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دراسة توصيفية لانزيم الكلوكسيل ترانسفيريز الخاص بالمكورات الفموية من نوع *Streptococcus sobrinus* (Serotype G)

(التقديرات الوظيفية والمناعية)

رسالة

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Dedication

To . . .

My dearest family . . . Parents and brothers

Whom . . .

I still drink deep from the fountains of

their…

Love…

Warmth…

Comforts…

and Security.

Nada

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Chapter One

1.1 Introduction

The mouth is the only site in human body where such hard solid surfaces are found (i.e. the surface of the teeth) and it is an open growth system in which the nutrients and microbes are repeatedly introduced to and removed from it (Lynche and Poole, 1979). Saliva not only provides nutrients to the microbiota, but also buffers acidic fermentation products of the flora and removes waste products, so it acts as a reservoir for ions facilitating remineralization of teeth (Mandle, 1982). The flow rates of saliva are so high that only successful organisms can adhere to the surface of the oral cavity or those which can retain their ability in pits and fissures, this means that the oral cavity harbors a unique microbiota and most of its members are not able to colonize any other sites of the human body (Marsh, 1993).

Dental plaque is an adherent deposit of bacteria and their products, which forms as a white greenish or even yellow film on all tooth surfaces (Marsh, 1994). Dental plaque accumulates naturally at stagnant or retentive sites formed after one to two days with no oral hygiene (Kidd and Joyston-Bechel, 1997). The following steps explain how it is formed:

- Proteins that are present in saliva adhere to the enamel surface and form the pellicle.
- Certain types of bacteria attach themselves to the hard surfaces on the pellicle.
- If the bacteria are not removed, they multiply and grow in number until they form colony.
- Other types of bacteria then attach to this colony which continues to grow on the tooth enamel (Marsh, 2004).

When plaque masses increase (such as in case of frequent consumption of fermentable dietary carbohydrates), saliva is less able to penetrate plaque and

protect enamel leading to shift in the composition of the microflora as well as in local environmental condition within the biofilm, for example sugar concentration and pH (Mandle, 1982; Li and Burne, 2001). Such diets lead to arise in the proportions of mutans streptococci and *Lactobacilli* with a *Streptococcus oralis* group which includes *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus mitis* (Loesche, 1986). The metabolism of plaque also changes from a heterofermentative pattern to one in which sugars are converted primarily to lactic acid (Marsh, 1994). All these changes are associated with an increased risk of dental caries, periodental disease and gingivitis (Marsh, 1994; Kidd and Joyston-Bechal, 1997).

Dental caries (tooth decay) have plagued human since the dawn of civilization and still constitutes one of the most common human infectious disease in different parts of the world (WHO, 1997). Although this disease is not life threating and currently decreasing in severity in more advanced countries, epidemiological studies in our countries revealed an increase in severity as well as prevalence of dental caries among children and adolescent (Al-Sammarai, 1989; Al-Azawi, 2000; Al-Damerchi, 2001).

Dental caries is a multifactorial disease stems from a variety of interactive elements. These factors include the resistance of the host, nutritional status and diet (sugar intake) and the presence of the cariogenic microflora (Jackson *et al*., 1990; Koch *et al*., 1994).

Mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) are the most important bacteria in the pathogenesis of dental caries due to many epidemiological, experimental and animal studies (Wyatt *et al*., 1988; Narhi *et al*., 1994; Sullivan *et al*., 1996). Glucosyltransferase is an extracellular enzyme produced by mutans streptococci responsible for polymerizing the glucose moiety of sucrose to form glucan which plays an important role in caries formation process (Tsumori and Kuramtisu, 1997; Wunder and Bowen, 1999).

The identification of caries as truly infectious disease caused by identifiable microorganisms made scientists and dentists to increase their efforts towards its prevention through:

1. Increasing the interest of people and specially children in communities for the continuous usage of oral hyagine preventive procedures such as brushing with fluoride supplemented tooth paste, the usage of supplemented fluoride drinking water and chemoprophylactic agents like chlorohexidine in order to decrease number of mutans streptococci and lactobacilli in their oral cavity (Hajishengallis and Michalek, 1999; Balakrishnan *et al*., 2000).
2. Interfering with transmission of mutans streptococci bacteria via vaccine against dental caries (Balakrishnan *et al*., 2000).

Streptococcus mutans virulence factors antigens such as (AgI/II, Glucosyltransferase enzyme and glucan binding protein) have been considered primary candidate to develop a safe human caries vaccine effective in inducing immunity against *Streptococcus mutans* infection and especially for children between 1-2 years (Michalek *et al*., 2003). The production of an effective, safe and readily deliverable vaccine may not only help against pain and health issues associated with caries but also saves billions of dollars that are currently spent for restorative procedures (Cromie, 2001).

1.2 Aims of The Study

1. Isolation and characterization of mutans streptococci.
2. Purification and characterization of glucosyltransferase enzyme from the isolates and studying the effects of some inhibitors on the activity of glucosyltransferase enzyme as well as on the growth of mutans streptococci bacteria.
3. Immunizing experimental animals with the above enzyme to induce production of antibodies against dental caries.

List of Abbreviations

Ag I/II	Antigen I/II
BSA	Bovine Serum Albumin
CAT	Catalytic binding domain
(C-G)	Cytosine-Guanine
CHX	Chlorohexidine
CMIS	Common Mucosal Immune System
CTB	Cholera Toxin type B
CWS	Clarified human Whole Saliva
EDTA	Ethylene Diamine Tetra Acetic acid
EMP	Embeden Myerhove Pathway
GBP	Glucan-Binding Protein
GIF	Glucosyltransferase-Inhibitory Factor
GLU	Glucan-Binding region of GTF
GTF	Glucosyltransferase enzyme
gtf	Glucosyltransferase gene
IEF	Isoelectrical Focusing
IG	Insoluble Glucan
K_m	Michalis Menten Kinetic constant
LTA	Lipotenchoic Acid
MAbs	Monoclonal Antibodies
MS	Mitis-Salivarius agar
MSB	Mitis-Salivarius Bacitracin agar
PAGE	Polyacrylamide- Gel Electrophoresis
PAS	Periodic Acid-Schiff base
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
S	*Streptococcus*
SDS	Sodium Doedecyl Sulfate
SG	Soluble Glucan

SHA	Saliva Coated Hydroxyapatite
S-IgA	Secretory- Immunoglobulin A
Spp.	Species
TTC	2, 3, 5-Triphenyl Tetrazolium Chloride
V_{max}	Maximum Velocity

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Chapter Two

Literature Review

2.1 Dental Caries

2.1.1 Historical Background

Along centuries, many ideas about caries etiology , were started first from the ancient Sumerian text. From that old time, people think that dental caries as a disease originating within the tooth itself, analogous to bone gangrene (Newbrun, 1989). Parmly (1819) proposed an unidentified "chemical" agent that was responsible for caries, while Erdle (1843) described a filamentous parasites in the surface membrane (plaque) of the teeth and suggested that such parasites cause decomposition of the enamel and then dentin (Al-Hayali, 2002). The more acceptable theory of caries etiology was described by Miller in (1890) and called "Chemo-Parasitic theory" which proposed that caries is caused by acid producing microorganisms of the mouth (Rothman, 1976).

Early in the last century, Clark (1924) isolated the bacterium, *Streptococcus mutans*, upon which modern dental research has cast its brightest light. In the latter half of the twentieth century, research efforts in Scandinavia confirmed the cariogenic properties of this organism, demonstrated it's transmissibility, and described its world wide distribution (Loesche, 1986). Later, others identified many of its virulence characteristics and unrevealed its biochemistry. Ultimately, the complete genome sequence of *Streptococcus mutans* was reported in 2002 (Ajdic *et al*., 2002). Although molecular, biological and cultural techniques have also incriminated other bacteria in the process and extension of dental caries in various dental habitats, *Streptococcus mutans* continues to be public enemy, especially for early childhood dental disease (Loesche, 1986).

2.1.2 Definition and Causes of Dental Caries

Dental caries is a bacterial disease of the dental hard tissues which occurs in certain localized sites of the dentitions. These sites are in order of frequency of attack the pits and fissure particularly those on the occusal surfaces of the teeth, the proximal contacting surfaces and those labial, buccal and lingual surfaces of the dentition adjacent to the gingiva (Nolte, 1982).These sites are protected from the cleaning action of saliva, the tongue, and the oral musculature. They are the regions where food is retained and where bacteria, salivary protein and other oral debris readily collected. The tenaciously adhering and loose deposits of bacteria and proteins in these non self cleaning regions are commonly referred to as the dental plaque without which the caries process can not occur (Simmonds *et al*., 2000). Plaque develops naturally, and is generally considered of benefit to the host because of its ability to prevent colonization by exogenous and often pathogenic microorganisms. The microflora at a site remains relatively stable overtime and this stability terms "microbial homeostasis" (Marsh, 1989; Marsh and Bradshaw, 1997).

The regulatory factor forces influencing oral ecosystem can be divided into three major categories: host related, microbe related and external factors. Among host factors, secretory IgA constitutes the main specific immune defense mechanism in saliva and other secretions which may play an important role in the homeostasis of the oral microbiota by reducing adherence of bacteria to the oral mucosa and teeth. However, homeostasis can be broken down leading to the shift in balance of the microflora and the two major diseases caries and periodontal disease can occur (Schhultz Hadt, 1964; Marcotte and Lavoie, 1998).

Dental caries is associated with an increasing frequency of consumption of fermentable sugars in diet, which results in an increase in the isolation and proportion of acidogenic and aciduric species such as *Streptococcus*, *Actinomyces*, gram-negative bacilli and *Lactobacillus* to produce acid. However

most importantly is the lactic acid that demineralize the inorganic matrix of the teeth causing decay to hundreds of species that inhabit human oral cavity, and also posses many toxic factors making them pathogenic under certain conditions; for example, enzymatic activities (e.g hyalouronidase, proteases, collagenase and glucosyltransferase) exerted activities on the host (Whiley *et al*., 1992). Dissolution by acid of the surface enamel exposes the underlying avascular mineralized connective tissue matrix of dentine, which is prone to invasion. This occurs by migration of the bacteria to the network of tubules occupied by processes of pulpal odontoblasts. Early stages of invasion involve *Lactobacillus*, *Actinomyces spp*., *Veillonellae* and other oral *Streptococcal* species including mutans streptococci (Love and Jankinson, 2002). This phase is followed by the invasion of a more diverse group of microorganisms including gram-negative anaerobes (Love *et al*., 2000).

Lactobacilli have been reported to occur in high numbers in both superficial and deep caries (Hanh *et al*., 1991), though they are not suspected of being involved in bacterial invasion of non exposed dental pulp (Hoshino *et al*., 1992).Martin *et al*.,(2002), indicated by cultivating *Lactobacillus* from dental caries samples and using quantitative molecular technique (Real-Time PCR) that *Lactobacillus acidophilus* was numerically dominant, although *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Lactobacillus fermentum* were also present in many samples.

Streptococci constitutes between 30 and 60% of the total oral bacterial flora. A group of related oral bacteria, collectively known as "mutans streptococci", is implicated as the primary etiological agents of human caries, within this group, *Streptococcus sobrinus* and frequently *Streptococcus mutans* were the species most commonly isolated from human (Loesche, 1986).

2.2 The Genus *Streptococcus*

Genus *Streptococcus* belongs to the family *Streptococaceae*, which includes the pyogenic, oral and the anaerobic groups of streptococci, as well as a group of other streptococci. The cells are spherical or ovoid, 0.5-2.0 μm in diameter, occurring in pairs or chains when grown in liquid media, and stain gram positive, non motile, non sporeforming. Some species are encapsulated, facultative anaerobic, catalase and oxidase negative (Holt *et al*., 1994). Chemoorganotrophs, requiring nutritionally rich media for growth and sometimes 5% CO_2. The metabolism is fermentative producing mainly lactate but no gas. They attack commonly red blood cells with either greenish discolored (α-hemolysis) or complete clearing (β-hemolysis). Optimum temperature for growth is 37°C, and growth is usually restricted between 25-45°C.

Streptococci constitutes a major population in the oral cavity with several different species colonizing various etiological niches of the mouth. Differentiation between the pyogenic, oral and anaerobic group may be laborious and combined information is needed for classification (Holt *et al*., 1994).

The oral group has sometimes named the viridans streptococci referring to the partial clearing of the erythrocytes around the colony. However, the term is not interchangeable when some species classified as viridans streptococci are not detected in the oral cavity. As showing figure (2-1), current classification of the oral streptococci places the bacteria into four species groups, the anginosus, mitis, mutans and salivarius. The classification is based on chemotaxonomic and genotyping data, especially DNA-DNA base pairing and 16S rRNA gene sequence analysis (Russell, 2000).

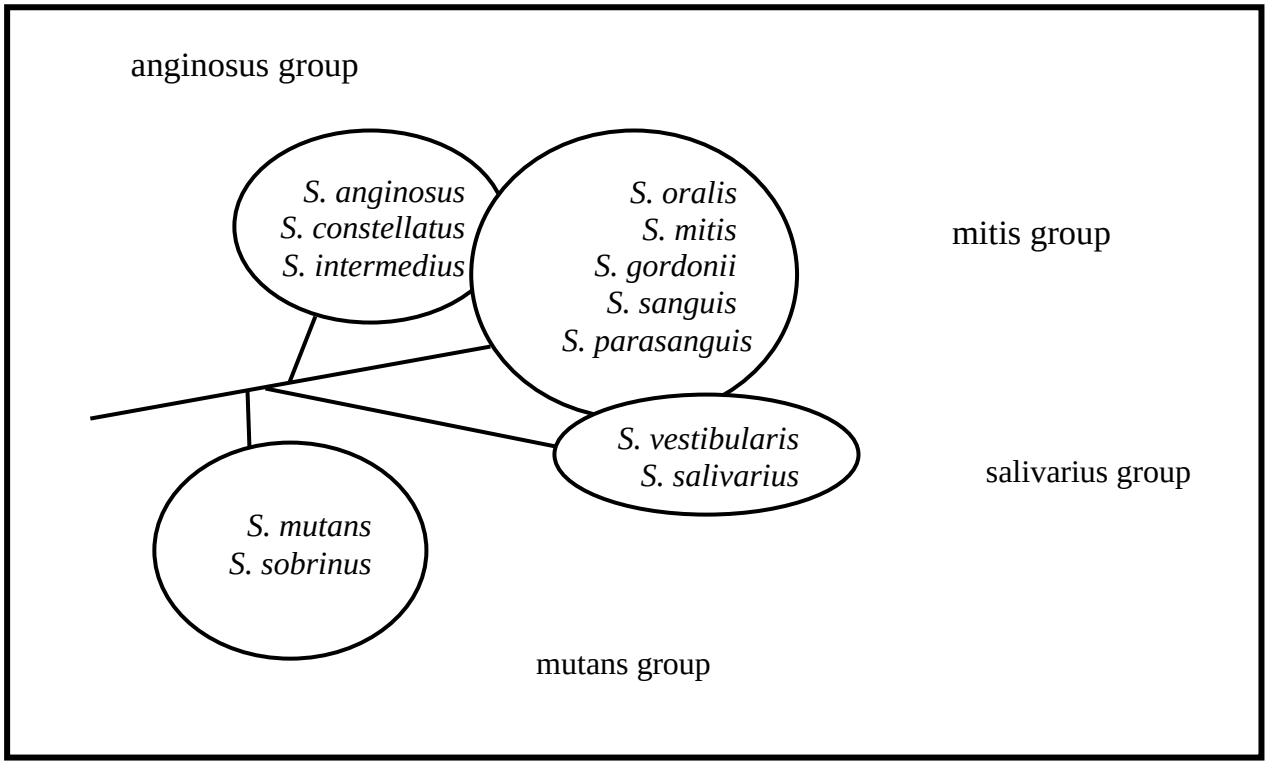

Figure 2-1: Streptococci commonly found in the human mouth phylogenetic relationships among groups (Russell, 2000)

Viridans streptococci is usually but invariable found in plaque of individual with high caries activity where ammonia and aerobic environment favor it's growth, their numbers increase with caries onset and decrease when open cavities are restored. They increase when salivary flow is reduced and exhibited stronger correlations with caries development when plaque rather than saliva is sampled for their presence (Menaker, 1980; Nolte, 1997). They are not entratransmitted between persons nor entertransmitted among teeth (Menaker, 1980). Moreover, viridans streptococci are phenotypically homogenous species, however, they exhibit a great degree of biochemical and antigenic heterogenecity (Igarashi *et al*., 1996).

Viridans streptococci are the most prominent members of normal flora of the human respiratory tract, and cause disease only when settle on abnormal heart valves (subacute infective endocarditis), in the meninges, or in the urinary

tract of humans, when mutans streptococci have been identified as the pathogens primarily for elderly patients with heart disease (Ullman *et al*., 1988).

Several other bacterial infections have also been related to oral microbes. For example, bacteremia may occur after dental treatment with vigorous tooth brushing especially in patients with periodentitis in which mutans streptococci are most dominant (Rams and Slots, 1992).

2.3 Mutans Streptococci Group

2.3.1 Classification, Identification and Detection of Mutans Streptococci Group

Generic name of *Streptococcus mutans* is of Greek origin, the species name "mutans" means variation in form, while *Streptococcus* meaning a chain of berries. Because of their role in the etiology of dental caries, *Streptococcus mutans* has received a great interest and originally isolated from carious human teeth by Clark (1924) shortly after war, it was discovered from a case of infective endocarditis (Loesche, 1986).

Streptococcus mutans cells are about 0.5-0.75 μm in diameter, occurs in pairs or in short- or medium length chains without capsules. Under acid condition in broth and on some solid media, these cocci may form short rod 1.5-3.0 μm in length. Rod-shaped morphology may be evident on primary isolation from oral specimens. *Streptococcus sobrinus* are about 0.5 μm in diameter, occurs in pairs and chains. The word sobrinus means male cousin on mother's side and refers to "distant relationship" between this species and *Streptococcus mutans*, *Streptococcus rattus, Streptococcus ferus.* While cells of *Streptococcus cricetus* are about 0.5 μm in diameter, occurring in pairs or chains (Hardie, 1986).

The mutans streptococci group can be separated into eight serotypes according to their surface antigens in their cell wall (Beighton *et al*., 1991). The

classification series is ranging between serotype **a** to **g** and / or **h**. For serotypes **a**, **d** and **g**, the antigenes are composed of glucose, galactose and rhamnose for serotypes **c**, **e** and **f** the antigens comprise glucose and rhamnose, While those for serotype **b** are galactose and rhamnose (Coykendall, 1989). These serotypes can in turn be classified into four groups according both to other biochemical properties (Shklair and Keen, 1974) and to the specificities of their enzymes in extracellular polysaccharide synthesis (Kametaka *et al*., 1987). Thus serotypes **d**, **g** and or **h** (*Streptococcus sobrinus*) form one group, serotype **a** (*Streptococcus cricetus*) another, serotypes **c**, **e** and **f** (*Streptococcus mutans*) the third, and serotype **b** (*Streptococcus rattus*) the fourth group. Mechanisms of synthesis of extracellular polysaccharides in each group seem to be different (Yamashita *et al*., 1988).

Formation of the insoluble extracellular polysaccharide from sucrose is an essential characteristic of cariogenic bacteria which plays an important role in the adherence and build up of those bacteria on tooth surface forming a protective matrix against adherence of other bacterial species. It may act as a barrier for the diffusion of acids from plaque resulting in increasing acid concentration in proximity to the tooth surface (Al-Obaidi, 1993; Jenkinson and Lamont, 1997).

The most commonly isolated species of mutans streptococci from human is usually *Streptococcus mutans* serotypes **c**, **e**, and **f,** followed by *Streptococcus sobrinus* serotypes **d** and **g** (Carlsson, 1988). Serotype **c** of mutans streptococci, is the most frequent comprising about 70% of the clinical isolates, this may be due to its ability to produce bactriocins more frequently than other serotypes (Menaker, 1980). *Streptococcus rattus* (serotype **b**), although it was first isolated from rats (Coykendall, 1989), but has also been detected in specimens from humans, most commonly African population (Kilian *et al*., 1979). *Streptococcus cricetus* (serotype **c**) was first recovered from hamsters (Coykendall, 1989), but has also been isolated from humans (Bratthall, 1972).

Streptococcus ferus (serotype **c**) comes from wild rats and *Streptococcus macacae* and *Streptococcus downeii* (serotype **c** and serotype **h**) respectively, are isolated from monkeys (Coykendall, 1989). There is non reported isolation of the latter three species from humans.

Mutans streptococci form a heterogeneous group when their biochemical and genetic properties were studied (Coykendall, 1974; Scheifer *et al*., 1984). Coykendall (1974) divided mutans streptococci into four genotypes (geno species) depending on their DNA contents. The first group was mutans streptococci subsp. mutans (*Streptococcus mutans*) which contains 36-38% cytosine-guanine (C-G), the second group was mutans streptococci subsp. rattus (*Streptococcus rattus*) containing 41-43% (C-G), the third group called mutans streptococci subsp. sobrinus (*Streptococcus sobrinus*) with 44-46% (C-G), while the fourth group named mutans streptococci subsp. cricetus (*Streptococcus cricetus*), which contains 42-44% (C-G). These groups were described also by Russell (1976) by using SDS-polyacrylamide gel electrophoresis.

Mutans streptococci were also classified into five biotypes according to their ability to ferment mannitol, sorbitol, raffinose, milibiose, salicin or inuline. They are unlike other streptococci, ferment mannitol but may vary in their ability to ferment other sugars and in their ability of producing ammonia from arginine (Marhart and Fitzgerald, 1980; Newbrun, 1983).

Epidemiological studies implicated *Streptococcus mutans* as the primary pathogen in the etiology of enamel caries in children and young adults (Hardie and Bowden, 1974; Hamada and Slade, 1980). This is due to their ability of rapid lactic acid formation from dietary carbohydrates, mainly sucrose and glucose. It was demonstrated that the rate of lactic acid producing by *Streptococcus mutans* is faster than that of other acidogenic bacteria including *Actinomyces*, *Lactobacillus* and other *Streptococcus* species such as *Streptococcus sanguis* and *Streptococcus mitior* (Van Hout *et al*., 1991).

Principle identification or diagnosis of mutans streptococci is usually made from the characteristic morphology of its colonies on 5% sucrose containing culture media. Mitis-Salivarius medium, is usually used, which is composed of mitis salivarius agar with sucrose and potassium tellurite. It has the ability to inhibit growth of most bacteria, except streptococci, because it contains trypan blue and crystal violet, which suppress the growth of gram-negative organisms (Carlsson, 1967). MS-agar is a logically starting point for the development of further selective culture media. Van Houte (1973) modified MS-agar by adding 0.2 U/ml bacitracin and increasing the sucrose concentration to 20%, to inhibit growth of *Streptococcus sobrinus* and *Streptococcus cricetus*. This medium is called "Mitis-Salivarius bacitracin" (MSB) agar (Gold *et al*., 1973).

On the MS-agar, *Streptococcus mutans* colonies are small, raised, irregularly margined and adherent, while *Streptococcus sobrinus* colonies are surrounded by a zooglea with a gelatinous consistency (Hamada and Slade, 1980).

Further identification of mutans streptococci after culturing on the selective and non-selective agar media is, gram-staining, distinctive cell shape on light microscopy and biochemical test (including antibiotic-sensitivity test, the ability to tolerate 4% NaCl, the ability to produce dextran or levans, and sugar fermentation and enzymatic patterns). Mutans streptococci can also be further identified by the use of a commercial biochemical test system like API 20 Strep and the Lancefield grouping kit (Friedrich,1981).

Various types of *Streptococcus mutans* can also be characterized by bacteriocin production and susceptibility profiles to the bacteriocin. It has been suggested that this could be useful, a part from identification tests, in epidemiological and ecological studies (Rogers, 1975). There are indications to believe that the bacteriocins (mutacins) from *Streptococcus mutans* contains at least two kinds of inhibitory substances ranging from molecules with low molecular weight to high-molecular weight, and protein-lipid complexes.

Production of, and susceptibility to, the bacteriocin-like substances which has been determined to be influenced by the medium might be closely related to the colonial variation of *Streptococcus mutans* (Yamomoto *et al*., 1975).

Monoclonal antibodies (MAbs) directed against specific species of mutans streptococci, and also against cell markers and enzymes have been developed. The MAbs have been used in a number of studies on mutans streptococci for detection in epidemiological studies and in studies on microbial mechanisms (Shi *et al*., 1998).

Presently, molecular approaches have been developed for the detection and identification of mutans streptococci. These methods have a high discriminatory ability and reproducibility since they do not examine the gene expression but rather the DNA of the microorganisms to be studied (Olive and Bean, 1999). Among these methods are plasmid analysis, restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE) and arbitrarily primed polymerase chain reaction (PCR) (Caufield *et al*., 1982; Caufield and Walker, 1989; Saarela *et al*., 1993; Alaluusua *et al*., 1994; Arbeit, 1999; Olive and Bean, 1999).

Sato *et al*., (2001) reported a method using restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes to identify *Streptococcus mutans* and *Streptococcus sobrinus*, followed by culturing and isolation of colonies on agar plates. However, this method is time-consuming and requires culturing and isolating target bacteria on the agar-plates.

Most oral streptococci, posses glucosyltransferase (GTF) enzyme that use sucrose as a substrate to synthesize extracellular polysaccharides (Monchois *et al*., 1999), which facilitates the accumulation and adhesion of the oral bacterial cells on the tooth surface. A comparison of *Streptococcal* GTF's (GTF-D from *Streptococcus mutans*, GTF-T from *Streptococcus sobrinus*, GTF-K from *Streptococcus salivarius* and GTF-G from *Streptococcus gordonii*) revealed a non-conserved region located just down stream of the signal peptide. In

addition, there is a report in sues that the 5\-terminal region of *gtf* gene was conserved in the *Streptococcus mutans* (Fujiwara *et al*., 1998) and *Streptococcus oralis* (Fujiwara *et al*., 2000) species. Thus, this region of the *gtf* gene was thought to be a species-specific sequence for the oral streptococci and useful for their classification (Hoshino *et al*., 2004).

A direct PCR method has been developed to detect mutans streptococci directly from dental plaque samples or from saliva samples using the specific PCR primers without the need to culture and isolate the bacteria. Oligonucleotide primers specific for portions of the glucosyltransferase genes (*gtfB* of *Streptococcus mutans* and *gtf I* of *Streptococcus sobrinus*) were designed and used for the amplification of these two genes and identifing the mutans streptococci in the saliva samples using two stages PCR strategy (Oho *et al*., 2000; Igarashi *et al*., 2000; Rupf *et al*., 2003). However, specific bacterial DNA amplification may be influenced by the presence of other bacterial DNA extract from dental plaque samples and this may result in reduced sensitivity (Bamford *et al*., 1998; Sugita *et al*., 2001). Most of the PCR-based diagnosis systems reported are qualitative analysis and are therefore unsuitable for accurate evaluation of caries susceptibility or caries activity.

Quantitative analysis is essential in oral specimens, such as dental plaque and saliva. A real-time PCR assay with the Taq Man system based on the 5\-3\ exonuclease activity of Taq polymerase has been developed for the quantitative detection of DNA copy number (Holland *et al*., 1991). Yoshida *et al*., (2003) described a method for the absolute and relative quantification of human cariogenic bacteria including *Streptococcus mutans* and *Streptococcus sobrinus* from oral specimens by a Taq Man PCR assay.

2.3.2 Primary Acquisition and Transmission of Mutans Streptococci

Mutans streptococci are found mainly in dental plaque. Molar teeth are more heavily colonized than interior teeth and fissures in these regions. Teeth are more susceptible to colonization than proximal, buccal or lingual surfaces. Also, except for the occusal surfaces, teeth with restorations harbor more mutans streptococci than those with sound surfaces (Lindquist and Emilson, 1991).

Normally, the fetus is sterile before birth. The inoculation of the human oral cavity starts with the first tactile contacts with the mother and other persons present at the parturition as well as contact with air and equipment. The oral cavity of the toothless child contains only epithelial surfaces, and the first colonizers are species not requiring a nonshedding surfaces. Early colonizers include some streptococci, *Veillonellae*, *Actinomyces*, *Fusobacterium* and a few gram-negative rods (Könönen *et al*., 1994). *Streptococcus salivarius* is among the first permanent colonizer, when colonizing the dorsum of the tongue in the edentulous infant (Socransky and Manganiello, 1971). In the first months, most of the detected strains are transient colonizers. *Streptococcus sanguis* and mutans streptococci are stably colonized only after the first tooth has erupted (Berkowitz *et al*., 1975). After extraction of the teeth, mutans streptococci disappear, but reappear upon use of dentures (Carlsson *et al*., 1969a). Mutans streptococci may also be isolated from pre-dentate infants who wear obturators (Berkowitz *et al*., 1975). These observations suggest hard surfaces are essential for mutans streptococcal colonization.

In general, acquisition of microorganisms by human body occurs through transmission directly from one host to another, or indirectly by means of another living agent (vector). Pathogens can also be transmitted by in animate objects and disease vehicles such as food and water (Madigan *et al*., 1997b).

The mother is considered to be the most important source of infection to the baby. First indication of this is obtained by using a phenotyping technique of

Streptococcus mutans, bacteriocin typing (Masuda *et al*., 1985). Li and Caufield, (1995) detected by molecular typing of mutans streptococcus a homology of strains in 71% of mothers and their children, while Kozai *et al*., (1999) found that 51.4% of mutans streptococcal genotypes found in children were also identified in their mothers. Fathers and infants have a far lower strains match (Li and Caufield, 1995). The time period when most children gain mutans streptococci in their oral flora is when the primary teeth are erupting, i.e. between 8 month and 3 years of age (Caufield *et al*., 1993). The probability of colonization with mutans streptococci is frequent and the microbial cell count is at least 10^5 per ml saliva (Berkowitz *et al*., 1981). Another suggested prerequisite for early colonization is that the baby's diet includes frequent intake of refined carbohydrates (Alaluusua, 1991).

Recently, it has been shown that mothers can diminish the probability of transmitting mutans streptococci to their children by using xylitol chewing gum (Soderling *et al*., 2000). The exact mechanism of action regarding xylitol is unknown. The sugar substitute xylitol is not fermented by mutans streptococci into cariogenic acid end-products, and in the oral environment, xylitol presumably selects for mutans streptococci with a weakened virulence (Trahan, 1995).

It has previously been shown that in mothers harboring high numbers of mutans streptococci in their saliva preventive measures aimed at decreasing mutans Streptococcal counts also results in decreasing caries counts in their children, even after 15 years (Bratthall, 1997).

2.4 Molecular Pathogenesis of Dental Caries

Demineralization of enamel which results from acid production, and ability of mutans streptococci to adhere then colonize the teeth plays an equally important role in virulence (Loesche, 1986; Kuramitsu, 1993). Figure (2-2) shows that tooth colonization by these oral bacteria appears to be a two-step

process consisting of a sucrose-independent initial adherence to the acquired salivary pellicle, followed by sucrose-dependent cellular accumulation (Hajishengallis and Michalek, 1999).

