of maltooligosaccharides (Figure 4.5) suggest that the type of glucosidic linkage (in the maltooligosaccharides) is changed upon MEE addition. A change also observed in glucan formation in the presence of MEE (Table 4.2).

In summary, it is clear that the formation of glucan and maltooligosaccharide by *S. mutans* glucosyltransferase-D (GTF-D) can be engineered by to bis-2-methoxyethyl ether (MEE) addition. By adding 20% (v/v) of the cosolvent MEE, an increase in the formation of reducing sugars and high molecular weight glucans was observed. Besides the increase in absolute amounts of glucan, also a change in the type of glucosidic linkages formed, was observed. In the presence of maltose, only the formation of short maltooligosaccharides was observed upon MEE addition. The addition of the cosolvent MEE provides an alternative for the screening of various glucansucrases for the formation of specific glucan and maltooligosaccharide products.

5

Thioglucosidase activity from *Sphingobacterium* sp. strain OTG1

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S. Hartmans

ABSTRACT

Screening for novel thioglucoside hydrolase activity resulted in the isolation of *Sphingobacterium* sp. strain OTG1 from enrichment cultures containing octylthioglucoside (OTG). OTG was hydrolysed into octanethiol and glucose by cell free extracts. Besides thioglucoside hydrolysis, several other glucoside hydrolase activities were detected in the *Sphingobacterium* sp. strain OTG1 cell free extract. By adding β-glucosidase inhibitors it was possible to discriminate between these different activities. Ascorbic acid and D-gluconic acid lactone inhibited the hydrolysis of *p*-nitrophenyl β-glucoside, but did not affect octyl- and octylthioglucoside hydrolase activity. Besides OTG, various other thioglucosides were hydrolysed by the novel thioglucosidase, with almost the same activities regardless of the nature of the aglycone, including the myrosinase model substrate sinigrin (a glucosinolate). Sinigrin could also be used as a growth substrate by *Sphingobacterium* sp. strain OTG1, although at concentrations exceeding 0.15 mM degradation was not complete.

INTRODUCTION

In nature, three types of glycosidic linkages occur, the *O*-, *N*- and *S*-glycosidic linkage. Especially *O*- and *N*-glycosides have been subjects of extensive research. *S*-glycosides however, have received relatively little attention.

The *S*-type glycosidic linkage mainly occurs in glucosinolates, plant metabolites present in species of the order Caparales (Rask et al., 2000). These glucosinolates may be a sink for nutrients like nitrogen and sulphur. To our knowledge, only one enzyme capable of hydrolysing the *S*-glycosidic linkage has been characterised, myrosinase (β-thioglucosidase EC 3.2.3.1). After glucosinolate hydrolysis, the aglycone becomes unstable and undergoes several rearrangements depending on its structure. The products of glucosinolate hydrolysis, which probably play an important role in the plant defence system, have a characteristic flavour and may also benefit human health (Fenwick et al., 1983; Mithen et al., 2000; Rask et al., 2000). Although myrosinases have mainly been isolated from a number of plant sources (Fenwick et al., 1983; Rask et al., 2000), there are also reports concerning myrosinase activities from *Escherichia coli* and *Aspergillus niger* (Oginsky et al., 1965), *Enterobacter cloacae* (Tani et al., 1974), *Aspergillus clavatus, Fusarium oxysporum* (Smits et al., 1993) and *Lactobacillus agilis* (Palop et al., 1995).

The stability of thioglycosides, other than glucosinolates, towards enzymatic hydrolysis is striking. Reports concerning almond β-glucosidase show that it is not able to hydrolyse the *S*-glucoside of octanethiol or β-mercaptoethanol (Saito and Tsuchiy 1984; Dintinger et al., 1994; Meulenbeld et al., 2001). This is in contrast with the *O*-glucosides of β-mercaptoethanol and octanol, which are hydrolysed by almond β-glucosidase. Therefore, enzymes from microbial origin that can hydrolyse thioglycosides other than glucosinolates are interesting.

In general, investigations concerning the enzymatic synthesis of glycosidic linkages have focused extensively on the formation of the *O*- and *N*-types glycosidic linkage (Ichikawa et al., 1992; Prasad et al., 1999). The only enzyme reported so far capable of forming (*in vitro*) *S*-glycosides is the almond β-glucosidase (Dintinger et al., 1994). This enzyme glucosylates by the reversed hydrolysis mechanism. This is in contrast with the biosynthesis of glucosinolates, which are thought to be formed by the UDP-glucose transferring enzyme, thiohydroximate glucosyltransferase (Fenwick et al., 1983; Reed et al., 1993; Mithen et al., 2000). Screening for thioglucoside hydrolytic enzymes might eventually result in the isolation of new (*S*)-glycosidases applicable to the synthesis of (thio)glycosides.

We screened for enzymes with the ability to hydrolyse thioglucosides, by searching for microorganisms capable of growth using octylthioglucoside as a carbon source. Here we report the initial characterisation of novel bacterial thioglucoside hydrolase activity.

MATERIALS AND METHODS

Enzymes

Almond β-glucosidase, *Caldocellum saccharolyticum* β-glucosidase and *Sinapis alba* β -glucosidase (myrosinase) were obtained from Sigma. *Aspergillus niger* β-glucosidase was obtained from Fluka. *Pyrococcus furiosus* β-glucosidase was kindly provided by T. Kaper (Laboratory of Microbiology, Wageningen University). All enzymes were used without further purification.

Chemicals

Ascorbic acid, castanospermine, 1-deoxynojirimycin, D-gluconic acid lactone, D-glucose β-D-thioglucoside, isopropyl β-D-thiogalactoside, isopropyl β-D-thioglucoside, *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl α-D-glucoside, octanethiol (OT), phenyl β-D-thiogalactoside and sinigrin were obtained from Sigma. N-Bromosuccinimide was obtained from Acros. Octylthioglucoside (1-octyl β-D-thioglucoside, OTG), octylglucoside (1-octyl β-D-glucoside) were obtained from Boehringer. 1-Heptanethiol, 1-heptyl β-D-thioglucoside and 2-propanethiol were obtained from Fluka.

Isolation of thioglucoside degrading microorganisms

The screening for microorganisms with thioglucoside hydrolase activity was performed by mixing 2 g of soil from various sources with 5 mL of mineral salts medium pH 7 (Hartmans et al., 1991) and 2 mM of octylthioglucoside (OTG) in 100 mL serum flasks. All growth experiments were performed at 30°C. Growth of the enrichment cultures was followed by determining the formation of CO_2. A 500 µL amount of positive enrichment cultures was inoculated in 250 mL serum flasks containing 50 mL of mineral salts medium pH 7 supplemented with 2 mM OTG. After growth was observed, 50 µL of these cultures was plated on agar plates containing 1 mM OTG and 0.01% (w/v) yeast extract (YE, GibcoBRL). Separate colonies were selected and inoculated in 100 mL serum flasks containing 10 mL of mineral salts medium supplemented with 1 mM OTG and 0.02% (w/v) YE. After growth, supernatant and cell free extracts were screened for thioglucoside hydrolase activity. All enzyme assays were performed at room temperature.

Growth conditions and cultivation of *Sphingobacterium* sp. strain OTG1

The isolated microorganism *Sphingobacterium* sp. strain OTG1 is deposited in the Industrial Microbiology Culture Collection of the Wageningen University (CIMW no. 410B). *Sphingobacterium* sp. strain OTG1 was maintained on agar plates containing 0.5% (w/v) cellobiose and 0.02% (w/v) YE. *Sphingobacterium* sp. strain OTG1 was grown aerobically in 250 mL serum flasks containing 50 mL of mineral salts medium pH 7 supplemented with cellobiose (2 g/L) and 0.02% (w/v) YE at 30°C. Growth was followed by measuring the optical density (OD) at 600 nm.

Preparation of cell free extracts

Cultures were harvested during the exponential growth phase by centrifugation (39,100 × *g*, 60 min, 4°C). The cells were washed with Tris-HCl buffer (20 mM, pH 7.5) and resuspended in the same buffer. After sonication (Sonifier 250, Branson: duty cycle 30% output control 3) the lysate was centrifuged (39,100 × *g*, 60 min, 4°C). Finally the supernatant was filtered using a 0.2 μm sterile filter (Schleicher and Schuell) and was used as cell free extract.

CO_2 measurements

CO_2 concentrations were determined by analysing 100 μL gas phase samples on a Hewlett Packard HP 6890 GC-system containing a Chrompack Poraplot Q column.

Protein determination

Protein concentrations were determined using the Bio-Rad DC Protein Assay. Bovine serum albumin was used as the standard.

Thioglucoside hydrolase activity

The thioglucoside hydrolase activity was determined by measuring the initial (0-60 min) formation of octanethiol (OT). For each separate data point, cell free extract was incubated with 2 mM OTG and 20 mM Tris-HCl buffer pH 7.5 in a total volume of 110 μL at room temperature. OT was extracted from the reaction mixture by adding 200 μL of n-hexane. The n-hexane was supplemented with 4 μL n-decane per 100 mL as internal standard. The amount of OT in the n-hexane phase was measured by analysing 2 μL samples on a Hewlett Packard HP 6890 GC-system, with a ChromPack 7351 CP-porabond Q Capillary column (25.0 m × 320 μm) using N_2 as carrier gas at a flow rate of 2.4 mL/min. Octanethiol (detection limit about 6 μM) and heptanethiol were measured at 250°C. 2-Propanethiol and octanol were measured at respectively 190°C and 175°C. The identity of reaction products was confirmed by comparison of the GC-retention times with the commercially available compounds.

Thioglucosidase hydrolase activity in culture supernatants was determined after 10-fold concentration using an Amicon YM10 system.

β-Glucosidase activity

β-Glucosidase activity present in the cell free extract was measured by following the increase in *p*-nitrophenolate (*p*NP) concentration at 405 nm. Cell free extract was incubated with 2 mM *p*-nitrophenyl β-glucoside (β*p*NPG) and 20 mM Tris-HCl buffer pH 7.5 in a total volume of 1 mL at room temperature. The molar extinction coefficient for *p*NP under these conditions was 15900 $M^{-1} \times cm^{-1}$. The molar extinction coefficient using 20 mM potassium phosphate buffer pH 6.0 was 1500 $M^{-1} \times cm^{-1}$. To determine the α-glucosidase activity, *p*-nitrophenyl α-glucoside (α*p*NPG) was used.

Sinigrin

The amount of sinigrin was determined by monitoring the absorbance at 227 nm in 20 mM Tris-HCl pH 7.5 (molar extinction coefficient 7200 $M^{-1} \times cm^{-1}$).

Dinitrosalicylic acid assay

The amount of glucose formed during the hydrolysis of the (thio)glycosides was determined using the dinitrosalicylic acid (DNS) method (Miller 1959). Enzyme solutions and thioglycosides were incubated in 20 mM Tris-HCl buffer pH 7.5 in a volume of 110 µL. To stop the enzyme reaction, 100 µL of the DNS solution was added and the complete reaction mixture was heated at 100°C for 5 min. After cooling, 1 mL of water was added and the absorption at 575 nm was measured spectrophotometrically. Glucose was used as the standard. The DNS solution (100 mL) contained 1.6 g NaOH, 1.0 g 3.5-dinitrosalicylic acid, 30.0 g KNa-tartrate, 0.2 g phenol and 50 mg Na_2CO_3.

RESULTS

Screening for thioglucoside hydrolase activity

In the initial stages of screening for thioglucoside hydrolase activity, we were able to isolate five microorganisms capable of using octylthioglucoside (OTG) as a carbon source from various sources of soil. These isolates were able to form CO_2 in the presence of 2 mM OTG (in liquid cultures at 30°C) compared to controls containing only yeast extract. However, we could detect enzymatic thioglucoside hydrolase activity in the cell free extract of only one isolate. No extracellular thioglucoside hydrolase activity was observed.

This isolated strain was identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as a bacterium belonging to the genus *Sphingobacterium* (the cellular fatty acid profile is typical for the genus *Sphingobacterium*, data not shown). Partial 16SrDNA sequences show similarities of 96.7-98.7% to members of the species *Sphingobacterium multivorum* (data not shown). However, some of the physiological tests do not correlate with the description of *S. multivorum*, therefore the isolated microorganism was designated as *Sphingobacterium* sp. strain OTG1.

Sphingobacterium sp. strain OTG1 was isolated using an enrichment culture with 2 mM OTG as the carbon source. However, in pure culture, growth (determined by measuring the OD 600 nm) using 2 mM OTG was inhibited compared to cultures with 0.5 mM OTG. Consequently, high amounts of biomass could not be obtained using OTG as a growth substrate. Besides OTG, strain OTG1 is also able to use other glycosides for growth (Table 5.1).

Using these glycosides for *Sphingobacterium* sp. strain OTG1 growth (0.5% w/v and 0.02% w/v YE), the initial rates of hydrolysis of several glucosides (2 mM of respectively octylglucoside [OG], octylthioglucoside [OTG] and *p*-nitrophenyl α- and β -glucoside [α*p*NPG and β*p*NPG]) in cell free extracts were determined. The highest OTG hydrolysis activity in cell free extract was obtained using cells grown on

cellobiose. Therefore *Sphingobacterium* sp. strain OTG1 was grown on cellobiose during further experiments.

Table 5.1 Glucosidase activities of *Sphingobacterium* sp. strain OTG1 cell free extract.

growth substrate	glucosidase activity (nmol.min^{-1}.mg^{-1}) [1,2]			
	OTG	OG	βpNPG	αpNPG
octylglucoside	2.2	3.4	6.7	3.8
glucose	4.1	8.2	5.4	5.6
cellobiose	4.8	9.1	3.8	5.4
maltose	3.8	6.7	3.6	16.2

1 Glucosidase activities were determined using 2 mM of glycosides, 20 mM Tris-HCl buffer pH 7.5 at room temperature.
2 Substrates (2 mM) respectively octylthioglucoside (OTG), octylglucoside (OG), *p*-nitrophenyl β- and α-glucoside (β*p*NPG, α*p*NPG).

Enzymatic thioglucoside hydrolase activity

Using the *Sphingobacterium* sp. strain OTG1 cell free extract, the hydrolysis of OTG to equimolar amounts of OT and glucose was observed and expressed as the formation of OT per min per mg of protein (Figure 5.1). Inactivation of cell free extract (by heating at 100°C) resulted in the total loss of hydrolase activity.

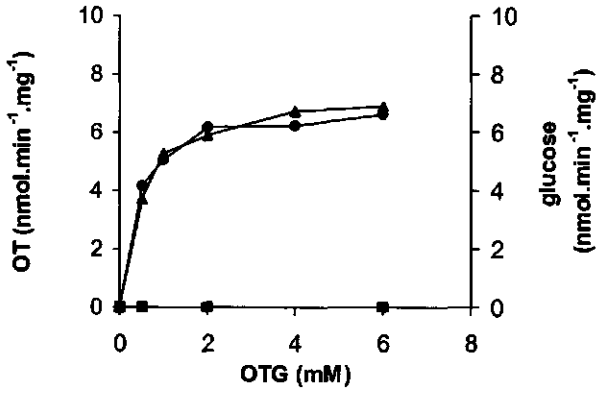


Figure 5.1 The hydrolysis of octylthioglucoside (OTG) by *Sphingobacterium* sp. strain OTG1 cell free extract at various initial OTG concentrations. The specific rates of octanethiol (OT, ●) and glucose formation (▲) were determined using *Sphingobacterium* sp. strain OTG1 cell free extract and inactivated cell free extract (■) in 20 mM Tris-HCl buffer pH 7.5 at room temperature.

The thioglucosidase hydrolase activity in cell free extract is stable for several days at 4°C (data not shown). Incubating the cell free extract for one hour at a fixed temperature (20-60°C, in 20 mM Tris-HCl pH 7.5) showed that OTG hydrolysis activity (determined at room temperature) was stable between 20 and 30°C, but at higher

temperatures activity was lost (Figure 5.2). The pH optimum of OTG hydrolysis (2 mM) was between pH 7 and 8 (Figure 5.2) and was independent of the type of buffer used. Consequently all experiments using cell free extract were performed at pH 7.5 (20 mM Tris-HCl buffer) and at room temperature.

