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Oligosaccharides from Cell Surface Glycoproteins of Thermoplasma acidophilum, an Archae

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Oligosaccharides from Cell Surface Glycoproteins of Thermoplasma acidophilum, an Archae

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ABSTRACT

Thermoplasma acidophilum is a member of the newly recognized kingdom of organisms known as Archae. Evolutionarily placed between prokaryotes and eukaryotes, these organisms possess no peptidoglycan cell wall but synthesize glycoproteins which coat their surfaces. Archae have no nuclear membrane, endoplasmic reticulum or Golgi, therefore the entire secretory and glycosylation pathway occurs in an unknown system on the plasma membrane. It is our intent to characterize the carbohydrates comprised of 1-4 sugars from the external glycoprotein to examine their relationship to eukaryotic glycoproteins and to determine in this way which synthetic enzymes (glycosyl transferases) must be present as targets for further study.

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List of Abbreviations

AMAC	2-aminoacridone
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
FACE	Fluorescent-Assisted-Carbohydrate-Electrophoresis
GC	Gas chromatography
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
MALDI-TOF	Matrix-Assisted-Laser-Desorption-Ionization Time-of-Flight
MS	Mass spectrometry
TA	*Thermoplasma acidophilum*
TAGP	*T. acidophilum* glycoproteins ultracentrifugated at 100,000*g*
TAP	*T. acidophilum* glycoproteins ultracentrifugated at 100,000*g* and treated with pronase

1. INTRODUCTION

An abundance of molecular and physiological data over the past years has led to the proposal of a third kingdom classified as Archae. The numerous new findings and ideas have provided support for unique biochemical and molecular features of the *Archae* [1]. A large number of various proteins with diverse biological function and origin are known to contain covalently linked carbohydrates called glycoproteins [2]. Many carbohydrates components serve as receptors for viruses, hormones, intercellular recognition, adhesion, and antibodies [1]. Although numerous in eukaryotes, glycoproteins are rare in prokaryotes. However, N-linked oligosaccharides were reported for the first time in Archae by Mescher [3]. Experimental studies on the Archae will provide an understanding of (a) evolutionary links between prokaryotes and eukaryotes, and (b) their position in a genealogical system of organisms. No Archae lives in a normal environment, being halophiles, thermophiles, acidophiles or combinations of these characteristics. Archae carbohydrates and proteins must be stable in extreme environment, therefore we are specifically concerned with the characterization of the carbohydrates from the external Archae glycoprotein to examine structural characteristics which may contribute to stability and its relationship to eukaryotic glycoprotein structure and biosynthesis.

A thermophilic mycoplasma, *Thermoplasma acidophilum* , grows optimally at pH 2.0 and 56^{o}C [4]. Evolutionarily placed between prokaryotes and eukaryotes, these organisms posses no peptidoglycan cell wall, instead they synthesize glycoproteins which coat their surface [4, 6, 7]. The lack of murein in *Archae* and the presence of chemically diverse murein cell wall polymers on some groups of the *Archae* are basic evidence of biochemical variation between the *Archae* and bacteria [5]. Archae have no nuclear membrane, endoplasmic reticulum, or Golgi. These organelles are necessary in eukaryotic secretion and the N-glycosylation synthetic pathway. Therefore the entire secretory and glycosylation pathway is an unknown system on the plasma membrane. However, Archae have been shown to glycosylate Asparagine using the consensus

glycosylation sequence first discussed in eukaryotes (Asn-X-Thr/Ser). It has been reported that for the first time N-glycosydically linked, mannose-rich glycoproteins appear on "prokaryotes" [6]. Preliminary experiments have indicated that glycosylation of certain membrane proteins may contribute to the survival ability of *T. acidophilum* at extreme environment conditions [6]. The pathways of central metabolism provide the metabolic links between the catabolic (degradative) and anabolic (biosynthetic) routes in living organisms, and also serve as their major pathways of energy generation.

While eukaryotic glycoprotein and glycolipid synthesis has been extensively studied, little is known about the biosynthesis of prokaryotic glycoconjugates. There has been little research done on the glycosylation mechanisms of *T. acidophilum* for the last 18 years. The archaebacterial glycosylation system is interesting from the standpoint of exploring the early evolution and development of the endoplasmic reticulum and Golgi.

Furthermore, protein glycosylation in the endoplasmic reticulum occurs through membrane-bound sugar carriers generically known as dolichols. Dolichol-phosphate-mannose (Dol-P-Man) synthase is a membrane-bound enzyme found in the endoplasmic reticulum of most eukaryotic organisms. Dol-P-Man synthase catalyzes the formation of Dol-P-Man from Dol-P and GDP-Man [15]. Dolichol phosphate mannose ($C_{85\text{-}105}$ isoprenyl) participates in N-glycosylation of protein, a process highly conserved among eukaryotic cells. It is also an important glycosyl donor in biosynthesis of asparagine-linked oligosaccharides chains of glycoproteins, for the synthesis of O-linked mannosyl units in yeast glycoproteins, and for the synthesis of the phosphatidylinositol anchor found on many membrane proteins [16]. The first glycosyl transferase system enzyme homologous to eukaryotes from archae was reported by Zhu and Laine in 1996 [17]. Also, in the synthesis of peptidoglycan in prokaryotes, C_{55} polyisoprenols are utilized to transport sugars from the cytoplasm to the murein layer [18].