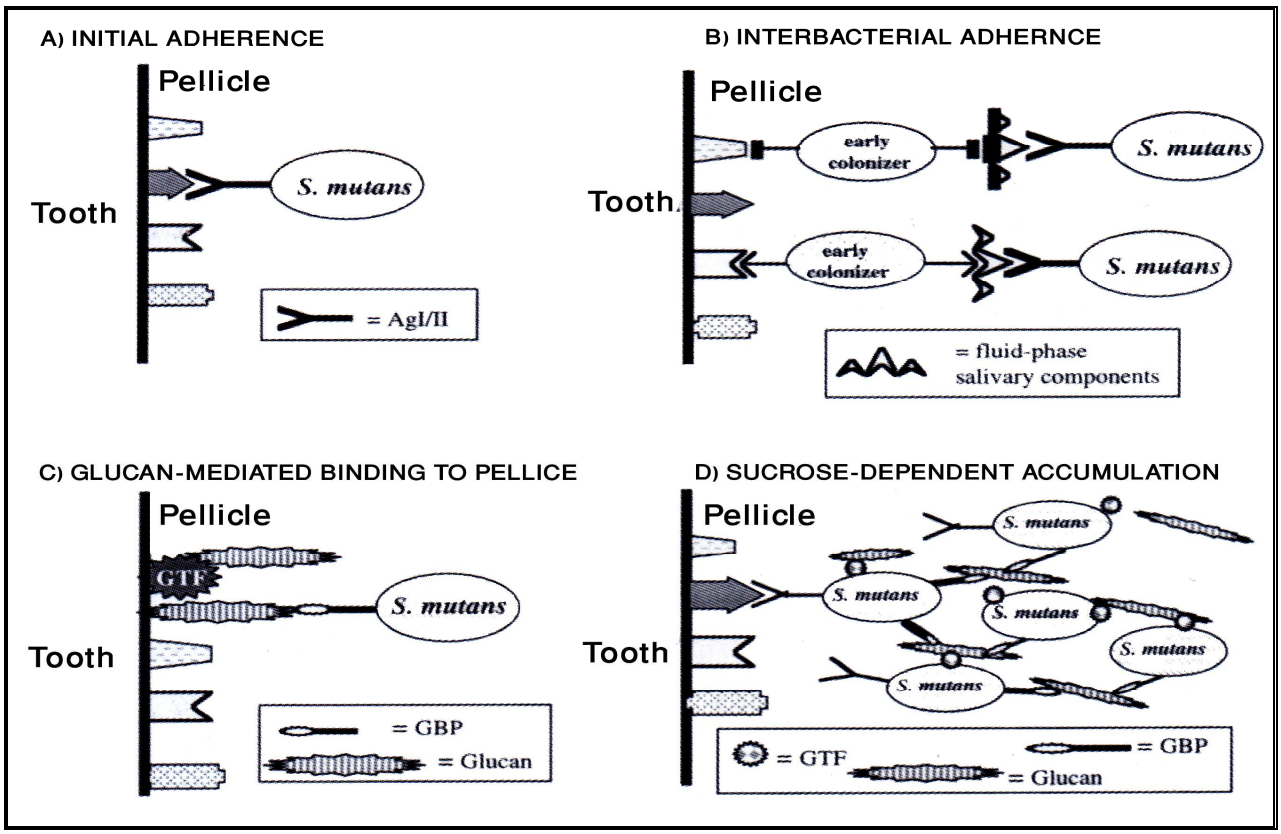

Figure 2-2: Colonization mechanisms of mutans streptococci (Hajishengallis and Mechalek, 1999).

The tropism of *Streptococcus mutans* and *Streptococcus sobrinus* for dental surfaces depends on the function of cell-surface salivary adhesins and extracellular biosynthetic enzymes, which should be accessible and thus vulnerable to block or inhibit by S-IgA antibodies in saliva. Specifically, a surface fibrillar protein originally termed antigen I / II (Ag I / II) and a group of secreted or cell-associated glucosyltransferases (Figure 2-2) (Smith, 2002) are implicated as key players in molecular pathogenesis of dental caries (Kuramitsu, 1993).

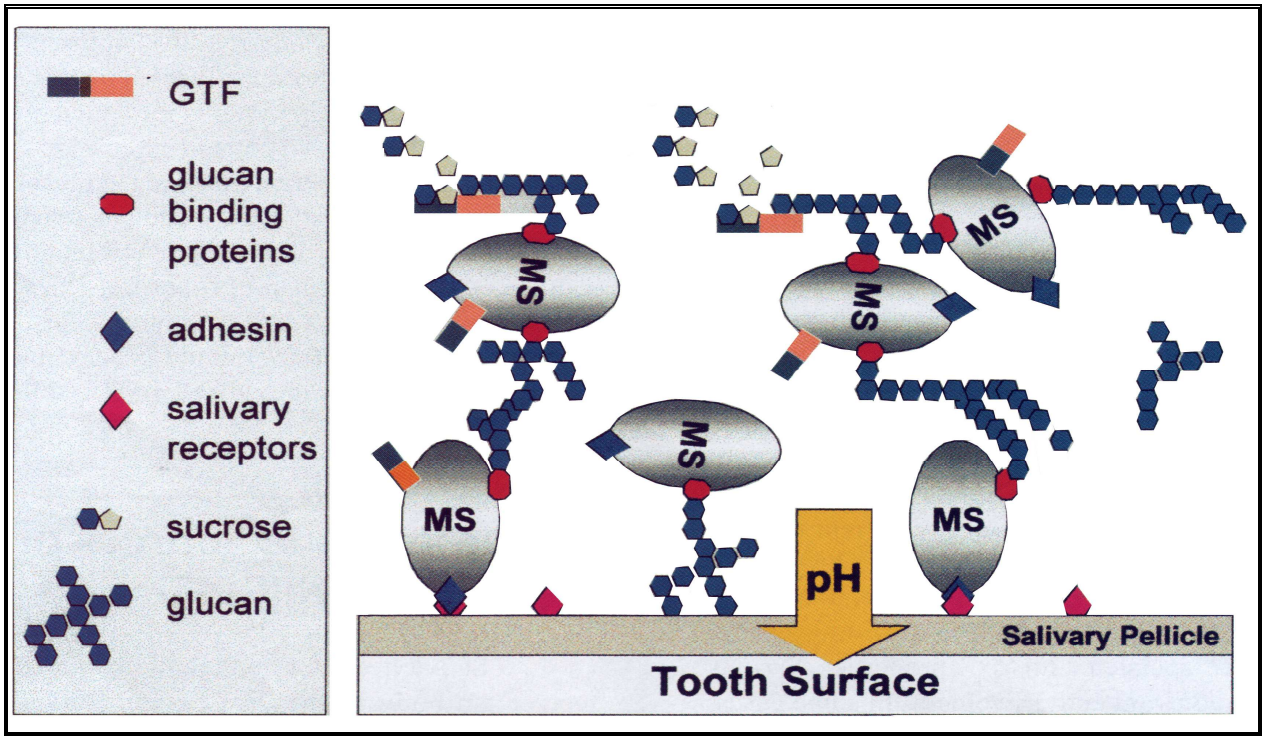

Figure 2-3: Molecular pathogenesis of mutans streptococci to the teeth (Smith, 2002)

Molecular pathogenesis of mutans streptococci involves several phases, each of which is very important in the dental caries formation. These acidogenic streptococci require hard surfaces furnished by teeth to sustain colonization and accumulation. To colonize the oral cavity, these streptococci must first bind to pre-existing receptors within dental biofilms. Initial attachment to the tooth occurs by the interaction of bacterial proteins with host-derived components in the dental pellicle covering the tooth surface. Such bacterial adhesins which referred to as antigen I / II in *Streptococcus mutans*, bind acidic, mucin-like glycoprotiens found in parotid and submandibular saliva. These components are found in salivary pellicles that coat both tooth surfaces and early colonizing bacteria such as *Streptococcus sanguis* and *Actinomyces spp.* (Russell and Lehner, 1978).

The ultimate pathogenecity of mutans streptococci occurs through erosion of the hydroxyapatite like mineral in dental enamel by lactic acid, a metabolic end product of bacterial growth. However, significant distractive concentrations

of this acid are required for the substantial accumulation of these acidogenic streptococci in dental plaque. Such accumulation process is initiated by the activity of extracellular glucosyltransferase (GTF) of which several are constitutively secreted by mutans streptococci (Russell *et al*., 1987; Shiroza *et al*., 1987). In presence of dietary sucrose, GTFs synthesize several forms of high molecular weight branched extracellular glucan. GTFs that synthesize insoluble forms of glucan (*S. mutans* GTF-B and GTF-C) have been most closely associated with pathogenecity. These glucose polymers provide scaffolding for the aggregation of mutans and other oral streptococci through interaction with bacterial cell-associated glucan binding proteins. Furthermore, glucans modify the porosity of the dental biofilm, thus increasing the availability of nutrients for continued bacterial metabolism.

Several glucan-binding proteins have been described in mutans streptococci (Sato *et al*., 1997). Although each of these glucan-binding proteins (GBPs) has the ability to bind to certain forms of glucan and some have been shown to be cell-associated, their unique contributions to *in vivo* plaque development are as yet unclear. GTFs also contain glucan binding domains. The interactions of glucans with cell-associated glucan-binding domains of (GTFs) and (GBPs) combine to cause extensive accumulation of mutans streptococci in the dental biofilm (Figure 2-2). Since GTFs and GBPs are also secreted into the extracellular environment, their specific or nonspecific incorporation into the salivary pellicle would also provide binding sites for mutans streptococci (Smith, 2002).

Theoretically, next phase of the pathogenesis results from the metabolic activities of these masses accumulated mutans streptococci (and possibly of other accumulation associated microorganisms). Mutans streptococci are the most prolific producers of lactic acid in these accumulation, although other “low pH bacteria” may also contribute (Van Ruyven *et al*., 2000). Dental caries

ultimately in sues because resulting increase in the lactic acid concentration can not be sufficiently buffered to prevent enamel dissolution (Smith, 2002).

2.5 Virulence Factors of Mutans Streptococci

The term virulence describes the capacity of a parasite (a microorganism) to cause disease to its host. This property is quantitative and expresses the degree of pathogenecity and the ability to inflict damage to the host. The relationship between host and parasite is dynamic and depends on their individual characteristics and interrelationship as well as on the external factors. Virulence consists of bacterial properties required for the interaction between host and parasite. Factors that promote entry, colonization and growth of the pathogen within the host, including those required for opposing host defences and for nutrient acquisition (Madigan, 1997b).

The initial information on virulence factors of *S. mutans* was obtained from studies involving isolation and biochemical characterization of extracellular and cell wall-associated components of this microorganism that appeared to be involved in pathogenesis (Hamada and Slade, 1980). Genetic approaches employing mutants of *S. mutans* have been extremely helpful in delineating not only virulence components but also mechanisms involved in the virulence of *S. mutans* (Loesche, 1986). Recombined DNA techniques, availability of transformable *S. mutans* strains and methods of transforming *S. mutans* are facilitating studies at the molecular level to determine the number of genes contributing to a specific phenotype and mechanisms involved in their regulation (Hensel and Holden, 1996).

Based on the accumulating information from biochemical, genetic, and immunological studies, a number of components of *S. mutans* have been identified for their potential roles in virulence and use in vaccine development (Krass, 1988).

Recognized virulence factors of mutans streptococci are adhesin-like cell surface proteins, acid tolerance, acid production and production of glucosyltransferases, mutacin and intracellular polysaccharide which promot their colonization and survival in the biofilm as well as the dental plaque that covers the tooth surfaces (Kuramitsu, 1993). In addition to the recognized virulence factors, other properties of microorganisms may influence virulence. One of the suggested virulence factors is the proteolytic activity of mutans streptococci (Jackson *et al*., 1997). *S. mutans* has been shown to produce two extracellular proteases, possibly metalloproteases, capable of degrading both gelatin and collagen-like substrates (Harrington and Russell, 1994).Under debate, is whether metalloproteases detected in connection with mutans streptococci are produced by the microorganisms or whether they are host-derived (Tjäderhane *et al*., 1998). Regarding S-IgA protease activity of mutans streptococci, the presiding view is that the organisms themselves do not produce this protease (Marcotte and Lavoie, 1998). However, other investigators were able to demonstrate the ability of *Streptococcus* for production of S-IgA-protease and prone their functions (Al-Mudallal, 2002; Marcotte and Lavoie, 1998). Another trait enabling survival is the ability of mutans streptococci to rapidly adapt to the environment by microbial genetics phenomena. This property has been suggested to be an essential element in the dominance of *S. mutans* in cariogenic dental plaque (Burne, 1998). As a rule, the ability of cells to take up exogenous DNA and regulating the natural genetic competence in bacteria is indicated by nutritional conditions and cell-to-cell signaling (Solomon and Grossman, 1996).

2.5.1 Acidogenicity and Acidouricity

Mutans streptococci ferment many different sugars. They appear to metabolize sucrose to lactic acid more rapidly than other oral bacteria. This is thought to be related to the multitude enzyme systems catalyzing the reactions of

transport and metabolism of sucrose expressed by these organisms (Kuramitsu, 1993). It seems that lactic acid is the most important acid involved in the etiology of dental caries. Dietary sugars other than sucrose (e.g. glucose and lactose) can also induce acid formation. However, these sugars are less cariogenic than sucrose, because, in addition to being converted to acidic metabolites, sucrose is also uniquely utilized for extracellular polysaccharide synthesis. Starch is less cariogenic than other dietary sugars because it does not readily diffuse into plaque and is less readily hydrolyzed (Horton *et al*., 1985).

Despite that mutans streptococci can grow at relatively low pH values, some strains are able to grow in a pH value lower than 4. These streptococci produce large amounts of a membrane-associated ATPase, capable of functioning at low pH, which helps to pump H^+ ions from the cell and thus reduce intracellular acidification (Bender *et al*., 1986; Carlsson, 1989). However, *S. sobrinus* is more acidogenic and highly cariogenic than other mutans streptococci (de Soet *et al*., 1989)

2.5.2 Bacteriocins

Bacteriocins are proteinaceous antibacterial substances produced by some bacteria to interfere with the growth of others, generally closely related bacteria. Bacteriocins are produced in addition to other inhibitory substances, including bacteriolytic enzymes and metabolic-by-products, such as (organic acid, diacetyl and hydrogen peroxide), which are formed during bacterial growth (Jack *et al*., 1995).

Bacteriocins are ribosomally synthesized and usually require extensive posttranslational modification for their activity. The genes involved in the synthesis and modification of bacteriocins are often carried by a plasmid or transposon (Madigan *et al*., 1997a). Bacteriocins are frequently named according to the bacterial species producing them, those who produced by mutans streptococci are called mutacins (Hamada and Ooshima, 1975). Mutacin

production is usually not plasmid encoded (Caufield *et al*., 1990). Mutacin typing include both susceptibility to and production of mutacin, has used for epidemiological typing of isolates. Berkowitz and Jordan (1975) were the first to bind between the mutacin typing method and the transmission of the microorganisms from the mother to her child. Clinically, mutacins have been considered important for the establishment and equilibrium of bacteria in dental plaque. The mutacin-producing strains might colonize more easily and suppress non-producing strains (Hillman *et al*., 1987). Most strains of mutans streptococci are able to produce mutacin on agar, but very few produce them in liquid culture (Hamada and Ooshima, 1975). Several mutacins have been purified and biochemically characterized (Chikindas *et al*., 1995).

2.5.3 Adhesins:

Adhesin is a cell surface fibrilliar protein of molecular weight 185 KDa, it is also known as protein B (Russell, 1979), P_1 (Forester *et al*., 1983), PAc (Okahashi *et al*., 1989); MSL-1 (Demuth *et al.,* 1990), SR and antigen (Ag I / II) in *S. mutans*, and also known as (SPaA or PAg) in *S. sobrinus* (Smith, 2002). Ag I / II was found in the supernatant of *Streptococcus mutans* culture and bind selectively to human-saliva coated hydroxyapatite (SHA), which stimulates pellicle-coated enamel. While isogenic Ag I/II-deficient mutants of *S. mutans* lack the protein fuzzy coat on the cell surface and bind poorly to experimental salivary pellicles (Lee *et al.,* 1989). Ag I/II has also been shown to bind collagen and make *S. mutans* able to invade human root dentinal tubules (Love *et al*., 1997). Also it has the ability to bind not only to collagen but to various other extracellular matrix molecules (Sciotti *et al*., 1997). Thus, it appears to possess versatile binding properties and interacting with matrix protein may contribute to the progression of dentinal carious lesions and initiation of endodontic infections. Moreover, to the dissemination of bacteria under pathological

conditions that expose matrix molecules in the oral cavity (Hajishengallis and Michalek, 1999).

Ag I/II consists of a protease-sensitive N-terminal region, AgI, and protease-resistant Ag II, which represents the C-terminal and membrane-proximal one-third of the molecule (Russell *et al.,* 1980; Kelly *et al.,* 1990). Molecular studies have shown that the genes encoding Ag I/II- like proteins are highly conserved both within and between serotypes of mutans streptococci, and share homology with genes for surface proteins from other species of oral streptococci (Brady *et al.,* 1991a; Ma *et al.,* 1991)

2.5.4 Glucan-Binding Proteins

Glucan is a polymer of glucose synthesized from dietary sucrose and possibly additional oligosaccharides, by glucosyltransferases enzymes produce by oral streptococci (Hamada and Slade, 1980; Loesche, 1986; Vacca-Smith *et al.,* 1996a,b). It is not a simple linear chain, but rather a branched structure composed of glucose units joined by both α (1-6) and α (1-3) glucosidic linkages. The α (1-3) linkages occur sparely in soluble dextrans, where as the relatively insoluble forms termed mutans, contain considerably increased proportions of α (1-3) linkages, and hence significantly more branching (Yamashita *et al.,* 1993). It has also been suggested that glucan synthesized by *S. mutans* may play additional role in the cariogenesis other than serving as a mean of enhancing the attachment of the organisms to the tooth surfaces (van Houte *et al*., 1989). Thus, glucan may serve as permeability barriers within the dental plaque biofilm, which could affect the transport of sugars into plaque and / or the diffusion of acids from plaque.

The ability of mutans streptococci to bind glucan is presumed to be mediated, at least in part, by cell-wall associated glucan-binding proteins (GBP). Many proteins with glucan-binding properties have been identified in *S.mutans* and *S. sobrinus* (Smith *et al.,* 1998). Each glucan-binding proteins has the ability

to bind α 1-6-glucan, through other glucan linkage potentially may impart higher binding contents. *S. mutans* secretes at least three distinct proteins with glucan-binding activity: GBP-A (Russell, 1979), GBP-B (Smith *et al.,* 1994) and GBP-C (Sato *et al.,* 1997).GBP-A has a deduced sequence of 563 amino acids (Banas *et al.,* 1990). The molecular weight for the processed protein is 59 KDa. The carboxy-terminal two-thirds of GBP-A sequence have significant homology with putative glucan-binding regions of mutans Streptococcal glucosyltrans-ferases (Haas and Banas, 2000). GBP-A has a greater affinity for water-soluble than for water-insoluble glucan. The expressed GBP-B protein is 431 residues long and has a calculated molecular weight of 41.3 KDa (Mattos-Graner *et al*., 2001). Its sequence is unrelated to those reported for other *S. mutans* (GTFs) or GBPs, paralleling the lack of reaction of anti-GBP-B antibody with those proteins. The N-terminal third contains several immunodominant regions, which may explain the significant apparent immunogenicity of this protein in human (Smith *et al*., 1998) and animal (Smith and Taubman, 1996). Although the function of this protein in the native environment is as yet unresolved, biofilm formation on plastic surfaces by strains of *S.mutans* is directly correlated with expression of GBP-B (Mattos-Graner *et al*., 2001), suggesting a role for GBP-B in this process. The third *S. mutans* non-enzymatic glucan-binding protein GBP-C, is composed of 583 amino acids, this protein has a calculated molecular weight of 63.5 KDa. The GBP-C protein, detected when *S. mutans* cultures are stressed during growth, is associated with dextran-dependent aggregation (Smith, 2002).

2.5.5 Glucosyltransferase (GTF)

Glucosyltransferases (GTFs) are extracellular enzymes that bind and synthesize extracellular glucan, which are important for the establishment of cohesive mutans Streptococcal masses on the tooth surfaces and subsequent caries development (Kuramitsu, 1993; Lilijemark and Bloomquist, 1996). These

enzymes are essential virulence factors to facilitate the ability of this bacterium and other microorganisms for colonizing the tooth when sucrose is present in the host diet (Yamashita *et al.*, 1993). They play a significant role in the initiation, development and maturation of dental plaque, and are essential in the formation of smooth surface lesions at least and contribute to the formation of sulcal surface lesions (Schilling and Bowen, 1992; Kuramitsu, 1993; Yamashita *et al.*, 1993).

Glucosyltransferase of mutans streptococci is able to catalyze the hydrolysis of glycosidic linkage of sucrose resulting in the release of glucose and fructose and transfering the glycosyl moiety to a terminal site on the growing glucan molecule. The catalytic or sucrose-binding and splitting region is located at the N-terminal one-third of the glucosyltransferase (Mooser *et al*., 1991; Kato *et al*., 1992; Tsumori *et al*., 1997), while the glucan binding region at the C-terminal one-third of the molecule (Kato and Kuramitsu, 1990; Abo *et al.*, 1991). The glucan binding domain is essential for the transferase activity (Kato and Kuramitsu, 1990), and as shown in (Figure 2-4) for binding enzyme to the cell surface (Smith, 2002). *Streptococcal* GTFs are moderately sized proteins (approximately 1.500 amino acids) synthesized with an additional peptide extension (signal peptide) of about 30 amino acids which facilitate export across the cell membrane.

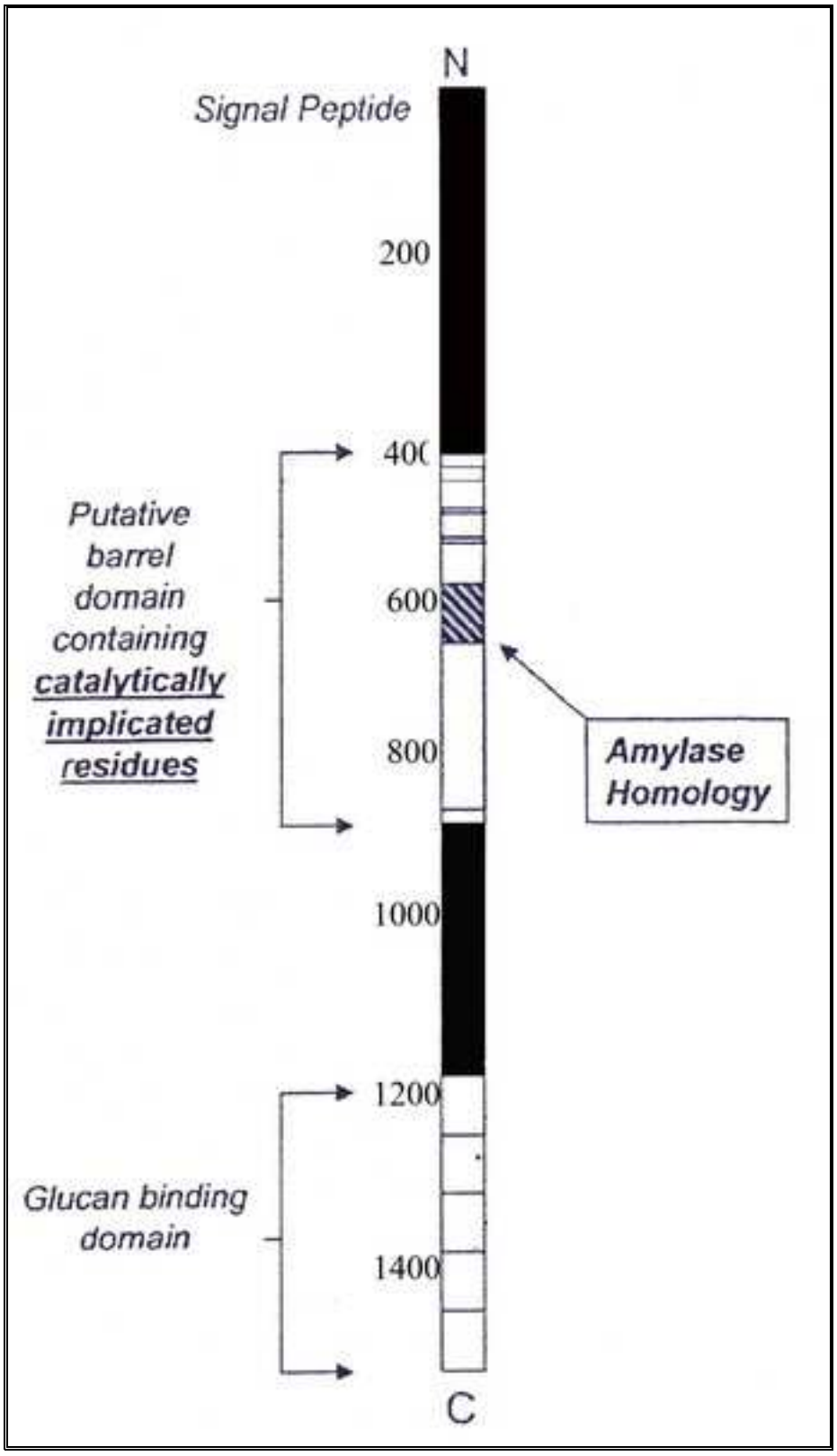

Figure 2-4: Structure of Glucosyltransferase (Smith, 2002)

The 200 or so amino acids adjacent to the signal peptide vary significantly among GTFs from different species, and comprise part of the catalytic domain of the enzyme. Amino acid sequencing reveals the presence of repeating glucan binding units, each of approximately 23 amino acids requiring 3 to 6 times interspersed with other sequences within the glucan binding domain of the enzyme (Banas *et al.,* 1990). Thus, having evolved a useful functional module, the evolutionary process duplicates and spaces them in order to subtly alter the glucan-binding properties of different GTF species. Evidence that this region or unit has glucan binding function is obtained from observation revealed large C-terminal, tryptic fragments of GTF which retain the ability to bind α 1, 6 glucan (Wong *et al.,* 1990; Abo *et al.,* 1991). The glucan binding potential of these repeating GTF sequences is also supported by the observation that amino acids deletions in this region remove glucan binding activity or decrease the efficiency of insoluble glucan synthesis (Konishi *et al.,* 1999).

The catalytic activity of GTF appears to be associated with several, sequentially separate, residues in the N-terminal third of the molecule. These residues have been identified by a variety of methods, including the labeling of catalytic intermediates and site-directed mutagenesis (Tsumori *et al.,* 1997; Monchois *et al.,* 2000). Insight into catalytically important residue identification, which have revealed significant homology between GTF and alpha amylase with respect to several invariants residues important to the catalytic activity of the alpha amylase family, suggesting that the amylase (β,α) 8 barrel element may be also a feature of the GTF catalytic domain (MacGregor *et al.,* 1996; Devulapalle *et al.,* 1997).

Streptococcus mutans (serotypes **c**, **e** and **f**); *S. sobrinus* (serotypes **d** and **g)**, *S. downeii* (serotype **c**), *S. salivarius* and *S. sanguis* express several glucosyltransferase enzyme types which differing in chain length, content of α-1,3 and α-1-6 glucosyl linkages, degree of branching and solubility (Shiroza *et al.,* 1987; Hamada *et al.,* 1989). GTF which synthesizes soluble glucan from

mutans streptococci (serotypes **a** and **d**) would be similar to the enzyme from mutans streptococci (serotype **g**), since these enzymes are immunological identical or partially identical to each other. On the other hand, GTF from mutans streptococci (serotypes **b**, **c**, **e** and **f**) may be considerably different from the enzymes of (serotypes **a**, **d**, and **g**), since the former group have no immunological relationship with the latter enzyme (Mukasa *et al.,* 1982).

Molecular weight of *S. mutans* and *S. sobrinus* GTF enzymes are between (140-160KDa). There optimum pH is 6.5 and temperature at 37^{o}C (Hamada *et al.,* 1989). The reason behind the interest of studying GTF enzymes of *S. mutans* (serotypes **c**, **e**, and **f**) and *S. sobrinus* (serotypes **d** and **g**) is due to their ability to elicit protective immunity against experimental dental caries caused by implanted mutans Streptococcal species (Taubman and Smith, 1977).

At least three GTFs enzymes have been identified from *S. mutans*. GTF B has a molecular weight of 148 KDa which synthesizes α1,3-linked water insoluble glucan, GTF D with a molecular weight of 143 KDa synthesizes α 1, 6-linked water soluble glucan and GTF-C with a molecular weight of 138KDa which synthesizes a mixture of water-soluble and insoluble glucan (Aoki *et al.,* 1986; Hanada and Kuramitsu, 1988; 1989). GTF-B and C enzymes are cell wall associated and share approximately 75% identity (Ueda *et al.,* 1988), while GTF D enzyme (a secreted enzyme) is 50 % identical to GTF B enzyme (Honda *et al.,* 1990).

Streptococcus sobrinus (serotypes **d** and **g**) secretes four kinds of glucosyltransferase enzymes; one a water-insoluble glucan synthase [(1→3-α-D-glucan synthase)] and three water-soluble glucan synthases [S-GTases, (1→6)-α-D-glucan synthase] (Hanada and Takehara, 1987). Three of these enzymes have required an exogenous primer or acceptor (such as dextran) for full expression of their essential activities (Shimamura *et al.,* 1983). It is thought that the fourth glucosyltransferase of *S. sobrinus* which has a molecular weight of (139 KDa) supplied the primer for the other three GTFs enzymes and has a

significant role in the formation of extracellular polysaccharide of these bacteria (Yamashita *et al.*, 1988).

Streptococcus sanguis is an early colonizer of the tooth surfaces and is found abundantly in the mature dental plaque (Frandsen *et al.,* 1991). Results from *in vitro* studies have shown that this organism binds to specific salivary molecules and may also coaggregate with other oral microbes (Bleiweis, 1993), suggesting multiple cooperative events allowing its presence on the hard tissues of the oral cavity (Kohlenbrander *et al.,* 1983). *S. sanguis* also produces a single glucosyltransferase enzyme (GTF-S) that gives primarily soluble glucan with both α-1,6 and α-1,3 linkages (Beeley and Black, 1977), also it may contribute to the over- all polysaccharide contents of dental plaque (Carlsson *et al.,* 1969b). Optimum pH of the GTF-S enzyme in solution ranges between 5.2 and 7.0, and optimal activity at a temperature of 45^{o}C. It is different from those GTF enzymes produced by mutans streptococci (Wunder and Bowen, 2000).

Analysis of the encoding gene sequences reveals that the number of GTF genes in each species of mutans streptococci varies. Three genes encoding for glucosyltransferase activity have been cloned and sequenced from *S.mutans* (serotypes **c**, **e** and **f**), *gtf* B is responsible for the insoluble glucan synthesis (Shiroza *et al.,* 1987), *gtf* C for soluble and insoluble glucan synthesis (Ueda *et al.,* 1988); and *gtf* D for soluble glucan synthesis (Honda *et al.,* 1990). Animal studies with defined mutants of *S. mutans,* in which single or multiple *gtf* genes have been inactivated, showed that insoluble glucan synthesis is more important for cariogenicity than soluble glucans (Munro *et al.,* 1991; Yamashita *et al.,* 1993). *S. sobrinus* (serotypes **d** and **g**) GTFs are encoded by two genes (*gtf* I) (Russell *et al.,* 1987) and *gtf* S (Gilmore *et al.,* 1990) genes which are responsible for synthesizing insoluble and soluble enzymes respectively.

S. downei and *S. salivarius* have four genes, while *S. gordonii* appears to have only one gene encoded for GTF enzyme activity. The individual GTF genes are often located adjacent to one another on the bacterial chromosome

(Giffard *et al.,* 1991), and these arrangements probably results from the duplication of a single ancestral gene. Once duplicated, the individual genes can evolve new characteristics independently of each other through successive mutation and evolution selection (Simmonds *et al.,* 2000).

2.5.5.1 Determination of GTF Activity

Active GTFs have been identified in whole human saliva and salivary pellicle and also detected in experimental pellicle (Rölla *et al*., 1983; Scheic *et al*., 1987; Vacca-Smith *et al*., 1996b).

GTF-B, GTF-C and GTF-D of *S. mutans*, were enzymatically active both in solution and in an experimental pellicle, are formed by adsorbing GTF onto surface of clarified human whole saliva (CWS)-coated hydroxyapatite (HA) (Venkitaraman *et al*., 1995).