To allow purification of *Sphingobacterium* sp. strain OTG1 thioglucoside hydrolase, we first studied the stability of the thioglucoside hydrolase activity during dialysis experiments. Cell free extract was dialysed against 20 mM of Tris-HCl buffer pH 7.5 at 4°C for several days and the rates of hydrolysis of both β*p*NPG and OTG were determined (data not shown). While the β*p*NPG hydrolysis activity remained almost unchanged the thioglucoside hydrolase activity using OTG decreased with dialysis time. This loss of OTG hydrolysis activity could not be prevented or restored by the addition of a variety of ions (Ca^{2+}, Mg^{2+} and Mn^{2+}). The loss of OTG hydrolysis activity was also observed using various chromatographic purification steps (data not shown). Consequently no further attempts were made to purify the OTG-hydrolase.

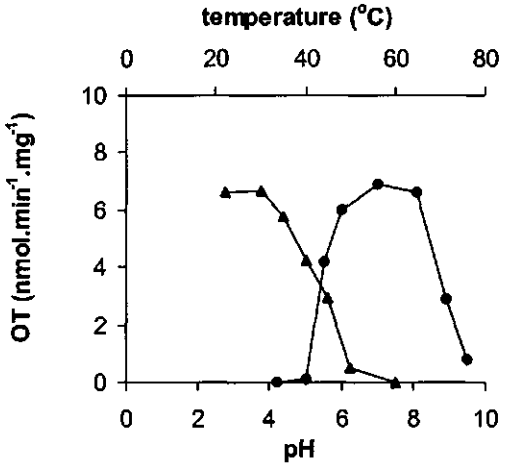


Figure 5.2 Effect of pH on the hydrolysis of octylthioglucoside (OTG) by *Sphingobacterium* sp. strain OTG1 thioglucosidase and its thermal stability. The formation of octanethiol (OT) using 2 mM OTG was monitored using different buffers (20 mM sodium acetate pH 4, 5 and 6, potassium phosphate pH 5.5, 6, 6,5 and 7, Tris-HCl pH 7, 8, 8.5 and 9) at room temperature (●). For the temperature stability experiment, cell free extract was incubated at various temperatures in 20 mM Tris-HCl buffer pH 7.5 for one hour prior to the analysis of OTG-hydrolysis (2 mM) at room temperature (▲).

Discrimination between glucosidase activities in *Sphingobacterium* sp. strain OTG1 cell free extract

We observed that during growth of strain *Sphingobacterium* sp. OTG1 on cellobiose the ratio of two hydrolase activities (formation of *p*-nitrophenolate, *p*NP, from *p*-nitrophenyl β-glucoside and OT from OTG) changed. During the lag phase the ratio varied between 1.8 and 1.0, while in the exponential growth phase the *p*NP/OT ratio was 0.6-0.8. The variation in the ratio of these two activities was especially evident when cell free extracts of strain OTG1 grown on different carbon sources were compared (Table 5.1). This suggests the presence of more than one enzyme responsible

for the hydrolysis of β*p*NPG and/or OTG. Partial inactivation of β*p*NPG hydrolase activity due to the addition of inhibitors could demonstrate this more clearly. Therefore we incubated the cell free extract of strain OTG1 with 1 mM of ascorbic acid, N-bromosuccinimide, castanospermine, 1-deoxynojirimycin or D-gluconic acid lactone and determined the initial rate of hydrolysis of β*p*NPG, OTG and octylglucoside (OG) (Table 5.2). Ascorbic acid, 1-deoxynojirimycin and D-gluconic acid lactone inhibited the hydrolysis of β*p*NPG while the rate of OTG hydrolysis remained almost unchanged. This lack of inhibition of OTG hydrolysis using three different β-glucosidase inhibitors, strongly suggests the presence of a specific thioglycoside hydrolase.

Table 5.2 Effect of glucosidase inhibitors on *Sphingobacterium* sp. strain OTG1 cell free extract hydrolase activity.

compound (1 mM)	relative glucosidase activity		
	β*p*NPG (%)[1]	OTG (%)[2]	OG (%)[3]
-	100	100	100
ascorbic acid	23	85	95
N-bromosuccinimide	0	0	nd [4]
castanospermine	100	100	nd
1-deoxynojirimycin	60	90	nd
D-gluconic acid lactone	17	83	85

1 Reaction solution containing 2 mM β*p*NPG in 20 mM Tris-HCl buffer pH 7.5 at room temperature. 100 % Corresponds to the formation of 4.4 nmol *p*-nitrophenolate $min^{-1}.mg^{-1}$.
2 Reaction solution containing 2 mM OTG in 20 mM Tris-HCl buffer pH 7.5 at room temperature. 100 % Corresponds to the formation of 6.6 nmol octanethiol $min^{-1}.mg^{-1}$.
3 Reaction solution containing 2 mM OG in 20 mM Tris-HCl buffer pH 7.5 at room temperature. 100 % Corresponds to the formation of 11.2 nmol octanol $min^{-1}.mg^{-1}$.
4 Not determined.

Screening of β-(thio)glucosidases for OTG hydrolase activity

To demonstrate the novelty of the *Sphingobacterium* sp. strain OTG1 thioglucoside hydrolase, several commercially available glycosidases were screened for OTG hydrolase activity (Table 5.3). β-Glucosidases from respectively Almond, *Aspergillus niger, Caldocellum saccharolyticum* and *Pyrococcus furiosus* were used and also the *Sinapis alba* β-thioglucosidase (myrosinase). First, the specific hydrolase activities towards β*p*NPG were determined. Next the enzymes were screened for OTG-hydrolase activity using 0.08 mU/mL of enzyme based on the hydrolysis of β*p*NPG at pH 7.5 (20 mM Tris-HCl at room temperature). Because of the low activity of *S. alba* thioglucosidase, only 0.02 mU/mL was used. The specific activity of the glucosidases at pH 6.0 was significantly higher than at pH 7.5, therefore the same screening experiments were also performed at this lower pH value. Nevertheless, no enzymatic hydrolysis of OTG was observed during 24 h under the described experimental conditions.

Table 5.3 Enzymatic hydrolysis of *p*-nitrophenyl β-glucoside (β*p*NPG) and n-octylthio β-glucoside (OTG) by various β-(thio)glucosidases.

enzyme origin	glycosidase activity			
	βpNPG[1]		OTG[2]	protein
	pH 6.0	pH 7.5		(mg/mL)[3]
Almond	1650	45	$< 4 \times 10^{-3}$	1.8
Aspergillus niger	410	10	$< 9 \times 10^{-4}$	7.8
Caldocellum saccharolyticum	710	7	$< 6 \times 10^{-4}$	11.4
Pyrococcus furiosus	9600	120	$< 10 \times 10^{-3}$	0.7
Sinapis alba (thioglucosidase)	0.15	0.044	$< 2 \times 10^{-5}$	480

1 Initial hydrolysis activity (nmol.min^{-1}.mg^{-1}) using 2 mM β*p*NPG in respectively 20 mM KPi buffer pH 6.0 or Tris-HCl buffer pH 7.5 at room temperature.
2 Hydrolysis activity (determined after 24 h, nmol.min^{-1}.mg^{-1}) using 2 mM OTG in respectively 20 mM KPi buffer pH 6.0 or Tris-HCl buffer pH 7.5 at room temperature.
3 Protein concentration used for OTG hydrolysis.

Substrate specificity of *Sphingobacterium* sp. strain OTG1 thioglycoside hydrolase

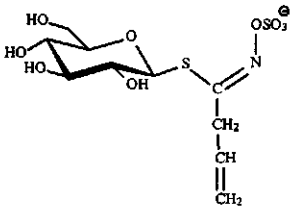
In order to examine the substrate specificity of *Sphingobacterium* sp. strain OTG1 thioglucoside hydrolase, cell free extract was incubated with different thioglycosides (2 mM) in 20 mM Tris-HCl pH 7.5 at room temperature. Activities were determined by measuring the amount of glucose liberated (DNS-method, Table 5.4). The different β-thioglycosides used were stable during the glucose analysis. From the data presented it is clear that only the thioglucosides are hydrolysed. The thiogalactosides were not hydrolysed al all. This indicates the presence of a thioglucosidase in strain OTG1 cell free extract.

Table 5.4 Substrate specificity of thioglycosidase hydrolase in cell free extract of *Sphingobacterium* sp. strain OTG1.

thioglycoside	thioglycosidase activity (nmol.min^{-1}.mg^{-1})[1,2]
octylthioglucoside	4.1
heptylthioglucoside	3.5
isopropylthioglucoside	2.6
isopropylthiogalactoside	0
phenylthiogalactoside	0
D-glucose thioglucoside	2.9
sinigrin	3.2

1 Reaction solution containing 2 mM thioglycoside in 20 mM Tris-HCl buffer pH 7.5 at room temperature.
2 Formation of reducing sugars determined using the DNS method.

Interestingly the glucosinolate sinigrin (2-propenyl glucosinolate, Scheme 5.3, Fenwick et al., 1983), which is generally used as model substrate in myrosinase assays, was also hydrolysed by strain OTG1 cell free extract (Table 5.4).



Scheme 5.3 Structure of sinigrin.

Because of the interest in the hydrolysis of glucosinolates (see Introduction), we tested the consumption of sinigrin by strain OTG1. Incubation of *Sphingobacterium* sp. strain OTG1 with 0.15 mM of sinigrin in a mineral salts medium supplemented with 0.02% (w/v) YE at 30°C resulted in growth (Figure 5.4). At the same time, sinigrin was almost completely consumed. The sinigrin absorbance at 227 nm decreased significantly and monitoring spectra between 200 and 350 nm showed that no other products accumulated. The data presented (Figure 5.4) are corrected for growth using a control without sinigrin, this YE-control reached an OD 600 nm value of 0.1 after 9 h.
Growth could not be significantly enhanced by increasing the sinigrin concentration. After approximately 0.15 mM of sinigrin was hydrolysed, growth of *Sphingobacterium* sp. strain OTG1 stopped.

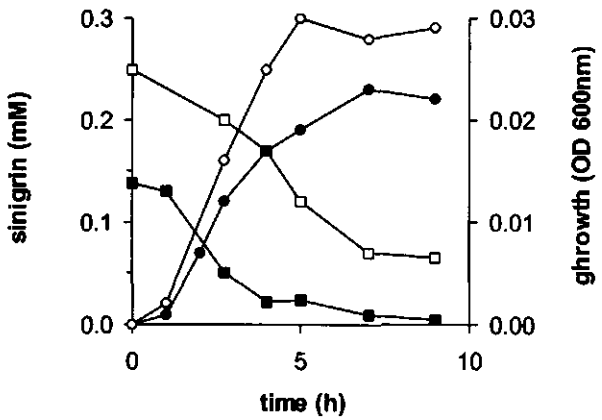


Figure 5.4 Growth of *Sphingobacterium* sp. strain OTG1 on sinigrin. Using 0.15 mM (closed symbols) and 0.25 mM (open symbols) sinigrin (■), the growth (●, OD 600 nm) corrected for control without sinigrin, of *Sphingobacterium* sp. strain OTG1 was determined in mineral salts medium pH 7 at 30°C.

DISCUSSION

In this report we describe the search for microbial thioglucoside hydrolases. By using octylthioglucoside (OTG), rather than glucosinolates present in, for example mustard seed (Ohtsuru et al., 1973; Tani et al., 1974), we hoped to find novel thioglucoside hydrolases in stead of the known myrosinases. This approach resulted in the isolation of a *Sphingobacterium* sp. strain OTG1. We were able to quantify the hydrolysis of OTG to octanethiol (OT) and glucose (both products were quantitatively detected) with only one of the five isolates obtained from the enrichment cultures (Figure 5.1). Nevertheless, the wide spread occurrence of microorganisms with thiohydrolase activity in soil was demonstrated by a dilution experiment. Growth (determined as the formation of CO_2) with OTG was observed with an inoculum as small as 2 μg of soil, while growth on octylglucoside was still observed in the dilution containing 20 ng of soil.

Sphingobacterium sp. strain OTG1 was selected from enrichment cultures for its ability to grow on OTG (2 mM) in a medium supplemented with yeast extract. However, in pure cultures growth was inhibited by OTG concentrations exceeding 0.5 mM. As a consequence, *Sphingobacterium* sp. strain OTG1 was cultivated using cellobiose to obtain cell free extract with thioglucoside hydrolase activity (Table 5.1). The inhibition of growth by higher concentrations of OTG was not caused by inhibition of the thioglucoside hydrolase activity. Hydrolysis activity in cell free extract was still observed using 6 mM OTG (Figure 5.1) and almost all of the OTG added was hydrolysed (data not shown). Higher OTG concentrations were not tested because of the critical micelle concentration of OTG (Saito and Tsuchiya, 1984). Experiments using glucose as carbon source supplemented with (0.5 mM) octanol or OT showed complete inhibition of growth, indicating the toxicity of octanol and OT. Apparently in the enrichment cultures with 2 mM OTG other microorganisms were present that consumed the OT formed during OTG hydrolysis.

To determine whether strain OTG1 expressed a single glucosidase with a broad substrate specificity or a specific thioglucosidase (for *S*-glycosides) and a β-glucosidase (for *O*-glycosides), the ratio of *p*NP and OT formation was determined under various conditions. This ratio is defined as the ratio between the formation of *p*-nitrophenolate (*p*NP) from *p*-nitrophenyl β-glucoside (β*p*NPG) and OT from OTG. While this *p*NP/OT formation ratio depended on several factors like growth substrate, growth phase and dialysis (of cell free extract), suggesting the existence of a specific thioglucosidase, the use of β-glucosidase inhibitors proved to be conclusive (Table 5.2). The hydrolysis of β*p*NPG was almost completely inhibited using ascorbic acid and D-gluconic acid lactone and partially inhibited by 1-deoxynojirimycin. The hydrolysis of OTG was not affected by these β-glucosidase inhibitors, making it clear that strain OTG1 cell free extract contains a specific thioglycosidase.

Under the typical β-glucosidase inhibitory conditions, the hydrolysis of octylglucoside was not inhibited (Table 5.2), suggesting that the substrate specificity of the novel thioglucosidase is not restricted to *S*-glucosides only. The ratio of OTG hydrolysis and

octylglucoside hydrolysis only varied between 1.6 and 2.0 in the different cell free extracts (Table 5.1). This further supports the assumption that both substrates are hydrolysed by the same enzyme. The *Sphingobacterium* enzyme was further characterised as a thioglucosidase because the glucose moiety could not be replaced by other sugars (Table 5.4).

The only thioglucosidase reported so far is a myrosinase that is activated by ascorbic acid (Botti et al., 1995; Chen and Halkier, 1999) and inhibited by low concentrations of castanospermine (Chen and Halkier, 1999). However, varying the ascorbic acid concentration did not stimulate OTG hydrolysis and the addition of castanospermine did not result in the inhibition of OTG hydrolysis in cell free extract of cellobiose grown cells. The absence of ascorbic acid stimulation and castanospermine inhibition suggests that *Sphingobacterium* OTG hydrolase and myrosinase are different enzymes.

Various (commercially) available β-glucosidases were also screened for their ability to hydrolyse the *S*-glucosidic linkage present in OTG at either pH 6 or pH 7.5. However, hydrolysis of OTG was not observed. Myrosinase (β-glucosidase) was also not able to hydrolyse OTG, despite its ability to hydrolyse various glucosinolates regardless of the nature of the side chain (Botti et al., 1995; Fenwick et al., 1983). This screening (Table 5.3) therefore demonstrates the unique properties of strain OTG1 thioglucosidase.