2. Preliminary Studies on Oligosaccharides from the Cell Surface Glycoproteins of the Archae: *T. acidophilum*

2.1 Isolation and Purification

Method for isolation and purification of the *T. acidophilum* glycoprotein was adapted from Yang and Haug in 1979. *T. acidophilum* were grown under optimal conditions at 56°C and pH 1.2. The plasma membrane along with the membrane-bound glycoproteins was isolated by ultracentrifugation. Oligosaccharides were isolated from the *T. acidophilum* membrane-bound glycoprotein using pronase digestion. Size-exclusion-Chromatography was used to purify the carbohydrate variety. The separation by size-exclusion-chromatography is based on the fact that smaller molecules are retained in the gel pore of the eluting column [Figure 1 and 2]. Detection of the carbohydrates with phenol/sulfuric acid assay and measurement of the absorbance at 490 nm using the spectrophotometer was used to graph an elution diagram. The chromatogram showed three distinct peaks [Figure 3]. Using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry, we determined the approximate average molecular weight of each of the peaks. The molecular weight of the first peak was found to be 8457.43 while the second peak had an average molecular weight of 1523.67 [Figure 4 and 5]. We found oligosaccharides with 1-4 sugars along with some larger saccharides.

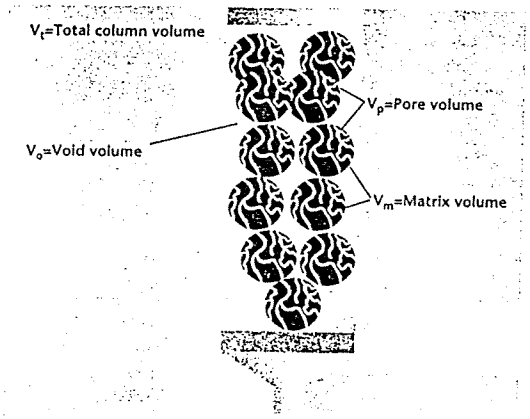


Figure 1. Schematic representation of size exclusion matrix

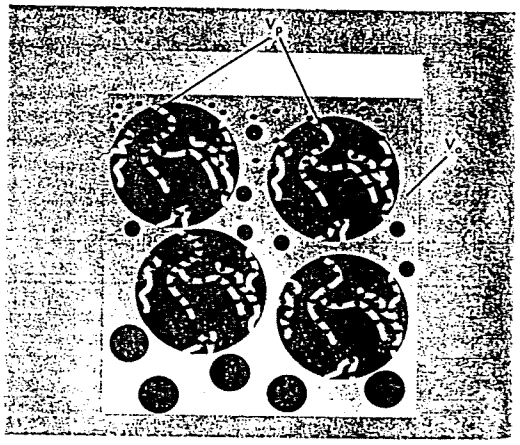


Figure 2. Mechanism of size exclusion chromatography. Large molecules do not penetrate the pores of the support, eluting in the void volume (V_O); medium-sized molecules penetrate the support to some degree and elute in a volume (V_e) between V_O and V_t; small molecules penetrate the pore volume (V_p) of the support completely and elute in the total volume (V_t).