Reviews of previous studies indicate that research in general types, structure and function of GTFs concentrated on assay for enzyme activity Mukasa *et al*., (1982) determined GTF activity in broth of *S. mutans* (serotype **c**) by collecting the synthesized glucan from the supernatant after incubation with sucrose substrate and precipitating with 75% ethanol then determination by phenol / sulfuric acid method (Dubois *et al*., 1956). The synthsized glucan could be measured by gas-liquid chromatography using a WCOT column of OV-101 (Mukasa *et al*., 1982). One international unit (IU) of enzyme was defined as the amount of glucosyltransferase catalyzing the incorporation of 1 μmol of glucose from sucrose under the conditions of experiment (Mukasa *et al*., 1982).However, others determined activity depending on measurement of reducing sugar release from sucrose at 500 nm (Somogyi, 1945) and accordingly, One unit of the total activity is corresponded to the amount of glucosyltransferase releasing 1 μmol of reducing sugar from sucrose under the conditions of experiments (Mukasa *et al*., 1982).

On the other hand, some studies includes SDS-PAGE in measurement of GTF activity. They were able to differentiate between the enzyme which is responsible for producing soluble glucan from that produce insoluble type through incubation the polyacrylamide gel with the substrate over night at 37°C (Furuta *et al*., 1983; Mukasa *et al*., 1985).

Shimamura *et al*., (1983) estimate GTF activity of *S. sobrinus* by determining soluble and insoluble-glucans synthesized by three GTFs, which are easily distinguished from each other by isoelectrical focusing (IEF) gel after the application of suitable amount of culture supernatant on a filter paper on the gel surface. After IEF process, the gel was incubated at 37°C for 22 hours in 5% sucrose. The activity of GTF water-insoluble polysaccharide was directly detected as a white-band, and water-soluble one was stained by the periodic acid-schiff base (PAS) method (Kapitany and Zebrowski, 1973).

Recently, GTF activity of *S. mutans* and *S. sanguis* was determined by incorporation of C^{14}-labled glucose from sucrose into glucans produced by diluting GTFs in a standard substrate buffer solution which contains a suitable amount of sucrose and dextrans. All reactions were carried out at 37°C and stopped after 4 hours by the addition of ice-cold 70% ethanol. An amount of the precipitated glucan which was collected on the surface of gas fiber filter is quantified by liquid scintillation spectrometry (Venkitaraman *et al*., 1995; Vacca-Smith *et al*., 2000; Wunder and Bowen, 2000).

Venkitarman *et al*., (1995) determined activity of GTF on the surface of hydroxyapatite. Hydroxyapatite beads were incorporated with the clarified human whole saliva (CWS) for 30 min at 37°C. After pH was maintained at 6.5, the enzyme was added, and unadsorbed materials were removed by washing with buffer, then the enzyme substrate was added and the reaction was stopped after 4 hours by the addition of ice-ethanol. The glucans were separated from unreacted sucrose by filtration, and total glucan product was calculated as

micromoles of glucose incorporated into glucan by use of known ratio of labeled to unlabeled sucrose .

2.5.5.2 Purification of GTF

Several methods have been used for purification of mutans streptococci GTF enzyme. Challacombe *et al*., (1973) purified GTF enzyme from culture fluid of *S. mutans* by the use of hydroxyapatite column chromatography. Stepwise elution in 0.2 M and 0.5 M phosphate buffer resulted in two pools of activity as determined by isoelectrical focusing of this preparation revealed it to be a mixture of at least seven GTFs.

Other purification procedures were made from 20L culture supernatant of *S. mutans* by filtration through different ultrafiltration membranes in an Amicon Ultrafiltration cells in order to concentrate the enzyme and to remove any contaminating D-glucan. Polyacrylamide gel electrophoresis was used in order to quantitate the enzyme acitivity and the degree of purification (Ciardi *et al*., 1977; Figures and Edwards, 1978).

Smith *et al*., (1979) purified *S. mutans* GTF by gel filtration on a column containing 8% agarose. The GTF fractions were concentrated by negative pressure and further purified on Sepharose 4B.

McCabe (1985), purified GTF from *S. mutans* cell free supernatant fluid by precipitation with 50% saturated ammonium sulfate to obtain a crude extracellular protein preparation, affinity chromatography on Sephadex G-50 in order to separate the glucan binding proteins from the crude extracellular preparation. Proteins were further fractionated by ion-exchange chromatography on a bed of Tris acryl-M-DEAE-affinity chromatography yielded two peaks containing proteins, then GTF activity was determined by polyacrylamide gel electrophoresis which indicate the presence of two types of GTF represented by each peak, also the presence of the third one named (GTF-I isozymes) in both peaks.

Taubman *et al.*, (1988) purified two types of GTF enzymes from *S. sobrinus* using SDS-PAGE and named them GTF-I and GTF-S with molecular weights of 153 KDa and 148 KDa, respectively. Purification procedures were performed by Sephadex G-100 column, Sepharose 4B-CL, then the two enzymes were separated on a column of DEAE-Bio gel A as two peaks. The yield of total GTF from the column was approximately 13% of the activity applied.

Yamashita *et al*., (1989) purified four types of GTF enzymes from *S. sobrinus* by salt precipitation, affinity chromatography using dextran-agarose affinity column, high-pressure liquid chromatography column with a TSK-gel phenyl-5PW column, DEAE-cellulose chromatography column, ultrafiltration and the use of immunsorbent column. The degree of purification and molecular weight of these enzymes were determined by isoelectrical focusing and SDS-PAGE.

Purification of *S. sobrinus* GTF enzymes was also done from culture supernatant of this bacteria by chromatography on Sephadex G-100. The GTF-rich pools were then subjected to fast protein liquid chromatography on Superose 6. The gel filtration step separates non-GTF and other glucan-binding proteins as demonstrated by (SDS-PAGE). *S. sobrinus* GTF preparation obtained after gel filtration on Superose 6 contained a mixture of water (GTF-I)-insoluble glucan product [IG], (GTF-U) primer stimulated soluble glucan [SG] product and (GTF-S) primer independent SG-product (Taubman *et al*., 2000; Smith *et al*., 2003).

Wunder and Bowen,(2000) purified GTF-B, GTF-D and GTF-C of *S. mutans*, as well as GTF-S enzyme of *S. sanguis* by the use of salt precipitation, affinity chromatography column containing protein G conjugated with anti-GTF IgG (Anti-serum raised in rabbit against GTF enzyme), hydroxyapatite chromatography column using Macroprep Ceramic hydroxyapatite type 1 column. The final preparation of GTF-D, GTF-C and GTF-S was purified 250,

300 and 100 fold ,respectively, over the starting material as determined by usage of silver-stained (SDS-PAGE).

2.6 Effect of Inhibitors on GTF Enzyme and Mutans Streptococci

Many compounds were found to have the ability to reduce plaque formation *in vivo* (Gjermo *et al*., 1973; Lobene and Soparker, 1973) and inhibiting the activity of GTF enzymes in solution (Ciardi *et al*., 1978; Thaniyavarn *et al*., 1981, Kawabata *et al*., 1993). These compounds include: quaternary ammonium salts, bis-biguanides, phenolic-compounds and aliphatic amines. However such earlier studies were performed using mixtures of GTF enzymes in undetermined ratios with inconsistent results.

Vacca-Smith and Bowen (1996) revaluate the effects of some previously studied GTF inhibitors and some common mouthrinses on each of glucan formation by individual, they purified GTF enzyme both in solution and on the surface of Saliva-Coated Hydroxyapatite (SHA). It was found that chlorohexidine digluconate at concentration (1.25mM) reduce glucan formation by GTF-C of *S. mutans* in solution. It also may concern that glucan formation by a mixture of GTFs from *S. mutans* adsorbed onto bare hydroxyapatite beads was also effected by chlorohexidine diglucanate (Scheie and Kjeilen, 1987).The clinical effectiveness of chlorahexidine could be due to the interaction of anti-plaque agent with GTF-C prior to adsorption of the enzyme onto tooth and apatitic surfaces, which inhibiting the enzyme before adsorption onto a tooth surface (Vacca-Smith and Bowen, 1996).

Al-Uqaili (2000) found that chlorohexidine at high concentration (0.25%) has a bactericidal activity against mutans streptococci and may act as detergent by damaging the cell membrane and precipitating cytoplasmic components.Sodium florids, Sodium-doedecylsulfate (SDS), hexetidine, iodine,

urea, zinc sulfate as wall as substitutes represented by sorbitol could alter the composition of plaque microflora widely by effecting the growth and acid production of *Streptococcus* species.

In addition to the adaptive or specific immunity that is mediated predominantly by secretory immunoglobulin A (S-IgA) antibodies, human saliva also contains an array of antimicrobial molecules whose presence does not depend on previous exposure to microbial antigens. These non immunoglobulin defense factors contribute to the protection of the dental and mucosal surfaces of the oral cavity by modulating colonization and metabolism (Levine *et al.,* 1987; Tenovuo, 1989; Scannapieco, 1994). Submandibular-sublingual mucins and other salivary glycoproteins such as the parotid salivary agglutinin, are capable of aggregating oral microorganisums from the mouth by swallowing (Rundegren, 1986; Tabak, 1990; Slomiany *et al.,* 1996).

Innate humoral defense factors present in saliva may act alone or with each other in a synergistic or antagonistic manner (Rundegren, 1986; Pruitt *et al.,* 1999). One type of interaction is via the formation of heterotypic complexes (e. g., mucins form complexes with various molecules, including lysozyme, cystatins and α-amylase), which in certain cases may have properties distinct from those of the individual components (Biesbrock *et al.,* 1991). Jespersgaard *et al.,* (2002), isolate and characterize GTF inhibitory factor (GIF) and identify it as a non immunoglobulin salivary component, and subsequently using chromatography purified and characterized as a glycoprotein-α amylase complex. The *in vitro* binding of this salivary factor to glucan-binding region (GLU) of GTF results in an interfering with the enzyme activity of GTF in a manner analogous to that of anti-GLU antibodies.

Propolis, a resinous substance collected by *Apis mellifera* bees from various plant sources and mixed with secreted bees-wax, is a multifunctaional material used by bees in the construction, maintenance and protection of their hives (Burdock, 1998). Flavonoids have been considered the main biologically

active compound in propolis (Bonhevi *et al.*, 1994). Ikeno *et al.*, (1991) demonstrated that topical application of propolis twice daily or it's inclusion in drinking water available ad Libtum reduce the incidence of dental caries in rats. Furthermore, two chemically distinct types of propolis from Brazil inhibited the activity of GTF and the growth of *S. mutans in vitro* (Koo *et al.*, 2000a; Koo *et al.*, 2000b).

Osawa *et al.*, (2001) found that Cacao bean husk has been shown to posses two types of cariostatic substances, one showing anti-glycosylation activity and the other antibacterial activity, and inhibit experimental dental caries in rats infected with mutans streptococci. It was found that polyphenolic-compounds and unsaturated fatty acid act as active components against GTF and *S. mutans* respectively,

Ooshima *et al.*, (1993) demonstrated that polyphenolic compound of Oolong tea (Semifermented tea leaves of *Camellia sinensis*) has an effect on insoluble glucan synthesis from sucrose by GTF of *S. mutans* and *S. sobrinus*. The administration of the Oolong tea polyphenolic compound into diet and drinking water resulted in significant reduction in caries development and plaque accumulation in rats infected with mutans streptococci.

2.7 Treatment and Vaccine of Dental Caries

Dental Caries is an infectious disease of bacterial etiology and is a prevalent and costly disease in both developing and industrial countries. After the demonstration of a molecular pathogeneses of mutans streptococci in caries formation, scientists and dentists effort to reduce the caries through many ways including:

- Temporary elimination of the established mutans streptococci population from the oral cavity by mechanical methods such as brushing, flossing and professional scaling of the teeth.

- Increasing acid-resistance of the teeth through the use of sodium fluoride and by controlling the carbohydrate composition of the diet.
- Using chemical means to fight plaque as well as the progress in the caries process through the application of mouthrinses specially chlorohexidine which is effective but causes teeth discoloration, and has a poor penetration abilities against plaque in pits, fissues and a proximal surfaces.
- Interfering with transmission of mutans streptococci through the use of vaccine (Balakrishnan *et al*., 2000).

Bowen (1969) discovered the modern area of vaccine therapy against dental caries through the use of *S. mutans* cell to intravenously immunize irus monkey, from that time researches focus towards the development of well tolerated and effective vaccine against this disease (Smith, 2002).

Several aspects be considered for the development of any dental caries vaccine:

- The vaccine must be safe for use in human.
- It must contain the appropriate virulence antigen (s) of mutans streptococci that includes antibodies, which can prevent the bacterium from causing disease.
- The vaccine must be given via the appropriate route to induce the desired response.
- The composition of vaccine must be such that it is effective in reaching immune inductive sites and capable of potentiating the desired response.
- Furthermore, a cost-effective vaccine would be practical for widespread-application (Michalek *et al*., 2001).

An earliest attempts in the 1970s demonstrated that local injection of heat-killed or formalinized *S. mutans* in complete Freund's adjuvant resulted in a local antibody response (Taubman and Smith, 1974; Bowen *et al*., 1975; McGhee *et al*., 1975; Challencombe and Lehner, 1980; Linzer *et al*., 1981 and Michalek *et al*., 1983) such as the local injection of this type of antigen into the salivary gland region of rats. These resulted in local salivary antibody response; or the orally administration of antigen which in itself resulted in the stimulation of lymphoid cells and subsequent IgA antibody responses in external secretions via the common mucosal immune system (CMIS).

In order to understand the mechanisms of adherence and cariogenecity of *S. mutans* and *S. sobrinus*, more specific antigen preparations have been used. Such single components vaccines have the advantages of providing much greater control over product standardization and safety (Smith and Taubman, 1990).

From previous studies, several molecular components had been used as antigens to stimulate the immune system against cariogenic bacteria. These antigens include many types of cell wall proteins of mutans streptococci such as adhesin Ag I/II, P_1, SPA, PAc, antigen "A" and glucan binding protein; lipotechoic acid such as (LTA), a polymer of glycerol and phosphate covalently linked to a glycolipid and some specific enzymes specially GTF (Gregory *et al*., 1990; Ogier *et al*., 1990; Russell and Wu, 1990; Brady *et al*., 1991b; Bleiweis *et al*., 1992; Taubman *et al*., 2001).

Abundant *in vitro* and *in vivo* evidence, using a variety of active and passive immunization approaches, indicates that antibodies with specificity for mutans streptococci adhesins can interfere with bacterial adherence and subsequent dental caries. But this type of vaccine is used in animal only due to it's potential cross reactivity with human heart diseases (Ma *et al*., 1987; Russell and Wu, 1990; Ackermans *et al*., 1991; Filler *et al*., 1991; Harrington and Russell, 1993; Oho *et al*., 1999; Balakrishnan, 2000; Smith, 2002).

Mutans streptococci fimberial structure was also described as a long hair like extracellular appendages observed clearly in electronmicroscope. It seems to function as an adhesin that enables these bacteria to bind to salivary pellicle coated tooth surfaces (Fontana *et al*., 1999). It may also be possible to prevent colonization of oral bacteria by immunization with a vaccine consisting of purified fimbrial adhesins (Perrone *et al*., 1997).

The major group of antigens considered as potential vaccine candidates are the glucosyltransferases (Taubman and Smith, 1992). GTF preparations are attractive possible vaccines that may constitute an important target of the antibacterial mechanism of the immune response and have the advantage of not eliciting heart reactive antibodies and also offer cross-protection between different strains of mutans streptococci (Kato and Kuramitsu, 1992; Eto *et al*., 1999; Jespersgaard *et al*., 2000).

In vitro experiments had been shown that antibodies which inhibit GTF activity, especially that responsible for the formation of insoluble glucan, can inhibit plaque formation (Evans and Genco, 1973; Wunder and Bowen, 1999). In these experiments, GTF were coupled with complete Freund's adjuvant to enhance the response of the immune system and given through (intravenously, subcutaneously or in the vacinity of the parotid and submandibular glands route). These results were encouraging more studies to focus on GTF due to it important in dental caries.

Later, it was found that gastric intubation of rats and subcutaneous injection of rabbits with GTF (bound to insoluble polysaccharide or to aluminum hydroxide) resulted in higher salivary IgA immune responses than were seen in the orally immunized with GTF alone. So that enhancement of the immune system may increase in presence of the insoluble form of GTF as compared with the soluble one (Ebersole *et al*., 1983; Wunder and Bowen, 2000).

Recent studies with GTF antigen (contributed to the development of the oral vaccine and their effective deliver) used liposomes, a virulent *Salmonella*

and B subunit of the Cholera toxin (CTB) as an adjuvant (Michalek *et al*., 1984; Michalek *et al*., 1986; Childers *et al*., 1994; Michalek *et al*., 2003; Smith *et al*., 2003). In these studies which had been done on both animals or human either the native molecules or peptides were used representing functional domains of the molecules such as the catalytic (CAT) or glucan binding domain (GLU) of (GTF).

Although, the early experimental oral immunization studies with mutans Streptococcal antigens showed the induction of protective immune responses , in most instances complete protection against caries formation was not attained (Childers *et al*., 1996). Therefore, the use of a mucosal vaccine which consisting of Ag I/II and GTF synthetic peptides may induce responses. These responses confer greater protection than would be obtained with an antigen from a single virulence factor (Jespersgaard *et al*., 1999; Taubman *et al*., 2000; Michalek *et al*., 2001).

Cromie (2001) described the efforts of Taubman and Smith in the development of vaccine for children between 18 month to 2 years old. After the babies had replaced their permanent teeth, the two scientists gave them their boosting when they start school. Hopeful, that process gave them a protection for a life. The vaccine consists of GTF with suitable adjuvant suitable for human health; both packaged into microparticles made of the same material used for surgical sutures that eventually absorbed by the body. Such packaging seems to stimulate a stronger protective effect than the enzyme alone. Both scientists gave their vaccine as swallowing capsules or by dripped it on the inside surface of their lips or it may given as a nasal spray.

Chapter Three

Materials and Methods

3.1 Materials

3.1.1 Chemicals

The following chemicals were used in this study:

Chemicals and Media	Company (Origin)
Agar, acetone, brain heart infusion agar, brain heart infusion broth, bromophenol blue, dextrose, dipotassium phosphate, ethanol (absolute), ethylene diamine tetraacetic acid (EDTA), glucose, glycerol, hydrochloric acid, iodine, methanol, polyethylene glycol (4000), potassium iodide, phosphoric acid, sodium azide, safranine, sodium deodecyle sulfate (SDS), sodium chloride, sodium fluoride, sulfuric acid, tryptose, trypan blue.	**BDH-England**
Bacitracin, Optochin, Vancomycin	**Bioanalyse-Turkey**
Hydroxyapatite beads	**Bio-Rad – USA**
Crystal violet, phenol, yeast-extract.	**Fluka-Switzerland**
2,3,5 triphenyl-tetrazolium chloride	**H and W –England**
Coomasie-brilliant blue G-250	**LKB-Switzerland**
Blood agar base, protease-peptone, trypton, Todd-Hewitt broth.	**Oxoid-England**
Agarose, sephacryl S-200, DEAE-cellulose.	**Pharmacia-Sweden**
Freund's complete adjuvant and Freund's in complete adjuvant.	**PIE-RCE – England**
Hydrogen peroxide, mannitol, sorbitol, inulin, Tris-hydroxymethyl aminomethan, Muller-Hinton agar.	**Sigma-USA**

3.1.2 Pastorex® Strep (Bio-Rad-France):

Latex test for grouping streptococci belonging to groups A, B, C, D, F and G. Kit for 50 to 60 tests containing the following:

a) Six vials, each of which contains 1ml of latex suspension, specific for group A, B, C, D, E, F and G streptococci respectively. The latex particles are coated with group-specific rabbit immunoglobulin in suspension in a glycin buffer, pH 8.2, containing (0.01%) thimerosal and (0.1%) sodium azide as the preservatives.

b) Two vials of freeze-dried extraction enzyme in Tris containing (0.01%) thimerosal.

c) One vial containing (1.5 ml) polyvalent positive control antigen, composed of a mixture of lancefield extracts of group A, B, C, D, E, F and G streptococci and (0.02%) thimerosal as the preservative.

d) Two x 125 rods.

e) Sixty disposable agglutination cards.

3.1.3 Solutions

A) Solutions for Isolation of *Streptococcus spp.*

I. Normal Saline:

It was prepared by dissolving (8.5g) of sodium chloride in (750 ml) of distilled water, pH was adjusted to (8.0), and the volume was completed to 1L by distilled water, sterilization was made by autoclaving at 121°C.

B) Solutions for Identification of *Streptococcus spp.*

I.Sodium Acetate (10%):

It was prepared by dissolving (10g) of sodium acetate in (100 ml) of distilled water.

II. Iodine (1%) (Atlas *et al.*,1995):

It was prepared by dissolving (0.3g) of iodine and (6.6g) of potassium iodide in (100 ml) of distilled water, then filtration was done through filter paper (Whattman No.1) and put in a dark bottle.

III. Crystal Violet Stain (Atlas *et al*., 1995):

It was prepared by dissolving (2.0g) of crystal violet in (20 ml) of (absolute) ethanol, the volume was completed to (100 ml) with distilled water with continuous stirring, filtration was done through filter paper (Whattman No.1).

IV. Safranin Stain (Atlas *et al.*, 1995):

It was prepared by dissolving (0.25g) of safranin in (10 ml) of absolute ethanol, then the volume was completed to (100 ml) with distilled water with continuous stirring. The solution was allowed to stand for several days then filtrated through filter paper (Whattman No.1).

V. 2,3,5-triphenyltetrazolium chloride (TTC) (4%):

It was prepared by dissolving (4g) of (TTC) in (100 ml) of distilled water in dark bottle and sterilized by autoclaving.

VI. Mannitol (10%):

It was prepared by dissolving (10g) of mannitol in (100 ml) distilled water and sterilized by filteration.

C) Solutions for Determination of GTF Activity

I. Sucrose (50%):

It was prepared by dissolving (50g) of sucrose in (100 ml) of distilled water and sterilized by filtration.

II. Tris-HCl Buffer (0.025 M):

It was prepared by dissolving (3.025g) of Tris-hydroxymethylamino methane in (750 ml) of distilled water, pH was adjusted to (8.3) and the volume was completed to 1L by distilled water.

III. Sodium Chloride (0.1M):

It was prepared by dissolving (2.92g) of sodium chloride in (500 ml) of Tris-HCl buffer (0.025M) as described in (3.1.3.C.II)

IV. Sodium Hydroxide (1M):

It was prepared by dissolving (40g) of sodium hydroxide in (1L) of distilled water.

V. Phenol (5%):

It was prepared by dissolving (5g) of phenol in (100 ml) of distilled water. The container was wrapped in aluminum foil and stored.

VI. Glucose (120 µg/ml):

It was prepared by dissolving (0.012g) of glucose in (100 ml) of distilled water. From this stock solution, the concentrations (110, 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 µg/ml) were prepared by diluting the stock with Tris-HCl buffer (0.025M) as described in (3.1.3.C.II).

D) Solutions for Determination of GTF Protein Concentration

I.Bovin Serum Albumin (BSA) (20 µg/ml):

It was prepared by dissolving (0.0025g) of (BSA) in (100 ml) of distilled water. From this stock solution the concentrations (19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5 4, 3, 2 and 1 µg/ml) were prepared by diluting the stock in distilled water.

I. Phosphoric Acid (85%):

It was prepared by the addition of (15 ml) distilled water to (85 ml) of phosphoric acid.

II. Sodium Hydroxide (1M):

It was prepared as described previously in (3.1.3.C.IV).

III. Coomassie Brilliant Blue G-250 (Bradford,1976):

It was prepared by dissolving (0.1g) of Coommasie-brilliant blue G-250 in a solution composed of (100 ml) of phosphoric acid (85%) (3.1.3.D.II) and (50 ml) of (absolute) ethanol, then the volume was completed to (1L) by distilled water. The solution was filtrated through filter paper (Whattman No.1).

E) Solutions for Purification of GTF

I. Purification by Adsorption Chromatography Using Hydroxyapatite Beads

1) Phosphate Buffer (0.05 M) (pH 7.5):

It was prepared by dissolving (8.709g) of dipotassium hydrogen orthophosphate (K_2HPO_4) in (750 ml) of distilled water, pH was adjusted to (7.5), and the volume was completed to 1L by distilled water.

2) Phosphate Buffer (0.15 M) (pH 7.5):

It was prepared by dissolving (13.063g) of K_2HPO_4 in (250ml) distilled water. pH was adjusted to (7.5) and the volume was completed to (500) ml by distilled water.

3) Phosphate Buffer (0.3 M) (pH 7.5):

It was prepared by dissolving (26.127g) of (K_2HPO_4) in (250 ml) distilled water. pH was adjusted to 7.5and the volume was completed to (500ml) by distilled water.

II. Purification by Ion Exchange-Chromotography (DEAE-Cellulose) Column

1) Sodium Hydroxide (0.25 M):

It was prepared by dissolving (1g) of sodium hydroxide in (100 ml) of distilled water.

2) Hydrochloric Acid (0.25 M):

It was prepared by the addition of (498 ml) distilled water to (1.8 ml) of concentrated hydrochloric acid, the volume was completed to (500 ml) by distilled water.

3) Sodium Chloride (0.25M):

It was prepared by dissolving (7.3 g) of sodium chloride in (500 ml) distilled water.

4) Phosphate Buffer (0.05 M) (pH 7.5):

It was prepared as described previously in (3.1.3.E.I.1).

5) Phosphate Buffer (0.3 M) (pH 7.5):

It was prepared as described previously in (3.1.3.E.I.3).

III. Purification by Gel Filtration Chromatography (Sephacryl S-200 Column) and Determination of GTF Molecular Weight:

1) Phosphate Buffer (0.3 M) (pH 7.5):

It was prepared as previously described in (3.1.3.E.I.3)

2) Solution for Blue Dextrane 2000 (2.5 mg/ml):

Five gram of blue dextrane was dissolved in (2ml) of (0.3 M) phosphate buffer (pH 7.5) (3.1.3.E.I.3).

3) Solution for Standard Proteins:

A standard protein's solution was prepared by dissolving (5 mg) of each Bovine Serum Albumin, Aldolase, Catalase, Ferritin and Theroglobulin A in (2 ml) of (0.3M) phosphate buffer (pH 7.5) (3.1.3.E.I.3).

F) Solutions for Determination of Purified GTF Activity

I. Sucrose (50%):

It was prepared as described previously in (3.1.3.C.I)

II. Phosphate Buffer (0.3M):

It was prepared as described previously in (3.1.3.E.I.3).

III. Phenol (5%):

It was prepared as described previously in (3.1.3.C.V)

G) Solutions for (GTF) and Bacterial Inhibitors

I. Sodium Fluoride (20mM):

It was prepared by dissolving (1g) of sodium fluoride in (100 ml) of phosphate buffer (0.3M) (3.1.3.E.I.3). From this stock solution, the concentrations (18, 16, 12, 8, 4, 2, 1 and 0.5 mM) were prepared by diluting with phosphate buffer (0.3 M) (pH 7.5) as in (3.1.3.E.I.3).

II. Chlorohexidine Dichloride

A stock solution of chlorohexidine dichloride (20 mM) was obtained from (Al-Mansour Factory). From this stock, the concentrations (15, 10, 5 and 3 mM) were prepared by diluting with phosphate buffer (0.3M) (pH 7.5) (3.1.3.E.I.3).

III. ZAK (Mouthrinse)

This solution is a combination of (12 mM) chlorohexidine dichloride with (0.05%) sodium fluoide it was obtained from (Al-Safa-Factory).

From this stock solution the concentrations (10, 5 and 3 mM/ml) was prepared by diluting with phosphate buffer (0.3M) (pH 7.5) (3.1.3.E.I.3).

IV. EDTA (50mM)

It was prepared by dissolving (1.86g) of EDTA in (100 ml) of phosphate buffer (0.3M) (3.1.3.E.I.3). From this stock solution the following concentrations (25, 20, 15, 10, 5 and 3mM) were prepared by diluting with phosphate buffer (0.3M) (pH 7.5) (3.1.3.E.1.3).

H) Solution for Double Immunodiffusion Assay

I. Phosphate Buffer Saline (PBS) (Coli *et al*., 1996)

It was prepared by dissolving the following ingredients in (750 ml) of distilled water:

NaCl	8.0g
KCl	1.15g
K_2HPO_4	1.15g
KH_2PO_2	0.2g

pH was adjusted to (8.0), then the volume was completed to 1L by distilled water and sterilized by autoclaving at 121 ^{0}C for 15 minutes.

I. Solution for the measurement of GTF Kinetic constants

I. Sucrose (50%):

It was prepared as described previously in (3.1.3.C.I). From this stock solution the concentrations (0.01, 0.02, 0.025, 0.04, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2 and 0.25mM) were prepared by diluting with (0.3M) phosphate buffer pH (7.5) as described previously in (3.1.3.E.1.3).

3.2 Methods

3.2.1 Culture Media Preparation

A- Brain Heart Infusion Agar

This medium was prepared by dissolving (52g) of brain heart infusion agar in (1L) of distilled water, then sterilized by autoclaving at 121°C for 15 minutes.

B- Brain Heart Infusion Broth

This medium was prepared by dissolving (35g) of brain heart infusion broth in (1L) of distilled water, then sterilized by autoclaving at 121°C for 15 minutes.

C- Blood Agar

This medium was prepared by dissolving (37.5g) of blood agar base in (1L) of distilled water, then sterilized by autoclaving at 121°C for 15 minutes and cooled to (45°C), then (5%) human blood was added and poured into a sterile plates.

D- Mitis Salivarius (MS) Agar (Gold *et al*., 1973)

A selective medium for *Streptococcus* was prepared according to the instructions described by (Gold *et al*., 1973) as follows:

Tryptose	10g
Protease-peptone	5g
Dextrose	1g
Sucrose	200g
Dipotassium phosphate	4g
Trypan blue	0.075g
Crystal violet	0.0008g
Agar	15g

To prepare one litter, (800 ml) of distilled water was added to the above constituents, pH was adjusted to (7.0), then the volume was completed to 1L by distilled water and sterilized by autoclaving at 121°C for 15 minutes.

E- Muller Hinton Agar

This medium was prepared by dissolving (36g) of Muller Hinton agar in (1L) of distilled water, and then sterilized by autoclaving at 121°C for 15 minutes.

F- TYS Broth (Guthof, 1970)

It was prepared according to the instructions described by (Guthof, 1970) as follows:

Trypton	15g
Yeast extract	10g
Sodium chloride	5g
Sucrose	30g

To prepare one liter of this broth, (800 ml) of distilled water was added to the above constituents, pH was adjusted to (7.0), the volume was completed to 1L by distilled water and sterilized by autoclaving at 121°C for 15 minutes.

G- Todd Hewitt Broth

This medium was prepared by dissolving (36.4g) of Todd-Hewitt broth in (1L) of distilled water, then sterilized by autoclaving at 121°C for 15 minutes.

3.2.2 Samples collection

Fifty plaque samples were collected from stuff of Biotechnology Department in College of Science, Al-Nahrain University, in steriled tubes containing 2ml normal saline (3.1.3.A.I). Samples were stored in a cool place then transported to the laboratory.

3.2.3 Isolation of Mutans Streptococci Bacteria

One hundred microliter of undiluted samples were spread on the surface of MS-agar plates (3.2.1.D) using sterile swabs. Cultures were incubated anaerobically for 48 hrs at 37°C and aerobically overnight at 37°C. Count of more than 250 colonies (10^4 cells/ml) was considered as positive samples (Friedrich, 1981).

3.2.4 Growth Measurements

Measurement of bacterial growth was made by estimation of optical density of three days incubated culture, using spectronic (Spectrophotometer-20) at wave length of (600 nm).

3.2.5 Identification of Isolates

Colonies grown on Ms-agar medium was spread on the surface of blood agar plates (3.2.1.C) and incubated anaerobically for two days. Subcultures was repeated several times in order to obtain pure cultures. Identification of the isolates was done using the schematic diagram suggested by (Friedrich,1981) criteria used for identification of the isolates at the genus level were:

a. Colonial shape and form on MS-agar and blood agar.
b. Gram-staining and microscopic examination.
c. Catalase test.
d. Dextrane production test.