In summary, we screened for thioglycosidase activity and were able to isolate *Sphingobacterium* sp. strain OTG1 that expresses a thioglucosidase with a broad substrate specificity. One of our objectives was to synthesise new (thio)glucosides using novel thioglucosidases. Strain OTG1 thioglucosidase was incubated under reversed hydrolysis conditions. However, synthesis of OTG from OT and glucose was not detected. Considering other possible applications of the novel thioglucosidase, the hydrolysis of sinigrin is perhaps the most promising because of the interest in manipulating the glucosinolate levels in vegetables (Mithen et al., 2000). Although *Sphingobacterium* sp. strain OTG1 is able to use OTG as a carbon source, the hydrolysis of sinigrin by strain OTG1 is readily inhibited, most likely due to the accumulation of sinigrin breakdown products.

6

Enzymatic synthesis of thioglucosides using almond β-glucosidase

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ABSTRACT

A selection of different glycosidases was screened for the glycosylation of 1-propanethiol. The β-glucosidases from almond, *Aspergillus niger* and *Caldocellum saccharolyticum* were capable of 1-propanethioglucoside (1-PTG) formation. The almond β-glucosidase showed the highest activity in this reversed hydrolysis type of reaction using glucose as glucosyl donor. Besides 1-propanethiol, also thioglucosides of 2-propanethiol and furfuryl mercaptan were formed by the almond β-glucosidase. The substrate specificity of the almond β-glucosidase with respect to thioglucosylation is restricted to primary and secondary aliphatic thiols. Once the thioglucosides are formed, they are not hydrolysed at a significant rate by almond β-glucosidase. As a consequence the synthesis of 1-PTG could be observed at very low aglycone concentrations (0.5% v/v based on the reaction solution) and high yields (68% based on 1-PT and 41% based on glucose) were obtained. An excess of aglycone, otherwise frequently applied in reversed hydrolysis glycosylation, is therefore not necessary in the glucosylation of 1-PT.

INTRODUCTION

Enzymatic glycosylation of compounds that would otherwise be too volatile or have a low solubility in aqueous systems is of interest to the pharmaceutical, cosmetics and food industries. In general, the synthesis of *O*- and *N*-glycosides have been subjects of extensive research, while *S*-glycosides have received relatively little attention.

In Nature, the *S*-glycosidic linkage mainly occurs in glucosinolates that are plant metabolites. Glucosinolates may be a sink for nutrients like nitrogen and sulphur and the products of glucosinolate hydrolysis probably play an important role in the plant defence system and have a characteristic flavour. The biosynthesis of these glucosinolates involves the use of the UDP-glucose transferring enzyme thiohydroximate glucosyltransferase (Fenwick et al., 1983). Thioglycosides in general, are also useful as specific enzyme inhibitors (Bock et al., 1983), as detergents (e.g. octylthioglucoside, Saito and Tsuchiya, 1984) or as glycosyl donors in oligosaccharide synthesis (Fügedi et al., 1987).

Considering the well known benefits of biocatalysis compared to the classic chemical synthesis routes, it is surprising that (*in vitro*) enzymatic thioglucosylation has only been reported using almond β-glucosidase (Dintinger et al., 1992). Using β-mercaptoethanol as aglycone, both *O*- and *S*- glucosides were formed.

Therefore we screened a comprehensive selection of different glycosidases for thioglycosylating activities and characterised the substrate specificity of the enzyme with the highest activity in more detail.

MATERIALS AND METHODS

Enzymes

β-Galactosidase from *Aspergillus oryzae*, α-glucosidase from *Saccharomyces cerevisiae*, β-glucosidase from almond and *Caldocellum saccharolyticum*, α-mannosidase from Jack beans and the β-thioglucosidase (myrosinase) from *Sinapis alba* were obtained from Sigma. β-Glucosidase from *Aspergillus niger* and α-glucosidase from yeast were obtained from Fluka. *Bacillus circulans* β-galactosidase was obtained from Daiwa Kasai K.K. *Pyrococcus furiosus* β-glucosidase (1.4 mg/mL) was kindly provided by T. Kaper (Wageningen University, Department of Microbiology). All enzymes were used without further purification.

Chemicals

Ethyl β-D-thioglucoside and 2-propane β-D-thioglucoside were obtained from Sigma. Furfuryl mercaptan, heptyl β-D-thioglucoside, 2-methyl 2-propanethiol, 1-propanethiol, 2-propanethiol and thiophenol were obtained from Fluka. 1-Octyl β-D-glucoside and 1-octyl β-D-thioglucoside were obtained from Boehringer.

Enzyme activity

The activities of β-glucosidase and thioglucosidase, α-glucosidase, β-galactosidase, α-*mannosidase* were determined using respectively *p*-nitrophenyl β-D-glucoside (β*p*NPGlu), *p*-nitrophenyl α-D-glucoside (α*p*NPGlu), *o*-nitrophenyl β-D-galactoside (β*o*NPGal) and *p*-nitrophenyl α-D-mannoside (α*p*NPMan). The enzymes were incubated in 50 mM potassium phosphate buffer pH 6.0 at 30°C in a total volume of 1 mL. The glycosidase activity was measured by following the increase in *p*-nitrophenolate (*p*NP) or *o*-nitrophenolate (*o*NP) concentration at 405 nm. The molar extinction coefficients for *p*NP or *o*NP at this pH were respectively 1500 $M^{-1} \times cm^{-1}$ and 760 $M^{-1} \times cm^{-1}$.

One unit (U) of enzyme activity was defined as the amount of enzyme that caused the release of one μmol of the corresponding nitrophenolate per minute in 50 mM potassium phosphate buffer pH 6.0 at 30°C.

Thioglycosylation

A typical thioglycosylation reaction was performed in a 2 mL reaction mixture containing a second phase of 1.5 mL 100% v/v of thiol (75% v/v_{total}) and 0.5 mL aqueous phase (25% v/v_{total}). The aqueous phase contains enzyme and the corresponding glycosyl compound (300 mM glucose, galactose or mannose) in 50 mM potassium phosphate buffer pH 6.0 at 30°C. Note that concentrations are based on this aqueous phase. The reaction was terminated by mixing 20 μl of the reaction sample (aqueous phase) with 380 μl of methanol (100% v/v). The formation of thioglycosides was monitored by high performance liquid chromatography (HPLC).

High performance liquid chromatography

The HPLC system was composed of a Gyncotek pump and autosampler, a Spherisorb S5-amino column (150 × 4.6 mm) and a Sedex evaporative light scattering detector. The samples (20 μl injection volume) were eluted with an acetonitril (AcN) / water gradient (1 mL/min): 0-4 min 85% AcN, 5-10 min 70% AcN, 11-17 min 85% AcN.

A multiport streamswitch valve from Spark Holland was added to the HPLC system for preparative purification of the glucoside. In this case the injection volume was 50 μl.

Product concentrations were calculated using an external standard of ethyl thioglucoside.

Gas chromatography

The amount of 1-propanethiol was measured by analysing 100 μl gas-phase samples on a Hewlett Packard HP 6890 GC-system equipped with a Chrompack CP-WAX 52CB column (30 m × 0.32 mm).

Dinitrosalicylic acid assay

The amount of glucose formed during (thio)glycoside hydrolysis was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). Enzyme and thioglycosides were incubated in 50 mM potassium phosphate buffer pH 6.0 in a volume of 100 μl. To stop

the enzyme reaction, 100 µl of the DNS solution was added and the complete reaction mixture was heated at 100°C for 5 min. After cooling, 1 mL of water was added and the absorption at 575 nm was measured spectrophotometrically. Glucose was used as the standard.

Mass spectroscopic analysis

Mass spectra were measured using a Thermoquest LCQ spectrometer with direct injection.

NMR spectroscopic analysis

^{1}H NMR measurements were carried out on a 200 MHz Brücker AC 200 and a 400 MHz Brücker AC 400 spectrometer.

Identification of thioglucosides

The (thio)glucoside fractions (structure and atom numbering Scheme 6.2) from the preparative HPLC experiments (not optimised) were collected and evaporated under reduced pressure at 40°C.

1-thiopropyl β-D-glucopyranoside (1-propanethioglucoside, 1-PTG):
White powder; ESI-MS: 477 (dimer + H), 277 (M + K); ^{1}H NMR (200 MHz, D_2O): δ 0.74 (t, J = 7.35 Hz, 3H, H-3'), 1.40 (t, J = 7.31 Hz, 2H, H-1'), 2.53 (m, 2H, H-2'), 3.12 (t, J = 8.67, 1H, H-2), 3.22 (br s, 1H, H-5), 3.42 (m, 2H, H-3, H-4), 3.64 (br s, 2H, H-6), 4.30 (d, J = 9.74, 1H, H-1).

2-thiopropyl β-D-glucopyranoside (2-propanethioglucoside):
White powder; ESI-MS: 477 (dimer + H), 277 (M + K); ^{1}H NMR (400 MHz, D_2O): δ 1.14 (d, J = 9.25, 6H, H-1', H-3'), 3.11 (m, 1H, H-2), 3.25 (m, 2H, H-5, H-1), 3.40 (m, 2H, H-3, H-4), 3.54 (m, 2H, H-6), 4.44 (d, J = 9.82, 1H, H-2').

furan-2-methylsulfanyl β-D-glucopyranoside (furfuryl mercaptanthioglucoside):
White powder; ESI-MS: 575 (dimer + Na), 299 (M + Na); ^{1}H NMR (400 MHz, D_2O): δ 3.26 (m, 3H, H-2, H-1'), 3.55 (br s, 1H, H-5), 3.68 (m, 2H, H-3, H-4), 3.70 (s, 2H, H-6), 4.26 (d, J = 9.81, 1H, H-1), 6.24 (d, J = 25.66, 2H, H-2', H-3'), 7.34 (s, 1H, H-4').

RESULTS AND DISCUSSION

Screening for thioglycoside formation

In order to screen for enzymes with thioglycoside forming activities (thioglycosylation), various glycosidase type of enzymes were selected (Table 6.1). This selection was motivated by the (commercial) availability of the enzymes and their reported glycosylation activities (in oligosaccharide synthesis) (Ichikawa et al., 1992). In analogy with the thioglycosylation experiments performed by Dintinger et al. (1992), we used an aliphatic model substrate. To facilitate the purification and characterisation of any

glycoside formed, 1-propanethiol (1-PT, which contains one thiol group) was selected as model glycosyl acceptor (aglycone).

Prior to the thioglycosylation screening experiment, the specific activities of the selected enzymes towards the corresponding nitrophenyl glycosides were determined (Table 6.1). In the thioglycosylation screening experiment a standardised amount of enzyme, 90 U/mL with respect to the hydrolysis of the corresponding nitrophenyl glycoside, was added to each reaction mixture. Using 75% (v/v_{total}) of 1-PT, reversed hydrolysis conditions were applied. After 48 h of incubation, the concentration of 1-propanethioglucoside (1-PTG) was determined (Table 6.1). Three enzymes, β-glucosidase from almond, *Aspergillus niger* and *Caldocellum saccharolyticum* showed thioglycosylation activity (formation of 1-PTG). With the other enzymes, thioglycoside formation was not observed. Some loss of enzyme activity was observed during the 48 h incubation. However, residual activities (formation of nitrophenolate) were still at least 30% of the initial amount added (residual activity, Table 6.1).

Because of the low specific activity of the potentially interesting *Sinapis alba* thioglucosidase (3.0 $nmol.min^{-1}.mg^{-1}$), this enzyme was not used in the thioglycosylation screening experiment.

Table 6.1 Screening for 1-propanethiol glycosylation activity.

enzyme	origin	specific activity[1]		1-PTG[3]	residual activity
		(substrate)[2]	(value)	(mM)	(%)[4]
β-glucosidase	Almond	β*p*NPGlu	3.0	46	90
	Aspergillus niger	β*p*NPGlu	0.25	0.6	60
	Caldocellum saccharolyticum	β*p*NPGlu	2.7	4.8	43
	Pyrococcus furiosus	β*p*NPGlu	27.5	0	32
α-glucosidase	*Saccharomyces cerevisiae*	α*p*NPGlu	16.5	0	72
	Yeast	α*p*NPGlu	70.2	0	43
β-galactosidase	*Aspergillus oryzae*	β*o*NPGal	3.5	0	44
	Bacillus circulans	β*o*NPGal	1.2	0	38
α-mannosidase	Jack beans	β*p*NPMan	13.0	0	43
thioglucosidase	*Sinapis alba*	β*p*NPGlu	0.003	-	-

1 Specific activity ($\mu mol.min^{-1}.mg^{-1}$) using 2 mM of the corresponding nitrophenyl sugar in 50 mM potassium phosphate buffer pH 6.0 at 30°C.

2 Substrates for specific activity measurements respectively *p*-nitrophenyl β-glucoside (β*p*NPGlu), *p*-nitrophenyl α-glucoside (α*p*NPGlu), *o*-nitrophenyl β-galactoside (β*o*NPGal) and *p*-nitrophenyl α-mannoside (β*p*NPMan).

3 Thioglycosylation reactions were performed using 75% (v/v_{total}) 1-propanethiol, 300 mM glucose or galactose or mannose, 90 U/mL of enzyme in 50 mM potassium phosphate buffer pH 6.0 at 30°C in a total volume of 2 mL. Formation of 1-PTG (1-propanethioglycoside) was analysed after 48 h.

4 Residual activity (after 48 h) expressed as the percentage of the initial activity (90 U/mL).

Thioglucosylation by almond β-glucosidase

As the almond β-glucosidase showed the highest thioglucosylation activity, further experiments were performed using this enzyme.

At first the optimal pH for thioglucosylation was determined. Using different buffers (sodium acetate, pH 4-6; potassium phosphate, pH 6-7; Tris-HCl, pH 7-8) the glucosylation of 1-PT was analysed (Figure 6.1). No significant loss of enzyme activity was observed (hydrolysis of β*p*NPG, data not shown). The pH optimum for thioglucoside formation was pH 6, which is in agreement with the pH frequently applied in the synthesis of *O*-glucosides using almond β-glucosidase.

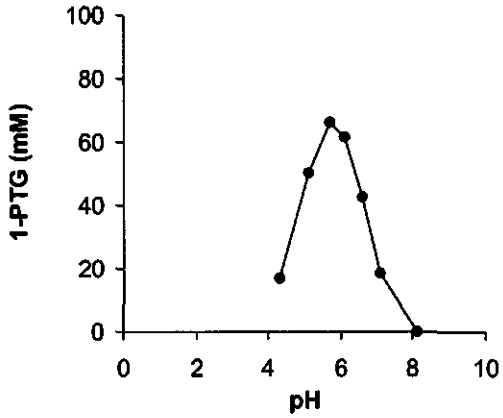
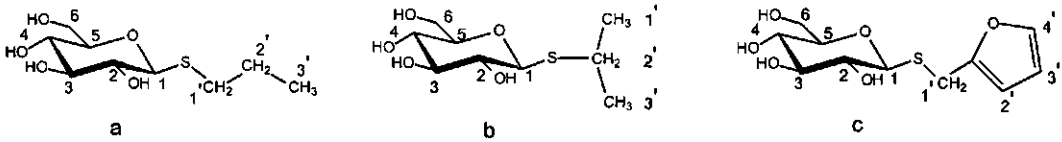


Figure 6.1 The pH optimum of 1-propanethiol glucosylation. Using 75% (v/v_{total}) of 1-propanethiol and 25% (v/v_{total}) aqueous phase containing 300 mM glucose and 90 U/mL of almond β-glucosidase in 50 mM potassium phosphate buffer at 30°C, the formation of 1-propanethioglucoside (1-PTG) was determined at different pH-values after 48 h.

Next the substrate specificity of almond β-glucosidase was analysed in more detail. Besides 1-propanethiol (primary thiol, 1-PT) also 2-propanethiol (secondary thiol, 2-PT), 2-methyl 2-propanethiol (tertiary thiol), thiophenol (aromatic thiol), and furfuryl mercaptan (an aliphatic thiol flavour compound, Burdock, 1995) were tested. Using the same experimental conditions as described in Table 6.1, reaction samples were taken during 100 h of incubation and were analysed by HPLC. The almond β-glucosidase activity (with respect to β*p*NPG hydrolysis) did not significantly decrease during the entire incubation period. With 1-PT, 2-PT and furfuryl mercaptan, a linear formation rate of the thioglucosides was observed during the 100 h period (Table 6.2). These three thioglucosides were recovered from the reaction mixture and identified by ^{1}H NMR and mass spectroscopy (Scheme 6.2).