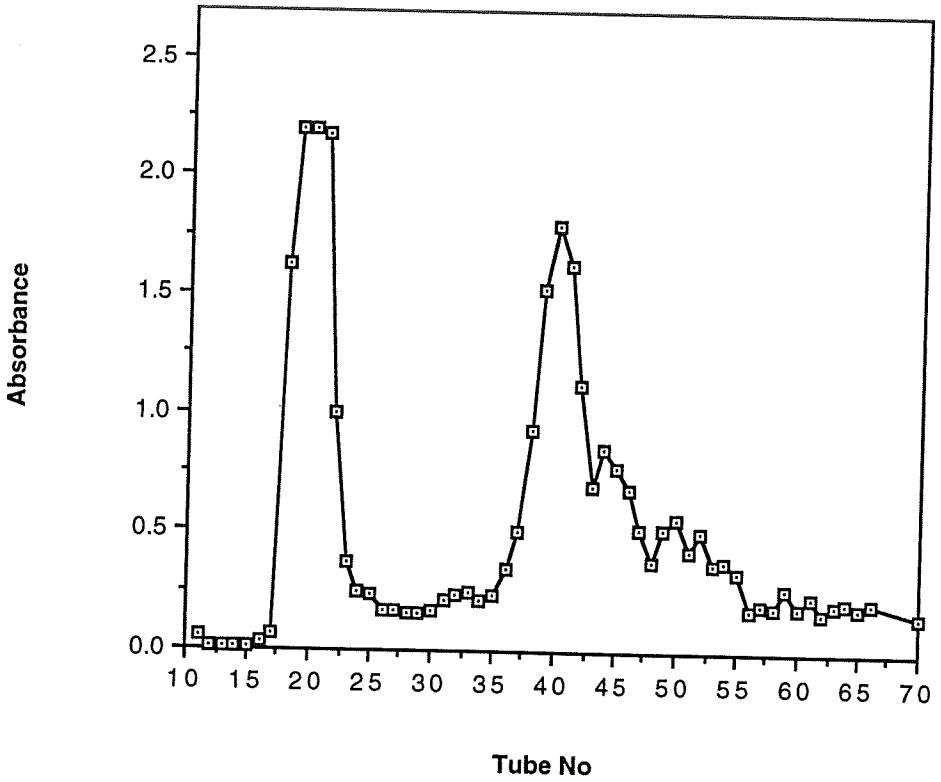


Figure 3. Elution pattern of isolated *T. acidophilum* carbohydrates

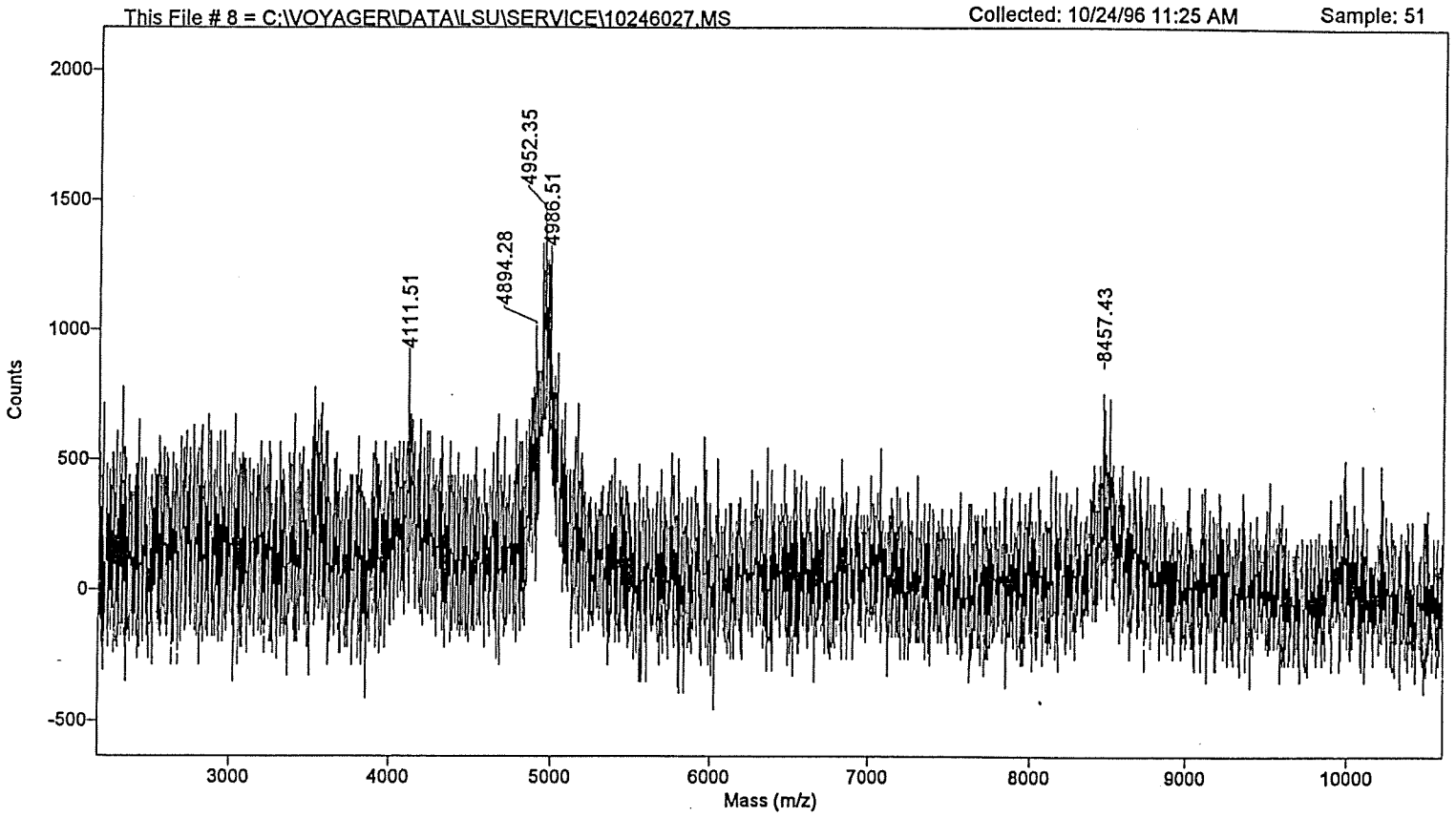


Figure 4. MALDI-TOF Mass spectrum of *T. acidophilum* isolated carbohydrates from peak I