Dextran production test was done following the method of (Guthof, 1970) as follows:

A (2 ml) of TYS broth medium (3.2.1.F) was inoculated with loopfull of bacterial culture then incubated anaerobically at 37°C for two days to yield a visible turbid growth. Each culture tube was centrifuged at (2500xg) for 10 minutes, and (0.1 ml) of the supernatant of each culture was added to each three

tubes and mixed with (0.3 ml) of (10%) sodium acetate (3.1.3.B.I). A (0.8-fold) volume (=0.32 ml) of acetone was added to tube 1, a (1.2-fold) volume (=0.48 ml) of ethanol to tube 2 and (1.5-fold) volume (=0.6 ml) of methanol was added to tube 3. Each tube was shaked well for 1-3 minutes and observed. Flocculation in all three tubes or only in acetone and turbidity in the alcohols indicated dextrane. If none of the tubes shows flocculation, an additional (1.3-fold) volume of ethanol was added to tube 2, and an additional (1.5-fold) volume of methanol to tube 3. After repeated shaking, flocculation in both tubes indicates the presence of levan.

3.2.6 Rapid Identification of Colonies

Identification of mutans streptococci from other streptococci like *S.sanguis* was done using the procedure described by (Carlsson, 1968, Gold *et al*., 1974) as follows:

Colonies developed on MS-agar after incubation anaerobically at 37^{0}C for two days were spread with test solution of (10%) mannitol (3.1.3.B.VI) and (4%) of 2,3,5-triphenyltetrazolium chloride (3.1.3.B.V) were spread on the plates. A change in color to a dark pink was developed due to hydolysis of mannitol to acid by the enzyme mannitol-1-phosphate dehydrogenase and a reduction of (TTC) which could taken as an indicator for the presence of mutans streptococci

3.2.7 Tolerance to High Concentration of Sodium Chloride

The susceptibility of bacterial isolates to (4%) NaCl was tested using TYS-broth medium (3.2.1.F) and incubated anaerobically at 37^{o}C for 48 hrs. Growth was monitored visually as compared with a control tube which did not contain this concentration of sodium chloride. Turbidity is an indicator for the ability of the bacterial isolates to tolerate this concentration of NaCl.

3.2.8 Utilization of Different Carbohydrates Sources

The ability of the bacterial isolates to utilize different carbohydrate sources was determined following the method described by (Fingold and Baron, 1986).

Brain heart infusion broth (3.2.1.B) supplemented with (10%) of each carbohydrate (sucrose, mannitol, sorbitol and Inulin). Sucrose was used as positive control and brain heart infusion broth medium as negative control, carbohydrates solutions were sterilized by filtration and added aseptically to the autoclaved brain heart infusion broth medium which contains a (0.02%) of phenol red, then, the suspended media was inoculated with the tested isolates and incubated anaerobically at 37^{o}C for 72 hrs. The change in the color of media from red to yellow as compared with the negative and positive control indicated the ability of these tested isolates to utilize these carbohydrates sources. For further conformation were made by measuring bacterial growth was measured using spectrophotometer and pH change using pH meter.

3.2.9 Antibiotic Sensitivity Test

Antibiotic sensitivity test was made using disk diffusion method following the method described by (Baron *et al.*, 1994). Brain heart infusion broth (10 ml) was inoculated with a loopfull of bacterial isolate, the culture was incubated at 37^{o}C to mid log phase. A (0.1 ml) of inoculated broth transferred to Muller-Hinton agar plates (3.2.1.E).A sterile cotton swab was used to streak the inoculum on the plate surface in three different planes. The inoculated plates were then placed at room temperature to allow absorption of excessive moisture. With sterile forceps selected antibiotic disks of (Vancomycin (30 μg), Bacitracin (30 μg) and Optochin (50 μg)) were placed on the inoculated plates and incubated at 37°C for 24 hrs in an inverted position. After this period of incubation, the diameters of inhibition zones were noted and measured by a ruler in (mm), results were compared with that described by the National Committee for Laboratory Standards (NCCLs, 2001).

3.2.10 Identification by Latex Test (PASTOREX® STREP)

This test is a rapid, sensitive agglutination test for grouping of (α or β) haemolytic streptococci (which were grown on the surface of blood agar plates) belong to the main lancefield groups. The test involves the use of latex suspensions specific for group A, B, C, D, F and G.

Identification of β or α-haemolytic streptococci based on group-specific polysaccharides requires previous extraction of these antigens from colonies obtained by primary blood agar cultures. With the PASTOREX® STREP system, this requires only 15 minutes at room temperature or 10 minutes at 37°C. Extraction is achieved by an active enzyme that causes lysis of the cell wall.

In the presence of the antigen, the latex particles coated with homologous antibodies agglutinate very rapidly. The speed of agglutination depends on the sensitivity of the latex particles suspensions, which are governed by the quality of the antisera raised in rabbits using lancefield's immunization protocol and by the amount of purified immunoglobulins adsorbed on the latex particles.

Test Procedure

A- Preparation of the Specimens:

The PASTOREX® STREP system should be used on colonies grown on blood agar and surrounded by an area of β-haemolysis or may reflect the α-haemolysis pattern. Following the characterization of gram-positive cocci on microscopic slides and a negative catalase test, further identification by direct grouping can be performed if a large enough number of primary culture colonies is available.

B- Preparation of the Extracts:

I. A (0.3 ml) of extraction enzyme solution (3.1.2.b) was placed in a haemolysis tube for each strain.

II. Approximately five colonies were picked off and dissociated in the enzyme solution. If the diameter of the colonies is less than 0.5 mm, the size of the inoculum must be increase until cloudiness is visible with the naked eyes.
III. Incubation must be performed at 37°C for ten minutes.

C- Grouping of the Extracts:

I. The contents of the vials which contained the latex particles were mixed by shaking vigorously for a few seconds.
II. One drop of each latex suspension was transferred to the center of an agglutination card.
III. A one drop of extract was transferred to the center of each agglutination card by using a pipette.
IV. The content of each circle was homogenized by using a rode. A different rod was used for each circle.
V. The card was rotated horizontally for one minute.
VI. The result was read. When the reaction is positive, the latex particles agglutinate within one minute at the most. The size and speed of development of the clumps varies with the concentration of the antigen in the extract, this concentration varies with the number and size of colonies picked from the agar.

D- Interpretation of Results:

I. Positive reaction: red clumps on a green background. Only marded, rapid agglutination with only one of the six latex suspensions convincingly establishes the group of *Streptococcus* bacteria.
II. Negative reaction: Uniform brown suspension.

E- Non-Specific Reactions:

I. Small clumps on a brown background.
II. Multiple agglutination can be caused by the presence of other bacteria harvested from the agar with (β or α)-haemolytic colonies (Mixture of

streptococci from different groups or presence of other bacteria yielding cross-reaction). When doubtful reactions of this type occur, the isolation procedure should be repeated.

III. Biochemical tests can be used to confirm the identification in some instances, for instance when the strain exhibits both group C and G antigens.

3.2.11 Maintenance of Isolates

Maintenance of bacterial isolates was done according to (Maniatis *et al*., 1982) and as follows:

A- Short-Term Storage:

Isolates were maintained for period of few weeks on the surface of MS-agar plates. The plates were tightly wrapped with parafilm and stored at 4°C.

B- Medium-Term Storage:

Isolates were maintained in slab culture for period of few months. Such culture were prepared in small-screw capped bottles containing (2-3 ml) of brain heart infusion agar medium (3.2.1.A) and stored at 4°C.

C- Long-Term Storage:

Isolates were maintained in slab culture for period of one year. Such cultures were prepared in small-screw capped bottles containing (5 ml) of brain heart infusion agar medium supplemented with (15%) of sterilized glycerol.

3.2.12 Determination of GTF Activity

GTF activity was determined through the estimation of the amount of glucan that was produced by the action of the enzyme, following the phenol-sulfuric acid method (Debois, 1956). A (1ml) of GTF (crude or purified) was mixed with (1ml) of (5%) phenol (3.1.3.C.V) with continuous shaking for (2-3 minutes). The reaction was stopped by the addition of (5ml) concentrated sulfuric acid and left to cool to 37°C. The absorbance was measured at (490nm).

A standard curve of glucose was determined also according to phenol-sulfuric acid method, using different concentrations of glucose (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 µg/ml) as in (3.1.3.C.VI). Each was prepared in duplicate in sterile test tubes.

The estimated absorbance was plotted against the corresponding concentration of glucose as in figure (3-1).

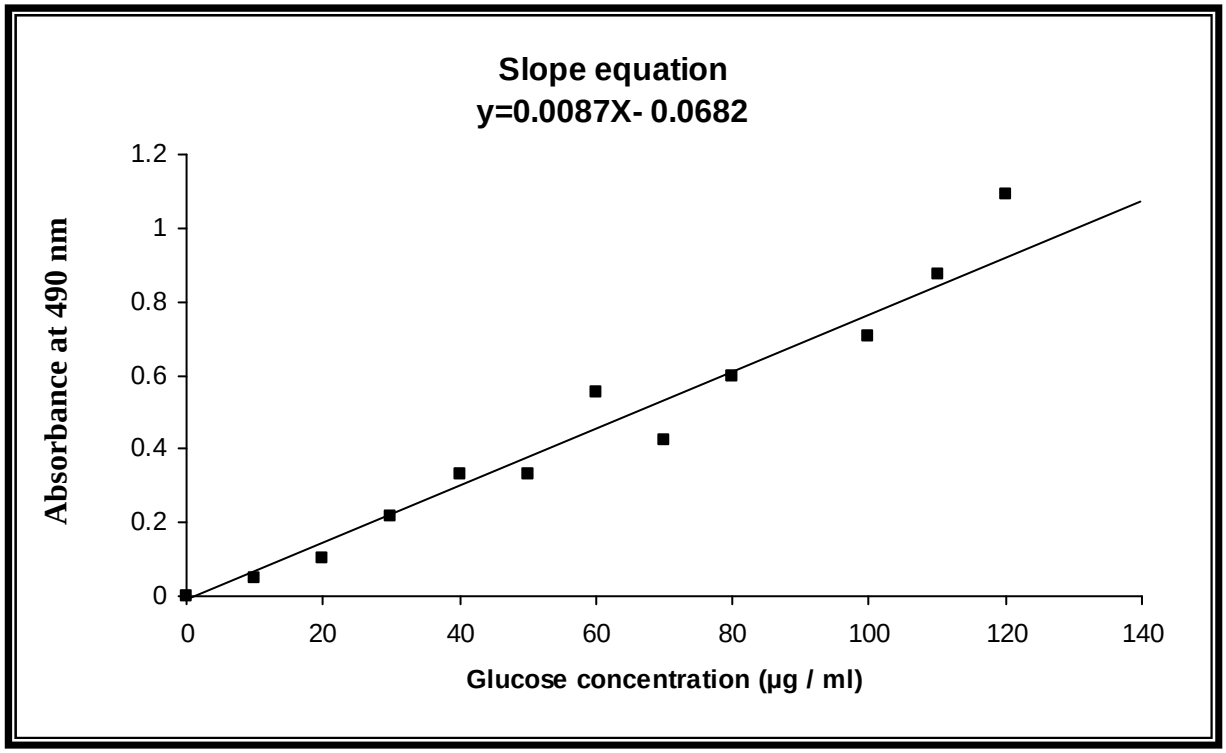

Figure (3-1) A standard curve of Glucose

The estimated GTF absorbance was plotted on the glucose standard curve and one unit (1U) of GTF was defined as the amount of enzyme catalyzing the incorporation of 1µmol of glucose from sucrose under the conditions of experiment (Mukasa *et al.*, 1982).

3.2.13 Determination of Protein Concentration

Protein concentration was carried out using the method of Bradford, (1976) as follow:

A (20µl) of GTF (crude or purified) was mixed with (50 µl) of (1M) NaOH (3.1.3.D.III) with continuous shaking for (2-3minutes) then (1ml) of Coomasie Brilliant Blue G-250 as in (3.1.3.D.IV) was added with shaking. The absorbance was measured at (595 nm) by spectrophotometer.

A standard curve bovine serum albumin was carried out using different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,17, 18, 19 and 20 µg / ml) as in (3.1.3.D.I) each was pipetted in duplicate in sterilized test tubes, then protein concentrations were measured using the method of Bradfored, (1976).The absorbency was plotted against the corresponding concentration of bovine serum albumin (Figure 3-2).

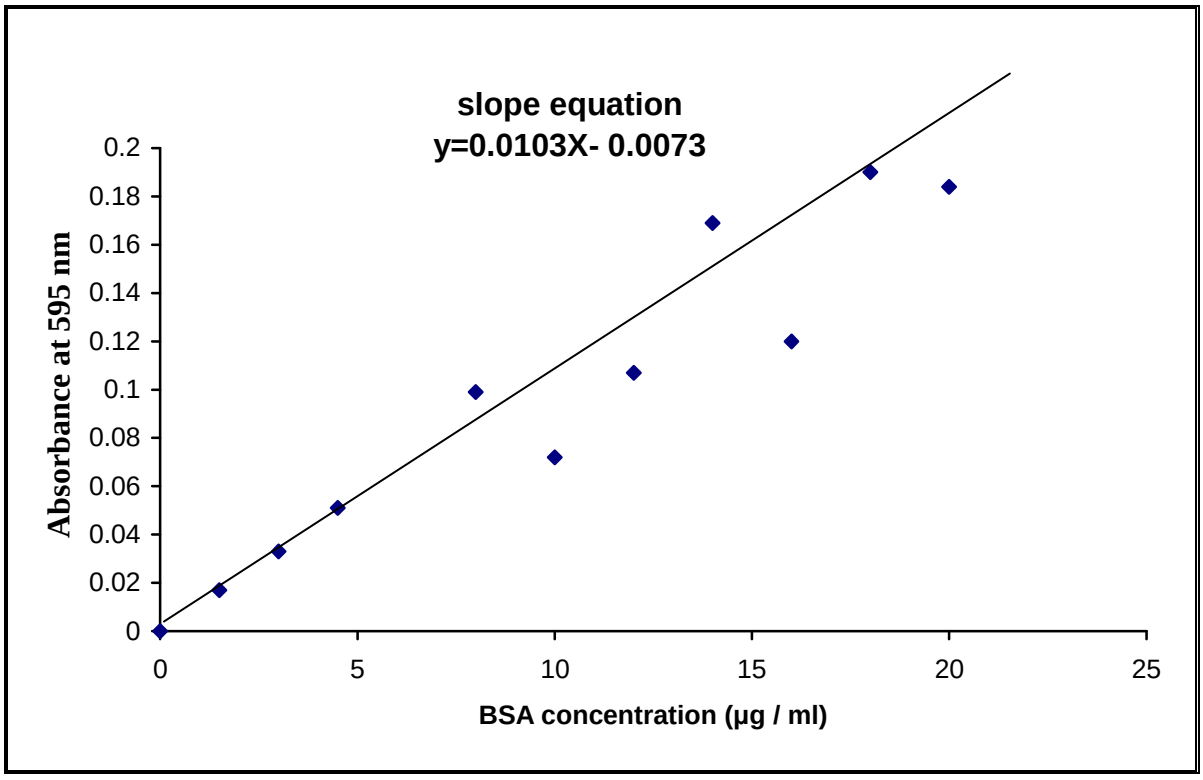

Figure (3-2) A standard curve of bovine serum albumin.

3.2.14 Extraction, Characterization and Purification of GTF from Mutans Streptococci:

A. Growth of Bacteria:

Bacterial isolate was grown on the surface of blood agar medium (3.2.1.C) and incubated anaerobically at 37^{o}C for 48 hrs.

A stock culture suspension was prepared by incubating a separated colony in (5ml) brain heart infusion broth (3.2.1.B) and incubated anaerobically at 37°C to (0.D= 0.25). Total viable count was estimated by making a serial dilution of bacterial growth (10^{-1}-10^{-6} cells), a (100µl) of each dilution was spreaded on the surface of brain heart infusion agar (3.2.1.A) and incubated at 37^{o}C for 24 hrs. Bacterial colonies were calculated for each dilution.

A (2.5 ml) of this stock culture was inoculated into (250 ml) Todd-Hewitt broth (3.2.1.G) medium containing (1.8%) glucose and incubated anaerobically for 18-24 hrs at 37^{o}C (Christine *et al*., 1979).

B. Extraction of GTF

The Todd-Hewitt broth culture was centrifuged at (5000 Xg) for 30 minutes at 4°C. Protein concentration was estimated in the supernatant following the method of (Bradford, 1976) as described in (3.2.13), pH of the supernatant was adjusted to (6.5) with (1M) (NaOH) (3.1.3.C.IV), then supernatant was incubated with (2.5 %) of (50%) stock solution of sucrose (12.5 ml) (3.1.3.C.I) with (0.02%) sodium azide for about (18 hrs). The mixture was centrifuged at (5000 Xg) for 30 minute at (4°C). A (200ml) of cold absolute ethanol was added to the supernatant, then left in the refrigerator for about (4 hrs). Centrifugation was also performed at (5000 Xg) for (30 minute) at (4°C). The precipitate was dissolved in (5ml) Tris-HCl buffer (0.025 M) containing (0.1M) of NaCl as in (3.1.3.C.III).

The amount of glucan produced by GTF was estimated following the method of phenol-sulfuric acid as described in (3.2.12). The blank included

contains Todd-Hewitt broth medium with (2.5%) of (50%) sucrose and treated with the same procedure that had been used for the extraction of GTF .

C. Production Phase and GTF Activity:

In order to determine production phase a (500 ml) flasks containing (250 ml) of Todd-Hewitt broth medium supplemented with (1.8%) glucose, each were inoculated with (2.5 ml) of bacterial stock culture had an (O. D= 0.25) and incubated anaerobically at 37°C. At the beginning (at the time of inoculation) and after each 60 minutes time interval, the absorbency of the culture using (Spectronic-20) spectrophotometer at wavelength (600 nm) was measured, and also the activity of (GTF) was determined as in (3.2.12) in (5 ml) of the supernatant.

D. Preservation and Concentration of Crude GTF:

For further purification steps and immunization protocol, a preservation and concentration of crude GTF was done later. A (350 ml) of Todd-Hewitt broth medium containing (1.8%) glucose was inoculated with a stock bacterial culture (O.D.=0.25) and incubated anaerobically for 24 hrs. at (37°C). Centrifugation was done at (5000 Xg) at (4°C) for 30 minutes, and pH of the supernatant was calibrated to be 6.5. The supernatant was divided into two parts. The first part (125 ml) which was in role divided into five equal halves (25 ml) for each. The protein concentration and GTF activity were determined in one portion as described in (3.2.13) and (3.2.12) respectively. The remainders were preserved at (-20°C). GTF activity and protein concentration were determined for each after 1, 2, 3 and 7 days. The second part (225 ml) which divided into two parts. The first one (125 ml) was divided into 5 portions (25 ml), then a (15 %) glycerol about (3.75ml) was added to each then stored at (-20^{o}C). The GTF activity and protein concentration were determined for each of them after 7, 14, 21, 25 and 30 days. The second part (100 ml) which was divided in role into four equal halves (25 ml) for a half. The first half was concentrated to (12.5 ml)

by the use of polyethylene glycol powder (PEG 4000), the second one was lyophilized into (2 ml) by the use of lyophilizer (Freez/dry system/Freezone 4-5/Labconco-USA), the third one was concentrated to about (12.5 ml) by the use of sucrose powder and the final half was concentrated to (10 ml) by (Ultra filtration–cell method) using an Amicon-filter P50 (Amicon corp., Danvers MASS, USA, model 82800).

The protein concentration and GTF activity were estimated for each concentrated crude GTF. For the concentrated enzyme via the (Ultrafiltration-cell method) the enzyme activity and protein concentration were estimated for the (In-part) (the suspension that appeared on the filter) and for (Out-part) (the liquid that discarded outside the filter).

E. Large Scale Production of GTF

A large scale production of GTF was done from the chosen bacterial isolate after growing in (750ml) Todd-Hewitt broth medium. Total viable count was determined for the stock bacterial culture (O.D.=0.25) by making a serial dilutions of the bacterial growth (10^{-1}-10^{-6}) as described in (3.2.14.A). Extraction of GTF from bacterial culture was done as in (3.2.14.B) then GTF activity and protein concentration as well as specific activity were determined as described in (3.2.12) and (3.2.13) in (10 ml) of bacterial suspension.

The crude GTF was passed through an Amicon-filter P50 in (Ulterafiltration-cell) and concentrated to (40 ml). GTF activity, protein concentration and specific activity were also determined in (10 ml) of the concentrated suspension.

F. Purification of GTF by Adsroption Chromatography:

Purification of GTF was done using adsorption chromatography on hydroxyapatite beads. Preparation and packing of the column with the enzyme was done according to the instructions described by the manufacturing company (Bio-Rad-USA) and following the batch-wise method described by Scopes, (1972). A (10g) of hydroxyapatite beads powder was suspended in (250 ml) of distilled water and left for a time to precipitate. The floating partricles were removed away from the solution until the upper layer was cleared. The hydroxyapatite particles (beads) were then suspended in (250 ml) of (0.05M) phosphate buffer (pH 7.5) (3.1.3.E.I.1) and left for a time in order to precipitate and gain the pH of the buffer. The buffer was removed from the hydroxyapatite particles and (40 ml) of crude concentrated GTF enzyme was added at (5°C) with a gentle stirring and left for a time to allow adsorption of the enzyme on the beads. This mixture was then transferred to the surface of Whattman No.1 filter paper in Buchnner funnel and filtrated under pressure. The remaining precipitates on the filter was washed with (25 ml) of (0.15M) phosphate buffer (pH 7.5) (3.1.3.E.I.2) and fractions of (5 ml) were collected from the out-part of the filter. Then (25 ml) of (0.3M) phosphate buffer (pH 7.5) (3.1.3.E.I.3) was used to elute the protein from the remaining precipitate on the filter then fractions of (5 ml) were collected.

The presence of GTF was estimated by measuring GTF activity (3.2.12) for all fractions which represented the washing and elution parts then after collection of active GTF fractions together, protein concentration, GTF activity and specific activity were determined as in (3.2.13) and (3.2.12) respectively. .

G. Purification of GTFs by Ion Exchange Chromatography:

The exchanger DEAE-cellulose was prepared and packed into a column following the method described by Whitaker, (1972) as follows:

A (25 g) of DEAE- cellulose was suspended in (1000 ml) of distilled water and left for a time to precipitate. The floating flakes were removed a way from the solution until the upper layer was cleared, then filtrated through buchnner funnel with Whatman No. 1 filter paper under vacuum. The precipitate was suspended in (500 ml) of (0.25 M) NaCl (3.1.3.E.II.3) under vacuum, then washed several times with distilled water. A (500 ml) of (0.25 M) NaOH (3.1.3.E.II.4) was passed through the precipitated exchanger under vacuum and washed several times with distilled water. The exchanger was suspended also with (0.25 M) HCl solution (3.1.3.E.II.2) and washed several times with distilled water. The DEAE-cellulose was suspended in (0.05 M) phosphate buffer pH (7.5) (3.1.3.E.II.1) until the pH of the exchanger was reached (7.5). The DEAE-cellulose was packed into the column (7.5x3.5 cm), then the column was equilibrated with the same buffer overnight.

Partially purified concentrated GTFs (12 ml) were separately passed after loaded onto the column carefully. Then (100 ml) of (0.05M) phosphate buffer pH (7.5) was added. Proteins were eluted by using (200 ml) of a gradient from (0.05-0.3 M) phosphate buffer (pH 7.5). Fractions of (5 ml) were collected and absorbency was monitored at (280 nm). The presence of the GTFs were estimated from each fraction of the major peaks as in (3.2.12) then protein concentration and specific activities were determined for the collected active fractions as in (3.2.13).

H. Purification of GTFs by Gel Filtration Chromatography

Sephacryl S-200 column (67x2.1cm) was prepared and packed according to the instruction of the manufacturing company (Pharmacia- Sweden). The column was equilibrated with (0.3 M) phosphate buffer (pH 7.5) (3.1.3.E.III.1) at a flow rate of (50 ml/hr).

A (3 ml) sample of each concentrated partially purified GTFs was added to the column, carefully using pasture pipette. Elutions of proteins were done with the application of (200 ml) of (0.3 M) phosphate buffer (pH 7.5). A (5 ml) fraction were collected for each GTF then protein contents were estimated by measuring the absorbency at (280 nm), The major peaks for each GTF were determined by plotting the absorbency of protein fractions versus the elution volumes. GTF activity was determined for each fraction of the major peaks. Protein concentrations and specific activities were also determined for the collected fractions of the major peaks of the different GTFs.

I. Determination of Molecular Weights of GTFs by Gel Filtration Chromatography:

I. Determination of the Void Volume of the Column:

Sephacryl S-200 column (67x2.1 cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden). The column was equilibrated overnight with (0.3 M) phosphate buffer (pH 7.5) with a flow rate of (50 ml /hr).

A (2ml) of blue dextrane 2000 solution (3.1.3.E.III.2), was passed through the column, and (200 ml) of (0.3) phosphate buffer (pH 7.5) was added to the column (3.1.3.E.III.1). Fractions of (5 ml) were collected. The absorbency at (280 nm) for each fraction was measured. The column void volume (V_o) was determined by the estimation of total volume of fractions as characterized with start point movement of the blue dextrane to that of climax of absorbency of the blue dextrane.

II. Determination of GTFs Elution Volumes (V_o):

Sephacryl S-200 column (67x2.1cm) was prepared, packed and equilibrated for a second time as described previously in (3.2.14.I.I).

A (3ml) of concentrated purified GTFs samples were passed separately through the column, carefully, and (200 ml) of (0.3M) phosphate-buffer (pH

7.5), with a flow rate of (50 ml/hr) were passed through the column. Fractions of (5 ml) were collected. The elution volumes (V_e) were estimated separately for each separated and dissolved fractions of purified GTFs by following the absorbency at (280 nm).

III. Measurment of Standard Proteins Elution Volumes (V_e):

Different standard proteins table (3-1) (3.1.3.E.III.3) were applied through sephacryl S-200 column, then eluted with (0.3M) phosphate buffer (pH 7.5) with a flow rate of (50 ml/hr).

Table (3-1) High molecular weights standard proteins

Standard Proteins	Molecular Weight (Dalton)
Thyroglobulin	660000
Ferritin	440000
Catalase	230000
Aldolase	158000
Bovine serum albumin	67000

The elution volume was estimated for each standard protein by following the absorbency for the separated fractions at wave length (280 nm). The (V_e/V_o) ratio was calculated for each standard protein and for the dissolved fractions and separated fractions of purified GTFs, then standardization was done, by plotting the elution volume (V_e) of each standard protein to the void volume (V_o) of the blue dextran 2000 (V_e/V_o) versus the log of each standard protein molecular weight (Whitaker, 1963; Stellwagen, 1990). The GTFs molecular weights were accordingly calculated.

3.2.15 Immunological Studies

A- Antigen Preparations:

The antigen used in this experiment was GTF which was prepared and purified as described previously, and stored with (15%) glycerol then refrigerated at (-20°C) until the time of immunization. The antigen was dialyzed in (1L) distilled water overnight, and protein concentration was estimated as described in (3.2.13).

The preparation of the first dose of the immunization and the second one was done following the method described by (Wunder and Bowen, 2000) as follows:

For first dose, equal amounts of Freund's complete adjuvant (1.5 ml) were mixed before immunization with (1.5 ml) of purified GTF (0.15 mg/ml), which was injected to the experimental animal groups, or with phosphate buffer saline which was injected to the control animal group.

In the second dose (boosting one), which consists of (1 ml) of Freund's incomplete adjuvant with (1 ml) of purified GTF (0.15 mg/ml) and also another (1 ml) of Freund's incomplete adjuvant was mixed with (1 ml) of phosphate buffer saline to immunize the control animal group.

B- Immunization of Rabbits

The immunization of rabbits was done following the method of Wunder and Bowen (2000) as follows:

Four Newzeland rabbits about 2.5 kilograms in weight were used in this experiment, their age ranged between (6-7 months), these rabbits were divided into two groups as shown bellow:

i. The first group (controls) were immunized subcutaneously with phosphate buffer saline with either Freund's complete adjuvant (in the first dose) or Freund's incomplete adjuvant in (the second dose).

ii. The second group (experimental) received all their injection subcutaneously in their backs in eight areas with purified GTF either with Freund's complete adjuvant (for first dose) or Freund's incomplete adjuvant for the second dose.

The immunization schedules were consist of two doses of purified GTF, the first one at the zero time or at the beginning of the experiment, while the second dose was given after twenty two days as a booster dose.

C- Collection of Serum

After 14 days from the boosting dose, blood was collected from all groups. Blood sampling and serum preparation were started as described by (Garvey, 1977) as following:

i. Five milliliter of blood was collected from rabbits (either control or experimental) by ear vein puncture in sterile test tubes, which were put inclined position at room temperature for 1 hr to induce clotting.
ii. The adhesion between clot and the walls of test tubes was removed carefully by needle, and the test tubes were left in refrigerator for 24 hrs to enhance clot shrinkage and isolated from serum.
iii. Serum samples were centrifuged at (3000 Xg) for 5 minutes then the supernatants stored in the refrigerator for further usage.

D- Double Immunodiffusion Assay

Immunodiffusion assay was done following the method of Ochterlony (1958), the method based on antigen-antibody diffusion reaction in semihard media like agarose, as follow:

A (1g) agarose was dissolved in phosphate buffer saline (PBS) as in (3.1.3.H.I) by boiling on a hot plate for a short period until became clear and left it cool to about 45°C, plates were poured with approximately (20 ml) of agarose solution and permitted to solidify for 1 hr at room temperature. Separated holes

about (0.5-2 cm) a part were prepared using gel puncture, and removed the plugs of agarose from the holes using a Pasteur pipette. In the center hole a (25µl) of serum anti-GTF antibody was pipetted, while in the peripheral holes, an equal amounts of separated fractions of purified GTF enzymes and serum control were pipetted . Plates were put in the humid chamber for 24 hrs at room temperature, in the second day, plates were studied under the sun light and any precipitated lines between the center and peripheral holes were recorded.

The protein concentration of anti-GTF antibody were calculated as described in (3.2.14)

3.2.16 Effects of Inhibitors and Anti-GTF on Mutans Streptococci Growth

The effects of sodium fluoride, chlorohexidine, EDTA, ZAK (mouthrinse) and anti-GTF antibody on the growth of bacteria were estimated by the following methods:

a-Susceptibility of bacterial growth toward these inhibitors and anti-GTF antibody was determined by a broth-dilution method (Carpenter, 1972). Brain heart infusion broth was supplemented with different concentrations of chlorohexidine (20, 15, 10, 5 and 3mM) (3.1.3.G.II), sodium fluoride (18, 16, 12, 8, 4, 2, 1 and 0.5mM) (3.1.3.G.I), EDTA (25, 20, 15, 10, 5 and 3mM) (3.1.3.G.IV) and ZAK (mouthrinse) (12,10,5and 3mM) (3.1.3.G.III) then autoclaved at 121°C for 15 minutes. Also a sterile brain heart infusion broth supplemented with different concentrations of anti-GTF antibody was also prepared. These broth cultures were inoculated with a stock culture of bacterial broth (O.D.=0.25) for each concentration of the inhibitors and anti-GTF antibody and incubated at 37°C for 48 hrs, then the effects of these inhibitors and anti-GTF antibody on the growth and metabolism of the bacteria were determined using the spectrophotometer at wave-length of (600 nm) with the use of broth culture media containing the

desired inhibitors agents with different concentrations and anti-GTF antibody with different concentration but without any inoculum as a blank.

b- Also the susceptibility of bacterial growth against these previous inhibitors and anti-GTF antibody with the same concentrations was tested by diffusion method on a solid media following the method of Silva *et al*., (1987). A (0.1 ml) of brain-heart infusion stock bacterial culture (O.D.=0.25) was spreaded on surfaces of brain heart infusion agar plates by the use of swab and left it to stand for 30 minutes at 37°C. A (50 µl) from each concentration of inhibitors and anti-GTF antibody was pipetted into prepared holes on the same brain heart infusion agar plates and incubated for (24-48 hrs) at 37°C. The diameters of clearance zone area were measured for each concentration and dilution.