The glucosylation rate with the secondary thiol (0.056 mU/mg) was 10-fold lower than with the primary thiol (0.57 mU/mg) while the tertiary thiol was not glucosylated at all. The same was observed with *O*-glucosylation, where tertiary alcohols are not glucosylated due to steric hindrance (Vic et al., 1995). Glucosylation of the aromatic thiol group of thiophenol was not detected, which is in line with the observations by

Vic et al. (1995) that aromatic hydroxyl groups were not glucosylated.



Scheme 6.2 Structure and atom numbering of 1-propanethioglucoside (a), 2-propanethioglucoside (b) and furfuryl mercaptan (c).

Thioglucoside stability towards enzymatic hydrolysis

We and others, have observed that thioglucosides are very stable towards enzymatic hydrolysis (Saunders and Timell, 1968; Saito and Tsuchiya, 1984; Meulenbeld and Hartmans, 2001). Consequently, once thioglucosides are formed (e.g. in the screening for thioglucosylating enzymes, Table 6.1) they are not easily hydrolysed. This stability of thioglucosides towards enzymatic hydrolysis has been attributed to the lower basicity of the sulphur atom, which results in a lower degree of protonation. (Saunders and Timell, 1968 and references cited herein).

Table 6.2 Formation and hydrolysis of thioglucosides by almond β-glucosidase.

reaction	compound	thioglucoside (mM)	activity ($nmol.min^{-1}.mg^{-1}$)
thioglucosylation[1]	1-propanethiol	103	0.57
	2-propanethiol	10	0.06
	2-methyl 2-propanethiol	0	0
	thiophenol	0	0
	furfuryl mercaptan	117	0.65
(thio)hydrolysis[2]	octylthioglucoside		< 0.50 [3]
	ethylthioglucoside		< 0.50
	2-propanethioglucoside		< 0.50
	octylglucoside		87
	hexylglucoside		42
	p-nitrophenyl β-glucoside		3000

1 The formation of thioglucosides after 100 h of incubation using 300 mM glucose, 75 % (v/v_{total}) of the corresponding thiol and 90 U/mL of almond β-glucosidase in 50 mM potassium phosphate buffer pH 6.0 at 30°C in a total volume of 2 mL.

2 Hydrolysis rate of 2 mM thioglucoside using 90 U/mL almond β-glucosidase in 50 mM of potassium phosphate buffer pH 6.0 at 30°C. Hydrolysis was determined using the DNS-method.

3 Values below the detection limit of the DNS-assay.

We analysed the stability of various (commercially available) thioglucosides (octylthioglucoside, ethylthioglucoside and 2-propanethioglucoside) towards the

hydrolytic activity of almond β-glucosidase (Table 6.2). After 48 h of incubation, no hydrolysis products (glucose) could be detected. Because almond β-glucosidase hydrolyses long chain aliphatic *O*-glucosides (octylglucoside, hexylglucoside, Table 6.2), the inability of the enzyme to hydrolyse thioglucosides is clearly due to the presence of the *S*-atom.

Reversed hydrolysis thioglucosylation

In general, glycosylation reactions performed under reversed hydrolysis conditions are thermodynamically controlled. In order to increase the formation of glycosides the equilibrium must be shifted towards synthesis. This can be accomplished by lowering the water activity or by adding an excess of aglycone and/or glycosyl moiety (Vic et al., 1995). In our experiment an almost constant rate of 1-PTG formation was observed during the initial 100 h of incubation (Table 6.2). After 600 h of incubating almond β-glucosidase under standard thioglucosylation conditions, the rate of 1-PTG formation decreased but an equilibrium could still not be observed (data not shown).

Because of the absence of thiohydrolase activity of the almond β-glucosidase (Table 6.2), the requirement for high amounts of aglycone (normally applied in reversed hydrolysis glycosylation) in order to shift the equilibrium towards synthesis may not be necessary. We therefore tried to glucosylate 1-PT using low aglycone concentrations (approx. 0.5% v/v based on the reaction solution), to demonstrate that thioglucoside formation is not depending on an excess of aglycone.

This experiment was performed in closed 25 mL glass vials to overcome possible loss of 1-PT (due to evaporation). The reaction was monitored by analysing the decrease of 1-PT in the gas-phase. The reaction solution (2 mL) contained 300 mU/mL almond β-glucosidase, 120 μmol 1-PT (0.5% v/v based on the reaction solution) and 100 mM glucose in 50 mM potassium phosphate buffer pH 6.0 at 30°C. By measuring the amount of 1-PT in the headspace (1-PT partition coefficient 0.26 ± 5%, 30°C) and comparing this with the initially added amount of 1-PT, the amount of 1-PTG was calculated assuming that all of the 1-PT that was consumed is converted into 1-PTG (Figure 6.3). After no further decrease in 1-PT concentration was observed, the amount of 1-PTG was determined using HPLC.

The results as presented in Figure 6.3 show that almond β-glucosidase is able to thioglucosylate 1-PT at low aglycone and glucose concentrations. As a result the final yield of 1-PTG is high, 68% (based on 1-PT) and 41% (based on glucose) according to HPLC measurements. Calculations based on the CG-data result in even higher yields (80% based on 1-PT and 48% based on glucose). These high yields can be obtained because of the lack of hydrolytic activity towards thioglycosides. Using 300 mM glucose the 1-PTG yield was also approximately 80% based on 1-PT (GC). This demonstrates that the formation of 1-PTG is not an equilibrium controlled reaction, normally observed in reversed hydrolysis reactions applied for the synthesis of *O*-glucosides.

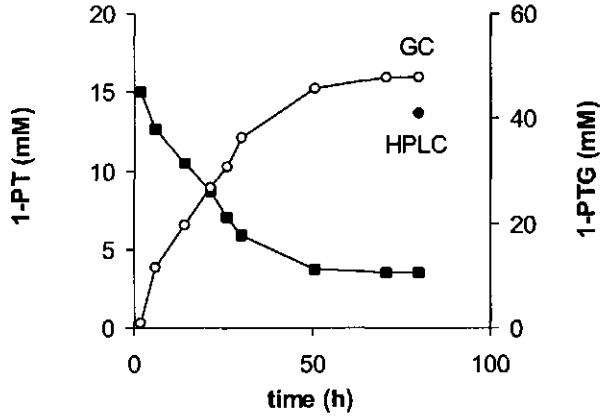


Figure 6.3 Glucosylation of 1-propanethiol, the time dependent decrease of 1-propanethiol (1-PT, ■, determined using GC) and the formation of 1-propanethioglucoside (1-PTG, ●, determined using HPLC). Based on the decrease of 1-PT the formation of 1-PTG (open symbol) was calculated. Reaction was performed in a total liquid volume of 2 mL containing 100 mM glucose, 300 U/mL of almond β-glucosidase and 0.5% (v/v) 1-propanethiol in 50 mM potassium phosphate buffer pH 6.0 at 30°C.

Transglucosylation conditions

The only other study that describes the glucosylation of thiols involves almond β-glucosidase and β-mercaptoethanol (Dintinger et al., 1992). In this study it was suggested that β-mercaptoethanol was glucosylated in a transglucosylation type of reaction when β*p*NPG was used as glucosyl donor, resulting in the formation of the corresponding *O*- and *S*-glucoside. However, based on the amount of almond β-glucosidase added, it can be calculated that the β*p*NPG would be completely hydrolysed long before significant amounts of glucosides were detected. This implies that the formation of the glucosides is a result of a reversed hydrolysis type of reaction, using the accumulated glucose, in stead of a transglucosylation type of reaction.

In our study the rate of β*p*NPG hydrolysis by almond β-glucosidase exceeds the rate of 1-PTG formation (using glucose as donor) by about 5 thousand times (Table 6.2). We also confirmed that the rapid hydrolysis of β*p*NPG (by almond β-glucosidase) was not affected by the presence of various thiols (data not shown). Therefore the use of β*p*NPG in a transglucosylation type of (thioglucoside forming) reaction using almond β-glucosidase seems irrelevant.

In summary, from a variety of glycosidases tested three β-glucosidases showed thioglucosylation activity. The almond β-glucosidase showed the highest thioglucosylation activity towards 1-propanethiol. Only primary and secondary thiols are glucosylated by this enzyme. The formation of furfuryl mercaptan thioglucoside is the most interesting considering the possible applications as a flavour precursor. The absence of thioglucoside hydrolase activity of the almond β-glucosidase prevents the hydrolysis of thioglucosides once formed. Consequently high aglycone concentrations, as normally applied in reversed hydrolysis, are not necessary in order to obtain

thioglucosides.

7

Concluding Remarks

ENZYMATIC GLUCOSYLATION:
sucrose glucosyltransferases and glucosidases in *O*- and *S*-glucoside synthesis

The aim of the research described in this thesis was to explore enzymatic glucosylation of nonsaccharide acceptor molecules (aglycones). Restrictions initially imposed, concern the use of enzymes with transglucosylation activity with the ability to utilise economically feasible glucosyl donor substrates (e.g. sucrose). These restrictions led to the exploration of sucrose glucosyltransferases. In the second part of the thesis the attention was shifted towards glucosidase type of enzymes, especially with respect to the hydrolysis or formation of thioglucosides.

SUCROSE GLUCOSYLTRANSFERASES

Enzyme selection and screening

In nature, glycosides are mostly synthesised by highly specific Leloir type of glycosyltransferases, utilising sugar nucleotides as activated glycosyl donors. These biosynthetic routes have several drawbacks with respect to industrial applications. Alternative enzymes are the frequently applied glycosylhydrolase type of enzymes (glycosidases) and the non-Leloir glycosyltransferases, which are still relatively uncommon in the field of aglycone glycosylation. Based on the literature, three non-Leloir glycosyltransferases have been studied in aglycone glycosylation (Table 1.1), sucrose phosphorylase (SPase), cyclodextrin glucanotransferase (CGTase) and sucrose glucosyltransferase. SPase is an interesting enzyme, because it appears to be capable of glucosylating a wide range of (aromatic) alcohols. As SPase and CGTase have been the subject of earlier research, we focused on the third type of enzyme, sucrose glucosyltransferase (glucansucrases). These extracellular enzymes are mainly produced by *Streptococcus* species present in the oral flora (commonly termed glucosyltransferase) or the soil bacterium *Leuconostoc mesenteroides* (commonly termed dextransucrase). At the start of this research, aglycone glucosylation using glucansucrases had only been demonstrated using a mixture of different glucansucrases (Nakahara et al., 1995), indicating their glucosylation potential towards aglycones. The availability of several cloned glucansucrases enabled a more detailed study.

A potential source to screen for new non-Leloir glycosyltransferases, might be the lactic acid bacteria, capable of utilising lactose as an economical galactosyl donor. These microorganisms display a variety of glycosyltransferases involved in the production of exopolysaccharides. Although the majority of these enzymes are of the Leloir type, some supposedly might operate in a non-Leloir fashion.

In general, one of the problems encountered in the screening for enzymes with (trans)glycosylation activity, is the ubiquitous presence of glycosidases. These enzymes can hydrolyse the newly generated glycosides, hampering the detection of new glucosyltransferases or glycosidases. To avoid hydrolase activity in the screening for enzymes with (trans)glycosylation activity, thiols could be used (see later this chapter) or specific glycosidase inhibitors.

Acceptor specificity

The transglucosylation efficiency of several glucansucrases was examined using a complex model aglycone, catechin (a flavonoid) (Table 7.1). Glucosyltransferase-D (GTF-D) from *Streptococcus mutans* showed the highest transglucosylation efficiency (Chapter 2). Flavonoids are a group of about 4000 naturally occurring compounds that are ubiquitous in plants. Nowadays they are considered to be potentially beneficial to health by virtue of their antioxidant activities. Since most flavonoids are water insoluble or scarcely water soluble their use is limited. However, most of the flavonoids present in plants are glycosylated (Formica and Regelson, 1995; Di Carlo et al., 1999). Enzymatic glycosylation of flavonoids can also be used to reduce the bitter taste of flavonoids (Komentani et al., 1996; Sato et al., 2000). As catechin was efficiently glucosylated by GTF-D, it would be of interest to examine the acceptor specificity of GTF-D towards other (industrial relevant) flavonoids.

In calculating reaction yields, the data are usually based on the aglycone moiety, often not revealing the inefficient use of the glycosyl donor. A typical example is the yield of 81% reported for sucrose phosphorylase, which was obtained using 30-fold excess of sucrose. Consequently only 3% of the initially added amount of glucosyl donor is transferred to catechin. By comparing the glucoside yields based on catechin and sucrose, it is obvious that catechin is most efficiently glucosylated by GTF-D (Table 7.1).

Table 7.1 Yields of catechin glucosylation

enzyme (source)[1]	yield (%) based on:[2]	
	catechin	glycosyl donor
sucrose glucosyltransferase-D (*S. mutans*)		
standard incubation (Figure 2.4)	66	30
addition of *P. pastoris* (Figure 2.4)	80	13
addition of MEE (Figure 3.2)	37	55
sucrose phosphorylase (*L. mesenteroides*)	81	3.1
crude enzyme (*X. campestris*)	52	1.0
α-amylase (*B. subtilis*)	25 [3]	14
sucrose glucosyltransferase (*S. sobrinus*)	14	0.8
cyclodextrin glucanotransferase (*B. macerans*)	1.9 [4]	7.6 [4]
GSase, amylase (*B. subtilis*)	nd [5]	nd

1 For references see Table 1.1.
2 Yield (molar based), expressed as the ratio of moles of catechin glucoside formed to the amount of catechin or glycosyl donor initially added.
3 Assuming that the published efficiency is molar based.
4 Mass based.
5 No data available.

Furthermore, GTF-D efficiency can be engineered by modification of the reaction conditions. By adding *Pichia pastoris* higher catechin based yields can be obtained (Chapter 2), while addition of the cosolvent bis-2-methoxyethyl ether (MEE) results in

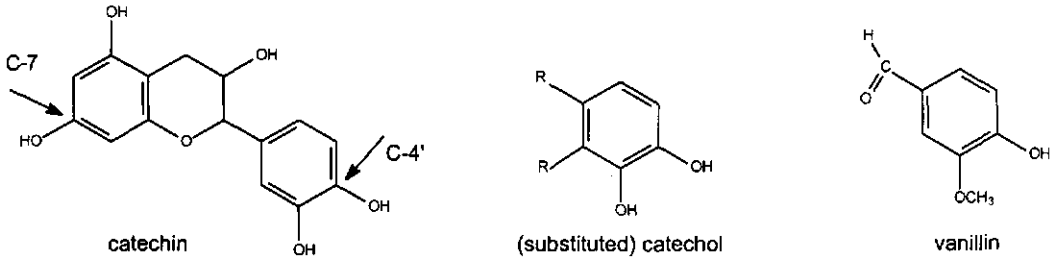
increased sucrose based yields (Chapter 3).

Because of the industrial relevance the acceptor specificity of GTF-D was examined in more detail based on the elucidated catechin glucoside structures and especially glucosylation on C-4'. Using a range of hydroxy aromatic compounds, only the dihydroxy aromatic compounds containing two adjacent aromatic hydroxyl groups are (α-) glucosylated by GTF-D (Chapter 3). This suggests that one hydroxyl group is involved in the interaction with GTF-D, while the other is available for glucosylation. Substituents next to the aromatic hydroxyl groups of catechol only affect the rate of transglucosylation by GTF-D, but not its specificity.

From an industrial point of view, vanillin (as a flavour compound) is an important aglycone, and was one of the initial target molecules of this research. Vanillin does not contain the dihydroxy aromatic structure, important in GTF-D transglucosylation. Another problem encountered in vanillin glucosylation is its low water solubility. To increase the water solubility of vanillin and a range of other potential aglycones, the addition of a cosolvent was examined (Chapter 3). By adding the organic cosolvent bis-2-methoxyethyl ether (MEE) the specific catechin (again used as model aglycone) transglucosylation activity could be increased 4-fold due to a 12-fold increase in catechin solubility. Despite the increase in vanillin concentration due to MEE addition, formation of vanillin glucosides was not observed.