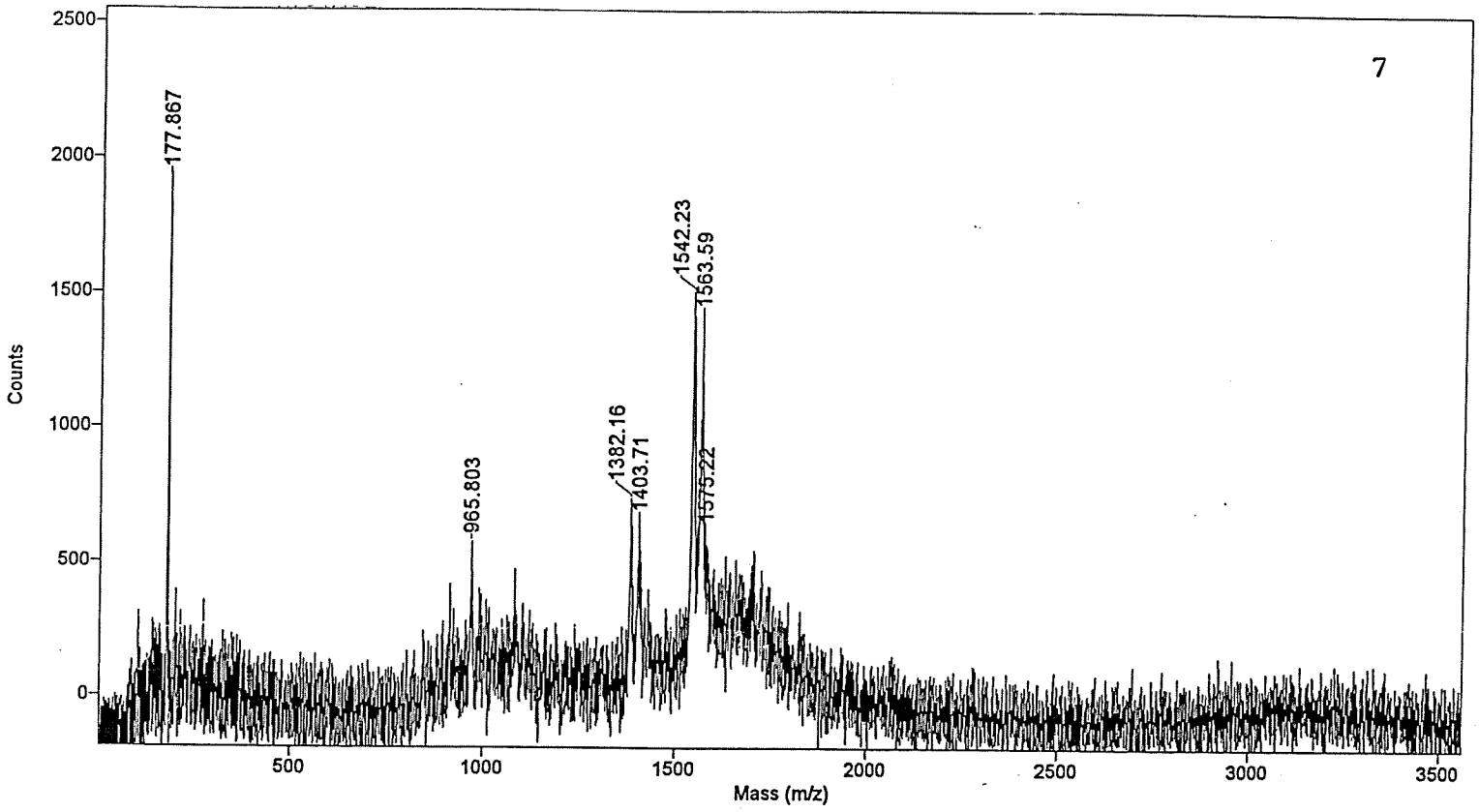


Figure 5. MALDI-TOF Mass spectrum of *T. acidophilum* isolated carbohydrates from peak II

2.2 FACE

Fluorescent-Assisted-Carbohydrate-Electrophoresis (FACE) technique is currently being performed to aid with the identification and quantification of the monosaccharides in the glycoprotein oligosaccharides. The oligosaccharides are hydrolyzed into component monosaccharides. The mixture of released monosaccharides is labeled with a fluorescent tag. Separation of the fluorophore labeled monosaccharides is done by polyacrylamide gel electrophoresis **[3].** The resulting banding patterns represent the monosaccharide composition of the starting material. A qualitative determination of the monosaccharides present on the sample can be seen using a UV light box. A quantitative determination of the monosaccharides present can be obtained using the FACE (Charged Coupled Device) Imaging System **[4].** The FACE technique will be used to quantify monosaccharide composition of *T. acidophilum* oligosaccharides.

The fluorophore labeling dye used in FACE, ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid), derivatizes the sugar molecule via reductive amination. The primary amine of the fluorescent tag and the C-1 aldehyde of the reducing sugar react to form a Schiff base, which is reduced to the

mixed aryl/aliphatic secondary amine by sodium cyanoborohydride **[5].** This reduction step is probably the overall rate-determining step of the labeling reaction [Figure 6]

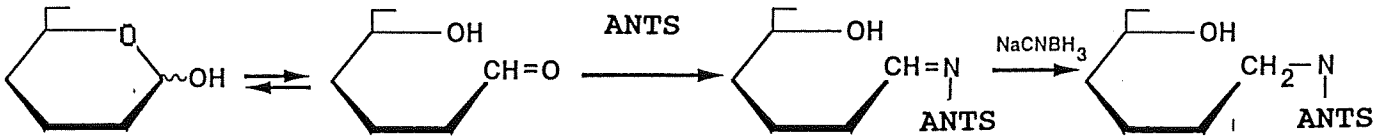


Figure 6. Fluorophore labeling of monosaccharides by reductive amination **[5]**

However, neutral and acidic saccharide derivatives were all negatively charged by the ANTS molecule. Therefore, they are not readily distinguishable electrophoretically as two distinct groups of molecules unlike those labeled with 2-aminoacridone (AMAC). The mechanism of the reductive amination using AMAC is the same as that of ANTS. In addition, the electrophoretic mobilities of derivatives of AMAC are much less influenced by the saccharide sizes but are much more strongly affected by the saccharide structures. As a result, numerous separations can be obtained with 2-aminoacridone but not with ANTS. An additional property of AMAC, in contrast to ANTS, is that, it can be used to derivatize N-acetylneuraminic acid **[6].**