3.2.17 Effects of Inhibitors and Anit-GTF antibody on Purified GTF

The effect of inhibitors and anti-GTF antibody on the GTF activity was estimated following the method described by Evans and Genco, (1973), as follow:

A mixture of (200 µl) of purified (GTF) with (200 µl) of each concentration of inhibitors and anti-GTF antibody as in (3.2.16.a) was incubated at 37°C for an hour. Then a (20 µl) of (50%) solution sucrose was added for each concentration and dilutions with (0.02%) sodium azide and incubated at 37°C for 18 hrs. Centrifugation was done at (5000 Xg) for 30 minutes and precipitation of glucan was done with an absolute ethanol then GTF activity was measured by phenol-sulfuric acid method as described in (3.2.12). The blank was prepared using the same procedure which contained the same amount of each concentration of the previous inhibitors and anti-GTF antibody with (200 µl) of phosphate buffer saline (0.3M) (pH 7.5) instead of enzyme with (20 µl) of (50%) sucrose.

3.2.18 The Measurement of GTF Kinetic Constants

Determinations of the Michalis Menton constants (K_m) and the maximum velocity (V_{max}) of the purified concentrated GTF were done following the method described by Segel (2002) as follows:

Different concentrations of substrate sucrose (0.01, 0.02, 0.025, 0.04, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2 and 0.25 mM) were prepared as described in (3.1.3.I.I). A (200 µl) of purified concentrated GTF (0.15 mg/ml) was mixed with (200 µl) of each concentration of sucrose (the substrate) then incubation was done anaerobically at 37°C for 18 hours, the reaction was stopped by the addition of the same volume of cold ethanol. GTF activity was measured for each concentration as described in (3.2.12). The blank was composed of (200 µl) of phosphate buffer (0.3M) pH (7.5) with the corresponding sucrose (the substrates) concentrations as with GTF. After calculation of the 1/[υ] which represented 1/[GTF activity] and 1/[S] which represented 1/[substrate] (at different concentrations) then plotting the 1/[υ] on the 1/[S] , the calculations of V_{max} and K_m of GTF were possible by Lineweaver Burk plot.

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Chapter Four

Results and Discussion

4.1 Isolation and Identification of Bacterial Isolates

Fifty plaque samples were collected from teeth of the staff of Biotechnology–Department, College of Science, Al-Nahrain University. Isolation of *Streptococcus* species was done using selective enrichment technique including culturing of plaque samples on MS-agar (Mitis–Salivarius agar), which promotes growth of streptococci and suppress other bacterial species (Carlsson, 1967). Accordingly forty five samples were considered to be positive, containing bacterial count about (10^4 cells/ml) (Friedrich, 1981).

To obtain pure cultures, single colonies from the surface of MS-agar were selected and subcultured on the surfaces of blood agar and MS-agar media.

Isolates were first identified to genus level depending on their gram-staining, microscopic examination and catalase test. The streptococci are gram-positive, individual cocci which are spherical or ovoid and arranged in chains under light microscope and considered as catalase negative bacteria as indicated by identification scheme of Friedrich (1981). Accordingly, forty three isolates were suspected to be belong to streptococci.

Further identification of the isolates were done depending on the colonial shape and form on the surface of MS-agar media. Isolates could be varied between, hard coherent, raspberry like high refractile, raised colonies that were identified as *S. mutans* which was considered as one of the most important etiological agent of dental caries (Loesche, 1986), while other colonies showed characteristics of zooglleic form, which were firmly attached to agar which were considered as *S. sanguis* depending on criteria reported by Colman and Williams, (1972) identification scheme. Other type of colonies produced a minute circular forms were considered as *S. salivarius*, the colonies that were

little raised with mucoid texture were considered as *S. milleri* following the information scheme of Buchanan and Gibbons (1974). Accordingly, thirty nine isolates were suspected to belong to mutans streptococci group and four isolates were belonged to *S. sanguis, S. salivarius* and *S. milleri*.

Characterization for the previous of species in accordance to their capability of producing specific type of exopolysaccharides as described in (3.2.5) as a criterion for separating species of mutans streptococci group bacteria and *S. sanguis* was done. Polysaccharide played a significant role as component of dental plaque matrix, enabling these cariogenic microorganisms to colonize and survive on the tooth surface, from that *S. salivarius* which produce polyfructan (levans). Results shown in table (4-1) indicate that only thirty seven isolates were capable of producing polyglucan, while the other six isolates were considered as levans producers, since no reaction was obtained with ethanol and methanol and which considered as a good sign for the polyfructan–production as reported by Guthof (1970).

A rapid identification of colonies belonged to mutans sterptococci group from that of other streptococci (*S. sanguis*) on MS-agar media following the method described in (3.2.6) which depend on staining the cells of mutans streptococci with 2,3,5-triphenylterazolium chloride (TTC) and changed to dark pink,due to mannitol-1-phosphate dehydrogenase-mediated hydrolysis of mannitol (Brown and Wittenberger, 1973) to the acid by these bacteria (Carlsson, 1968). Results indicated that thirty five isolates were stained with dark pink color and considered to be (positive result) and two isolates showed (negative result) no change in color.

Table (4-1): Ability of bacterial isolates to produce extracellular polysaccharide Dextran and Levans

No.	Symbols	Acetone	Ethanol	Methanol
1	N_1	+	+	+
2	N_2	+	+	+
3	N_3	+	+	+
4	N_4	+	+	+
5	N_5	+	+	+
6	N_6	+	+	+
7	N_7	+	+	+
8	N_8	+	+	+
9	N_9	+	+	+
10	N_{10}	+	+	+
11	N_{11}	+	+	+
12	N_{12}	+	+	+
13	N_{13}	+	+	+
14	N_{14}	+	+	+
15	N_{15}	+	+	+
16	N_{16}	+	+	+
17	N_{17}	+	+	+
18	N_{18}	+	+	+
19	N_{19}	+	+	+
20	N_{20}	+	+	+
21	N_{21}	+	+	+
22	N_{22}	+	+	+
23	N_{23}	+	+	+
24	N_{24}	+	+	+
25	N_{25}	+	+	+
26	N_{26}	+	+	+
27	N_{27}	+	+	+
28	N_{28}	+	+	+
29	N_{29}	+	+	+
30	N_{30}	+	+	+
31	N_{31}	–	–	–
32	N_{32}	+	+	+
33	N_{33}	+	+	+
34	N_{34}	+	+	+
35	N_{35}	–	–	–
36	N_{36}	+	+	+
37	N_{37}	+	+	+

38	N_{38}	+	+	+
39	N_{39}	–	–	–
40	N_{40}	–	–	–
41	N_{41}	–	–	–
42	N_{42}	–	–	–
43	N_{43}	+	+	+

+ =Turbidity or flocculent formation

– = No reaction

For further identification of the oral *Streptrococcus* species, other biochemical tests were included. One of those tests is the ability of the bacterial isolates to grow in a culture media containing (4%) NaCl as described in (3.2.7) Table (4-2) shows different patterns of growth, varied between a good growth with a percentage of (63%) to a weak growth with a percentage of (27%) and no growth with a percentage of (10%).

The tolerance of 4% NaCl was considered as criterion to differentiate species of the oral streptococci from that associated with dental disease (Holt *et al*., 1994). Only thirty three isolates with a percentage of (90%) (which gave the good and weak growth in 4% NaCl) were taken for further identification.

Table (4-2): The ability of the *Streptococcal* isolates to grow in the presence of 4% NaCl.

No.	Symbols	Growth in 4% NaCl
1	N_1	±
2	N_2	+
3	N_3	±
4	N_4	+
5	N_5	+
6	N_6	±
7	N_7	±
8	N_8	±
9	N_9	+
10	N_{10}	+
11	N_{11}	+
12	N_{12}	+
13	N_{13}	+
14	N_{14}	±
15	N_{15}	+
16	N_{16}	+
17	N_{17}	+
18	N_{18}	+
19	N_{19}	+
20	N_{20}	+
21	N_{21}	+
22	N_{22}	±
23	N_{23}	+
24	N_{24}	+
25	N_{25}	+
26	N_{26}	±
27	N_{27}	±
28	N_{28}	±
29	N_{29}	+
30	N_{30}	+
31	N_{32}	+
32	N_{33}	+
33	N_{34}	+
34	N_{36}	–
35	N_{37}	–
36	N_{38}	–
37	N_{43}	–

+ = Good growth; ± = weak growth; – = No growth

The ability of the isolates for utilizing various carbohydrates was carried out as described in (3.2.8) in a brain heart infusion broth medium supplemented with (10%) of the tested carbohydrates (mannitol, sorbitol and inulin) with sucrose as (a positive control). Results in table (4-3) show that most isolates were capable of reducing mannitol sugar with the exception of three isolates as compared with the sucrose (positive control) by changing the color of the media from red to yellow. Seven isolates were uncapable of fermenting sorbitol and inuline sugars.

According to results in table (4-3), the rapid utilization of the carbohydrates especially sucrose by the cariogenic bacteria is due to the ability of these bacteria to produce lactic acid during the Embeden Myerhove Pathway (EMP) which causes a rapid pH fall (the appearance of yellow color). Thus these bacteria were considered as acidogenic *spp*. which are capable to utilize sucrose to promote the production of polysaccharides and organic acid which represents major virulence factors that generated during fermentation process and facilitates the adhesion of these bacteria on the tooth surface (Burne *et al*., 1999).

Beighton *et al.* (1991) demonstrated that all mutans streptococci group that were isolated from humans are capable to ferment mannitol, sorbitol and inulin sugars except for *S. sobrinus* and *S. cricetus* in which 11-89% of strains are positive to sorbitol and inulin. So that three isolates did not belong to mutans streptococci group because of their inability to ferment mannitol, sorbitol and inulin and six isolates may belong to *S. sobrinus* and *S. cricetus* bacteria because of their ability to ferment mannitol and unability of fermenting sorbitol and inulin

Table (4-3): The ability of the *Streptococcal* isolates to ferment sucrose, mannitol, sorbitol and inuline

No.	Symbols	Sucrose (positive control)	Mannitol	Sorbitol	Inuline
1	N_1	+	+	+	+
2	N_2	+	+	+	+
3	N_3	+	+	+	+
4	N_4	+	+	–	–
5	N_5	+	+	+	+
6	N_6	+	–	–	–
7	N_7	+	+	+	+
8	N_8	+	–	–	–
9	N_9	+	+	+	+
10	N_{10}	+	+	–	–
11	N_{11}	+	+	+	+
12	N_{12}	+	+	–	–
13	N_{13}	+	+	–	–
14	N_{14}	+	+	+	+
15	N_{15}	+	+	+	+
16	N_{16}	+	+	+	+
17	N_{17}	+	+	+	+
18	N_{18}	+	+	–	–
19	N_{19}	+	+	+	+
20	N_{20}	+	+	+	+
21	N_{21}	+	+	+	+
22	N_{22}	+	+	+	+
23	N_{23}	+	+	+	+
24	N_{24}	+	+	+	+
25	N_{25}	+	+	+	+
26	N_{26}	+	+	+	+
27	N_{27}	+	+	+	+
28	N_{28}	+	–	–	–
29	N_{29}	+	+	+	+
30	N_{30}	+	+	+	+
31	N_{32}	+	+	+	+
32	N_{33}	+	+	+	+
33	N_{34}	+	+	–	–

+ = Changing the color of media from red to yellow;

_= no change in color

Antibiotic sensitivity test may be used as a criterion for separating oral *Streptococcus* from that belongs to *Streptococcus pneumoniae* and pediococcus species. Antibiotics like bacitracin, vancomycin and optochin were used following the disk diffusion method as described in (3.2.9).

Result shown in table (4-4) indicates that most isolates were resistant to bacitracin, optochin and vancomycin with the exception of seven isolates (N_1, N_3, N_6,N_7, N_8 ,$N_{26}$and N_{28}), three isolates (N_6, N_8 and N_{28}) and seven isolates (N_6, N_8, N_{22}, N_{25}, N_{28}, N_{30} and N_{34}) isolates were sensitive to these antibiotics respectively.

Dela Maza *et al.* (1997) identified oral streptococci group and separated them from other bacteria like *Pediococcus spp.* and *Streptococcus pneumonia* according to their susceptibility to vancomycin and resistance to optochin respectively. Whiley and Beighton (1998) recorded that bacitracin resistance was determined for all species of mutans streptococci that were isolated from human with the exception of *S. cricetus* bacteria.

Bacteria growing in dental plaque display increased resistance to antimicrobial agents (Pratten and Wilson, 1999). The mechanisms behind the increased resistance of dental plaque bacteria to these agents is due to mutations affecting the drug target of their cells, the presence of efflux pumps or to the production of modifying enzymes etc. (Marsh, 2004).

Table (4-4): Antibiotic sensitivity test of *Streptococcal* isolates.

No.	Symbols	Bacitracin	Vancomycin	Optochin
1	N_1	S	S	R
2	N_2	R	S	R
3	N_3	S	S	R
4	N_4	R	S	R
5	N_5	R	S	R
6	N_6	S	R	S
7	N_7	S	S	R
8	N_8	S	R	S
9	N_9	R	S	R
10	N_{10}	R	S	R
11	N_{11}	R	S	R
12	N_{12}	R	S	R
13	N_{13}	R	S	R
14	N_{14}	R	S	R
15	N_{15}	R	S	R
16	N_{16}	R	S	R
17	N_{17}	R	S	R
18	N_{18}	R	S	R
19	N_{19}	R	S	R
20	N_{20}	R	S	R
21	N_{21}	R	S	R
22	N_{22}	R	R	R
23	N_{23}	R	S	R
24	N_{24}	R	S	R
25	N_{25}	R	R	R
26	N_{26}	S	S	R
27	N_{27}	R	S	R
28	N_{28}	S	R	S
29	N_{29}	R	S	R
30	N_{30}	R	R	R
31	N_{32}	R	S	R
32	N_{33}	R	S	R
33	N_{34}	R	R	R

R: Resistance

S: Sensitive

Accordingly, three isolates (N_6, N_8 and N_{28}) are considered not belong to mutans streptococci bacterial group because of their sensitivity to bacitracin and optochin antibiotic and resistance to vancomycin. Four isolates (N_1, N_3, N_7 and N_{26}) may be related to *Streptococcus cricetus* (a species of mutans streptococci) because of their sensitivity to bacitracin and vancomycin and resistance to optochin antibiotics. The resistance pattern to vancomycin antimicrobial agent for the four isolates (N_{22}, N_{25}, N_{30} and N_{34}) is due to the reasons that described previousely by Marsh,(2004).

For further confirmation of results ande in order to group *Streptococcus* species, a Latex test (Pastorex^{R}Strep) kit was used. The test was done as well as results were recorded in accordance to the instructions reported by manufacturing company (Bio-Rad France).This agglutination test was used for grouping of (α or β) haemolytic streptococci belonged to the main Lancefield group depending on the activity of the latex suspension (antibody) which was specific for group A, B, C, D, F and G polysaccharide (antigen) that was found on the cell walls of these bacteria. Thirty three bacterial isolated were tested.

Result shown in table (4-5) and figure (4-1) indicate that most isolates were related to the genus *Streptococcus* with the exception of three isolates (N_6, N_8 and N_{28}), since no agglutination reaction was obtained for these isolates. These three isolates were represented a percentage of about (9.09%) from the whole thirty three isolates.

Grouping of the isolates was done as follow:

1. Six isolates were related to (serotype A) which represent (18.18%).
2. One isolate was related to (serotype B) and represents (3.03%).
3. Eight isolates were related to (serotype C) and represent (24.24%).
4. Eight isolates were belonged to (serotypes D) and represent (24.24%).
5. Two isolates were related to (serotype F) and represent (6.06%).
6. Five isolates were related to (serotype G) and represent (15.16%).

The identifiable bacteria by this test are not only related to the genus streptococci but also to the mutans streptococci bacteria because they were isolated from specimens of human dental plaque. Killian *et al*. (1983) found that mutans streptococci bacteria was responsible for the initiation of the dental plaque formation on the tooth surface even after four hours of cleaning the teeth.

Beighton *et al*., (1991) separated mutans streptococci group bacteria into eight serotypes according to their surface antigens in their cell walls. The classification series ranged from serotypes A to G and / or H. these serotypes can in turn be classified into four groups according to another biochemical properties (Shklair and Keen, 1974) and to the specificities of their enzymes in extracellular polysaccharide synthesis (Kametaka *et al*., 1987).

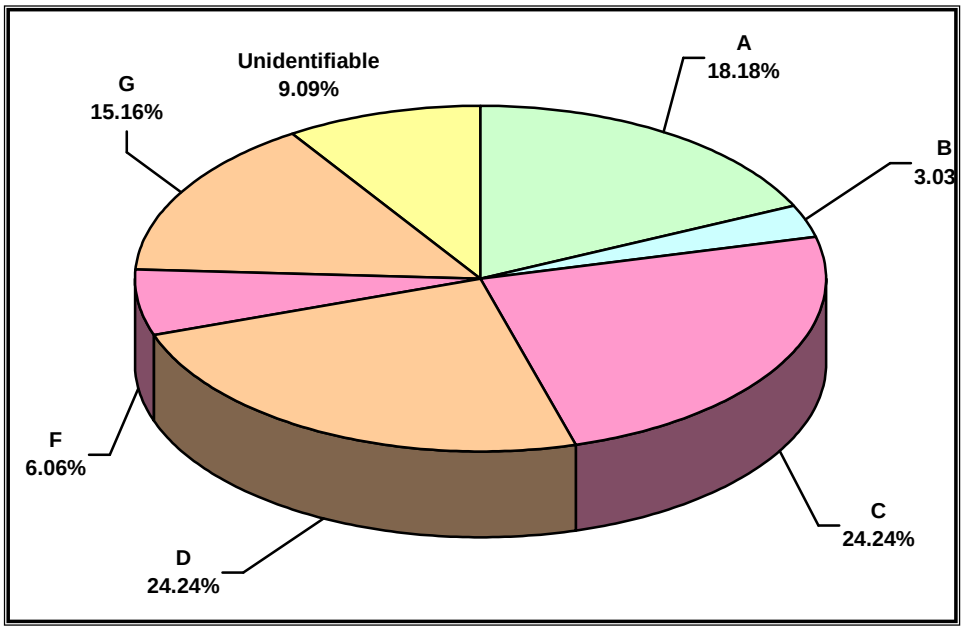

Figure (4-1): Percentages of each Lancefield group among the *Streptococcal* isolates.

Table (4-5): Latex test (Pastorex^{R}Strep) (Lancefield grouping) of *Streptococcal* isolates.

No.	Symbols	Lancefield group
1	N_1	A
2	N_2	C
3	N_3	A
4	N_4	D
5	N_5	F
6	N_6	–
7	N_7	A
8	N_8	–
9	N_9	C
10	N_{10}	G
11	N_{11}	C
12	N_{12}	D
013	N_{13}	D
14	N_{14}	B
15	N_{15}	G
16	N_{16}	F
17	N_{17}	C
18	N_{18}	G
19	N_{19}	C
20	N_{20}	D
21	N_{21}	G
22	N_{22}	**A**
23	N_{23}	D
24	N_{24}	G
25	N_{25}	C
26	N_{26}	A
27	N_{27}	A
28	N_{28}	–
29	N_{29}	D
30	N_{30}	D
31	N_{32}	C
32	N_{33}	C
33	N_{34}	D

Thus serotypes D, G and / or H (*S. sobrinus*) form one group, Serotype A (*S. cricetus*) another, serotype C, E and F (*S. mutans*) the third and serotype B (*S. rattus*) the fourth group. According to this information, isolates can in turn be classified into the following:

1. Six isolates were related to the mutans streptococci (*S. cricetus*) (serotype A) represent (18.18%).
2. One isolates was related to mutans streptococci (*S. rattus*) (serotype B) represents (3.03%).
3. Ten isolates were related to (serotype C) and (serotype F) which related to mutans streptococci (*S. mutans*) with a percentage of (30.302%).
4. Thirteen Isolates were related to (serotype D) and (serotype G) which belonge to mutans streptococci (*S. sobrinus*) with a percentage of (39.294%).

From these results, the most isolated species of mutans streptococci bacteria from the previously isolated dental plaque human specimens were (*S. sobrinus*) followed by (*S. mutans*) according to the percentage ratio of each of them. Carlsson (1988), classified the most commonly isolated species of mutans streptococci from human are usually *S. mutans* serotypes (C, E and F) followed by *S. sobrinus* serotypes (D and G).

4.2 Extraction, Characterization and Purification of GTF

4.2.1 Extraction of GTF

Prior to large scale production of the enzyme from the *Streptococcus* species screening of the isolates for their productivity was made. From thirty isolates only seventeen isolates was selected depending on their productivity and serotyping (Lancefield grouping). results show in table (4-6) indicate that all isolates were capable of producing an extracellular GTF enzyme and its production was not restricted in specific serotype group because isolates were related to different groups. Specific activities of GTF for isolates were ranged between (0.083-0.51 U/mg protein). The highest GTF productivity was found among (N_{10}, N_{23} , N_{32} and N_{19}) which belonged to serotypes (G,D, C and C) respectively with specific activities ranged between (**0.407**-**0.752** U/mg protein).

Table (4-6): GTF production from different mutans streptococci isolates.

No.	Symboles	Serotype	Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity (U/mg)
1	N_2	C	0.188	0.812	0.231
2	N_3	A	0.127	0.589	0.215
3	N_5	F	0.156	0.651	0.239
4	**N_{10}**	**G**	**0.518**	**0.690**	**0.752**
5	N_{14}	B	0.310	0.860	0.360
6	N_{16}	C	0.091	0.768	0.118
7	N_{17}	C	0.221	1.069	0.206
8	N_{18}	G	0.175	0.632	0.276
9	**N_{19}**	**C**	**0.310**	**0.764**	**0.407**
10	N_{21}	G	0.178	0.914	0.194
11	**N_{23}**	**D**	**0.437**	**0.656**	**0.667**
12	N_{25}	C	0.073	0.661	0.110
13	N_{26}	A	0.125	1.220	0.102
14	N_{30}	D	0.107	1.28	0.083
15	**N_{32}**	**C**	**0.375**	**0.691**	**0.544**
16	N_{33}	C	0.221	1.133	0.195
17	N_{34}	D	0.201	0.826	0.243

Glucosyltransferase is an extracellular enzyme that binds and synthesizes extracellular glucan which have a vital role in the establishment of cohesive mutans *Streptococcal* masses on the tooth surfaces and subsequent caries development (Lilijemark and Bloomquist, 1996). Hamada *et al*., (1989) found that mutans streptococci serotypes (A, B, C, D, F and G), *S. salivarias* and *S. sanguis* were capable to express several GTF types which differing in chain length, content of α 1,3 and α-1,6 glucosyl linkages, degrees of branching and solubility.

According to table (4-6) and this information all serotypes of mutans streptococci bacteria were capable to express GTF.

Because of the specific activities of GTF of isolates (N_{10}, $N_{23,}$ N_{32} and N_{19}) were very close to each others table (4-6), a conformation was made in order to choose the highest GTF producible bacteria among them. Extraction of GTF was repeated for them in (10 ml) Todd-Hewitt broth medium. GTF activity, protein concentration and specific activity were determined again for each isolate. Results shown in figure (4-2) indicated that mutans streptococci N_{10} (serotype G) has the highest GTF specific activity (0.752 U/mg protein) followed by N_{23} (serotype D) (0.667 U/mg protein) then N_{32} and N_{19} (serotype C) with specific activity of (0.544 U/mg protein) and (0.407 U/mg protein) respectively. From the previous discussion, bacteria which were related to serotypes (D and G) were named mutans streptococci. (*S. sobrinus*), so mutans streptococci N_{10} (*S. sobrinus*) was chosen for large scale production and characterization of GTF enzyme. In many studies, extraction of GTF that were used either for immunization protocol or characterization of this enzyme was done either from mutans streptococci (serotype C) or (serotypes G and D) which represented *S. mutans* and *S. sobrinus* bacteria because of their crucial role in the formation and metabolism of dental plaque and consequently in the induction of dental caries (Taubman *et al*., 1988; Wunder and Bowen, 2000).

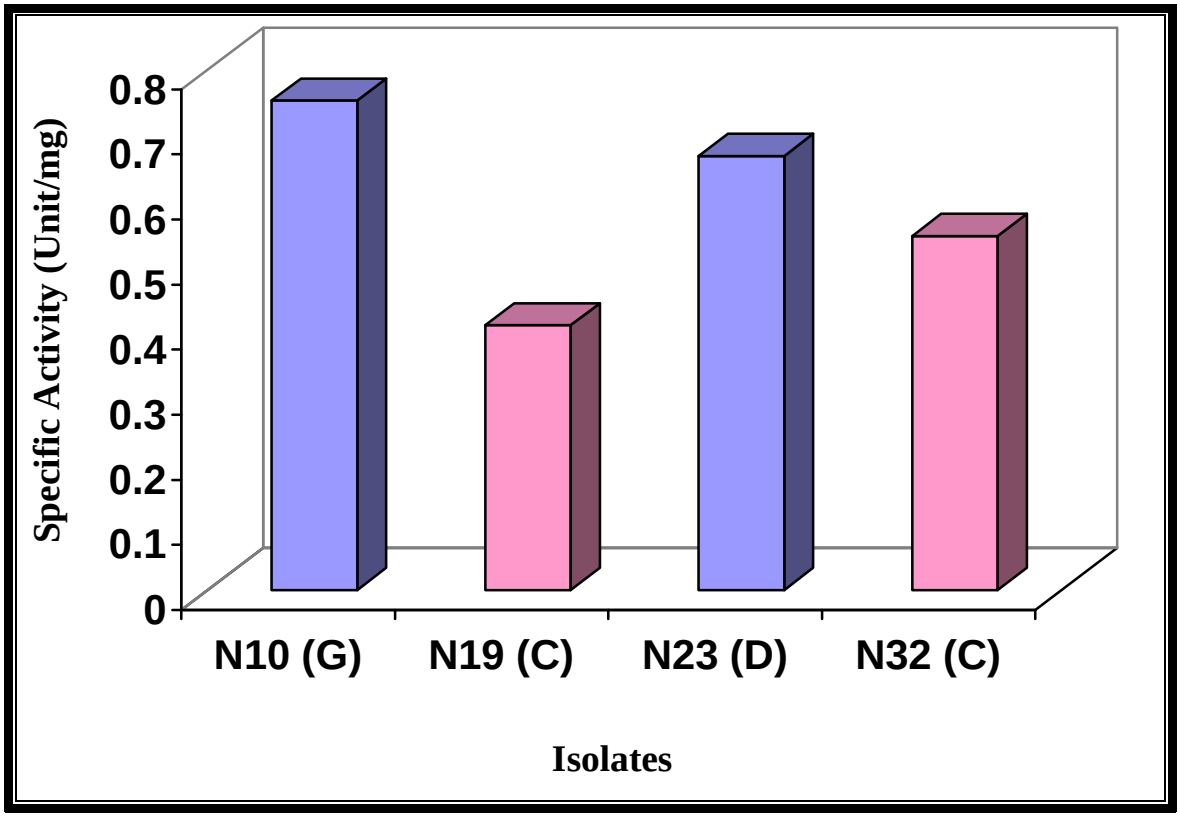

Figure (4-2): The abilities of the four mutans *Streptococci* bacterial isolates (N_{10}, N_{19}, N_{23} and N_{32}) to express GTF through their GTF specific activity.

4.2.2 Production phase and GTF Activity

In order to specify production phase for isolate $N_{10,}$ growth and GTF activity of the isolate was made as described in (3.2.14.C).

Results shown in figure (4-3) indicate that during the period of 0-6 hr. no change in optical density was noticed which is an indication of lag phase of growth followed by log phase of about 9hr.during which no productivity of GTF was found as indicated by measurement of GTF activity in culture broth. However, GTF activity was noticed after 20hr.and lasted to 40hr.of incubation which is a period of stationary phase.

Al-Fatlwai (2003) characterized the growth curve of *S. mutans* which was isolated from human dental plaque. This bacteria was entered in the lag-phase between (0-4 hrs) then between (4-10 hrs) an increase in cell numbers was recognized which characterized as a log-phase. The stationary phase was characterized by the bacterial constant number and occurred between (10-20 hrs).

Juarez and Steinson (1999) made a good linkage between growth of *Streptococcus gordonii* and the production of an extracellular protease. This enzyme was produced in chemically defined medium of growth (contain 30 mM glucose and some essential amino-acids) at an hour 5 (in the early log-phase) and continued until reach the maximum production at hour 15 (the mid-stationary phase) then it began to decrease until it became nil at hour 30. This was due to the loss of essential amino acids that were required for its secretion or production especially during the stationary-phase, due to the accumulation of the end products and toxic substances.

Simpson and Russell (1998) described the production of an intracellular α-amylase from *S. mutans* during the growth life of this bacteria in a chemically defined medium containing 2% glucose. The secretion of this enzyme began in the early log-phase (hour 3) and continued until it reached its maximum production value at (hour 24) (Late-stationary phase).

From these previous investigations about the intra and extracellular enzymes production from the *Streptococcus* bacteria, an extracellular GTF began its production at the stationary phase of the bacterial growth curve (hour 20) and reached it's maximum production also during this phase (hour 24). The reason behind the maximum production of this enzyme during this hour then the decrease in the activity or production until it became nil at hour 40 was due to the ability of the bacterial isolate to make a rapid metabolism of the carbohydrate substances that found in Todd-Hweitt broth medium (glucose) to produce lactic acid during EMP pathway which causes a rapid pH fall. Al-Uqaili (2000) found that the environmental pH value was reached to 4.5 after the growing of *S. mutans* for about 48 hrs. This pH value was unsuitable for the GTF production because the optimum pH value for GTF of *S. mutans*, *S. sobrinus* and *S. oralis* (which is a member of the oral streptococci family) was about 6.5 as described by (Hamada *et al*., 1989; Fujiwara *et al*., 2000).

Accordingly, especially for the large scale production of GTF, the extraction of this enzyme must be done after 24 hr. of the bacterial growth under anaerobic conditions and at 37°C. After the bacterial harvesting, pH of the supernatant must be calibrated in order to reach 6.5.