The experiments conducted with water miscible cosolvents (Chapter 3) suggest that aliphatic diols are also glycosylated. It would be interesting to examine this in more detail. The same accounts for the synthesis of glucosides using cyclohexane diols, possibly resulting in the formation of enantiomere pure glucosides.

GTF-D was selected on the basis of its catechin glucosylation efficiency. The acceptor specificity of other glucansucrases was not examined. In view of the distinct types of glucan formed by different glucansucrases, it is anticipated that the acceptor specificity of the different glucansucrases will vary.



Scheme 7.1 Analogy between vanillin, which is not glycosylated by GTF-D and acceptors catechin and (substituted)catechol.

Fructose conversion

Glucansucrase transglucosylation is complex and different reactions are possible. As the reaction proceeds the accumulation of fructose (increasingly) inhibits the glucosyl transfer to the aglycone as well as to the (growing) glucan chain. The inhibitory

influence of fructose was minimised by adding a yeast, *Pichia pastoris* that was able to directly consume the accumulating fructose and was incapable of utilising sucrose (Chapter 2). As a result of *P. pastoris* addition the duration of the maximum transglucosylation rate was increased and (in the case of catechin) higher glucoside yields were obtained. The glucosyl transfer to water, e.g. sucrose hydrolysis, was also significantly higher under these conditions. The economic benefit of fructose removal will therefore depend on the cost of the acceptor in comparison with sucrose and on the reaction conditions that will be applied.

Alternatively, the conversion of fructose towards more valuable compounds than *P. pastoris*, biomass and CO_2 may be of interest. One of the methods is the chemical reduction of fructose to sorbitol and mannitol. Both compounds do not affect GTF-D activity. The ability to reduce fructose to mannitol (by a mannitol dehydrogenase), as observed in *Leuconostoc mesenteroides* and *L. pseudomesenteroides*, could result in an integrated system of dextran production and fructose consumption (Vandamme et al., 1987; Grobben et al., 2001). Other, even more complicated (expensive) methods are the phosphorylation of fructose using hexokinase followed by conversion into glucose using phosphoglucose isomerase and the reduction of fructose to sorbitol using a novel NADPH-dependent ketose reductase from the silverleaf withefly (Salvucci et al., 1998).

In an attempt to broaden GTF-D acceptor specificity, the possibility of competition between fructose and potential acceptor molecules was examined. However, the removal of fructose, as a result of *P. pastoris* addition, did not appear to broaden GTF-D acceptor specificity. *P. pastoris* was able to withstand (the toxic) MEE for a period of time, without loss of fructose consuming activity. Addition of 2-methoxy-4-methylphenol (a vanillin analogue) to this MEE/*P. pastoris* system did not result in the formation of the corresponding glycoside, despite the consumption of fructose.

Glucan formation

Bis-2-methoxyethyl ether (MEE) exerts different effects on glucosyltransferase-D. Besides acting as a cosolvent (increasing the operational aglycone concentration) and reducing the inhibitory effect of high aglycone concentrations, an effect of MEE on glucan and maltooligosaccharide formation was also observed. By adding MEE, the formation rate and absolute amounts of glucan was increased and also a minor shift of the molecular weight distribution (also in case of maltooligosaccharides) and a change in the type of glycosidic linkage was observed (Chapter 4). With respect to engineering the glucan molecular weight distribution, it was suggested that addition of catechin also affects glucan synthesis. The presence of catechin probably results in the formation of shorter glucan chains (Chapter 2). Our experiments demonstrate that glucan (and maltooligosaccharide) formation by GTF-D can be engineered by MEE and aglycone addition. This provides an alternative for the screening of various glucansucrases to form specific glucan or maltooligosaccharide products.

To explain the influence of MEE in glucan formation, it was hypothesised that the apolar cosolvent MEE affects the glucan binding domain. Perhaps by a similar type of allosteric activation as is also observed in dextran formation, where dextran binding

supposedly induced an enzyme conformation favourable for (dextran) synthesis (Robyt and Corrigan, 1977; Robyt, 1995). MEE was also shown to stimulate GTF-D transglucosylation (Chapter 3) by changing the partitioning of the aglycone between the solvent and the protein. Combining the glucan and aglycone stimulation, it is hypothesised that addition of MEE exerts an effect on the quality of the solvent by creating a less polar environment. This change in solvent quality may influence the (inhibitory) aglycone concentrations at the solvent/protein interface and may change the enzyme conformation. Consequently resulting in glucosylation at otherwise inhibitory aglycone concentrations and the increased formation of high molecular weight glucans.
A promising new application of glucansucrases could be the conversion of sucrose to (soluble) glucans in fruit juices. In this way calorific sucrose would be converted into glucans. These glucans might have dietary fibre or prebiotic functions. The pH tolerance of GTF-D (Chapter 4) allows its use in the low pH environment as observed in different fruit juices. To overcome the inhibitory influence of fructose, yeasts could be added (Chapter 2).

Mechanism

The controversy in the literature about the precise mechanism of glucan chain elongation and aglycone glucosylation remains unsettled (Robyt, 1995; Monchois et al., 1999a). However, the stimulation of glucan formation by MEE tends to indicate that GTF-D uses a non-reducing end elongation mechanism. According to this mechanism, the glucan chain is elongated in a discontinuous manner. Hypothesising that MEE affects the GTF-D glucan binding domain, one could argue that chain elongation would be more influenced in the non-reducing end elongation mechanism, with the repeated release of the glucan chain, compared to the reducing end elongation mechanism where the glucan chain remains attached to the active site. Recent sequence analysis data seem to support chain elongation by the non-reducing end mechanism. These analysis show some resemblance between glucansucrase and the α-amylase family, operating by the non-reducing end mechanism (MacGregor et al., 1996; Monchois et al., 1999a). However, elucidation of the exact 3D-structure of glucansucrase is probably necessary to resolve the dispute.

GLUCOSIDASES

Screening for novel thioglucosidases

As mentioned earlier, the screening for novel glycoside forming enzymes is hampered by the presence of glycosidases. However, this seems only valid for *O*-glycosylation, because *S*-glycosides are very resistant towards enzymatic hydrolysis. This observation suggests that thioglycoside hydrolases could be (once isolated) very interesting enzymes, potentially expanding the pool of enzymes normally used in glycosylation.
Only a few thioglucosides (octylthioglucoside and isopropylthioglucoside) are commercially available for use as a carbon source in enrichment experiments. Using octylthioglucoside, thioglucosidase activity was detected in the cell free extract of

Sphingobacterium sp. strain OTG1 (Chapter 5). The relative ease, with which microorganisms were isolated using OTG, suggests that a more comprehensive screening could result in the isolation of other microorganisms containing thioglucosidase activity.

Glucosidase thioglucosylation

The screening for glycosylation activity of glycosidases using thiols resulted in the identification of three enzymes capable of forming 1-propane thioglycoside (Chapter 6). The three enzymes are β-glucosidases from different origins. Interestingly the frequently used β-glucosidase from almond, showed the highest yield. Only primary and secondary aliphatic thiols are (β-)glucosylated by the almond β-glucosidase. Possibly GTF-D, which was successively shown to glucosylate aromatic alcohols (Chapter 2 and 3), is also able to glucosylate aromatic thiols e.g. 2-thiophenol, which are not glucosylated by almond β-glucosidase.

Because of the difficulty of hydrolysing thioglucosides, the kinetic equilibrium in the reversed hydrolysis type of reaction, is automatically shifted towards synthesis (Chapter 6). Therefore *S*-glycosylation could be regarded as an apparent type of reversed hydrolysis, distinct from the normal reversed hydrolysis requiring an excess of acceptor or lowered water activity (Table 7.2).

Table 7.2 General characteristics of enzymatic aglycone glycosylation.

mechanism (enzyme)	aglycone	glycosyl donor
reversed hydrolysis		
•glycosylhydrolases (generally available)	▪high aglycone concentrations and/ or low water activity (organic phase) ▪wide range of aliphatic (alkyl /non-alkyl) aglycones ▪generally low yields	▲monosaccharides (not activated) ▲high concentrations required
apparent reversed hydrolysis[1]		
•glycosylhydrolases (generally available)	▪low aglycone concentration ▪only *S*-glycosylation (aliphatic thiols) ▪high yields	▲monosaccharides (not activated) ▲concentration independent
transglycosylation[2]		
•glycosyltransferases (Leloir and non-Leloir, generally less available) •glycosylhydrolases (generally available)	▪Leloir: highly specific (aromatic alcohols and saccharides) ▪non-Leloir and glycosylhydrolases: wide range of aromatic and aliphatic (alkyl/non-alkyl) aglycones ▪concentration independent ▪generally high yields	▲Leloir: sugar nucleotides ▲non-Leloir: disaccharides or phosphorylated sugars ▲glycosylhydrolases: disaccharides

1) Based on Chapters 5 and 6. 2) Based on Chapters 2-4.

INDUSTRIAL PROSPECTS

The need for industry to innovate initiated the research on enzymatic synthesis of glycosides. To stimulate the collaboration between universities and industry the Netherlands Ministry of Economic Affairs started the IOP program on catalysis (Innovation Oriented research Program). Within the IOP framework the research as described in this thesis (project number IKA96006) was performed in cooperation with the industrial partners, DSM Gist - Delft, Friesland Coberco Dairy Foods - Deventer, Quest - Bussum and Unilever - Vlaardingen. Some of the results described in this thesis justify the industrial interest and may encourage further research (Table 7.3).

Table 7.3 Enzymatic aglycone glycosylation, brief overview of industrial justification and areas of further (applied) research.

enzyme	
glucosyltransferase-D[1]	**industrial justification** •GTF-D can be used to transglucosylate aromatic alcohols in high yields using sucrose as glucosyldonor **further research** •application of aromatic alcohol glucosides (catechol derivatives and flavonoids) •screening and engineering of other glucansucrases (glucosyltransferase, dextransucrase, amylosucrase, levansucrase) for aglycone glycosylation •engineering by fructose conversion, cosolvent addition, genetic engineering and cloning in other (generally accepted) host •conversion of calorific sucrose to dietary glucan, especially in fruit juice
almond β-glucosidase[2]	**industrial justification** •almond β-glucosidase can be used to glucosylate primary and secondary thiols with high yields **further research** •application of thioglucosides (e.g. furfuryl mercaptan as flavour compound) and further optimisation of yields

1) Based on Chapters 2-4. 2) Based on Chapter 6.

References

Abo H, Matsumura T, Kodama T, Ohta H, Fukui K, Kato K, Kagawa H. 1991. Peptides sequences for sucrose splitting and glucan binding within *Streptococcus sobrinus* glucosyltransferase (water-insoluble glucan synthetase). J Bacteriol 173: 989-996.

Aoki H, Shiroza T, Hayakawa M, Sato S, Kuramitsu HK. 1986. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. Infect Immun 53: 587-594.

Barker PE, Ganetsos G, Ajongwen NJ. 1993. A novel approach to the production of clinical-grade dextran. J Chem Tech Biotechnol 57:21-26.

Bechthold A, Berger U, Heide L. 1991. Partial purification, properties, and kinetic studies of UDP-glucose: *p*-hydroxybenzoate glucosyltransferase from cell cultures of *Lithospermum erythrorhizon*. Arch Biochem Biophys 288: 39-47.

Bock K, Defaye J, Driguez H, Bar-Guilloux E. 1983 Conformations in solution of α,α-trehalose, α-D-glucopyranosyl α-D-mannopyranoside, and their 1-thioglycosyl analogs, and a tentative correlation of their behaviour with respect to the enzyme trehalase. Eur J Biochem 131: 595-600.

Botti MG, Taylor MG, Botting NP. 1995. Studies on the mechanism of myrosinase. J Biol Chem 270: 20530-20535.

Burdock GA. 1995. Fenaroli's handbook of flavor ingredients, 3rd edition. CRC Press, Inc.

Chen S, Halkier BA. 1999. Functional expression and characterization of the myrosinase MYR1 from *Brassica napus* in *Saccharomyces cerevisiae*. Prot Expression Purif 17: 414-420.

Côté GL, Robyt JF. 1982. Isolation and partial characterization of an extracellular glucansucrase form *Leuconostoc mesenteroides* NRRL B-1355 that synthesizes an alternating (1,6), (1,3)-α-D-glucan. Carbohydr Res 101:57-74.

Crittenden RG, Playne MJ. 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Science Technol 7: 353-361.

Crout DHG, Vic G. 1998. Glycosylation and glycosyl transferases in glycoside and oligosaccharide synthesis. Curr Opinion Chem Biol 2: 98-111.

Cui CB, Tezuka Y, Kikuchi T, Nakano H, Tamaoki T, Park JH. 1992. Constituents of a fern, *Davallia mariesii* Moore. IV. Isolation and structures of a novel norcarotane sesquiterpene glycoside, a chromone glucuronide, and two epicatechin glycosides. Chem Pharm Bull 40: 2038-2040.

Davis AL, Cai Y, Davies AP, Lewis JR. 1996. 1H and 13C NMR assignments of some green tea polyphenols. Magn Reson Chem 34: 887-890.

Devulapalle K, Goodman SD, Gao Q, Hemsley A, Mooser G. 1997. Knowledge-based model of a glucosyltransferase from the oral bacterial group of mutans streptococci. Prot Science 6: 2489-2493.

Devulapalle KS, Mooser G. 1994. Subsite specificity of the active site of glucosyltransferases from *Streptococcus sobrinus*. J Biol Chem 269: 11967-11971.

Di Carlo G, Mascolo N, Izzo AA, Capasso F. 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Science 65: 337-353.

Dintinger T, Dutheil-Bouëdec D, Bouchonneau M, Tellier C. 1994. Synthesis of β-mercaptoethyl-glycosides by enzymatic reverse hydrolysis and transglycosylation. Biotechnol Lett 16: 689-692.

Dordick JS. 1989. Enzymatic catalysis in monophasic organic solvents. Enzyme Microb Technol 11: 194-211.

Dordick JS. 1992. Enzymatic and chemoenzymatic approaches to polymer synthesis. Trends Biotechnol 10: 287-293.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350-356.

Ducret A, Giroux A, Trani M, Lortie R. 1995. Enzymatic preparation of biosurfactants from sugar alcohols and fatty acids in organic media under reduced pressure. Biotechnol Bioeng 48: 214-221.

Ebert KH, Schenk G. 1968. Mechanism of biopolymer growth, the formation of dextran and levan. Adv Enzymol 30: 179-310.

Eichel-Streiber C, Sauerborn M, Kuramitsu HK. 1992. Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and *Streptococcus mutans* glucosyltransferases. J Bacteriol 174: 6707-6710.

Endo T, Koizumi S. 2000. Large-scale production of oligosaccharides using engineered bacteria. Curr Opinion Struct Biol 10: 536-541.

Fenwick GR, Heaney RK, Mullin WJ. 1983. Glucosinolates and their breakdown products in food and food plants. Crit Rev Food Science Nutr 18: 123-202.

Ferretti JJ, Gilpin ML, Russell RRB. 1987. Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* Mfe28. J Bacteriol 169: 4271-4278.

Foo LY, Karchesy JJ. 1989. polyphenolic glycosides from douglas fir inner bark. Phytochemistry 28:1237-1240.

Formica JV, Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33: 1061-1080.

Fügedi P, Garegg PJ, Lönn H, Norberg T. 1987. Thioglycosides as glycosylating agents in oligosaccharide synthesis. Glycoconjugate J 4: 97-108.

Fukui K, Moriyama T, Miyake Y, Mizutani K, Tanaka O. 1982. Purification and properties of glucosyltransferase responsible for water-insoluble glucan synthesis from *Streptococcus mutans*. Infect Immun 37: 1-9.