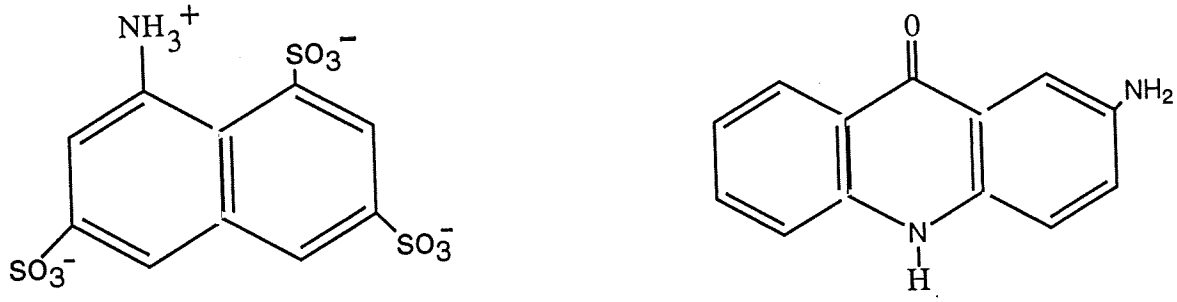


Figure 7. Structure of ANTS and AMAC, fluorophore labeling dyes

2.2.1 Hydrolysis of *T. acidophilum* carbohydrates

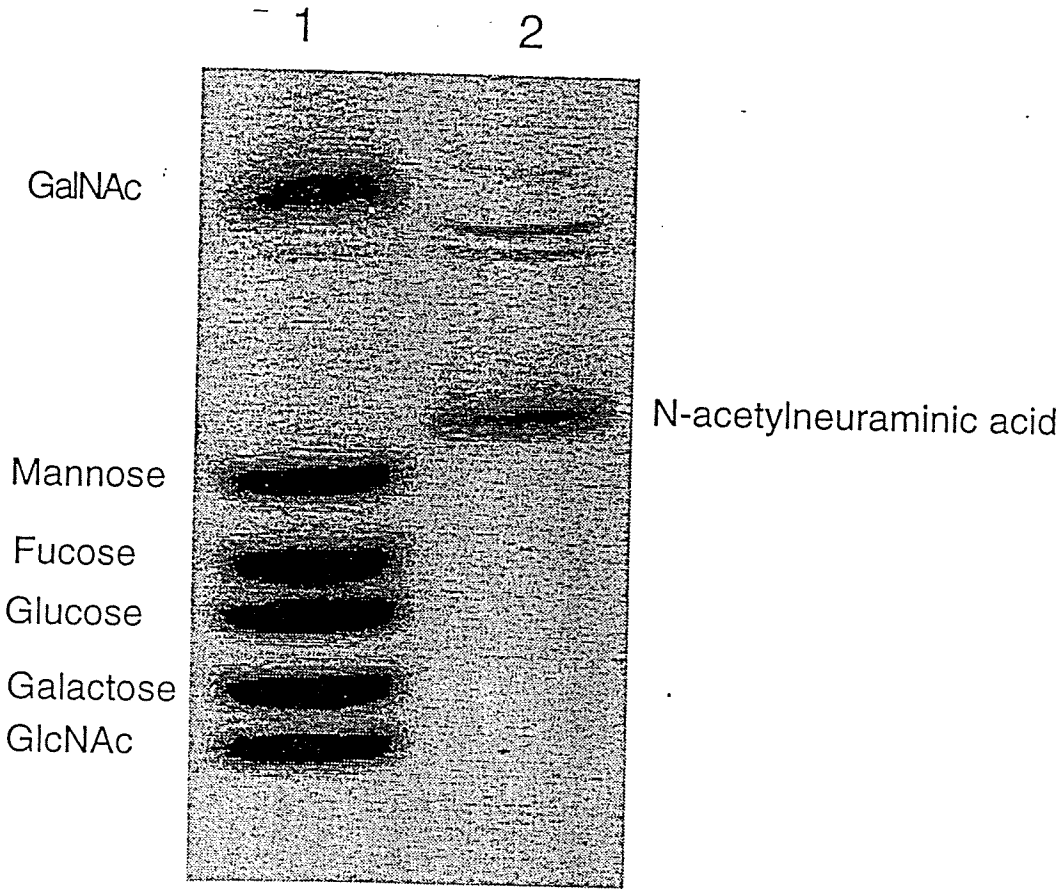
Procedure for hydrolysis of *T. acidophilum* carbohydrates was used from the FACE carbohydrate analysis kit. Three separate hydrolysis reactions were used for the isolated *T. acidophilum* carbohydrates using: **A)** 100 *u*l of 2M TFA and incubated at 100°C for 1 h for **neutral sugars, B)** 100 *u*L of 4 M HCl and incubated at 100°C for 30 min for **amine sugars**, and **C)** 100 *u*L of 0.1 M HCl and incubated at 80° for 30 min for **neuraminic (sialic) acids**. The N-acetylneuraminic acid was used as the control to monitor the efficiency of the hydrolysis, the reacetylation, and the labeling reaction. For amine hydrolysis reactions, a re-N-acetylation step must be performed. To the amine hydrolysis samples, 30 *u*L of 0.2 M $NaHCO_3$ pH 10.7 and 3 *u*L acetic anhydride were added. The samples were incubated in ice for 15 min and dried in a centrifugal vacuum evaporator (CVE).

2.2.2 Reductive Amination

Thermoplasma acidophilum carbohydrates were derivatized with the fluorophore AMAC using the procedure previously described by **Jackson [6].** Complete derivatization can be obtained using up to 100 nmol of sugar. A molar ratio fluorophore to reducing equivalents was maintained. This generally required addition of 5 *u*l of fluorophore (in 0.15 M acetic acid-water solution [3:17, v/v]) and 5 *u*L of $NaCNBH_3$ (1.0 M in DMSO). The sodium cyanoborohydride solution must be made fresh and used within a few minutes. The reaction was allowed to proceed for 16 h at 37° in an incubator and then redried in the centrifugal vacuum evaporator for about 1 h. Derivatized samples were resuspended in 10 *u*L of electrophoresis sample buffer DMSO-glycerol-water [2:1:7, v/v]. Two *u*L of each sample was then applied to the gel for electrophoresis. A 0.1 M tris-boric acid running buffer, pH 8.3 was used. Remaining samples were redried for 30 min on CVE and stored at -70°. Figure 8 and Figure 9 show the electropherograms obtained from the FACE gel electrophoresis.

Figure 8 shows the standard analysis provided by the FACE Monosaccharide System protocol and kit.