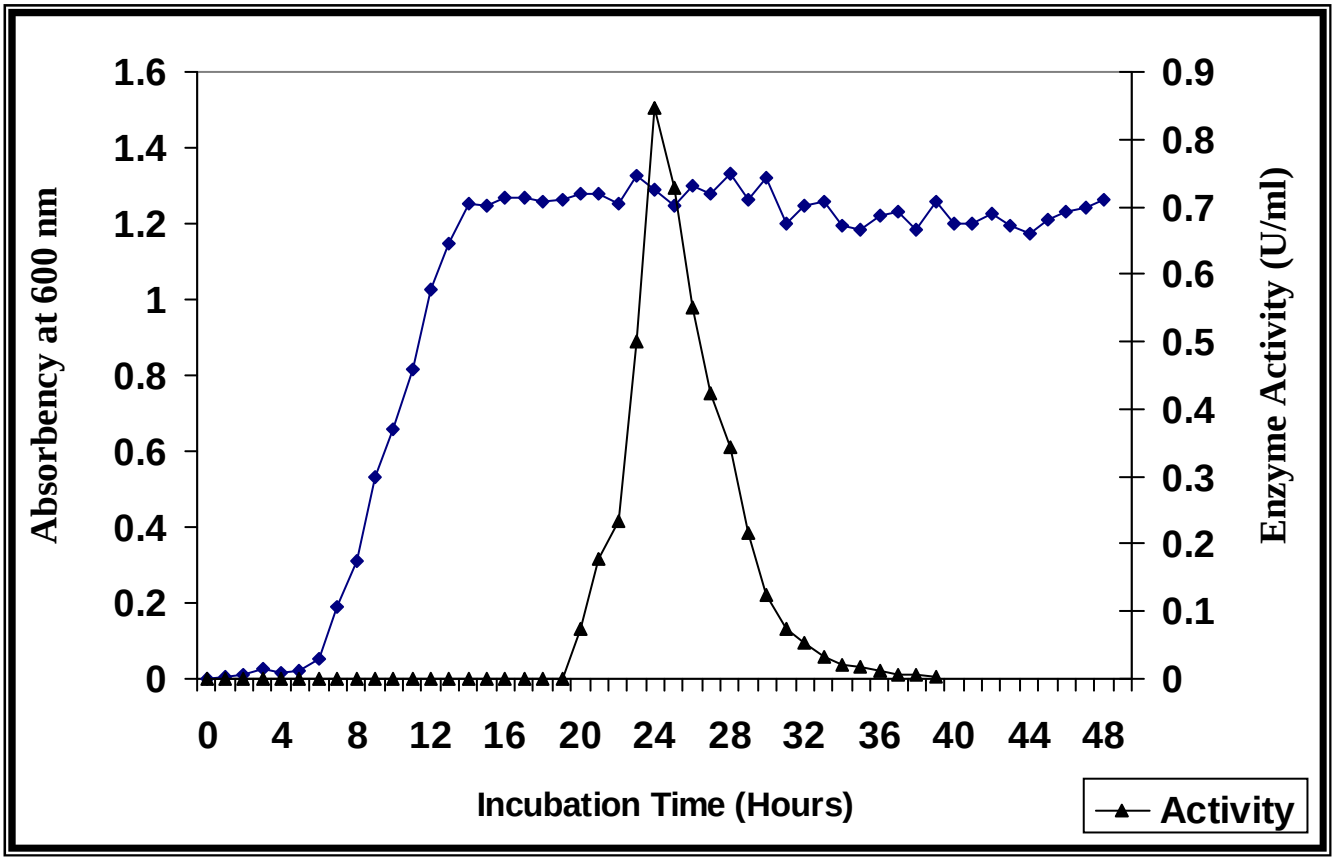

Figure (4-3): Production phase and GTF Activity of mutans streptococci (N_{10}) (Serotype G).

4.2.3 Preservation and Concentration of Crude (GTF)

To prepare the enzyme for the purification steps and immunization protocol that would be used later, preservation of GTF and the increase in the concentration and activity were estimated as described in (3.2.14.D). After the division of the bacterial supernatant (350 ml) into two unequal parts, results shown in table (4-7) indicated that for the first part (125 ml) which was in role divided into five equal halves. The protein concentration and GTF activity of the control half (25 ml) before the preservation process at (-20°C) were (0.7 mg /ml) and (0.337 U/ml). After preservation at (-20°C) the protein concentration and GTF activity for each one of the remaining four halves were (0.491 mg /ml), (0.177 U/ml); (0.150 mg/ml), (0.055 U/ml); (0.075 mg /ml), (.0059 U/ml), (0), (0) after (1, 2, 3 and 7 days) respectively.

Table (4-7): The effect of preservation at -20ºC on the activity and protein concentration of crud GTF enzyme.

Before preservation at -20ºC (Control)		After preservation at -20ºC (Without glycerol (15%))			
		1 day	2 days	3 days	7 days
Protein concentration (mg/ml)	0.7	0.491	0.150	0.075	0
GTF activity (unit/ml)	0.337	0.177	0.055	0.059	0

The remaining second part (225 ml) which was in role divided into two unequal parts, for the first one (125 ml) which was in role divided into five equal halves , after the addition of (15%) glycerol for each, results showed that after the preservation process at (-20^0C) ,the protein concentration and GTF activity for each one after (7, 14, 21, 25 and 30 days) were the same to the control (0.7 mg /ml) and (0.337 U/ml). So without the addition of the glycerol and after the

comparison with the control, the protein concentration and GTF activity were decreased after 1, 2, 3 days from the preservation at (-20°C) until they reached to their nil values at day 7. The reason behind this loss in protein concentration and GTF activity was due to the loss of protein integrity during freezing process. This interfacial phenomenon involving partial denaturation of protein at the ice / freezing process. Strambini and Gabellieri (1996) studied the phosphorescence life times of the tryptophan residues in several proteins and demonstrated that freezing of the aqueous solutions of these proteins were accompanied by loosening of the native fold and considerable loss of secondary and tertiary structure of the protein. After the addition of the glycerol (15%) protein concentration and GTF activity were remained stable after (7, 14, 21, 25 and 30 days) of preservation at (-20°C) this was due to the addition of glycerol which has the ability to reduce or eliminate the perturbation of the protein that was caused by freezing as described by Liu *et al*., (2005).

Wunder and Bowen (2000) described the long term storage of the GTF by mixing the samples (Crude or purified) with glycerol (final concentration at 15% v/v) and freezing at -20°C.

For the concentration protocol the second part of the bacterial supernatant (100 ml) which was divided in role into four equal halves (25 ml) for a half. Results show in table (4-8) indicated that, protein concentration and GTF activity as compared with the control for the first concentrated half to (12.5 ml) by the addition of the polyethylene glycol powder PEG (4000) were (0.85 mg/ml) with no activity, for the second half which was concentrated by lyophilization process to (2 ml) were no protein concentration and enzyme activity, for the third half which was concentrated to (12.5 ml) by the sucrose powder were (1 mg/ml) protein concentration, (16 U/ml) of enzyme activity and for the end half which was concentrated by an Amicon-filter P50 were (0.601 mg/ml), (11.076 U/ml) respectively.

Table (4-8): the effect of concentration by PEG, Sucrose, Lyophilization and filtration by Amicon on the activity and protein concentration of crude GTF enzyme.

Before concentration of GTF (Control)		After concentration of GTF			
		PEG	Lyophilization	Sucrose	Amicon filter
Protein concentration (mg/ml)	0.7	0.85	0	1	0.601
GTF activity (unit/ml)	0.337	0	0	16	11.076

For the concentrated half via the addition of the polyethylene glycol powder PEG (4000), the protein concentration was increased as compared with the control but the loss in activity was recognized. The reason behind the increasing in protein concentration was due to that, PEG is a polymer which dose not bind strongly to the protein so it does not able to alter the structure of the protein itself. PEG concentrated the protein via its ability to enhance the protein-protein interaction which capable to remove the protein surfaces area making a decrease in the volume from (25 ml) to (12.5 ml). The PEG molecules that excluded from the volume that surrounding the proteins can thoughts to exert an osmotic pressure which attempts to shrink the inaccessible volume, this information was described by Chinn *et al*. (2000). Chinn *et al*. (2000) described a good relationship between the PEG molecular weights and the ability of this polymer to protect the enzyme activity from loss. The large molecular weight PEG (8000-20000 dalton) was capable to protect many enzymes activities from loss. As shown in this study, GTF concentration experiment via PEG, (4000) which incapable to protect the enzyme activity from loss.

Wunder and Bowen, (2000) concentrated their GTF-D enzyme from *S. milliri* (which harbors the *gtfD* genes) by the use of PEG powder (20000) so that this high molecular weight capable to protect the GTF enzyme activity from loss.

As showed in the result for the concentration of crude GTF via the use of lyophilization process (Freezing-drying system). The protein concentration and GTF activity were lost after the concentration from (25 ml) to (2 ml). The reason behind this as described by Strambini and Gabellieri (1996), so the usage of the sucrose and glycine to avoid the denaturation of proteins during this process was also recommended by Lie *et al*. (2005).

For concentration of crude GTF by using sucrose powder, a great elevation in GTF activity (16U/ml) was recognized and increasing in the protein concentration (1mg/ml) was occurred. The reason behind the increase in protein concentration in which the sucrose powder capable to increase the protein-protein interactions so it was able to concentrate the enzyme as described for the PEG (Chinn *et al*., 2000). For the elevations in activity, the sucrose powder acted as a best substrate for the GTF because of its low K_m value. The GTF used the sucrose molecules as a high-energy glucosyl donner for the polysaccharide and oligosaccharide synthesis as described by Robyt, (1995). So the concentration of crude GTF cannot occur by using sucrose because GTF already acts on it to give its product.

Finally, concentration of crude GTF by using an Amicon-filter P50 in an (ultrafiltration-cell).The GTF activity was increased to (11.076 U/ml) and protein concentration was become (0.601 mg/ml) these results were found in the In-part (the suspension that appeared on the filter) while in the out-part no GTF activity was recorded and the presence of a very low protein concentration (0.059 mg/ml) was observed. So Amicon-Filter acts as a tool to concentrate the GTF, protects its activity and separates the enzyme from another protein that might pass the pores of the filter.

Figures and Edwards (1978) described the concentration of GTF enzyme from 20L bacterial supernatant through the usage of different ultrafiltration membranes in Amicon-Ultrafiltration cells. Meulenbeld *et al*.,(1999) described the ultrafiltration-process by Amicon filter as a tool to concentrate GTF-D enzyme of *S. sobrinus* after each purification process.

Accordingly, the best method for the long term storage of GTF enzyme is by the addition of (15%) glycerol then ice / freezing at (-20°C) and the best tool to concentrate the GTF is by using an Amicon-filter in (ultrafiltration-cell) system.

4.2.4 Large Scale Production of GTF

Large scale production of GTF was done from mutans streptococci (*S.sobrinus*) N_{10} bacteria as described in (3.2.14.E). After extraction of GTF, protein concentration, GTF activity then specific activity were determined in (10 ml) of bacterial suspension. Result showed that bacteria about ($1x10^8$ cells/ml) were able to produce (0.7 mg/ml) of crude GTF with an activity of (0.790 U/ml) which had a specific activity of (1.128 U/mg protein) after (1) fold of purification, when (750ml) crude GTF concentrated by an Amicon-filter P50 in (Ultrafiltration-cell) to (40ml), results shown in table (4-9), indicate that protein concentration and GTF activity were recorded to be (0.601 mg/ml) and (11.076 U/ml) with a specific activity of (18.42 U/mg protein) after (16.32) folds of purification which represented (74.77%) yield of enzyme.

Figure and Edwards (1981) concentrated GTF from three liters bacetrial culture (*S. mutans*) by using an Amicon "on-line" column effluent-concentration (Amicon-module (ECI) equipped with a PMI10 Ultrafiltration membrane). After concentration to (60 ml), protein concentration, GTF activity and specific activity were (0.660 mg/ml), (4 U/ml) and (12 U/mg protein) respectively.

Al-Hayali, (2002) began the purification procedure of GTF of mutans streptococci (BiotypeI- *S. mutans*) and mutans streptococci (Biotype IV-*S. sobrinus*) with precipitation by saturated ammonium sulfate from (450 ml) and (400 ml) respectively. After precipitation, protein concentrations, GTF activity and specific activity values for each were recorded to be (2 mg/ml), (2.8 mg/ml); (0.315 U/ml) (0.406 U/ml) and (0.16 U/mg protein), (0.15 U/mg protein) respectively.

Accordingly, concentration of GTF by using an Amicon-filter P50 in (Ultrafiltration-cell) is more suitable than precipitation with saturated ammonium-sulfate.

4.2.5 Purification of GTF by Adsorption Chromatography

Purification of GTF was done by Adsroption chromatography using hydroxyapatite beads as described in (3.2.14.F). Results showed that washing with (25 ml) of (0.15 M) phosphate buffer (pH 7.5) then elution with (0.3M) of the same buffer, GTF activity recorded in all fractions of the washing and elution parts. The collection of fractions of (the washing part) as well as for fractions of (the elution part) in a separated sterile containers was done. GTF activity, protein concentration and specific activity were determined for these separated parts. Results shown in table (4-9), indicate that washing with (0.15M) phosphate buffer produce a GTF activity of (6.699 U/ml), protein concentration of (0.359 mg/ml) with a specific activity of (18.56 U/mg protein) after (16.54) folds of purification which represented (57.47%) yield of enzyme. Elution with (0.3M) phosphate buffer produce a GTF activity of (6.922 U/ml), protein concentration of (0.365 mg/ml) and a specific activity of (18.96 U/mg protein) after (16.80) folds of purification which represented (57.47%) yield of enzyme.

Accordingly, adsorption chromatography (hydroxyapatite beads) is capable to produce two GTF enzymes with very close activity and protein concentration values. These two enzymes were named GTF-I and GTF-II. GTF-I represented the collection of fractions after washing and GTF-II represented the collection of fractions after elution. Partially purified GTF-I and GTF-II were passed separately through an Amicon-Filter and concentrated from (25 ml) for each to (12 ml).

Purification by hydroxyapatite column had been used widely before the application of ion-exchange chromatography due to its' high adsorption capacity with proteins (Scopes, 1972). Challacombe *et al*., (1973) purified GTF of *S. mutans* by hydroxyapatite column.After elution with (0.2 M) and (0.5 M)

phosphate buffer which resulted in appearance of two pools of activity (8.5 U/ml) and (14.7 U/ml) of GTF respectively. Isoelectric focusing of this preparation revealed it to be a mixture of at least seven GTF enzymes.

Koga *et al*., (1982) purified GTF of mutans streptococci (*S. sobrinus*) (serotype D) by hydroxyapatite column after precipitation with 50% saturated ammonium sulfate. GTFase-S and GTase-I were separated with specific activities of (3.7 U/mg protein) and (1.8 U/mg protein) respectively.

4.2.6 Purification of GTF by Ion-Exchange Chromatography

Purification of GTF enzymes (GTF-I and GTF-II) by ion-exchange chromatography (DEAE-cellulose column) was done as described previously in (3.2.14.G). The concentrated samples from previous step (GTF-I and GTF-II) (12 ml) for each were passed separately through the DEAE cellulose column. Results shown in figure (4-4) and (4-5) indicate that washing with (100 ml) of (0.05M) phosphate buffer (pH 7.5) allowed the presence of two peaks which were represented by fractions (9-16) for GTF-I and fractions (11-20) for GTF-II. Then after elution of proteins with (200 ml) of a gradient from (0.05M) to (0.3M) phosphate buffer (pH 7.5), two peaks were obtained for GTF-I which represented by fractions (20-26) and (32-38) and one peak was obtained for GTF-II which represented by fractions (47-52). Each fraction of GTF-I and GTF-II which represented the peaks after washing and elution processes were tested for GTF activity. Accordingly, only fractions (20-26) and (32-38) of GTF-I and fractions (47-52) of GTF-II were able to reflect GTF activity.

Fractions (20-26) and (32-38) of GTF-I and fractions (47-52) of GTF-II were pooled each separately and named GTF-I_a, GTF-I_b and GTF-II respectively. GTF activity, protein concentration and specific activity were determined for each one of them.

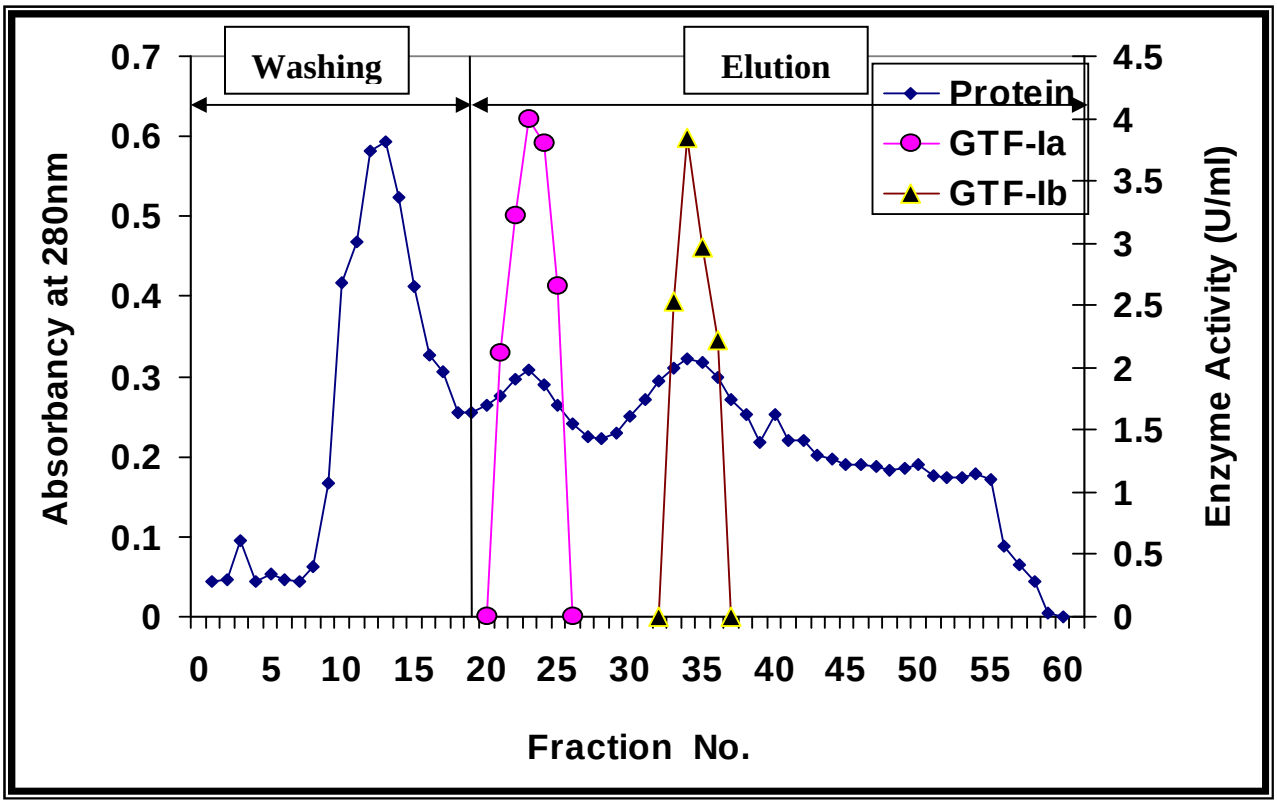

Figure (4-4): Purification of GTF-I enzyme by ion exchange chromatography (DEAE-Cellulose) column (7.5x3.5cm). The column was washed by using (0.05M) phosphate buffer pH (7.5), and then eluted by using a gradient of (0.05M) to (0.3M) phosphate buffer pH (7.5).

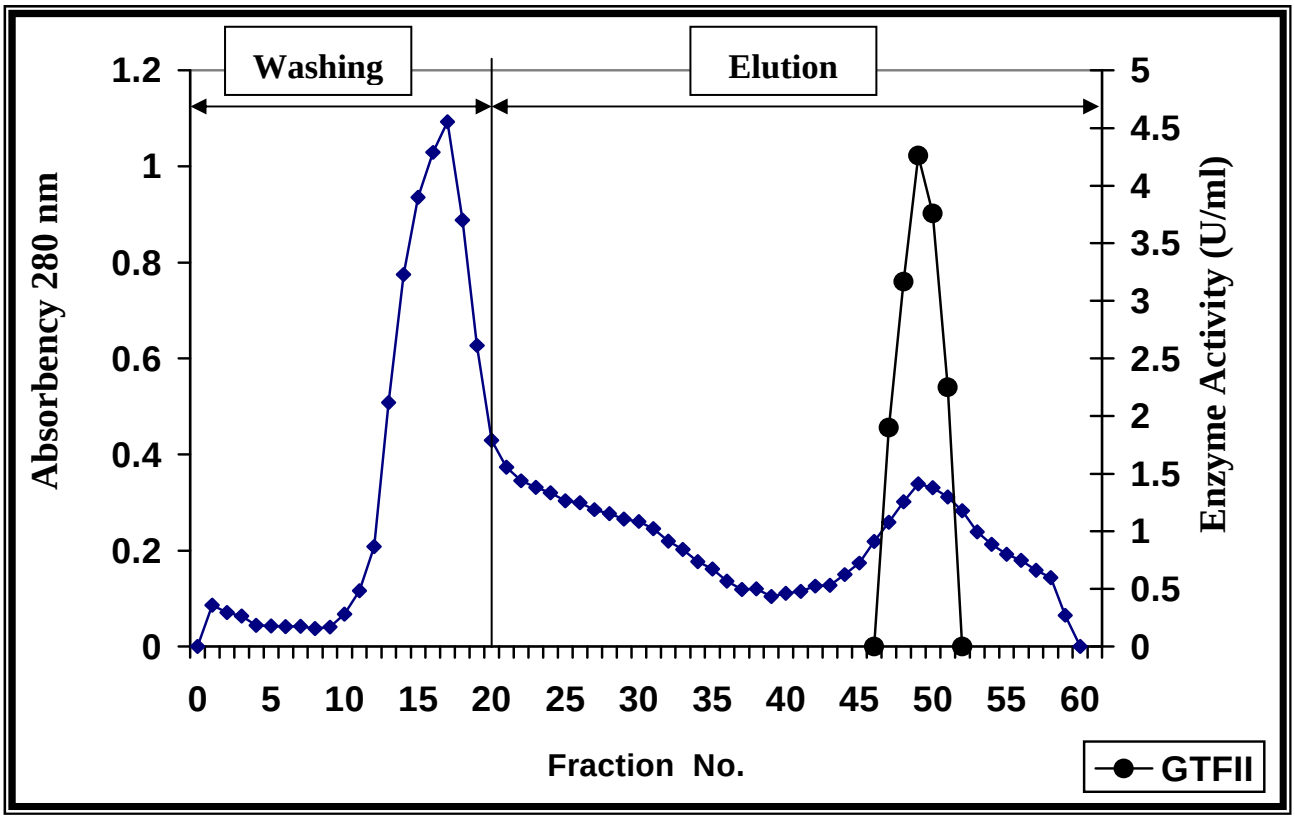

Figure (4-5): Purification of GTFII enzyme by ion exchange chromatography (DEAE-Cellulose) column (7.5x3.5cm).The column was washed by using (0.05M) phosphate buffer pH (7.5), then eluted by using a gradient of (0.05M) to (0.3M) phosphate buffer pH (7.5).

Result shown in table (4-9), indicated that the amounts of (3.576 U/ml), (3.931 U/ml) with a protein concentrations of (0.204 mg/ml), (0.175 mg/ml) and specific activities of (17.52 U/mg protein), (22.464 U/mg protein) with purification folds of (15.53), (19.91) and yield of (45.7%) were obtained respectively for GTF-I_a and GTF-I_b. For GTF-II an amount of (4.113 U/ml) with a protein concentration of (0.153 mg/ml) and a specific activity of (26.88 U/mg protein) was obtained with purification folds of (23.82) and yield of GTF of (45.71%).

Accordingly, three GTF enzymes (GTF-I_a, GTF-I_b and GTF-II) were obtained after purification with ion-exchange chromatography. Partially purified (GTF-I_a, GTF-I_b and GTF-II) were passed separately through an Amicon-Filter P50 in (Ultrafiltration-Cell) to concentrate them to (5 ml).

It has been suggested by several authors that various strains of mutans streptococci produced at least two GTF enzymes synthesizing different products (Ciardi *et al*., 1977; Fukushima *et al*., 1981; Robyt and Martin, 1983; Koga *et al*., 1983). Turchi and Edwards, (1985) proposed that an extracellular GTF system of mutans streptococci (serotype G) was a complex system with two or more enzymes worked in synchronous fashion to produce glucan.

Yamashita *et al*. (1989) described the purification of four glucosyltransferase from mutans streptococci (*S. sobrinus*) (serotype G) by DEAE-cellulose chromatography. GTF fractions were collected from the first DEAE-cellulose each separately and entered to the second DEAE-cellulose column. The specific activity, fold of purification and yield (%) for [P_2] (one kind of glucosyltransferase enzymes) after the first and second DEAE-cellulose were recorded to be (2.39 U/mg protein), (8.35 U/mg protein); (8.54) and (29.8) folds of purification with (43.6%), (27.0%) yields of GTF respectively.

4.2.7 Purification of GTF by Gel-Filtration Chromatography

Purification of GTF enzyme by gel-filtration chromatography (Sephacryl-S-200) column was done as described previously in (3.2.14.H). A (3 ml) of concentrated partially purified GTF-I_a, GTF-I_b and GTF-II each was added separately to the column. When elution of proteins was done by (200 ml) of (0.3M) phosphate buffer (pH 7.5). Results shown in figures (4-6), (4-7) and (4-8) indicate the presence of six peaks (two for GTF-I_a, three for GTF-I_b and one for GTF-II) represented by fractions (21-29), (30-33); (15-18), (24-30), (32-36) and (25-28) respectively. After the determination of GTF activity for all these peaks which indicate that fractions (30-33) of GTF-I_a, Fractions (32-36) of GTF-I_b and fractions (25-28) of GTF-II were able to produce GTF enzyme, as shown in figure (4-6), (4-7) and (4-8) respectively. Fractions (30-33) of GTF-I_a, fractions (32-36) of GTF-I_b and fractions (25-28) of GTF-II were pooled separately for each enzyme then GTF activity, protein concentration and specific activity were determined. Results shown in table (4-9), indicate that GTF-I_a, GTF-I_b and GTF-II were able to reflect GTF activity, protein concentration and specific activity of (5.531 U/ml), (4.320 U/ml), (6.760 U/ml); (0.175 mg/ml), (0.137 mg/ml), (0.102 mg/ml); (31.60 U/mg protein), (31.50 U/mg protein), (66.270 U/mg protein) after (27.59), (27.92), (58.75) folds of purification and yield of (42.05%) of GTF respectively.

Purified GTF-I_a, GTF-I_b and GTF-II were concentrated from (15 ml) for each to (5 ml) separately by passing through an Amicon-Filter P50 in (Ultrafiltration-cell).

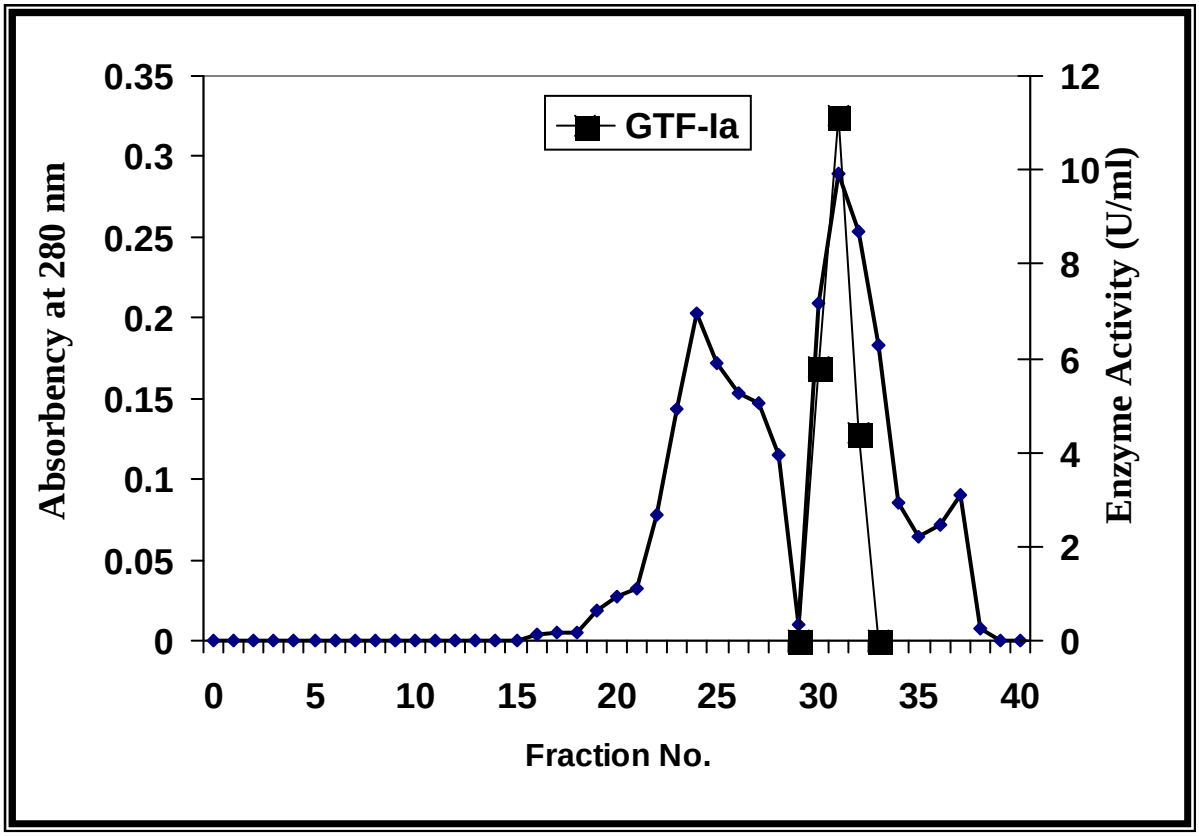

Figure (4-6): Purification of GTF-I_a by gel-filtration chromatography (Sephacryl S-200) column (67x2.1cm). Eluent: (0.3M) phosphate buffer pH (7.5) at a flow rate of (50ml/houre).

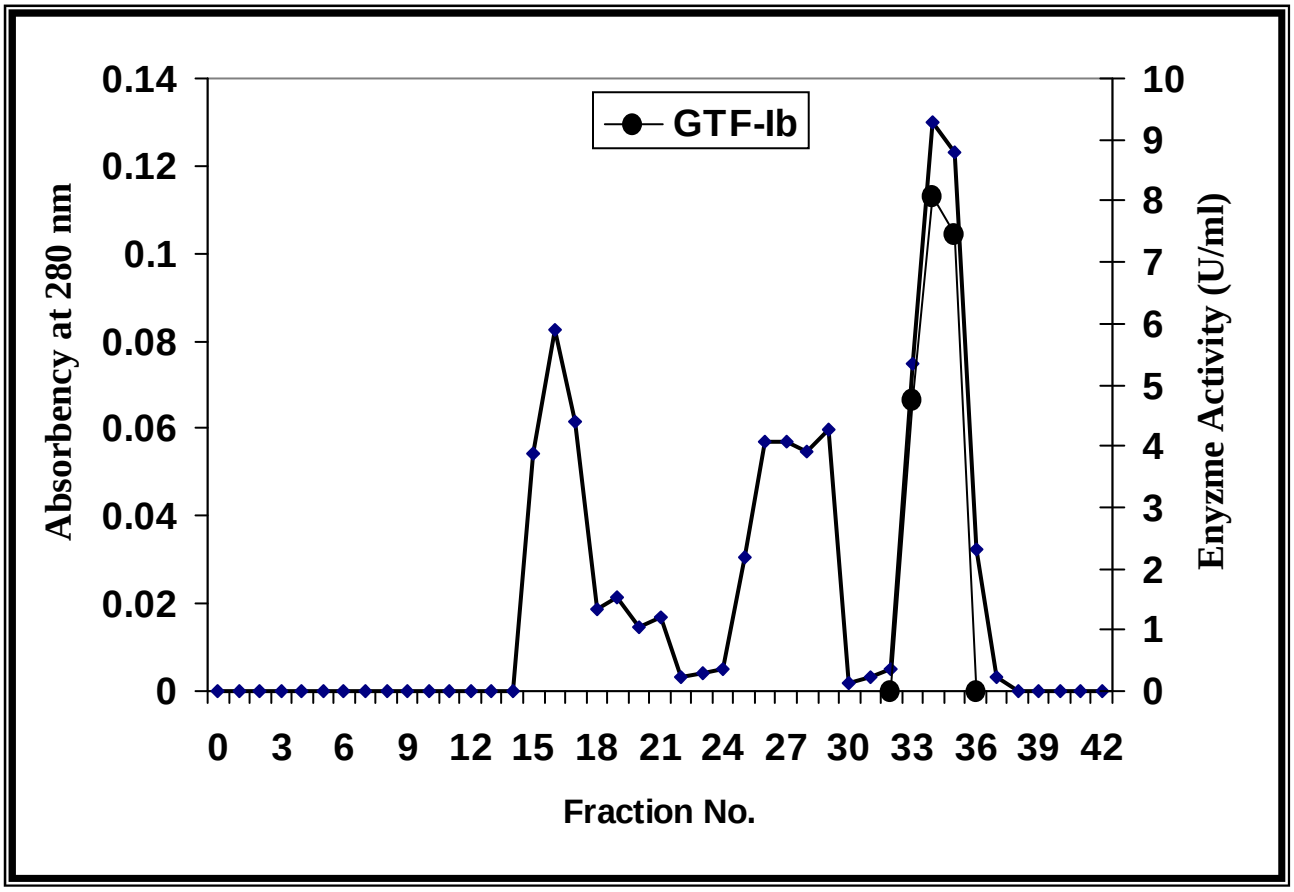

Figure (4-7): Gel filtration column chromatography (Sephacryl S-200) of GTF-I_b (67x2.1cm). Eluent: (0.3M) phosphate buffer pH (7.5) at a flow rate of (50ml/houre).