Funayama M, Arakawa H, Yamamoto R, Nishino T, Shin T, Murao S. 1994. A new microorganism producing a glucosyl transfer enzyme to polyphenols. Biosci Biotechnol Biochem 58: 817-821.

Funayama M, Nishino T, Hirota A, Murao S, Takenishi S, Nakano H. 1993. Enzymatic synthesis of (+)-catechin-α-glucoside and its effect on tyrosinase activity. Biosci Biotechnol Biochem 57: 1666-1669.

Furuta T, Koga T, Nisizawa T, Okahashi N, Hamada S. 1985. Purification and characterization of glucosyltransferases from *Streptococcus mutans* 6715. J Gen Microbiol 131: 285-293.

Furutani M, Iwaki M, Yagi T, Iida M, Horiike K, Nozaki M. 1988. A simple purification method for a glucosyltransferase complex from *Streptococcus mutans* OMZ 176 with a high yield. Int J Biochem 20:1327-1332.

Germaine GR, Chludzinski AM, Schachtele CF. 1974. *Streptococcus mutans* dextransucrase: requirement for primer dextran. J Bacteriol 120: 287-294.

Giffard PM, Allen DM, Milward CP, Simpson CL, Jacques NA. 1993. Sequence of the gtfK gene of *Streptococcus slaivarius* ATCC 25975 and evolution of the gtf genes of oral streptococci. J Gen Microbiol 139: 1511-1522.

Giffard PM, Simpson CL, Milward CP, Jacques NA. 1991. Molecular characterisation of a cluster of at least two glucosyltransferases genes in *Streptococcus salivarius* ATCC 25975. J Gen Microbiol 137: 2577-2593.

Girard E, Logy MD. 1999. Activity and stability of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F in the presence of organic solvents. Enzyme Microb Technol 24: 425-432.

Gonzalez R, Thiem J. 1999. Chemoenzymatic synthesis of space-linked oligosaccharides for the preparation of neoglycoproteins. Carbohydr Res 317: 180-190.

Grobben GJ, Peters SWPG, Wisselink HW, Weusthuis RA, Hoefnagel MHN, Hugenholtz J, Eggink G. 2001. Spontaneous formation of a mannitol-producing variant of *Leuconostoc pseudomesenteroides* grown in the presence of fructose. Appl Environ Microb 67: 2867-2870.

Gunstone FD. 1999. Enzymes as biocatalysts in the modification of natural lipids. J Science Food Agric 79: 1535-1549.

Hamada S, Slade HD. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol Rev 44: 331-384.

Hanada N, Kuramitsu HK. 1988. Isolation and characterization of the *Streptococcus mutans* gtfC gene coding for both soluble and insoluble glucan synthesis. Infect Immun 56: 1999-2005.

Hanada N, Kuramitsu HK. 1989. Isolation and characterization of the *Streptococcus mutans* gtfD gene coding for primer-dependent soluble glucan synthesis. Infect Immun 57: 2079-2085.

Hartmans S, Kaptein A, Tramper J, De Bont JAM. 1991. Characterization of a *Mycobacterium* sp. and a *Xanthobacter* sp. for the removal of vinyl chloride and 1,2-dichloroethane from waste gases. Appl Microbiol Biotechnol 37: 796-801.

Hassan MA, Ismail F, Yamamoto S, Yamada H, Nakanishi K. 1995. Enzymatic synthesis of galactosylkojic acid with immobilized β-galactosidase from *Bacillus circulans*. Biosc Biotechnol Biochem 59: 543-545.

Hehre EJ. 1953. Enzymatic synthesis of polysaccharides: a biological type of polymerization. Adv Enzymol 11: 297-337.

Honda O, Kato C, Kuramitsu HK. 1990. Nucleotide sequence analysis of the *Streptococcus mutans* gtfD gene encoding the glucosyltransferase-S enzyme. J Gen Microbiol 136: 2099-2105.

Ichikawa Y, Look GC, Wong CH. 1992. Enzyme-catalyzed oligosaccharide synthesis. Anal Biochem 202: 215-238.

Janeček Š, Svensson B, Russell RRB. 2000. Location of repeat elements in glucansucrases of *Leuconostoc* and *Streptococcus* species. FEMS Microbiol Lett 192: 53-57.

Jeanes A, Seymour FR. 1979. The α-D-glucopyranosidic linkages of dextrans: comparison of percentages from structural analysis by periodate oxidation and by methylation. Carbohydr Res 74: 31-40.

Johansson E, Hedbys L, Larsson PO. 1991. Enzymatic synthesis of monosaccharide-amino acid conjugates. Enz Microb Technol 13: 781-787.

Jones PR, Møller BL, Høj PB. 1999. The UDP-glucose: *p*-hydroxymandelonitrile-O-glucosyltransferase that catalyzes the last step in synthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. J Biol Chem 274: 35483-35491.

Kato C, Kuramitsu HK. 1990. Carboxyl-terminal deletion analysis of the *Streptococcus mutans* glucosyltransferase-I enzyme. FEMS Microbiol Lett 72: 299-302.

Kato C, Nakano Y, Lis M, Kuramitsu HK. 1992. Molecular genetic analysis of the catalyic site of *Streptococcus mutans* glucosyltransferases. Biochem Biophys Res Comm 189: 1184-1188.

Kim D, Robyt JF. 1994. Production and selection of mutants of *Leuconostoc mesenteroides* for glucansucrases. Enzyme Microb Technol 16: 659-664.

Kim D, Robyt JF. 1995. Dextransucrase constitutive mutants of *Leuconostoc mesenteroides* B-1299. Enzyme Microb Technol 17: 1050-1056.

Kitao S, Ariga T, Matsudo T, Sekine H. 1993. The synthesis of catechin-glucosides by transglycosylation with *Leuconostoc mesenteroides* sucrose phosphorylase. Biosci Biotechnol Biochem 57: 2010-2015.

Kitao S, Matsudo T, Sasakai T, Koga T, Kawamura M. 2000. Enzymatic synthesis of stable, odorless, and powdered furanone glucosides by sucrose phosphorylase. Biosci Biotechnol Biochem 64: 134-141.

Kitao S, Sekine H. 1992. Transglucosylation catalyzed by sucrose phosphorylase from *Leuconostoc mesenteroides* and production of glucosyl-xylitol. Biosci Biotechnol Biochem 56: 2011-2014.

Kitao S, Sekine H. 1994. α-D-Glucosyl transfer to phenolic compounds by sucrose phosphorylase from *Leuconostoc mesenteroides* and production of α-arbutin. Biosci Biotechnol Biochem 85: 38-42.

Kitao S, Sekine H. 1994. Synthesis of two kojic acid glucosides with sucrose phosphorylase from *Leuconostoc mesenteroides*. Biosci Biotechnol Biochem 85: 419-420.

Kitaoka M, Robyt JF. 1999. Mechanism of the action of *Leuconostoc mesenteroides* B-512FMC dextransucrase: kinetics of the transfer of D-glucose to maltose and the effects of enzyme and substrate concentrations. Carbohydr Res 320:183-191.

Klibanov AM. 2001. Improving enzymes by using them in organic solvents. Nature 409:241-246.

Kobayashi M, Matsuda K. 1980. Characterization of the multiple forms and main component of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Biochim Biophys Acta 614: 46-62.

Kobayashi M, Matsuda K. 1986. Electrophoretic analysis of the multiple forms of dextransucrase from *Leuconostoc mesenteroides*. J Biochem 100: 615-621.

Kobayashi M, Yokoyama I, Matsuda K. 1986. Substrate binding sites of *Leuconostoc* dextransucrase evaluated by inhibition kinetics. Agric Biol Chem 50: 2858-2590.

Koepsell HL, Tsuchiya HM, Hellman NN, Kazenko A, Hoffman CA, Sharpe ES, Jackson RW. 1951. Enzymatic synthesis of dextran: acceptor specificity and chain initiation J Biol Chem 200: 793-801.

Komentani T, Nishimura T, Nakae T, Takii H, Okada S. 1996. Synthesis of neosperidin glycosides and naringin glycosides by cyclodextrin glucanotransferase from an alklophilic *Bacillus* species. Biosci Biotechnol Bioeng 60: 645-649.

Komentani T, Terada Y, Nishimura T, Takii H, Okada S. 1994. Purification and characterisation of cyclodextrin glucanotransferase form an alkalophilic *Bacillus* species and transglycosylation at alkaline pH. Biosci Biotechnol Bioeng 58: 517-520.

Kretzschmar G, Stahl W. 1998. Large scale synthesis of linker-modified sialyl Lewis^x, Lewis^x and N-acetyllactosamine. Tetrahedron 54: 6341-6358.

Lee RT, Lee YC. 1974. Synthesis of 3-(2-aminoethylthio)propylglycosides. Carbohydr Res 37: 193-201.

Lee SJ, Kim JC, Kim MJ, Kitaoka M, Park CS, Lee SY, Ra MJ, Moon TW, Robyt JF, Park KH. 1999. Transglycosylation of naringin by *Bacillus stearothermophilus* maltogenic amylase to give glycosylated naringin. J Agric Food Chem 47: 3669-3674.

Leloir LF. 1974. Two decades of research on the biosynthesis of saccharides. Science 172: 1299-1303.

Lis M, Shiroza T, Kuramitsu HK. 1995. Role of the C-terminal direct repeating units of the *Streptococcus* glucosyltransferase-S in glucan binding. Appl Environ Microbiol 61: 2040-2042.

Ljunger G, Adlercreutz P, Mattiasson B. Enzymatic synthesis of octyl-β-glucoside in octanol at controlled water activity. Enzyme Microb Technol 16: 751-755.

Loesche WJ. 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 50: 353-380.

Ma Y, Lassiter MO, Banas JA, Galperin MY, Taylor KG, Doyle RJ. 1996. Multiple glucan-binding proteins of *Streptococcus sobrinus*. J Bacteriol 178: 1572-1577.

MacCregor EA, Jespersen HM, Svensson. 1996. A circularly permuted α-amylase-type α/β barrel structure in glucan-synthesizing glucosyltransferase. FEBS Lett 378: 263-266.

Matsuo I, Isomura M, Ajisaka K. 1999. The first synthesis of Neu5Acα2-3Galβ1-4ClcNAcβ1-2Manα1-Ser, a newly discovered component of α-dystroglycan. Tetrahedron Lett 40: 5047-5050.

Matsuo I, Isomura M, Miyazaki T, Sakakibara T, Ajisaka K. 1998. Chemoenzymatic synthesis of the branched oligosaccharides which corresponds to the core structures of N-linked sugar chains. Carbohydr Res 305: 401-413.

Meulenbeld GH, Hartmans S. 2000. Transglycosylation by *Streptococcus mutans* GS-5 glucosyltransferase-D: acceptor specificity and engineering of reaction conditions. Biotechnol Bioeng 70: 364-369.

Meulenbeld GH, Hartmans S. 2001. Thioglucosidase activity from *Sphingobacterium* sp. strain OTG1. Appl Microbiol Biotechnol 56: 700-706.

Meulenbeld GH, Zuilhof H, Van Veldhuizen A, Van den Heuvel RHH, Hartmans S. 1999. Enhanced (+)-catechin transglucosylating activity of *Streptococcus mutans* GS-5 glucosyltransferase-D due to fructose removal. Appl Environ Microbiol 65: 4141-4147.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426-428.

Minami Y, Yazawa K, Tamura Z, Tanaka Z, Yamamoto T. 1983. Selectivity of utilization of galactosyl-oligosaccharides by bifidobacteria. Chem Pharm Bull 31: 1688-1691.

Mithen RF, Dekker M, Verkerk R, Rabot S, Johnson IT. 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. J Science Food Agric 80: 967-984.

Monchois V, Arguello-Morales M, Russell RRB. 1999b Isolation of an active catalytic core of *Streptococcus downei* Mfe28 GTF-I glucosyltransferase. J Bacteriol 181: 2290-2292.

Monchois V, Lakey JH, Russell RRB. 1999d. Secondary structure of *Streptococcus downei* GTF-I glucansucrase. FEMS Microbiol Lett 177: 243-248.

Monchois V, Remaud-Simeon M, Russell RRB, Monsan P, Willemot RM. 1997. Characterization of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase (DRSR) and identification of amino-acid residues playing a key role in enzyme activity. Appl Microbiol Biotechnol 48: 465-472.

Monchois V, Reverte A, Remaud-Simeon M, Monsan P, Willemot RM. 1998. Effect of *Leuconostoc mesenteoides* NRRL B-512F dextransucrase carboxyl-terminal deletions on dextran and oligosaccharide synthesis. Appl Environ Microbiol 64: 1644-1649.

Monchois V, Vignon M, Russell RRB. 1999c. Isolation of key amino acid residues at the N-terminal end of the core region *Streptococcus downei* glucansucrase, GTF-I. Appl Microbiol Biotechnol 52: 660-665.

Monchois V, Vignon M, Russell RRB. 2000. Mutagenis of asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. Appl Environ Microbiol 66:1923-1927.

Monchois V, Willemot RM, Monsan P. 1999a. Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol Rev 23: 131-151.

Monchois V, Willemot RM, Remaud-Simeon M, Croux C, Monsan P. 1996. Cloning and sequencing of a gene coding for a novel dextransucrase from *Leuconostoc mesenteroides* NRRL B-1229 synthesizing only α(1,6) and α(1,3) linkages. Gene 182: 23-32.

Monsan P, Paul F. 1995. Enzymatic synthesis of oligosaccharides. FEMS Microbiol Rev 16: 187-192.

Mooser G, Hefta SA, Paxton RJ, Shively JE, Lee TD. 1991. Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* α-glucosyltransferases. J Biol Chem 14: 8916-8922.

Mooser G, Shur D, Lyou M, Watanabe C. 1985. Kinetic studies on dextransucrase from the cariogenic oral bacterium *Streptococcus mutans*. J Biol Chem 260: 6907-6915.

Murase H, Yamauchi R, Kato K, Kunieda T, Terao J. 1997. Synthesis of a novel vitamin E derivative 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol, by α-glucosidase-catalyzed transglycosylation. Lipids 32: 73-78.

Nakagawa H, Yoshiyama M, Shimura S, Kirimura K, Usami S. 1996. Anomer selective formation of *l*-methyl α-glucopyranoside by α-glucosidase catalyzed reaction. Biosci Biotechnol Biochem 60: 1914-1915.

Nakahara K, Kontani M, Ono H, Kodama T, Tanaka T, Ooshima T, Hamada S. 1995. Glucosyltransferase from *Streptococcus sobrinus* catalyzes glucosylation of catechin. Appl Environ Microbiol 61: 2768-2770.

Nakanishi K, Matsuno R, Torii K, Yamamoto K, Kamikubo T. 1983. Properties of immobilized β-D-glalactosidase from *Bacillus circulans*. Enzyme Microb Technol 5: 115-120.

Nishimura T, Komentani T, Takii H, Terada Y, Okada S. 1994. Purification and some properties of α-amylase from *Bacillus subtilis* X-23 that glucosylates phenolic compounds such as hydroquinone. J Ferment Bioeng 78: 31-36.

Nishimura T, Kometani T, Takii H, Terada Y, Okada S. 1994. Acceptor specificity in the glucosylation reaction of *Bacillus subtilis* X-23 α-amylase towards various phenolic compounds and the structure of kojic acid glucoside. J Ferment Bioeng 78: 37-41.

Nishimura T, Kometani T, Takii H, Terada Y, Okada S. 1995. Glucosylation of caffeic acid with *Bacillus subtilis* X-23 α-amylase and a description of the glucosides. J Ferment Bioeng 80: 18-23.

Oginsky EL, Stein AE, Greer MA. 1965. Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. Proc Soc Exp Med 119: 360-364.

Ohtsuru M, Tsuruo I, Hata T. 1973. The production and stability of intracellular myrosinase from *Aspergillus niger*. Agric Biol Chem 37: 967-971.

Palcic MM. 1999. Biocatalytic synthesis of oligosaccharides. Curr Opinion Biotechnol 10: 616-624.