Figure 8. FACE monosaccharide standard analysis



Lane 1- MONO Ladder Standard 2

Lane 2- NANA Labeling Control1

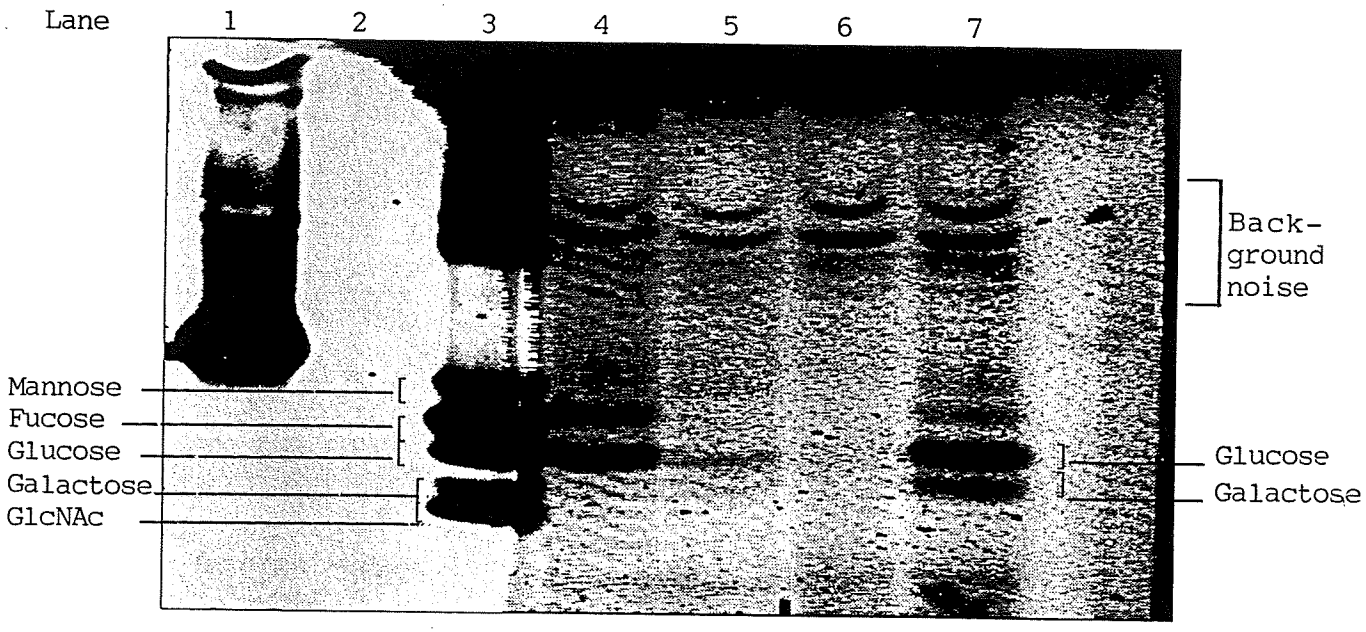


Figure 9. FACE electropherogram of *T. acidophilum* isolated carbohydrates from Peak I

Lane 1:	Sialic Acid Standard
Lane 2:	TA; hydrolysis reaction **C**
Lane 3:	Standard Sugars N-acetylgalactosamine (GalNAc), Mannose, Fucose, Glucose, Galactose, and N-acetylglucosamine (GlcNAc)
Lane 4:	N-acetylglucosamine (GlcNAc) standard; hydrolysis reaction **B**
Lane 5:	TA; hydrolysis reaction **B**
Lane 6:	TA; hydrolysis reaction **A**
Lane 7:	Lactose standard; hydrolysis reaction **A**

In Figure 9, lanes 1 and 2 were obtained by hydrolysis conditions **C** for sialic acid; lane 4 and lane 5 were obtained by hydrolysis conditions **B** for amino sugars; and lane 6 and lane 7 were obtained by hydrolysis conditions **A** for neutral sugars. [Refer to Section 2.2.1 Hydrolysis of *T.acidophilum* carbohydrates above for hydrolysis conditions.] Lanes 2, 5, 6 refer to *T. acidophilum* (TA) carbohydrates subjected to FACE analysis. The standard monosaccharides band separation [Lane 3] was not as clear as shown in Figure 8. Lane 1 was overloaded. The lactose standard [Lane 7], after hydrolysis, revealed a glucose and a galactose band. Lane 4 shows the presence of two bands. The N-acetylglucosamine was probably not re-N-acetylated adequately following the hydrolysis reaction for amine sugars. As a result, glucosamine separated on the polyacrylamide gel into its isomers, alpha- and beta- glucosamine, as seen by the presence of two bands on Lane 4. Lane 5 shows only one significant band, most likely glucose or glucosamine, obtained after the hydrolysis of amine sugars in *T. acidophilum.*

2.5 Amino Acid Analysis

An amino acid analysis was done on the isolated *T. acidophilum* carbohydrates. The chromatogram revealed the presence of various amino acids, of which aspartic acid had the greatest peak area. Refer to Table1 and Figure 10.

Peak Number	Retention Time	Component Name	Concentration Nm/50uL	Peak Area	Base Code	Response Factor
1	2.622		0.00	1.10222	BC8	0.0000
2	5.048		0.00	6.13777	BC8	0.0000
3	8.877	ASPARTIC	6.38	194.98004	BCV	0.0322
4	10.460	THREONINE	1.32	37.75841	VCV	0.0317
5	11.265	SERINE	0.84	23.69051	VC8	0.0308
6	14.671	GLUTAMIC	1.19	36.41662	BC8	0.0303
7	16.282	PROLINE	3.16	4.25755	BC8	0.6025
8	19.978	GLYCINE	4.27	143.02046	BCV	0.0239
9	21.462	ALANINE	1.31	39.84157	VC8	0.0305
	23.620	CYSTINE				
10	26.954	VALINE	0.63	18.47297	BC8	0.0315
11	29.493		0.00	1.47430	BC8	0.0000
	30.207	METHIONINE				
12	31.096		0.00	3.74241	BC8	0.0000
13	32.738	LEUCINE	0.58	12.95710	BCV	0.0325
14	33.768	ISOLEUCINE	0.61	13.43690	VC8	0.0325
	35.140	NORLEUCINE				
	36.672	TYROSINE				
	38.513	PHENYLALANINE				
15	43.990		0.00	1.84726	BC8	0.0000
16	45.793	HISTIDINE	0.46	5.61050	BC8	0.0358
17	50.659	LYSINE	1.91	54.09241	BC8	0.0309
18	56.219	AMMONIA	23.18	351.64841	EC8	0.0672
	63.668	ARGININE				
ALS			45.83	952.31555		