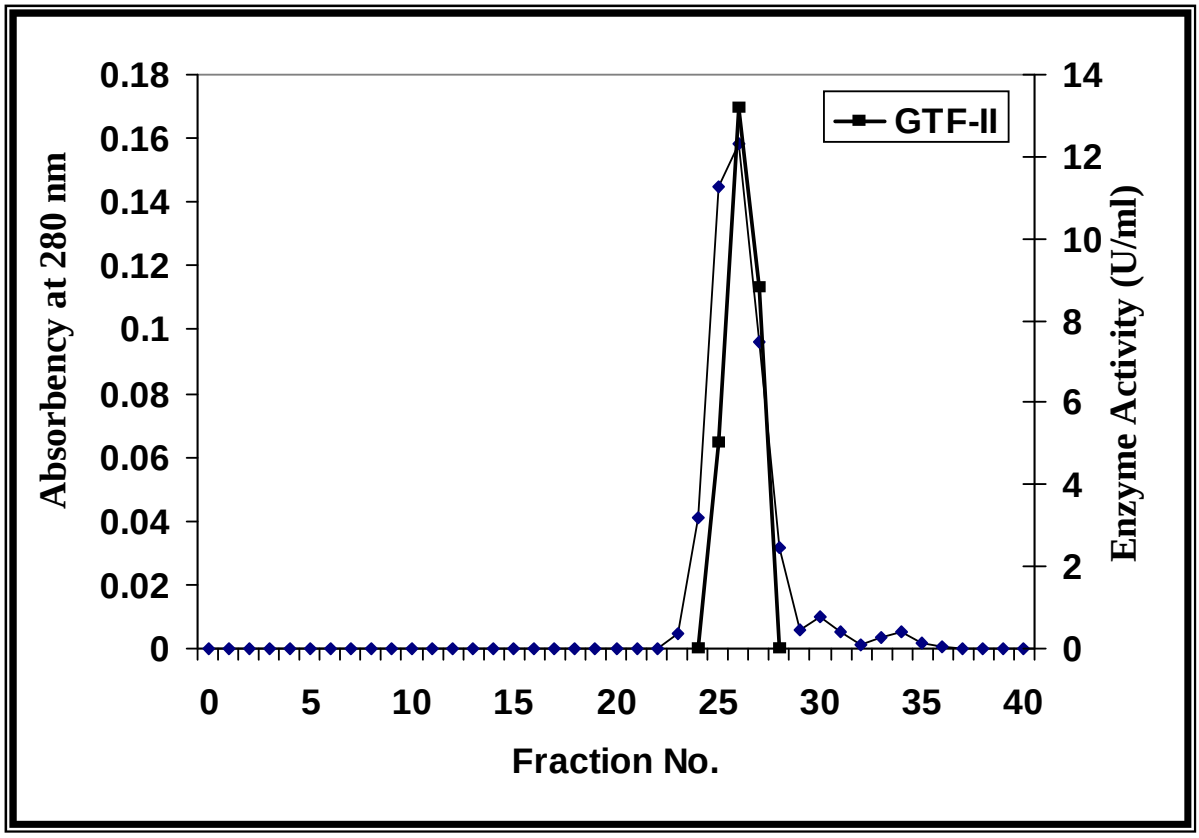

Figure (4-8): Purification of GTF-II enzyme by gel-filtration chromatography (Sephacryl S-200) column (67x2.1cm). Eluent: (0.3M) phosphate buffer pH (7.5) at a flow rate of (50ml/houre).

Turchi and Edwards (1985) characterized and purified GTF from *S. mutans* (serotype C). The last step in the purification procedure was Gel-filtration chromatography with the use of Bio-Gel A1.5cm. The purification scheme described the presence of three GTF enzyme and their GTF activity, protein concentration and specific activity were recorded to be (10 U/ml), (91 U/ml), (75U/ml); (0.26 mg/ml), (0.44 mg/ml) (0.42 mg/ml); (37 U/mg protein), (208 U/mg protein), (178 U/mg protein) after (25), (140) and (120) folds of purification with yield of (50%).

Al-Hayali, (2002) concluded that mutans streptococci (Biotype-IV-*S. sobrinus*) had the ability to produce three types of GTF after the purification step by gel-filtration chromatography using (Sepharose C1-6B). The purification scheme of this step for the third GTF reflected that GTF activity, protein concentration and specific activity of (0.208 U/ml), (0.09 mg/ml) and (2.3 U/mg protein) after (153.3) fold of purification with yield of (20.8%) respectively.

According to the specific activities, fold of purification and yields of enzyme, purification of GTF by gel filtration chromatography using (Sephacryl-S-200) column is more efficient than purification by (Sepharose C1-6B) and less efficient than purification by (Bio-Gel A1.5 cm).

Table (4-9): Purification scheme and yield of GTF enzymes from mutans streptococci (N_{10}) (*S. sobrinus*) (serotype G).

Steps	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	Protein concentration (mg / ml)	Specific activity (U /mg)	Purification factor (fold)	Yield (%)
Crude Enzyme	750	0.790	592.50	0.7	1.128	1	100
Concentrated crude GTF by Amicon Filter	40	11.076	443.05	0.601	18.42	16.32	74.77
Adsorption chromatography hydroxyapatite beads (Batch wiese)							
GTF-I	25	6.699	167.475	0.359	18.66	16.54	57.47
GTF-II	25	6.922	173.05	0.365	18.96	16.80	
DEAE-Cellulose column chromatography							
GTF-I_a	25	3.576	89.40	0.204	17.52	15.53	45.71
GTF-I_b	20	3.931	78.62	0.175	22.46	19.91	
GTF-II	25	4.113	102.82	0.153	26.88	23.82	
Gel-filtration Sepharcryl S-200 column chromatography							
GTF-I_a	15	5.531	82.96	0.175	31.60	27.59	42.05
GTF-I_b	15	4.320	64.80	0.137	31.50	27.92	
GTF-II	15	6.760	101.40	0.102	66.270	58.75	

4.2.8 Determination of Molecular Weight of GTF Enzymes by Gel-Filtration Chromatography

Molecular weights of purified GTF-I_a, GTF-I_b and GTF-II were determined using (Sephacryl S-200) column as described previously in (3.2.14.I). The void volume (V_o) of the column was calculated by estimating the void volume of blue dextran 2000 and the elution volume (V_e) for each standard proteins and for the dissolved and separated fractions of purified GTF-I_a, GTF-I_b and GTF-II each separately.

The ratio of the elution volume of each standard protein as well as the dissolved and separated fractions of each purified GTF-I_a, GTF-I_b and GTF-II to that of void volume of the blue dextran 2000 was calculated.

Results in table (4-10), show that the (V_e/V_o) ratios of purified GTF-I_a, GTF-I_b and GTF-II were about (2.4), (2.4) and (2.1) respectively. GTF-I_a and GTF-I_b had the same (V_e/V_o) ratio which located between Bovine Serum Albumin and Aldolase while GTF-II (V_e/V_o) ratio located between Aldolase and Catalase standard proteins.

Table (4-10): Standardization of GTF in accordance to the ratio of void volume and elution volume (V_e/ V_o) ratio.

Standard protein and purified GTF enzymes	Molecular Weight (Dalton)	(V_e / V_o) ratio
Bovine Serum Albumin	67000	3.05
Aldolase	158000	2.25
Catalase	232000	2
Ferritin	440000	1.3
Thyroglobulin	660000	1.2
GTF-I_a	–	2.4
GTF-I_b	–	2.4
GTF-II	–	2.1

Results, indicated that molecular weight of purified GTF-I_a and GTF-I_b was estimated as (128882 dalton) which located between Bovine Serum Albumin of (67000 dalton) and that of the Aldolase (158000 dalton). GTF II had a molecular weight of (186208 dalton) which located between Aldolase (158000 dalton) and Catalase (232000 dalton) Figure (4-9)

Mutans streptococci N_{10} (*S. sobrinus*) (Serotype G) was able to produce three GTF (GTF-I_a, GTF-I_b and GTF-II), two of them GTF-I_a and GTF-I_b were isozymes because of their same molecular weight and the third was GTF-II had a different molecular weight. These GTF enzymes are responsible for production of glucan product.

Ciardi *et al*. (1977) characterized and purified four GTF enzymes of mutans streptococci (*S. sobrinus*) (serotype G) each two were isozymes with molecular weights of (175000 and 150000 dalton) which were estimated by gel-Filtration chromatoghraphy and by SDS-PAGE.

Mukasa *et al*. (1982) described the extracellular glucosyltransferase of mutans streptococci (*S. mutans*) (serotype C) as a basic enzyme which was composed of three or more isozymes possessing the same molecular weight of (160000 dalton) as detected by SDS-PAGE.

Yamashita *et al*. (1989) purified four GTF enzymes from mutans streptococci (*S. sobrinus*) (serotype G), two of them isozyme to the basic GTF enzyme because of their molecular weight (152000 dalton) as well as to their specific activities (had the same specific activities after the last step of purification).

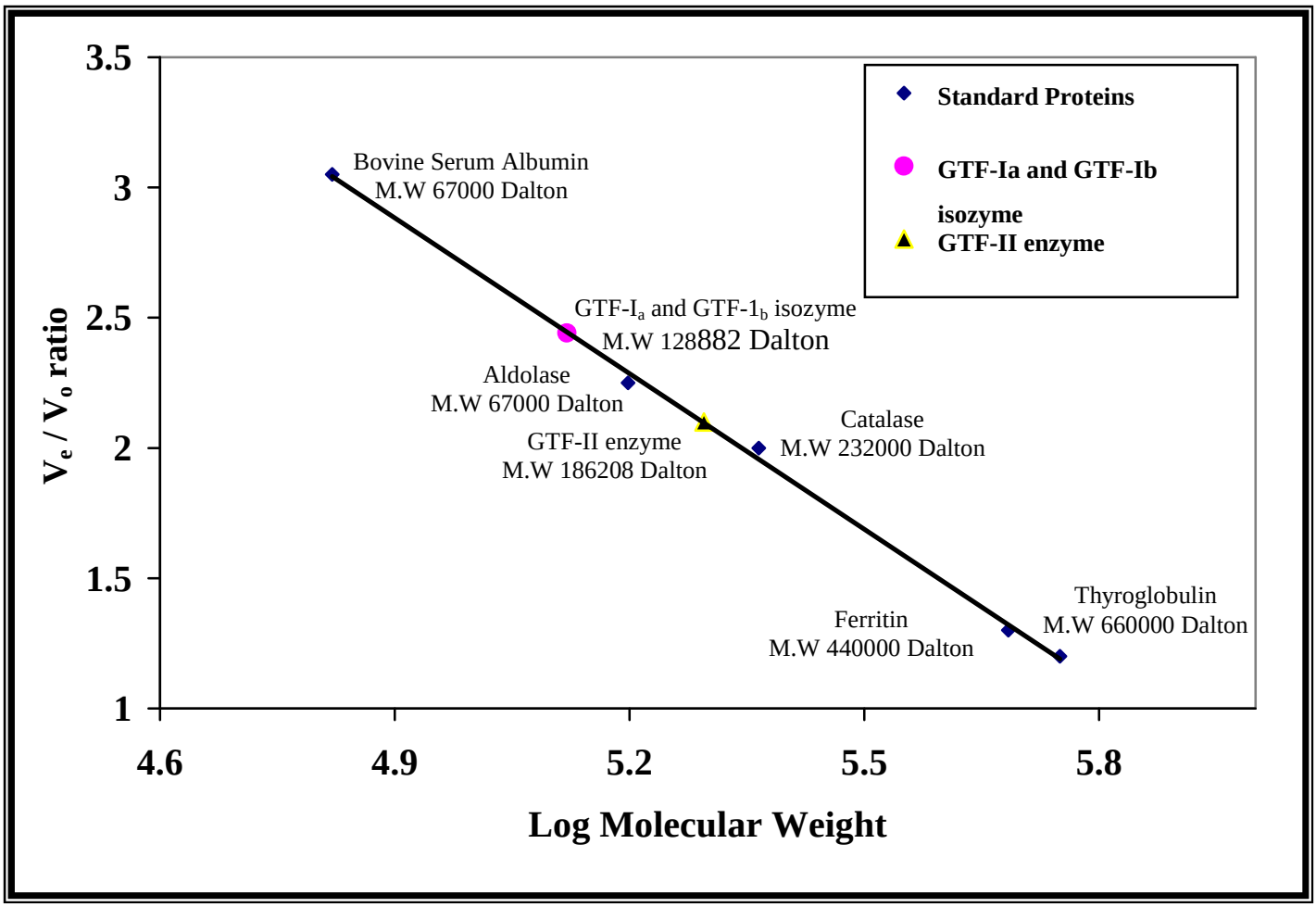

Figure (4-9): Standardization of GTF enzymes in accordance to the ratio of void volume and elution volume (V_e / V_o) ratio.

4.3 Immunological Study

The antigen that used in this experiment was a concentrated purified GTF-I_b with a protein concentration of (0.15 mg/ml). Results shown in figure (4-10), indicate the presence of the precipitation lines between anti-GTF-I_b antibody and purified concentrated GTF-I_b and GTF-I_a which were put in separated holes. No precipitation lines were indicated between anti-GTF-I_b antibody and GTF-II or between anti GTF-I_b antibody and the control serum. The concentration of the anti-GTF-I_b antibody was (0.2 mg/ml). This concentration was the same to the concentrations of GTF-I_b and GTF-I_a as well, so the producible precipitation lines were found in the middle distance between the antigen and antibody.

The subcutaneous route of immunization had been found to stimulate the systemic immunity which was occurred by production of circulatory immunoglobulins (IgG, IgM and IgA) (Russel and Johanson, 1987; Taubman *et al*., 1995) and these immunoglobulins were specific for GTF-I_b and GTF-I_a enzymes according to the appearance of the precipitation lines. The immunological responses similarities against GTF-I_a and GTF-I_b isozymes in spite of these antibodies were prepared only from purified concentrated GTF-I_b) were due to their similarities not only in molecular weights but also in their surfaces antigenic epitopes which capable to induce the same immunological response.

In this study there were no attempts to differentiate between the different types of immunoglobulins, due to the technical difficulties, but only the presence or absence of the immune response which was detected by double immunodiffusion test.

The production of an effective vaccine that inhibits the cariogencity of mutans streptococci was long been a goal to provide an immunotherapeutic approach to prevent dental caries (Taubman, 1993;

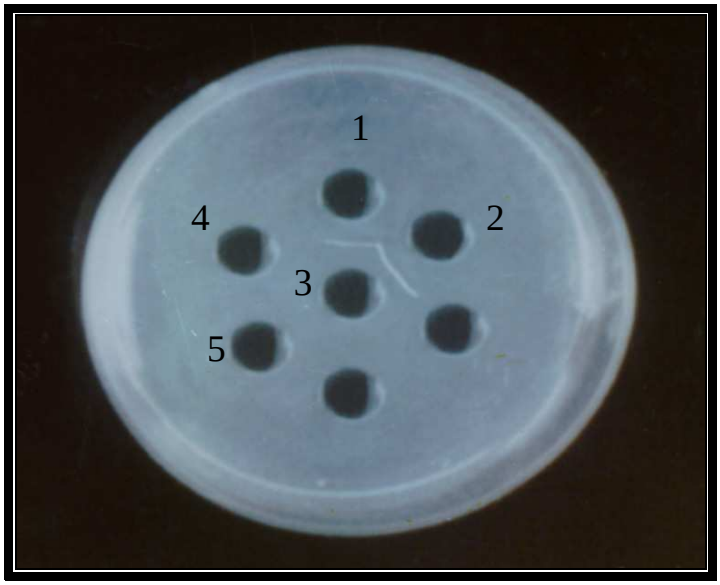

Figure (4-10): Double immunodiffusion (antigen-antibody reaction) between anti-GTF-I_b antibody and GTF-I_a, GTF-I_b and GTF-II antigens. 1. GTF-I_a antigen; 2. GTF-I_b antigen; 3. Anti-GTF-I_b antibody; 4. GTF-II; 5. Control sera.

Bowen, 1996). GTFs, the only proven virulence factors of mutans streptococci have been targets for such vaccine research (Yamashita *et al*., 1993). Most of the work involving the production of antibodies against GTF was made by the use of crude mixture of this enzyme (Chia *et al*., 1993; Cope and Mooser, 1993; Taubman *et al*., 1995; Laloi *et al*., 1996). In this study antibody were raised against specific type of GTF in animal as a trial to inhibit the sysnthesis of glucan *in vitro* as well as to inhibit GTF enzymes.

Wunder and Bowen (2000) investigate (GTF-C, GTF-B and GTF-D) of mutans streptococci (*S. mutans*) (serotype C) as well as on GTF-S of *S. sanguis*. The preparation of antibodies against these enzymes was done in the Newzeland-rabbits. They found that GTF-B or GTF-C antibodies were capable to inhibit the presence of GTF-B and GTF-C and incapable to inhibit the presence of GTF-D and GTF-S. Also GTF-D or GTF-S antibodies were capable to inhibit the presence of GTF-D and GTF-S and incapable to do so for GTF-C and GTF-B after examination by Western blots. Suggestion was made that GTF-

D and GTF-S and specially GTF-D because it was taken from (*S. mutans*) displays a number of structurally district epitopes that differ from the other two *S. mutans* GTFs. There was a similarity between the behavior of GTF-B, GTF-C and GTF-D and GTF-I_a, GTF-I_b and GTF-II in this study so that GTF-II displays a number of structurally distinct epitopes that differ from GTF-I_a and GTF-I_b so that no precipitation line was indicated.

Al-Haialy (2002) prepared antibodies against purified GTF enzyme by two immunization experiments. The first was subcutaneous rout of immunization in the back of rabbits to stimulate the systematic immunity. The second was carried out subcutaneously near the salivary gland to stimulate the local immunity (production of secretory IgA antibody). By making a comparison between the two experiments, the subcutaneous injection in the back of the rabbit gave a better result than local injection near the salivary gland after immunodiffusion experiment.

4.4 The Effect of Inhibitors and Anti-GTF antibody on Growth of Mutans Streptococci

The susceptibility of mutans streptococci N10 (*S . sobrinus*) (serotype G) to different concentrations of sodium fluoride, chlorohexidine dichloride, EDTA, ZAK (mouthrinse) and anti-GTF-I_b antibody with concentrations of ($1.5x10^{-3}$, $0.78x10^{-3}$, $0.51x10^{-3}$, $0.39x10^{-3}$, $0.31x10^{-3}$, $0.23x10^{-3}$, $0.21x10^{-3}$ M) were determined by two methods . For the first method, susceptibility of the bacterial growth against these inhibitors and anti-GTF-I_b antibody was estimated by a broth-dilution method as in (3.2.16.a). Results showed different concentrations of anti-GTF-I_b antibody and EDTA were incapable to inhibit the growth of bacterial isolate. The absorbance at 600 nm remained the same as a control. For the sodium fluoride, chlorohexidine (CHX), ZAK (mouthrinse), results shown in tables (4-11), (4-12) and (4-13) indicate that, these inhibitors were capable to inhibit the growth of bacteria at concentrations (0.5mM),

(3mM), and (3 mM) respectively. The minimal inhibitory concentrations in which these concentrations were found to give the lowest growth rate or effect were noticed at (2mM), (15mM), (12mM) respectively. Complete bacterial growth inhibition was noticed only with sodium fluoride and chlorohexidine at concentrations of (4mM) and (20mM) respectively.

Table (4-11) The effect of sodium fluoride (NaF) on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using broth dilution method.

Absorbance at 600 nm of the control	Concentration of NaF (mM)	Absorbance at 600 nm with NaF
0.940	–	–
	0.5	0.788
	1	0.459
	2	0.224
	4	–
	8	–
	12	–
	16	–
	18	–

–: no growth was detected.

Table (4-12) The effect of chlorohexidine dichloride (CHX) on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using broth dilution method.

Absorbance at 600 nm of the control	Concentrations of (CHX) (mM)	Absorbance at 600 nm with NaF
0.969	–	–
	3	0.704
	5	0.584
	10	0.292
	15	0.075
	20	–

–: no growth was detected.

Table (4-13) The effect of ZAK (mouthrinse) on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using broth dilution method.

Absorbance at 600 nm of the control	Concentration of ZAK (mM)	Absorbance at 600 nm with ZAK
0.959	–	–
	3	0.821
	5	0.633
	10	0.341
	12	0.174

For the second method in which susceptibility of bacterial growth against the previous inhibitors and different concentrations of anti-GTF-I_b antibody was tested as described previously in (3.2.16.b). Results shown in figure (4-11) and tables (4-14), (4-15), (4-16) indicated that, the highest zone of inhibition (50mm) was recognized with chlorohexidine at concentration of (20mM) followed by sodium fluoride with a zone of inhibition of (37mm) at concentration of (18 mM). ZAK (mouthrinse) at concentration of (12 mM) was capable to give zone of inhibition of (27.5 mm). Complete inhibition of the bacterial growth was unable to be recognized with any previous inhibitors at any concentration.

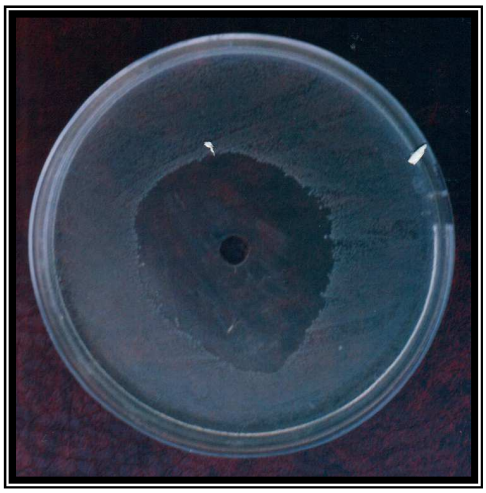

Figure (4-11) The highest zone of inhibition for the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) at (20 mM) of chlorohexidin (CHX).

Table (4-14) The effect of sodium fluoride (NaF) on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using diffusion method on solid medium.

Concentration of NaF (mM)	Inhibition zone diameter (mm)
0.5	3
1	5.5
2	7
4	10
8	17.5
12	25.7
16	30.9
18	37

Table (4-15) The effect of chlorohexidine (CHX) on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using diffusion method on solid medium.

Concentration of CHX (mM)	Inhibition zone diameter (mm)
3	14
5	20
10	29
15	37
20	50

Table (4-16) The effect of ZAK mouthrinse on the growth of mutans streptococci N_{10} (*S. sobrinus*) (serotype G) using diffusion method on solid medium.

Concentration of ZAK (mM)	Inhibition zone diameter (mm)
3	17
5	22
10	25
12	27.5

Accordingly, the best inhibitors of growth of bacteria by using the broth-dilution method were sodium fluoride at concentration of (4 mM) followed by chlorohexidine at concentration of (20 mM) in which complete inhibition of bacterial growth was noticed with a minimal inhibitory concentrations that gave the lowest growth rate at (2 mM) and (15 mM) respectively. ZAK (mouthrinse) has a good degree of inhibition on the growth of bacteria with minimal inhibitory concentration or effect on bacterial growth at concentration (12 mM) but no complete growth inhibition was recognized with this inhibitor. For the diffusion-method the best inhibitor for the bacterial growth was chlorohexidine at (20 mM) which capable to give the highest zones of inhibition on the agar followed by sodium fluoride with concentration of (18 mM). ZAK (mouthrinse) also capable to produce a zone of inhibition about (27.5 mM) at concentration of (12 mM).

In these methods no effect on the bacterial growth was detected by the use of anti-GTF-I_b antibody and EDTA inhibitor.

The susceptibility of the bacteria to the inhibitors was determined clearly by broth dilution method more than the diffusion-method in agar media because the first method give a quantitative assessment for the effective concentration of inhibitors which was given a complete inhibition (bactericidal or bacteriostatic action). Also the minimal inhibitory concentrations of the previous inhibitors can also be determined by this method.

The inability of the anti-GTF-I_b antibody to inhibit the growth of bacterial isolate was related to this antibody can decrease or inhibit the adsorption of these bacteria on the tooth surface *in vivo* or *in vitro* on the surface of saliva-coated hydroxyapatite. So this antibody was capable to block the synthesis of the insoluble-glucan which was very important for the adsorption and attachment only, so the bacteria was remained capable to grow and multiply (i.e. the number of the bacteria was remained the same). This information was recorded after an experiment in animal model *in vivo* and from the recognition of the behavior of bacteria on the surface of saliva-coated hydroxyapatite *in*

vitro after the usage of an antibody specific for GTF enzyme (Vacca-Smith *et al*., 1996a; Mattos-Graner *et al*., 2004).

Chlorohexidin had a bactericidal activity against both gram-positive and gram-negative bacteria. It was capable to reduce the number of mutans streptococci in the mouth greater than *S. sanguis* and lactobacilli (Emilson, 1994). Because chlorohexidin was positively charged, it was bind to various surfaces including enamel pellicle, hydroxyapatite and mucous membranes. It was also bind to the negatively charged bacterial surface and disrupt bacterial cytoplasmic membranes, including leakage of low molecular weight components and the precipitation of cell contents, chlorohexidine also inhibite the key metabolic enzymes such as glucosyltransferase and phosphoenolpypruvate (Scheie, 1994). The side effect of chlorohexidin was the discoloration of the teeth and taste disturbance. In spite of the poor penetration ability into plaque associated with pits, fissures and proximal surfaces, nowadays chlorohexidin is used in the form of chlorohexidin chewing gum or as a complement to tooth-brushing in order to increase the normal oral hygiene (Smith *et al*., 1996).

Fluoride was widely used as a highly effective anticaries agent. This action was related mainly to effect on the mineral phases of teeth and on the process of remineralization. Fluoride had important effects on the bacteria of dental plaque, which were responsible for the acidification of plaque that result demineralization. Fluoride can affect bacterial metabolism through a set of actions with fundamentally different mechanisms. It can act directly as an enzyme inhibitor ex: capable to inhibit the mutans streptococci enzyme enolase, leading to reduce the uptake of glucose through the phosphotransferase system, fluoride was capable to reduce the cariogenecity of dental plaque bacteria by enhancing the membrane permeability to protons and comprimisity the function of F-ATpases in exporting protons, thereby inducing cytoplasmic acidification and acid inhibition of glycolytic enzymes. Fluoride acted by reducing the aid tolerance of the bacteria. It was most effective at acid pH values, in the acidic

conditions of cariogenic plaque, fluoride at levels as low as 0.1mM can cause complete arrest of glycolysis by intact cell of *S. mutans* (Marquis, 1995; Balakrishnan *et al*., 2000). Fluoride in toothpaste and other oral products was believed to be the major reason for the substantial defect in caries incidence in many countries. Fluoride can be administrated systemically (Tabletes), applied topically (toothpastes or mouthwashes) or applied by dentists in the form of solutions, gels and varnishes. In some parts of the world, fluoride was added to drinking water (Balakrishnan *et al*., 2000).

It was found that the combination of chlorohexidin with fluoride or with metal ions such as Zn^{2+} capable to increase the anti-cariogenic activity. This action was bactericidal (Balakrishnan *et al*., 2000). In this study the bactericidal effect by ZAK mouthrinse on the growth of bacteria was unrecognized perhaps due to its low concentration as compared with chlorohexidin and sodium fluoride alone, or due to the interference between these material and other component of the mouthrinse capable to reduce its effect.

4.5 The Effect of Inhibitors and Anti-GTF antibody on The Activity of Purified GTF

The effect of different concentrations of EDTA, sodium fluoride, chlorohexidine dichloride, ZAK (mouthrinse) and anti-GTF-I_b antibody on the activity of purified concentrated GTF-I_b enzyme was done as described previously in (3.2.17). Results shown in figures (4-12), (4-13), (4-14) and (4-15), indicated that different concentrations of the inhibitors and anti-GTF-I_b antibody were capable to inhibit the GTF-I_b antibody activity with the exception of EDTA. Sodium fluoride at concentration (18mM) was capable to inhibit (81.64%) of the GTF-I_b enzyme activity, chlorohexidine at concentration of (20mM) was capable to inhibit (75.55%) of the GTF-I_b activity, ZAK (mouthrinse) at concentration of (12mM) was capable to inhibit (60.4%) of the GTF-I_b enzyme activity and anti-GTF-I_b antibody at concentration of ($1.5x10^{-}$

^{3}mM) was capable to inhibit (87.33%) of the GTF-I_b enzyme activity. No complete inhibition was recognized with any concentrations of these inhibitors and anti-GTF-I_b antibody.

Accordingly, anti-GTF-I_b antibody was considered as the best inhibitor on the activity of purified GTF-I_b enzyme followed by sodium fluoride, chlorohyexidine and ZAK (mouthrinse) respectively.

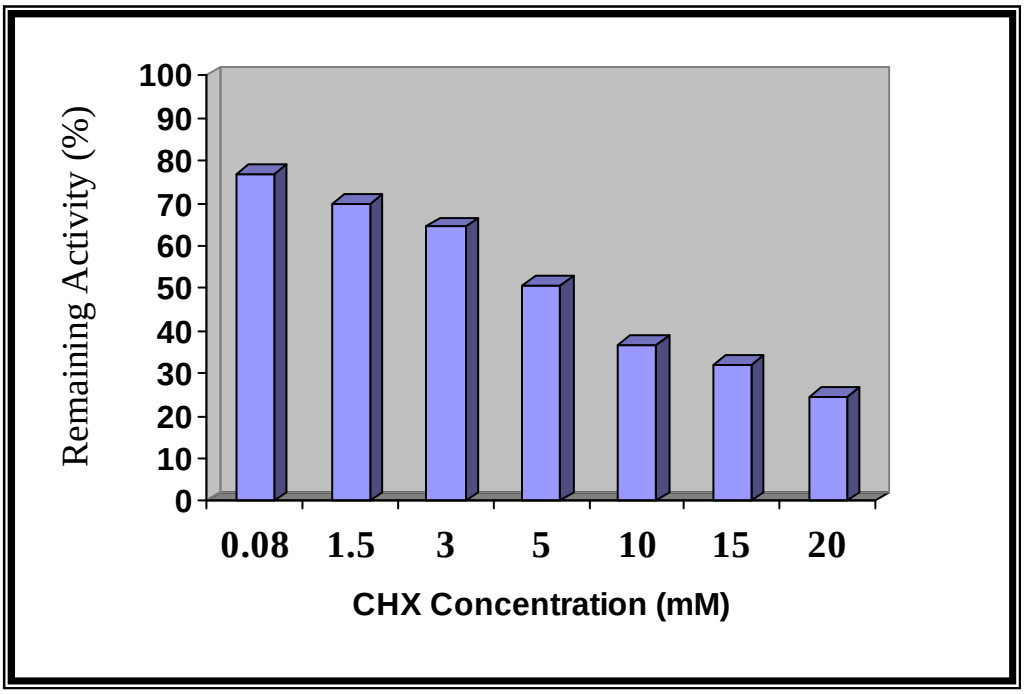

Figure (4-12): The effect of Chlorohexidine (CHX) mouthrinse on GTF-I_b activity.