Palop ML, Smits JP, Ten Brink B. 1995. Degradation of sinigrin by *Lactobacillus agilis* strain R16. Int J Food Microbiol 26: 219-229.

Panintrarux C, Adachi S, Araki Y, Kimura Y, Matsuno R. 1995. Equilibrium yield of n-alkyl-β-D-glucoside through condensation of glucose and n-alcohol by β-glucosidase in a biphasic system. Enzyme Microb Technol 17: 32-40.

Perez Oseguera MA, Guereca L, Lopez-Munguia A. 1996. Properties of levansucrase from *Bacillus circulans*. Appl Microb Biotechnol 45: 465-471.

Potocki de Montalk G, Remaud-Simeon M, Willemot RM, Planchot V, Monsan P. 1999. Sequence analysis of the gene encoding amylosucrase from *Neisseria polysaccharea* and characterisation of the recombinant enzyme. J Bacteriol 181: 375-381.

Prasad AK, Trikha S, Parmar VS. 1999. Nucleoside synthesis mediated by glycosyl transferring enzymes. Bioorg Chem 27: 135-159.

Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J. 2000. Myrosinase: gene family evolution and herbivore defense in *Brassicaceae*. Plant Mol Biol 42: 93-113.

Reed DW, Davin L, Jain JC, Deluca V, Nelson L, Underhill EW. 1993. Purification and properties of UDP-glucose: thiohydroximate glucosyltransferase from *Brassica napus* L. seedlings. Arch Biochem Biophys 305: 526-532.

Remaud-Simeon M, Willemot RM, Sarçabal P, Potocki de Montalk G, Monsan P. 2000. Glucansucrases: molecular engineering and oligosaccharide synthesis. J Mol Cat B: Enzymatic 10: 117-128.

Robyt JF. 1995. Mechanisms in the glucansucrase synthesis of polysaccharides and oligosaccharides from sucrose. Adv Carbohydr Chem Biochem 51: 131-168.

Robeson JP, Barletta RG, Curtiss R. 1983. Expression of a *Streptococcus mutans* glucosyltransferase gene in *Escherichia coli*. J Bacteriol 153: 211-221.

Robyt JF, Corrigan AJ. 1977. The mechanism of dextransucrase action. Arch Biochem Biophys 183: 726-731.

Robyt JF, Eklund SH. 1983. Relative, quantitative effects of acceptors in the reaction of *Leuconostoc mesenteroides* B-512F dextransucrase. Carbohydr Res 121: 279-286.

Robyt JF, Kim D, Yu L. 1995. Mechanism of dextran activation of dextransucrase. Carbohydr Res 266: 293-299.

Robyt JF, Kimble BK, Walseth TF. 1974. The mechanism of dextransucrase action. Arch Biochem Biophys 165: 634-640.

Robyt JF, Taniguchi H. 1976. The mechanism of dextransucrase action. Arch Biochem Biophys 174: 129-135.

Robyt JF, Walseth TF. 1978. The mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextransucrase. Carbohydr Res 61: 433-445.

Ruiz JMJ, Oßwald G, Petersen M, Fessner WD. 2001. The "natural strategy" for the glycosidase-assisted synthesis of simple glycosides. J Mol Cat B: Enzymatic 11: 189-197.

Saito S, Tsuchiya T. 1984. Characteristics of n-octyl β-D-thioglucopyranoside, a new non-ionic detergent useful for membrane biochemistry. Biochem J 222: 829-832.

Salvucci ME, Wolfe GR, Hendrix DL. 1998. Purification and properties of an unusual NADPH-dependent ketose reductase from the silverleaf whitefly. Insect Biochem Mol Biol 28: 357-363.

Sarney DB, Vulfson EN. 1995. Application of enzymes to the synthesis of surfactants. Trends In Biotechnol 12: 164-172.

Sato T, Nakagawa H, Kurosu J, Yoshida K, Tsugana T, Shimura S, Kirimura K, Kino K, Usami S. 2000. α-Anomer-selective glucosylation of (+)-catechin by the crude enzyme, showin glucosyl transfer activity of *Xanthomonas campestris* WU-9701. J Biosci Bioeng 90: 625-630.

Saunders MD, Timell TE. 1968. The acid hydrolysis of glycosides VII. Hydrolysis of 1-thio-β-D-glucopyranosides and 1-thio-β-D-glucopyranosiduronic acids. Carbohydr Res 6: 121-124.

Scheckermann C, Wagner F, Fischer L. 1997. Galactosylation of antibiotics using the β-galactosidase from *Aspergillus oryzae*. Enzyme Microb Technol 20: 629-634.

Schols HA, Posthumus MA, Voragen AGJ. 1990. Sructural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. Carbohydr Res 206: 117-129.

Seymour FR, Chen ECM, Bishop SH. 1979a. Methylation structural analysis of unusual dextrans by combined gas-liquid chromatography-mass spectrometry. Carbohydr Res 68: 113-121.

Seymour FR, Knapp RD, Bishop SH, Jeanes A. 1979b. High-temperature enhancement of ^{13}C-N.M.R. chemical shifts or unusual dextrans, and correlation with methylation structural analysis. Carbohydr Res 68: 123-140.

Seymour FR, Knapp RD, Chen ECM, Bishop SH. 1979c. Structural analysis of leuconostoc dextrans containing 3-O-α-D-glucosylated α-D-glucosyl residues in both linear-chain and branch-point positions, or only in branch-points positions, by methylation and by ^{13}C-N.M.R. spectroscopy. Carbohydr Res 74: 41-62.

Shimamura A, Nakano Y, Musaka H, Kuramitsu HK. 1994. Identification of amino acid residues in *Streptococcus mutans* glucosyltransferases influencing the structure of the glucan product. J Bacteriol 176: 4845-4850.

Shiroza T, Ueda S, Kuramitsu HK. 1987. Sequence analysis of the gtfB gene from *Streptococcus mutans*. Journal Bacteriol 169: 4263-4270.

Simpson CL, Giffard PM, Jacques NA. 1995. *Streptococcus salivarius* ATCC 25975 posesses at least two genes coding for primer independent glucosyltransferases. Infect Immun 63: 609-621.

Smits JP, Knol W, Bol J. 1993. Glucosinolate degradation by *Aspergillus clavatus* and *Fusarium oxysporum* in liquid and solid-state fermentation. Appl Microbiol Biotechnol 38: 696-701.

Stahl-Biskup E, Intert F, Stengele M, Schulz G. 1993. Glycosidically bound volatiles, a review 1986-1991. Flav Fragr J 8: 61-80.

Su D, Robyt JF. 1993. Control of the synthesis of dextran and acceptor-products by *Leuconostoc mesenteroides* B-512FM dextransucrase. Carbohdr Res 248:339-348.

Su D, Robyt JF. 1994. Determination of the number of sucrose and acceptor binding sites for *Leuconostoc mesenteroides* B-512FM dextransucrase, and the confirmation of the two-site mechanism for dextran synthesis. Arch Biochem Biophys 308: 471-476.

Tani N, Ohtsuru M, Hata T. 1974. Isolation of myrosinase producing microorganism. Agr Biol Chem 38: 1617-1622.

Tanriseven A, Robyt JF. 1992. Inhibition of dextran synthesis by acceptor reactions of dextransucrase, and the demonstration of a separate acceptor binding-site. Carbohydr Res 225: 321-329.

Terleckyj B, Willett NP, Shockman GD. 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect Immun 11: 649-655.

Thiem J. 1995. Applications of enzymes in synthetic carbohydrate chemistry. FEMS Microbiol Rev 16: 193-211.

Toone EJ, Simon ES, Bednarski MD, Whitesides GM. 1989. Enzyme-catalyzed synthesis of carbohydrates. Tetrahedron 45: 5365-5422.

Tsumori H, Kuramitsu HK. 1997. The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the gtfC enzyme. Oral Microbiol Immunol 12: 274-280.

Tsumori H, Minami T, Kuramitsu HK. 1997. Identification of essential amino acids in the *Streptococcus mutans* glucosyltransferases. J Bacteriol 179: 3391-3396.

Ueda S, Shiroza T, Kuramitsu HK. 1988. Sequence analysis of the gtfC gene from *Streptococcus mutans* GS-5. Gene 69: 101-109.

Vandamme EJ, Van Loo J, De Laporte A. 1987. Dynamics and regulation of sucrose phosphorylase formation in *Leuconostoc mesenteroides* fermentations. Biotechnol Bioeng 29: 8-15.

Van Geel-Schutten GH, Faber EJ, Smit E, Bonting K, Smith MR, Ten Brink B, Kamerling JP, Vliegenthart JFG, L Dijkhuizen. 1999. Biochemical and structural characterisation of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl Environ Microbiol 65: 3008-3014.

Varki A. 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3: 97-130.

Verbruggen MA, Beldman G, Voragen AGJ. 1995. The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions. J Cereal Sci 21: 271-282.

Vetter J. 2000. Plant cyanogenic glycosides. Toxicon 38: 11-36.

Vic G, Biton J, Le Beller D, Michel JM, Thomas D. 1995. Enzymatic glucosylation of hydrophobic alcohols in organic medium by the reverse hydrolysis reaction using almond β-glucosidase. Biotechnol Bioeng 46: 109-116.

Voragen AGJ, Rombouts FM, Hooydonk MJ, Pilnik W. 1971. Comparison of endgroup methods for the measurement of pectin degradation. Lebensm Wiss Technol 4: 7-11.

Vulfson EN, Patel R, Beecher JE, Anderws AT, Law BA. 1990. Glycosidases in organic solvents: I. alkyl-β-glucoside synthesis in a water-organic two-phase system. Enzyme Microb Technol 12: 950-954.

Watanabe N, Watanabe S, Nakajima R, Moon JH, Shimokihara K, Inagaki J, Etoh H, Asai T, Sakata K, Ina K. 1993. Formation of flower fragrance compounds from their precursors by enzymic action during flower opening. Biosci Biotechnol and Biochem 57: 1101-1106.

Wenzel TJ, Van den Berg MA, Visser W, Van den Berg JS, Steensma HY. 1992. Characterization of *Saccharomyces cerevisiae* mutans lacking the E1α subunit of the pyruvate dehydrogenase complex. Eur J Biochem 209: 697-705.

Wijsman MR, Hereijgers JLPM, de Groote JMFH. 1989. Selective enumeration of bifidobacteria in fermented dairy products. Neth Milk Dairy J 43: 395-405.

Winterhalter P, Schreier P. 1994. C_{13}-Norisoprenoid glycosides in plant tissues: an overview on their occurrence, composition and role as flavour precursors. Flav Fragr J 9: 281-287.

Wong CH, Haynie SL, Whitesides GM. 1982. Enzyme-catalyzed snthesis of N-acetyllactosamine with in sity regeneration of uridine 5'-diphosphate glalactose. J Org Chem 47: 5416-5418.

Yamashita Y, Hanada N, Takehara T. 1989. Purification of a fourth glucosyltransferase from *Streptococcus sobrinus*. J Bacteriol 171: 6265-6270.

Summary

Glycosylation is considered as a useful method for improving chemical properties like solubility and volatility of compounds with interesting organoleptic or physiological properties. The aim of the research described in this thesis was to explore the enzymatic glycosylation of aglycones (nonsaccharide acceptor molecules). The initial focus is on the glucosylation of aromatic alcohols by non-Leloir glucosyltransferases like sucrose glucosyltransferases.

Several streptococcal sucrose glucosyltransferases (glucansucrases) were screened for transglucosylation activity using the flavonoid catechin as model aglycone and sucrose as an economically feasible glucosyl donor substrate (Chapter 2). *Streptococcus mutans* GS-5 glucosyltransferase-D (GTF-D) glucosylated catechin most efficiently (90% catechin yield). Three different catechin glucosides were isolated of which two catechin glucoside structures were spectroscopically elucidated; catechin-4'-*O*-α-D- and catechin-4',7-*O*-α-di-D-glucopyranoside. The structure of the third glucoside remained unsolved, although hydrolysis studies using *Aspergillus niger* amyloglucosidase suggested that catechin-7-*O*-α-D-glucopyranoside was formed. The acceptor specificity of GTF-D towards aromatic aglycones is restricted to compounds containing two adjacent aromatic hydroxyl groups, e.g. (substituted) catechol(s) (Chapter 3). This suggests that one hydroxyl group is involved in the interaction with GTF-D, while the other is available for formation of the glucosidic linkage. Compounds containing one hydroxyl group like phenol, irreversibly inhibit GTF-D transglucosylation activity.

To facilitate the transglucosylation of less water soluble aglycones, the addition of water miscible organic solvents (cosolvents) was studied (Chapter 3). Bis-2-methoxyethyl ether (MEE) was selected as the most appropriate cosolvent. MEE addition resulted in a 4-fold increase in catechin transglucosylation activity due to a 12-fold increase in catechin solubility. Addition of MEE (10-30% v/v) also enabled the glucosylation of catechol aglycones at otherwise inhibitory concentrations (200 mM). This was explained by assuming that the partitioning of the aglycone between solvent and enzyme was changed upon MEE addition.

The addition of bis-2-methoxyethyl ether also affected the formation rate and absolute amounts of glucan formed (Chapter 4). An increase of 20% in reducing sugars was observed using 20% (v/v) MEE. Besides an increase in sucrose hydrolysis there was also an increase the formation of high molecular weight glucan chains (10^2-10^3 kDa). Linkage analysis showed that also the type of glucosidic linkage was affected upon MEE addition. Glucan formed in the presence of MEE contained an increased amount of α(1,3) linkages. It was hypothesised that MEE affected glucan formation through modifying the GTF-D glucan binding domain.

The accumulation of fructose was shown to inhibit aglycon glucosylation and glucan formation. To overcome this inhibition, the fructose consuming yeasts *Pichia pastoris* and the mutant *Saccharomyces cerevisiae* T2-3D were added (Chapter 2). Both yeasts are incapable of utilising sucrose. Due to the consumption of fructose during transglucosylation, an increase in glucoside yield and the maximum duration of catechin glucosylation was observed. Consequently, the consumption of sucrose by GTF-D increased. Eventually glucosylation yields by GTF-D could be engineered either by adding MEE or by fructose consuming yeasts (Chapter 2 and 3). Using MEE, the glucoside yield based on catechin decreased and the sucrose based yield increase. The addition of the yeasts resulted in an increased catechin based glucoside yield and a decreased sucrose based glucoside yield.

In the second part of this thesis the attention is focussed on glycosidases and the hydrolysis and formation of thioglucosides. The observed stability of thioglucosides towards enzymatic hydrolysis was used to screen for new thioglucoside active enzymes (Chapter 5). Using octylthioglucoside (OTG) as a carbon source for microbial growth, *Sphingobacterium* sp. strain OTG1 was isolated. In the cell free extract a novel thioglucoside hydrolase activity was observed, showing distinct characteristics compared to typical β- or thioglucosidases. Various thioglucosides were hydrolysed by the *Sphingobacterium* cell free extract, with almost the same activities.

In view of the aim of this thesis, the synthesis of glycosides, various glycosidases were screened for the glycosylation of 1-propanethiol (Chapter 6). The β-glucosidase from almond, *Aspergillus niger* and *Caldocellum saccharolyticum* showed thioglucosylation activity using glucose and 1-propanetiol. The almond enzyme showed the highest activity (3 $\mu mol.min^{-1}.mg^{-1}$). Only primary and secondary (10-fold slower reaction rate) aliphatic thiols were glucosylated. Of the different thiols examined, the glucosylation of furfuryl mercaptan is the most interesting because of the potential of the glucoside as a flavour precursor.

Because of the stability of thioglucosides towards most enzymatic hydrolases (glycosidases), an excess of aglycone otherwise frequently applied in reversed hydrolysis glycosylation is not necessary for thioglucosylation. Consequently, high yields up to 60% based on 1-propanethiol and 40% based on glucose were obtained for the synthesis of 1-propanethioglucoside.