Table1. Amino acid composition of the peak II isolated *T. acidophilum* carbohydrates

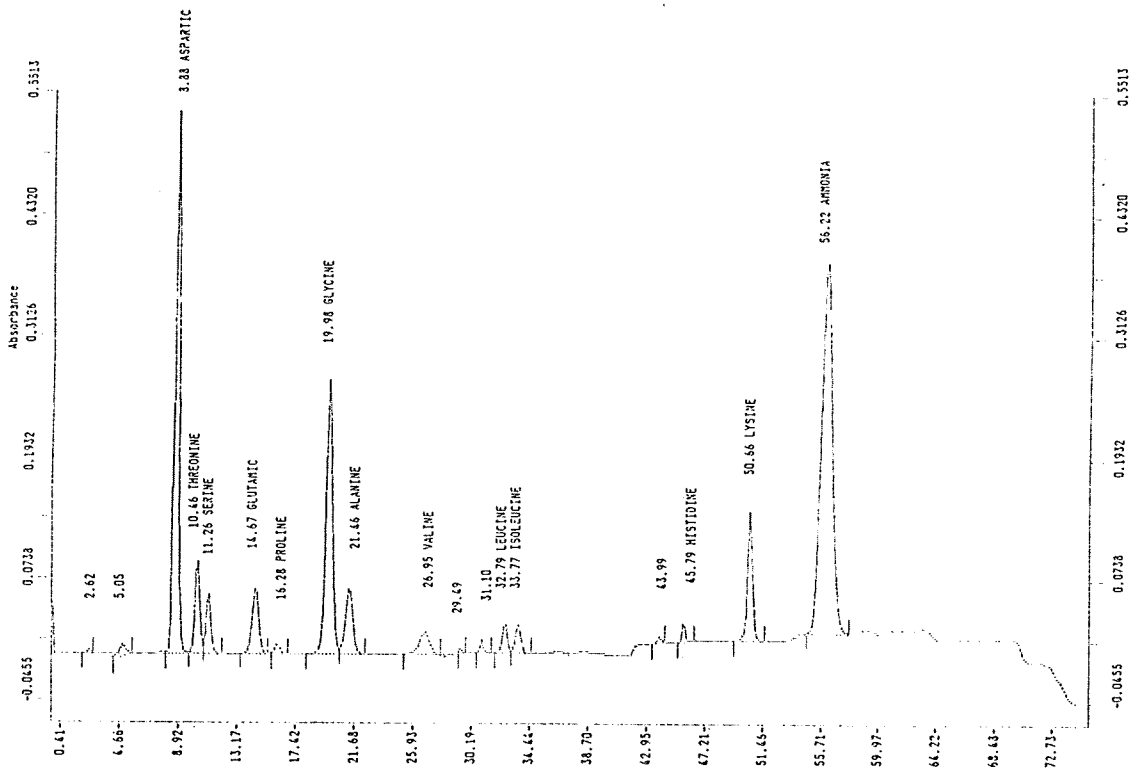


Figure 10. Amino acid analysis of Peak II isolated *T. acidophilum* carbohydrates

2.6 Hydrazinolysis

Exhaustive digestion of glycoproteins using protease such as pronase, and fractionation of the resulting glycoproteins have long been used to obtain samples for the structural study of sugar chains [7]. However, exhaustive protease digestion may be incomplete due to the steric effect of carbohydrates, and the resulting glycopeptides are mixtures containing oligosaccharides and peptides. Because of these problems, Hydrazinolysis of asparagine-linked sugar chains has been developed to produce free oligosaccharides [7]. Hydrazinolysis specifically cleaves the GlcNAc->Asn linkage and N-acetylglucosaminyl linkages. In order to cleave the glycosylamine linkage, the hydrazinolysis product is N-acetylated to preclude the stabilizing effect of the -NH_2 group by converting it to -NHAc [7]. Figure 11 shows the hydrazinolysis of asparagine-linked sugar chains. R represents either hydrogen or sugars.