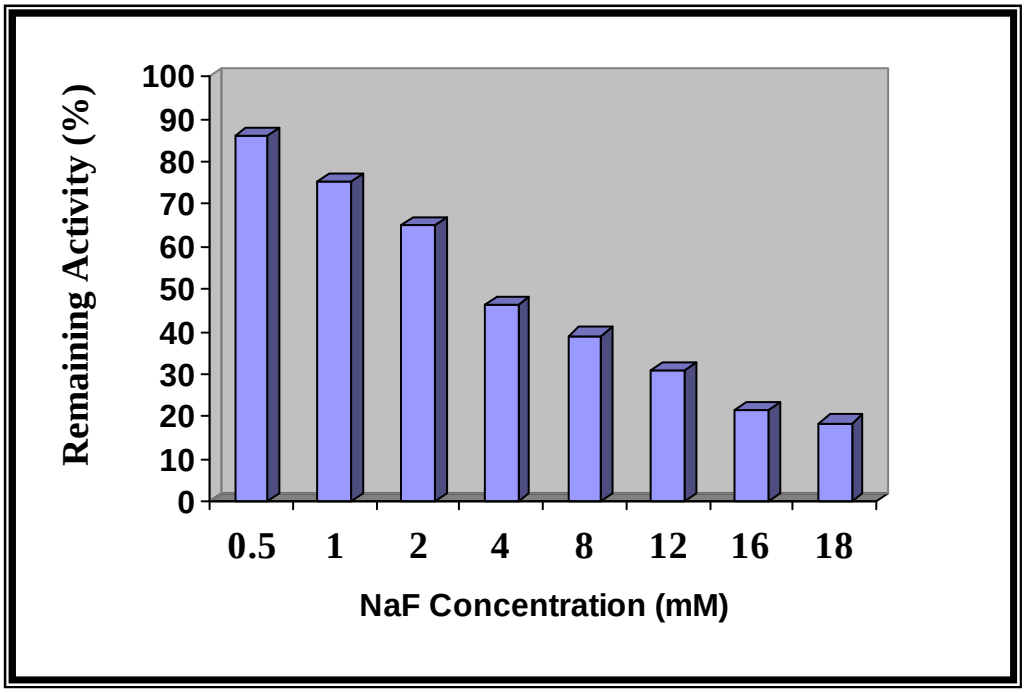

Figure (4-13): The effect of Sodium Fluoride (NaF) mouthrinse on GTF-I_b activity.

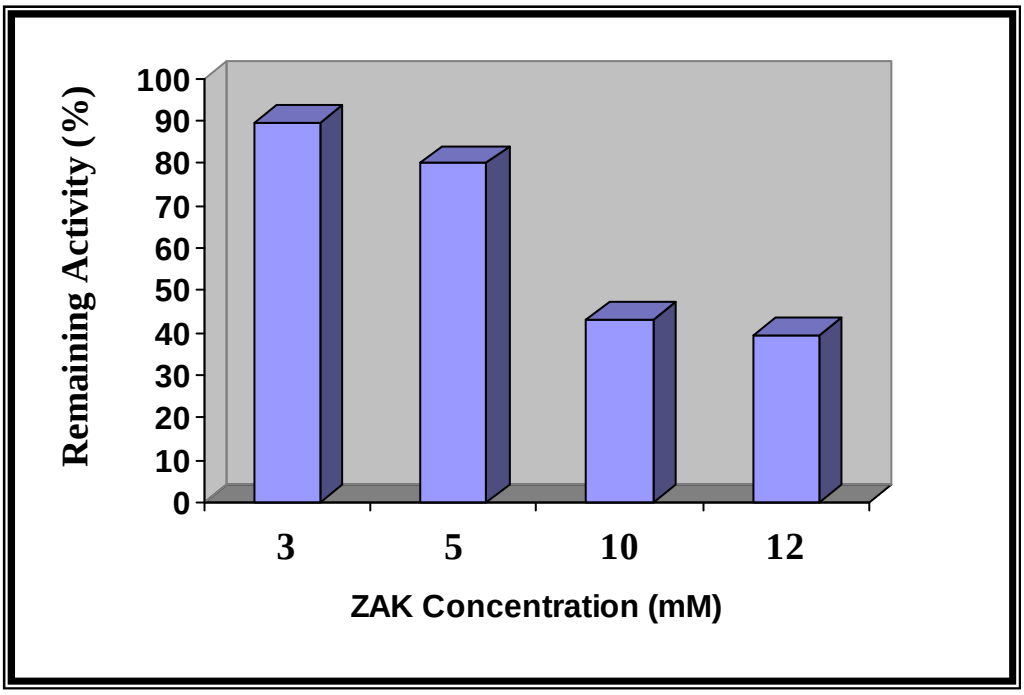

Figure (4-14): The effect of ZAK mouthrinse on GTF-I_b activity.

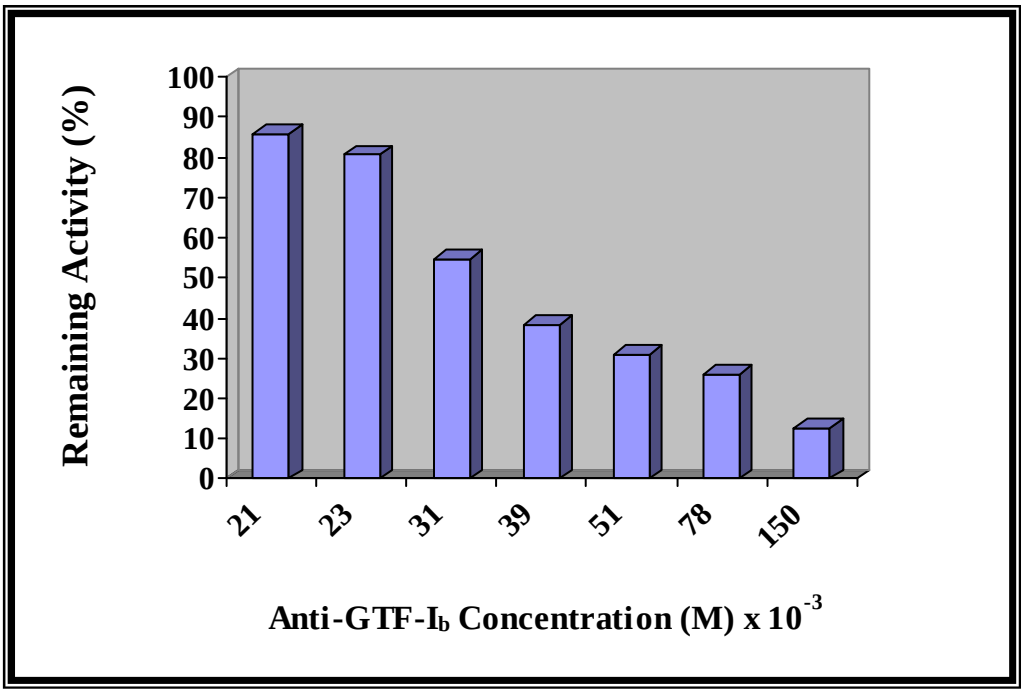

Figure (4-15): The effect of Anti-GTF-I_b mouthrinse on GTF-I_b activity.

Wunder and Bowen,(2000) demonstrated the inhibitory effect of antibodies specific for GTF-C, GTF-B and GTF-D enzymes of *S.mutans* on the activity of these enzymes in solution and on the surface of hydroxyapatite. Antibodies to GTF-B, GTF-C and GTF-D were found to inhibit (75%), (90%) and (90%) of the activity of these enzymes respectively. No complete inhibition was found with any one of these antibodies against the activity of GTF. These antibodies were capable to block the synthesis of insoluble and soluble glucan polymers. So by inhibiting the activity of GTF the adsorption of bacteria on the tooth surface will be prevented.

Fluoride was widely used as a highly effective anticaries agent (Emilson, 1994). It was found to inhibit the activity as well as the production of GTF enzymes from the mutans streptococci through a set of action with fundamentally different mechanisms. It inhibites the glycolytic enzyme enolase which had a vital role in the breaking of the glucose moieties during glycolysis

process, so it is capable to inhibit the formation of glucan. Another mode of action was involved in the formation of metal –fluoride complexes, most commonly A1F4-. These complexes were responsible for the fluoride inhibition of proton-translocation F-ATPases and were thought to act by mimicking phosphate to form complexes with ADP at reaction center of the enzyme. The linkage between the ATPs molecules and the F group was able to prevent the formation of glucan from the glucose-6-phosphate , so inhibition of GTF was recognized (Marquis, 1995)

Chlorohexidine was defined as an effective antiplaque agent (Emilson, 1994). Chlorohexidine in solution at concentration (1.25mM) was found to inhibit (100%) of GTF-C, (36%) of GTF-B and (15%) of GTF-D enzyme activities of *S. mutans* bacteria (Vacca-Smith and Bowen, 1996). The clinical effectiveness of chlorohexidine could be due to interaction of antiplaque agent with the GTF-C enzyme prior to the adsorption of the enzyme onto tooth and apatitic surfaces, thus inhibiting the enzyme before adsorption onto the tooth surface. This suggestion could be consistent with the staining frequency absorbed on the tooth surfaces of persons who used chlorohexidine (Le" and Schiott, 1970) (i.e chlorohexidin interacte with GTF-C in solution and bound to tooth surface with GTF-C).

Many potential anti-plaque agents might be ineffective when they became in the form of mouthrinse, because they were incompatible with other ingredients or materials that had found in the constituents of the mouthrinse (Garcia-Gody, 1989). ZAK (mouthrinse) although it contains sodium fluoride and chlorohexidine, it did not reflect the same effect as chlorohexidine or sodium fluoride alone.

In spite of the wide distribution of the antiplaque and anticaries agents, dental caries remained the most prevalent oral disease in different parts of the world especially in developing countries (WHO, 1997). Although the concept of using the GTF of mutans streptococci as a possible immunogen against

dental caries was attractive, several major problems continue to plague the field including:

1. Vaccination against caries was based on the idea that the same principles that applied to mucosal immunity were applicable for protection against caries.
2. Although the occurrence of dental plaque disease was not on a mucosal surface but on hard, non-shedding, largely non-reactive surfaces, protective antibody was required to react on a solid surface in a largely hostile environment with large variation in pH values, active proteases and limited diffusion into and out plaque.
3. Furthermore, Antibodies which were reacted with epitopes on putative protective bacterial proteins in solution might not identify the same epitopes when the proteins adsorbed to a surface undergo conformational changes. Such changes were known to occur for example with GTF adsorbed on saliva-coated hydroxyapatite (Vacca-Smith *et al*., 1996b). It was assumed that even partial inhibition of GTF by antibodies might be beneficial. However, it was cleared that the presence of antibody which partially inhibits or simply react with the enzyme, a glucan of novel structure might be formed there by providing a distinctive structure to which microorganism might bind (Bowen, 2002).

There had been an increasing interest in the possible use of topically applied antibodies as a mean of controlling dental caries (Ma *et al*., 1995). This approach certainly had attractions in that immunogens did not have to be administered systematically. Nevertheless, although the approach was technically feasibly, it shared many of the same problems mentioned above. In addition, depending on the method of administration, it might suffer from the same problem as many mourhrinse or topically application, in that it did not remain in the mouth for a sufficient time to exert its therapeutic effect. Perhaps

antibodies could be used as a "homing agent" to deliver therapeutic substance to specific area of the mouth (Bowen, 2002).

4.6 The measurement of GTF Kinetic Constant

The determination of the Mechalis Menton Kinetic constant (K_m) value and the maximum velocity (V_{max}) of the purified concentrated GTF-I_b enzyme was done with the presence of different concentrations of sucrose as described previously in (3.2.18). After the calculation of 1/[υ] and 1/[S] values, result shown in figure (4-16) indicate that the K_m and V_{max} values of purified GTF-I_b are (11 mM) and (0.05 mM/min^{-1}) respectively.

Mooser *et al*. (1984) made a kinetic study on the GTF of *S. mutans* bacteria. The K_m and V_{max} values were determined to be (2.83 mM and 0.0364 mM/min) respectively.

McCab, (1985) determined that the GTF-S of *S. mutans* in the presence of sucrose capable to reveal a K_m value of about (10 mM).

Vankitaraman *et al*. (1995) characterized the *S. mutans* GTF-B, GTF-C and GTF-D K_m values in solution and on the surface of hydorxyapatite. In solution, K_m values were about (30, 20 and 165 mM) respectively, while on the surface of hydroxyapatite the K_m values were (12, 10 and 22 mM) respectively.

Fujiwara *et al*. (2000) determined the K_m value of about (2.49 mM) of the GTF enzyme of *S. oralis* (a member of the oral streptococci family).

Vacca-Smith *et al*. (2000) determined the K_m values of GTF-S of *S. sanguis* bacteria in solution and on the surface of hydorxyapatite which were found to be (4.3 mM) and (5.0 mM) respectively.

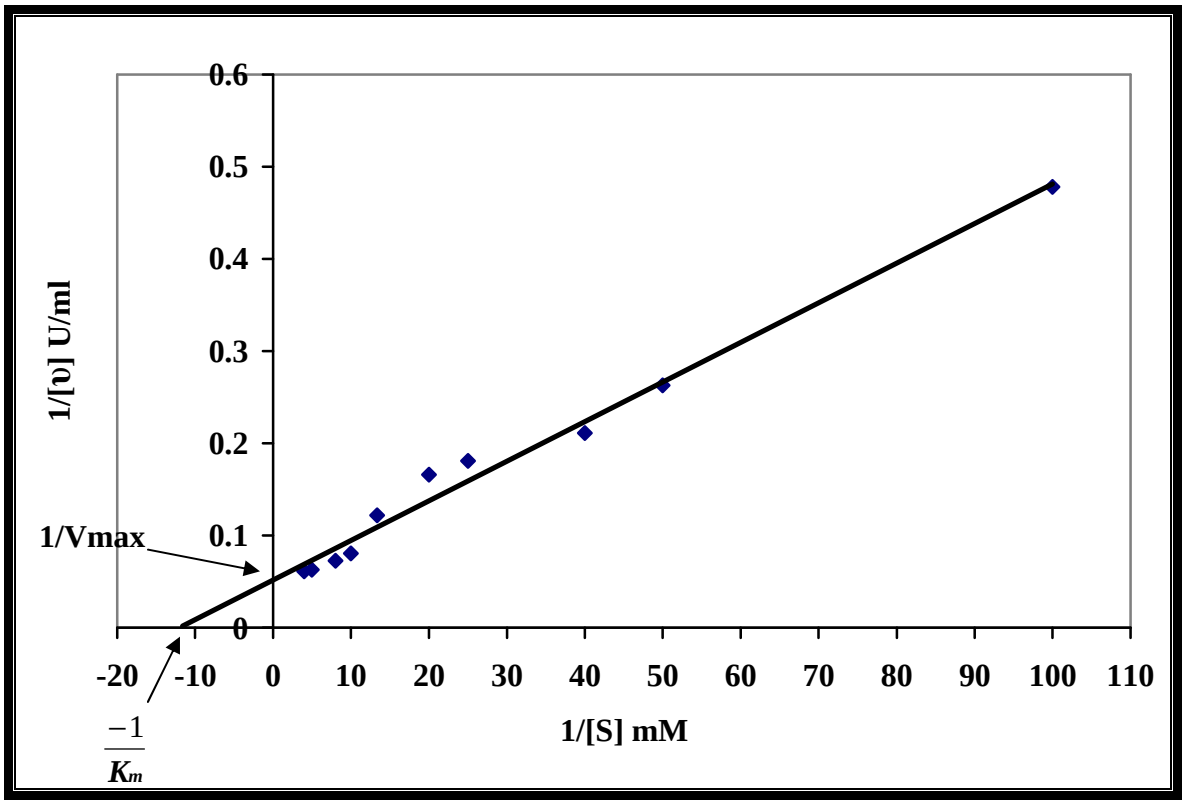

Figure (4-16): The Lineweaver Burk Plot (1/[υ] V_{max} / 1/[s]).

Conclusions

1. *S. sobrinus* (serotypes D, G) and *S. mutans* (serotypes C, F) were the most isolated bacterial species of mutans streptococci from the human dental plaque with the percentages of (39.29%) and (30.30%) respectively.

2. All serotypes of mutans streptococci bacteria (A, B, C, D, F and G) were capable of producing extracellular GTF enzymes and the highest producible bacteria was mutans streptococci N_{10} (*S. sobrinus*) serotype G with a specific activity of (0.752mg/ml).

3. Extracellular GTF were produced during the stationary phase of the bacterial growth life between hours (20-40) and their maximum production were recorded at hour 24.

4. The best tool to concentrate the GTF was an Amicon-filter P50 in (Ultrafiltration-cell).

5. Three purified GTF enzymes were produced from mutans streptococci N10 (*S. sobrinus*) serotype G , two of them were isozymes with a molecular weight of (128882dalton) and the third with a molecular weight of (186208 dalton) as determined by gel-filtration chromatography.

6. The same immunological response was indicated for the two GTF isozymes when one of them was used as an antigen in experimental animals.

7. Anti-GTF antibody and EDTA had no effect on the growth of bacteria mutans streptococci N10 (*S. sobrinus*) serotype G although EDTA had no effect on the activity of GTF enzyme.

8. Sodium fluoride and chlorohexidine at concentrations (18mM) and (20mM) respectively were capable to produce a complete bacterial growth inhibition.

9. Anti-GTF antibody enzyme at concentration of ($1.5x10^{-3}$mM) was capable to inhibit (87.33%) of GTF activity followed by sodium fluoride (18mM), chlorohexidine (20mM) and ZAK mouthrinse (12mM) which were capable to inhibit (81.64%), (75.55%), (60.4%) of GTF enzyme activity respectively.

10. K_m and V_{max} of GTF enzyme had been demonstrated to be (11mM) and ($0.05mM/min^{-1}$) respectively.

Recommendations

1. Simple and rapid detection of mutans streptococci (*S. mutans*) and (*S. sobrinus*) in human saliva or dental plaque samples by polymerase chain reactions (PCR).

2. An *in vitro* microbial caries model to study the efficacy of GTF or any other cell surface proteins antibodies against mutans streptococci in preventing dental caries.

3. The estimation of the specific types of immunoglobulin that had been produced after the immunization with GTF or any other surface proteins of mutans streptococci bacteria.

4. Oral passive immunization against dental caries in experimental animals by using Hen egg yolk antibodies specific for cell associated GTF of mutans streptococci bacteria.

Summary

Fifty plaque samples were collected from teeth. Forty five samples were considered to be positive bacterial isolates about (10^4 bacteria/ml) using selective Ms-agar (Mitis-Salivarius agar) medium. Thirty isolates were considered to be related to the genus *Streptococcus* and specially to the mutans streptococci of various group; *S. sobrinus* (serotype D, G), *S. mutans* (serotype C, F), *S. cricetus* (serotype A) and *S. rattus* (serotype B) with percentages of (39.29%), (30.30%), (18.18%) and (3.03%) respectively, depending on biochemical and Lancefield grouping identification systems. Seventeen isolates; related to different serotype groups, were tested for production of an extracellular Glucosyltransferase (GTF) through determination of their enzyme specific activity. All isolates were able to produce the enzyme. Mutans streptococci isolate N_{10} which identified as (*S. sobrinus* serotype G) was selected as the highest producible bacterial isolate for GTF with a specific activity of (0.752U/mg).

Productivity phase of growth and GTF activity for the isolate N_{10} (the chosen isolate) was specified prior to determine maximal productivity. It was found that GTF was produced during the stationary phase of growth (20-40 hr.) and its maximal productivity was recorded at 24 hr.

The effect of ice/freezing preservation at (-20^{o}C) on the activity of crude GTF with and without the presence of glycerol was done ,and the effect of concentration by polyethylene glycol (PEG) and sucrose powders, freezing/drying (lyophilization) system and an Amicone P50 in (ultrafiltation-cell system) on GTF activity and protein concentration was recorded . For long term storage of GTF, the addition of 15% glycerol then ice/freezing preservation at (-20°C) gives good results and the best method to concentrate

GTF was done by using an Amicon-filter P50 in (ultrafiltration-cell) which gave a specific activity of (18.42 U/mg protein).

Large scale production, concentration and purification of mutans streptococci (*S.sobrinus*) (serotype G) N_{10} GTF were done by ultrafiltration-method using an Amicone-filter P50, adsorption chromatography (hydroxyapatite beads), ion-exchange chromatography (DEAE-cellulose column) and gel-filtration chromatography using (Sephacryl S-200) column. Three purified GTF enzymes (GTF-I_a, GTF-I_b, GTF-II) were detected with a specific activity of (31.60U/mg protein), (31.50U/mg protein) and (66.270U/mg protein) after (27.59), (27.92) and (58.75) folds of purification with yield of (42.05%).

Determination of purified GTF enzymes (GTF-I_a, GTF-I_b, GTF-II) molecular weights was done by using gel-filtration chromatography (Sephacryl S-200) column with the presence of high-molecular weights standards proteins. Two GTF enzymes (GTF-I_a and GTF-I_b) were considered as isozymes with a molecular weight of (128882 dalton) and the third (GTF-II) had a molecular weight of (186208 dalton).

The ability of GTF to stimulate the immune system was tested in this study. The subcutaneous route injections of purified antigen (GTF-I_b) in the back of experimental rabbits were done. A double immunodiffusion test for detection of the immune response between anti-GTF-I_b enzyme antibody and purified (GTF-I_a, GTF-I_b and GTF-II) antigens were recorded. The same immunological response was indicated (by the appearance of the precipitation lines) on the surface of agarose-gel between the two GTF isozymes and the anti-GTF-I_b enzyme antibody.

The effect of different concentrations of inhibitors (EDTA, sodium fluoride, chlorohexidine) Zak (mouthrinse) and anti-GTF-I_b enzyme antibody on the growth of bacteria was tested using broth dilution method and diffusion method on solid medium. Anti-GTF-I_b enzyme antibody and EDTA had no

effect on the growth of mutans streptococci N_{10} (*S. sobrinus* serotype G), while sodium fluoride, chlorohexidine at concentrations (18mM) and (20mM) respectively were capable to produce a complete bacterial growth inhibition, ZAK (mouthrinse) at concentration (12mM) was capable to inhibit the growth of bacteria when broth-dilution method was used.

The effects of different concentrations of these inhibitors and anti-GTF-I_b enzyme antibody on the activity of purified GTF-I_b enzyme were also tested. Anti-GTF-I_b enzyme antibody at concentration 0f ($1.5x10^{-3}$mM) was capable to inhibit (87.33%) of the purified GTF-I_b enzyme activity followed by sodium fluoride (18mM), chlorohexidine (20mM) and ZAK (mouthrinse) (12mM) which were capable to inhibit (81.64%), (75.55%) and (60.40%) of the purified GTF-I_b enzyme activity respectively. However EDTA had no effect on the purified GTF-I_b enzyme activity.

Determination of the kinetic constants, Mechalis Menton constant (K_m) value and the maximum velocity (V_{max}) value for the purified GTF-I_b enzyme was specifies as (11mM) and (0.05mM/min^{-1}) respectively using (Lineweaver Burk plot).

Supervisors Certificates

We, certify that this thesis was prepared under our supervision at the Al-Nahrain University, College of Science, as a partial requirement for the degree of Doctor of Philosophy in Biotechnology.

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j

قالوا سُبحانَكَ لا عِلمَ لنا اِلاّ ما عَلَّمتنا

اِنَّكَ اَنتَ العَليمُ الحَكيمُ

صدق الله العظيم

سورة البقرة اية ٣٤

الخــلاصــة

عزلت خمسين عينة من الطبقة المغطية لسطح السن. خمس وأربعون عينة أظهرت نمواً إيجابياً للبكتريا حوالي (١٠^{٤} بكتريا/مل) باستخدام الوسط المخصص للنمو (Milis-Salivarius agar). ثلاثون عزلة إعتبرت عائدة لافراد البكتريا *Streptococcus* وخاصة للمكورات الفموية المتعددة الأنواع *S . sobrinus* (serotype D,G) و*S. mutans* (serotype C,F) و *S. cricetus* (serotype A) و*S. rattus* (serotype B) وبنسب (39.29%) و(٣٠,٣٠%) و(١٨,١٨%) و(٣,٠٣%) تباعاً والتي تم تشخيصها إعتماداً على الطرق البايوكيميائية وخصوصية عالق ال (Latex) المضاد والخاص بالمجاميع (A وB وC وD وF وG) المتعددة السكريدات (المستضاد) والموجودة على الجدار الخارجي لهذه البكتريا (Lancefied group) أو (Serotype group). سبعة عشر عزلة عائدة الى مجاميع (serotyes) مختلفة، إختبرت لإنتاج إنزيم الكلوكوسيل ترانسفيريز والمنتج الى خارج الخلية البكتيرية من خلال معاينة فعالياتهم النوعية الإنزيمية. جميع العزلات كانت قادرة على إنتاج هذا الإنزيم. واختيرت العزلة N_{10} والعائدة لبكتريا المكورات الفموية والتابعة للنوع (Serotype G *S. sobrinus*) كأحسن عزلة بكتيرية قادرة على إنتاج إنزيم الكلوكوسيل ترانسفيريز وبفعالية نوعية مقدارها (٠,٧٥٢ ملغم/مل).

تم تخصيص طورانتاجية النمو وفعالية إنزيم الكلوكوسيل ترانسفيريز للعزلة N_{10} (وهي العزلة المختارة) سابقة لتحصيل الانتاجية القصوى للكلوكوسيل ترانسفيريز. وقد وجد ان الكلوكوسيل ترانسفيريزقد انتج خلال الطور الثابت من النمو بين (٢٠-٤٠) ساعة وكانت أقصى إنتاجية له قد سجلت في الساعة (٢٤).

تمت معاينة تأثير الحفظ بواسطة التجميد/ الثلج بدرجة حرارة (-٢٠ °م) على فعالية إنزيم الكلوكوسيل ترانسفيريز الخام بوجود أو عدم وجود مادة الكليسيرول وقد تم تسجيل تأثير التركيز بواسطة مسحوقي البولي ايثيلين كلايكول (PEG) والسكروز ومنظومة التجميد/ التجفيف (lyophilizer) واستعمال مرشح ال(Amicon-P50) في منظومة الترشيح الخلوي العالي (Ultrafiltration-Cell System) على فعالية الكلوكوسيل ترانسفيريز وتركيزه البروتيني . وقد وجد أن اضافة (١٥%) من مادة الكليسيرول ومن ثم الحفظ بواسطة التجميد/الثلج في درجة حرارة (-٢٠°م) اعطت نتائج جيدة للخزن الطويل للكلوكوسيل ترانسفيريز وان افضل طريقة لتركيز

الكلوكوسيل ترانسفيريز قد تمت بواسطة استعمال مرشح (Amicon-P50) في منظومة الترشيح الخلوي العالي والتي أعطت فعالية نوعية مقدارها(18.42 وحدة /ملغم بروتين).

تم الانتاج المعياري الواسع والتركيز والتنقية للكلوكوسيل ترانسفيريز لبكتريا المكورات الفموية من النوع (*S . sobrinus*) (serotype G) العزلة N_{10} بواسطة طريقة الترشيح العالي باستخدام مرشح (Amicon-P50) وكروماتوغرافيا الادمصاص (حبيبات ال hydroxyapatite) وكروماتوغرافيا المبادل الايوني باستخدام عمود الـ(DEAE-cellulose) وكروماتوغرافيا الترشيح الهلامي باستخدام عمود الـ (Sephacryl S-200). تم تنقية ثلاث انزيمات من الكلوكوسيل ترانسفيريز ($GTF-I_a$ و$GTF-I_b$ وGTF-II) بفعالية نوعية مقدارها (٣١.٦٠ وحدة/ملغم بروتين) و(١.٥٠ وحدة/ملغم بروتين) و(٦٦.٢٧٠ وحدة/ملغم بروتين) وبعدد مرات تنقية (٢٧.٥٩) و(٢٧.٢٩) و(٨٥.٧٥) وبحصيلة انزيمية مقدارها (٤٢.٠٥%) تباعا.

تم تحديد الاوزان الجزيئية لانزيمات الكلوكوسيل ترانسفيريز النقية ($GTF-I_a$ و$GTF-I_b$ و-GTF II) بواسطة استعمال كروماتوغرافيا الترشيح الهلامي عمود الـ (Sephacryl S-200) وبوجود مجموعة من البروتينات القياسية ذات الوزان الجزيئية العالية. اثنان من انزيمات الكلوكوسيل ترانسفيريز ($GTF-I_a$ و$GTF-I_b$) اعتبرت متشابهة انزيميا (isozymes) وذات وزن الجزيئي (١٢٨٨٨٢ دالتون)، ووجد أن الإنزيم الثالث (GTF-II) يحمل وزناً جزئياً مقداره (١٨٦٢٠٨ دالتون).

كذلك في هذه الدراسة تم اختبار قابلية إنزيم الكلوكوسيل ترانسفيريز على استحداث الإستجابة المناعية. وتمت عملية الحقن تحت الجلد في ظهر الأرانب المختبرية باستخدام المستضاد الإنزيمي المنقى ($GTF-I_b$). سجلت الإستجابة المناعية بين المضاد الإنزيمي (anti-$GTF-I_b$) والمستضادات الإنزيمية ($GTF-I_a$ و$GTF-I_b$ وGTF-II) النقية باستخدام تفاعل الإستضداد المزدوج (المضاد مع المستضاد) على سطح هلام الأجاروز. تم الكشف عن نفس الإستجابة المناعية (بواسطة ظهور خطوط الترسيب) على سطح هلام الأجاروز بين إنزيمي الكلوكوسيل ترانسفيريز المتشابهين إنزيميا ($GTF-I_a$ و$GTF-I_b$) والمضاد الإنزيمي (anti-$GTF-I_b$).

وتم اختبار تأثيرات التراكيز المختلفة من المثبطات(EDTA, Sodium fluoride chlorohexidine) وسائل الغرغرة ZAK والمضاد الإنزيمي anti-$GTF-I_b$ على النمو البكتيري بواسطة التخفيف بوسط المرق السائل وبطريقة الإنتشار على سطح الوسط الصلب. وقد وجد أن المضاد (anti-$GTF-I_b$) و (EDTA) ليس لهما تأثير على نمو بكتيريا المكورات الفموية من نوع (*S.sobrinus* serotype G) العزلة N_{10} بينما ال Chlorohexidine, Sodium, Flouride بتراكيز (18mM) و(20mM) تباعاً لهما القابلية لإنتاج تثبيط كامل للنمو البكتيري،

وسائل الغرغرة ZAK بتركيز (12mM) له القابلية على تثبيط النمو البكتيري عند استعمال طريقة التخفيف بالمرق السائل.

كذلك تم اختبار تأثيرات تراكيز مختلفة من هذه المثبطاتا والمضاد الإنزيمي (anti-GTF-I_b) على الفعالية للإنزيم النقي GTF-I_b. وقد وجد أن المضاد الإنزيمي anti-GTF-I_b وبتركيز (١,٥×١٠$^{-٣}$مل مول) له القابلية على تثبيط (٨٧,٣٣ %) من الفعالية الإنزيمية لإنزيم (GTF-I_b) النقي ويتبعانه ال Sodium Fluoride (١٨مل مول) والـ Chlorohexidine (٢٠ مل مول) وسائل الغرغرة ZAK (١٢مل مول) الذين لهم القابلية لتثبيط (٨١,٦٤ %) و(٧٥,٥٥ %) و(٦٠,٤٠ %) من الفعالية الانزيمية لإنزيم (GTF-I_b) المنقى تباعا. التراكيز المختلفة من ال (EDTA) ليس لها تأثير على الفعالية لإنزيم الـ (GTF-I_b) النقي.

في هذه الدراسة تم أيضاً تعيين الثوابت الحركية، قيمة ثابت (Mechalis Menton) (K_m) وقيمة السرعة القصوى (V_{max}) للإنزيم النقي GTF-I_b والتي كانت تساوي (١١مل مول) و(٠,٠٥ مل مول \دقيقة $^{-١}$) تباعا باستخدام (Lineweaver Burk plot).

CHAPTER ONE

Introduction

CHAPTER TWO

Literature Review

CHAPTER THREE

Materials

&

Methods

CHAPTER FOUR

Results

&

Discussion

CHAPTER FIVE

Conclusions

&

Recommendation

Republic of Iraq
Ministry of Higher Education
and Scientific Research
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Characterization of Glucosyltransferase (GTF) of Mutans Streptococci *Streptococcus sobrinus* (Serotype G) (Functional and Immunological Assessment)

A Thesis
Submitted to the College of Science, Al-Nahrain University
In partial fulfillment of the requirements for the degree of Doctor of Philosophy of Science in Biotechnology

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(التقديرات الوظيفية والمناعية)

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