Samenvatting

Glycosylering is een bruikbare methode om een aantal chemische eigenschappen zoals oplosbaarheid en vluchtigheid van organoleptische en physiologische interessante verbindingen te verbeteren. Het doel van het onderzoek, zoals beschreven in dit proefschrift, was het verkennen van de enzymatische glycosylering van aglyconen (voornamelijk aromatische alcoholen). Hierbij ligt de nadruk op het gebruik van non-Leloir glucosyltransferases, zoals sucrose glucosyltransferases.

Verschillende *Streptococcus* sucrose glucosyltransferases (of glucansucrases) zijn gescreened op transglucosyleringsactiviteit, gebruikmakend van het flavonoid catechine als model aglycon en sucrose als economisch aantrekkelijk glucosyl donor substraat (Hoofdstuk 2). *Streptococcus mutans* GS-5 glucosyltransferase-D (GTF-D) bleek in staat het meest efficiënt catechine te glucosyleren (90% yield). Er zijn drie verschillende catechine glucosides geïsoleerd, waarvan er twee spectroscopisch zijn gekarakteriseerd; catechine-4'-*O*-α-D- en catechine-4',7-*O*-α-di-D-glucopyranoside. De structuur van het derde catechine glucoside is niet opgelost. Hydrolyse door *Aspergilles niger* amyloglucosidase duidt echter op de vorming van catechine-7-*O*-α-D-glucopyranoside. De acceptorspecificiteit van GTF-D met betrekking tot aromatische alcoholen is beperkt tot aglyconen met twee aromatische hydroxylgroepen, zoals in (gesubstitueerde) catechol(en) (Hoofdstuk 3). Waarschijnlijk is één hydroxylgroep betrokken bij de binding met GTF-D en maakt de andere onderdeel uit van de glucosidische binding. Verbindingen met een enkele hydroxylgroep, zoals phenol, remmen GTF-D irreversibel.

Om de transglucosylering van moeilijk in water oplosbare aglyconen te verbeteren, is de toepassing van organische oplosmiddelen (cosolvents) onderzocht (Hoofdstuk 3). Bis-2-methoxylethyl ether (MEE) werd geselecteerd als het meest geschikte cosolvent. Door toevoeging van MEE bleek de catechine transglucosyleringsactiviteit met een factor 4 toe te nemen door een 12-voudige toename in catechine oplosbaarheid. Bovendien bleek het mogelijk door toevoeging van MEE (10-30% v/v) aglyconen te glucosyleren bij concentraties (200 mM) die normalerwijze remmend werken. Wellicht dat de verdeling van het algycon tussen enzym en omgeving door MEE wordt beïnvloed.

Bis-2-methoxylethyl ether heeft ook een effect op de snelheid en op de absolute hoeveelheid glucan door GTF-D gevormd (Hoofdstuk 4). Zo treedt er een toename van 20% in reducerende suikers op door toevoeging van MEE (20% v/v). Deze toename in reducerende suikers wordt naast extra hydrolyse van sucrose verklaard door een toename van het hoog moleculaire glucan (10^2-10^3 kDa). Bindingsanalyse toonde aan dat MEE ook een effect heeft op de vorming van het type glucosidische binding in

glucan. Het glucan gevormd in aanwezigheid van MEE vertoonde een verhoogde hoeveelhied α(1,3) glucosidische bindingen. Waarschijnlijk wordt de vorming van glucan beïnvloed door invloed van MEE op het glucan bindings domein van GTF-D.
De accumulatie van fructose remt de glucoseoverdracht van sucrose naar zowel het aglycon als naar het glucan. Daarom zijn gisten toegevoegd, te weten *Pichia pastoris* en een mutant *Saccharomyces cerevisiae* T2-3D, welke in staat zijn fructose te consumeren zonder sucrose te gebruiken voor groei (Hoofdstuk 2). Door de consumptie van fructose tijdens de transglucosyleringsreactie kon een toename in de glucoside yield (catechine) en in de duur van de maximale glucosyleringsactiviteit worden waargenomen. Dit alles ging gepaard met een verhoogde consumptie van sucrose door GTF-D. Uiteindelijk bleek het mogelijk de yield van GTF-D glucosylering te beïnvloeden door toevoeging van MEE en de fructose consumerende gisten (Hoofdstuk 2 en 3). Het gebruik van MEE leidde tot een afname van de glucoside yield gebaseerd op catechine en een toename van de yield gebaseerd op sucrose. Toevoeging van de gisten had een tegenovergesteld effect tot gevolg.
In het tweede deel van dit proefschrift wordt de aandacht gericht op de hydrolyse en synthese van thioglucosides door glycosidases. De waarneming dat thioglucosides stabiel zijn ten opzichte van enzymatische hydrolyse is gebruikt om te zoeken naar nieuwe (thio)glucoside actieve enzymen (Hoofdstuk 5). Gebruikmakend van octylthioglucoside (OTG) als koolstofbron voor microbiële groei werd *Sphingobacterium* sp. strain OTG1 geïsoleerd. In het cel-vrije extract werd, in vergelijking met reeds bekende β- en thioglucosidases, nieuwe thioglucoside hydrolase activiteit waargenomen. Het *Sphingobacterium* thioglucoside hydrolase bleek in staat verschillende thioglucosides te hydrolyseren met identieke activiteiten.
Met betrekking tot de doelstelling van dit proefschrift, de synthese van glycosides, zijn verschillende glycosidases gescreened op thioglycosyleringsactiviteit (Hoofdstuk 6). β-Glucosidases van amandel, *Aspergillus niger* en *Caldocellum saccharolyticum* zijn in staat tot thioglucosylering, gebruikmakend van 1-propaanthiol en glucose. Het amandel enzym vertoonde de hoogste activiteit (3 $\mu mol.min^{-1}.mg^{-1}$). De acceptorspecificiteit van het amandel enzym beperkt zich echter tot primaire en secundaire (10-voudig langzamere reactiesnelheid) aliphatische thiolen. Van de verschillende thiolen welke onderzocht zijn, is de glucosylering van furfuryl mercaptaan het meest interessant. Dit vanwege de mogelijkheden van het glucoside als geurstofprecursor.
De stabiliteit van thioglucosides ten opzichte van de meeste hydrolyserende enzymen (glycosidases) maakt een overmaat aan aglycon, zoals normaal wordt toegepast tijdens reversed hydrolysis glycosylering, overbodig voor de glucosylering van thiolen. Dit heeft tot gevolg dat in geval van glucosylering van 1-propaanthiol hoge yields behaald worden; 60% gebaseerd op 1-propaanthiol en 40% gebaseerd op glucose.

Enzymatische glucosylering in vogelvlucht

Kort en bondig gaat het onderzoek in dit proefschrift over knippen en plakken, niet met schaar en lijm maar met enzymen. In het algemeen kunnen enzymen omschreven worden als zeer kleine biologische machines die overal in de natuur chemische reacties helpen uit te voeren. Tegenwoordig is men in enzymen geïnteresseerd omdat reacties zeer precies en op een natuurlijke wijze uitgevoerd kunnen worden. In dit proefschrift staat een enzymreactie met de naam glucosylering centraal.

Glucosylering laat zich omschrijven als het plakken van een suikermolecuul aan een andere molecuul, het aglycon. Suiker en aglycon aan elkaar gebonden is een glucoside en het plaksel (binding) tussen suiker en aglycon wordt de glucosidebinding genoemd. Door nu suiker aan het aglycon te plakken (sythetiseren) worden eigenschappen van het aglycon veranderd. Vaak zijn glucosides stabieler dan het aglycon, wat nuttig kan zijn voor bijvoorbeeld de toepassing van geur- en smaakstoffen.

Glucosides kunnen door enzymen op twee verschillende manieren gemaakt worden. De eerste methode is transglucosylering en vindt ook plaats in de natuur. Kenmerkend voor transglucosylering is dat het suikermolecuul geactiveerd is; het zit vastgeplakt aan een ander molecuul dan het aglycon. Eerst wordt het suikermolecuul vrijgemaakt (knippen) om vervolgens aan het aglycon geplakt te worden. Het suikermolecuul wordt als het ware getransporteerd. Deze methode heeft als voordeel dat hoge glucoside-opbrengsten mogelijk zijn. Een nadeel is dat het gebruik van de meeste geactiveerde suikermoleculen economisch niet rendabel is. De tweede glucosyleringsmethode wordt omschreven als omgekeerde hydrolyse en is eigenlijk een kunstmatige methode. Kenmerkend is dat het suikermolecuul niet geactiveerd is en daardoor direct aan het aglycon geplakt kan worden. Het nadeel is dat de glucoside-opbrengst laag is. Dit wordt veroorzaakt doordat de enzymen bij deze methode liever glucosides knippen (hydrolyseren) dan maken (plakken). Om toch glucosides te maken moet veel moeite gedaan worden de hydrolyse om te draaien, vandaar de term omgekeerde hydrolyse.

Gelukkig zijn enkele enzymen in staat goedkope geactiveerde suikermoleculen te gebruiken door middel van transglucosylering. Zo is het mogelijk op goedkope wijze efficiënt glucosides te maken. Dit idee vormt de basis voor de Hoofdstukken 2, 3 en 4 van dit proefschrift.

Allereerst is na een uitgebreide selectie gekozen om te werken met het enzym sucrose glucosyltransferase-D (afgekort GTF-D) uit de bacterie *Streptococcus mutans* (Hoofdstuk 2). Dit enzym kan sucrose als suikerbron voor glucosylering gebruiken. Sucrose bestaat uit twee delen, een molecuul glucose en een molecuul fructose. In de natuur plakt GTF-D de verschillende glucosemoleculen achter elkaar (via transglucosylering) tot een lange keten ontstaat, ook wel glucan genoemd. Dit glucan

speelt een rol bij de vorming van tandplak. GTF-D is echter ook in staat het suikermolecuul glucose aan verschillende aglyconen te plakken en wel op een zeer efficiënte manier. Door onderzoek is nu meer bekend over het type aglycon dat door GTF-D geglucosyleerd wordt (Hoofdstuk 3). Veel werk is verricht om de transglucosyleringsreactie te verbeteren. Zo bleek fructose, dat vrijkomt tijdens de glucose overdracht (transfer) van sucrose naar het aglycon, GTF-D te remmen. Deze remming werd opgeheven door het toevoegen van een micro-organisme, genaamd *Pichia pastoris*, dat in staat is fructose te consumeren. Hierdoor verdwijnt de remming van fructose en verloopt de transglucosylering efficiënter (Hoofdstuk 2). Een ander probleem vormt het moeilijke oplossen van aglyconen in water, wat niet gunstig is voor de efficiëntie van GTF-D. Dit probleem werd opgelost door het toevoegen van de hulpstof MEE aan GTF-D (Hoofdstuk 3). Het MEE heeft bovendien ook een interessant effect op de vorming van glucan (Hoofdstuk 4). Door toevoeging van MEE wordt niet alleen meer, maar ook een verschillend type glucan gevormd, waarin de glucosemoleculen onderling verschillend gebonden zijn. Hoe dit precies in zijn werk gaat is nog niet duidelijk, wellicht dat het MEE de vorm van GTF-D veranderd.

In de Hoofdstukken 5 en 6 is uit wetenschappelijke nieuwsgierigheid een kleine zijsprong gemaakt. Zoals er verschillende soorten plaksel zijn, zo zijn er ook verschillende mogelijkheden om suiker en aglycon aan elkaar te plakken (glucoside binding). Verreweg de meeste glucosidebindingen bestaan uit zuurstofmoleculen en worden *O*-glucosides genoemd, een klein deel bestaat echter uit zwavelmoleculen en worden *S*-glucosides of thioglucosides genoemd. Nu is gebleken dat thioglucosides heel stabiel zijn. Er zijn bijna geen enzymen in staat deze verbindingen te hydrolyseren (knippen). Indien een dergelijk enzym gevonden wordt, is dit wetenschappelijk gezien interessant en wellicht zou het toegepast kunnen worden in nieuwe (thio)glucosylerings-reacties. Gebleken is dat in de bacterie *Sphingobacterium* sp. strain OTG1 zo'n thioglucoside hydrolyserend enzym voorkomt (Hoofdstuk 5). Het is waarschijnlijk een nieuw enzym, verschillend van andere enzymen reeds beschreven in de literatuur.

In Hoofdstuk 5 ligt de nadruk op het knippen van thioglucosides (suiker en aglycon met zwavel aan elkaar geplakt). In Hoofdstuk 6 een groot aantal reeds bekende enzymen (werkend via de omgekeerde hydrolyse methode) getest op de mogelijkheid thioglucosides te maken (plakken). Gebleken is dat het enzym β-glucosidase afkomstig uit amandelen op efficiënte wijze verschillende thioglucosides kan maken. Dit β-glucosidase is in de literatuur al uitvoerig beschreven. Een nieuw aspect is dat tijdens de vorming van thioglucosides deze (bijna) niet door het amandel β-glucosidase gehydrolyseerd (knippen) worden. De vorming van thioglucosides verloopt dus eigenlijk niet via de normale omgekeerde hydrolyse methode, waardoor er sprake is van een variant op de omgekeerde hydrolyse methode.

Samenvattend staat in dit proefschrift enzymatische glucosylering beschreven gebruikmakend van sucrose glucosyltransferases en glucosidases om respectievelijk *O*- en *S*-glucosides te synthetiseren.

Nawoord

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Publications

HJ Heipieper, GH Meulenbeld, Q van Oirschot, JAM de Bont. Effect of environmental factors on the *trans/cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Applied and Environmental Microbiology, 1996, 62: 2773-2777.

MW Fraaije, F Drijfhout, GH Meulenbeld, WJH van Berkel, A Mattevi. Vanillyl-alchohol oxidase from *Penicillium simplicissimum*: reactivity with *p*-cresol and preliminary structural analysis. Flavins and Flavoproteins XII, 1997, pp. 261-264, University Press, Calgary.

GH Meulenbeld, H Zuilhof, A van Veldhuizen, RHH van den Heuvel, S Hartmans. Enhanced (+)-catechin transglucosylating activity of *Streptococcus mutans* GS-5 glucosyltransferase-D due to fructose removal. Applied and Environmental Microbiology, 1999, 65: 4141-4147.

GH Meulenbeld, S Hartmans. Transglucosylating activity of *Streptococcus mutans* GS-5 glucosyltransferase-D: acceptor specificity and engineering of reaction conditions. Biotechnology and Bioengineering, 2000, 70: 364-369.

GH Meulenbeld, S Hartmans. Thioglucosidase activity from *Sphingobacterium* sp. strain OTG1.
Applied Microbiology and Biotechnology, 2001, 56: 700-706.

GH Meulenbeld, BM de Roode, S Hartmans. Enzymatic synthesis of thioglucosides using almond β-glucosidase.
Biocatalysis and Biotransformation, 2001, accepted for publication.

GH Meulenbeld, EJ Bakx, HA Schols, AGJ Voragen, S Hartmans. Modifying the formation of glucan and maltooligosaccharides by *Streptococcus mutans* glucosyltransferase-D.
Submitted for publication.

Curriculum Vitae

Gerrit Hendrik Meulenbeld (Gerwin) werd op 3 februari 1973 geboren te Almelo, maar groeide op in Vriezenveen. Vanaf 1985 bezocht hij het Christelijk Lyceum in Almelo alwaar in 1991 het VWO diploma werd behaald. In hetzelfde jaar werd begonnen met de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit Wageningen. Na het behalen van de propadeuse werd in 1992 de overstap gemaakt naar de studie Bioprocestechnologie, oriëntatie cellulair/moleculair. In augustus 1996 werd deze studie afgesloten met afstudeervakken Industriële Microbiologie en Biochemie en een stage bij Diosynth in Oss. In de periode van januari 1997 tot augustus 2001 werkte hij aan zijn promotieonderzoek bij de sectie Industriële Microbiologie van Wageningen Universiteit.

The research described in this thesis was carried out at the Division of Industrial Microbiology, Department of Food Technology and Nutritional Sciences, Wageningen University, The Netherlands as a part of the Innovation Oriented research Program on Catalysis (IOP Katalyse, IKA 96006) sponsored by the Netherlands Ministry of Economic Affairs.