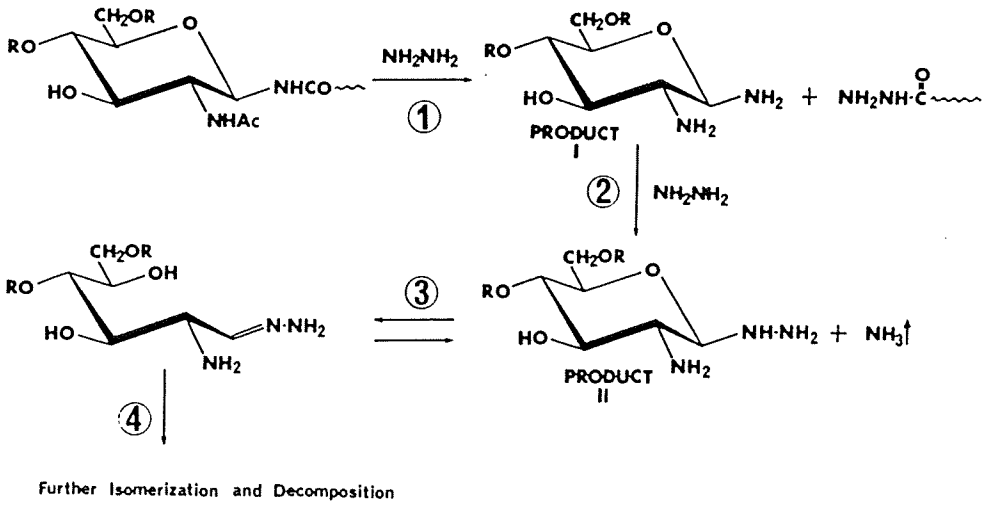
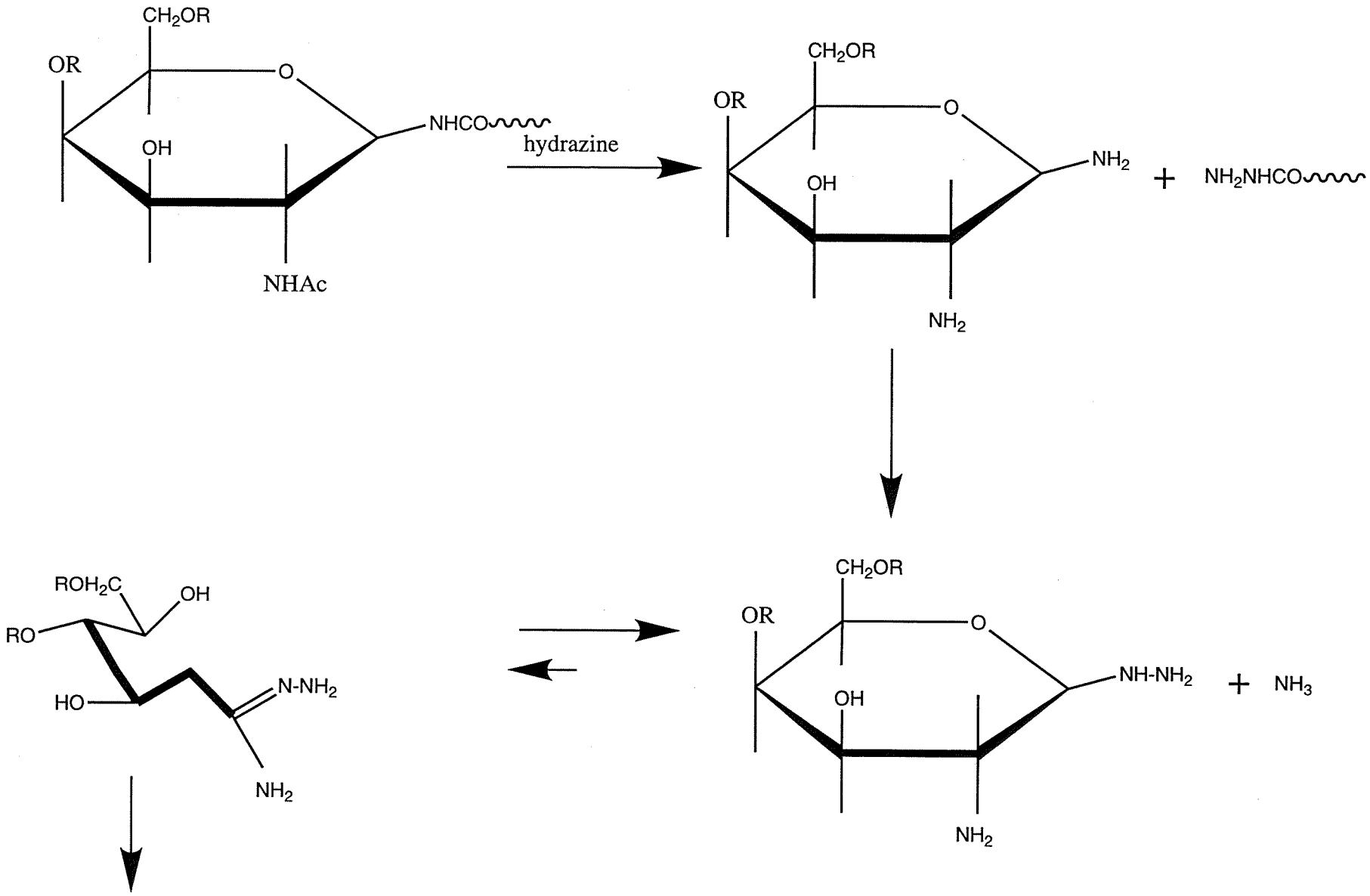


Figure 11. Hydrazinolysis of Asn-linked sugar chains [8].

Hydrazinolysis of Asn-linked Sugar Chains



Further Isomerization and Decomposition

(side reaction)

Procedure for the hydrazinolysis of *T. acidophilum* isolated carbohydrates was adapted from Takasi [7]. *T. acidophilum* isolated carbohydrates samples were suspended in 0.5-1 mL of distilled anhydrous hydrazine and heated in a sealed tube at 100^0C for 8-12 hr. The reaction mixture is evaporated to dryness under a lyophilizer. After drying, the samples were dissolved in saturated $NaHCO_3$ solution and 50 mL acetic anhydride was added to each sample. All free primary amino acids are acetylated by adding acetic anhydride. The reaction mixture was passed through a column of Dowex-50 (H^+) column. The column was then washed five times with bed volume to give a total of 15 mL which was combined with the sample. Samples were then frozen using dry ice and lyophilized overnight. After hydrazinolysis, most asparagine-linked sugar chains are completely released as an oligosaccharide mixture. Results of mass spectrometry performed on isolated *T. acidophilum* carbohydrates of peak II before and after hydrazinolysis are shown in Figure 12 and Figure 13.

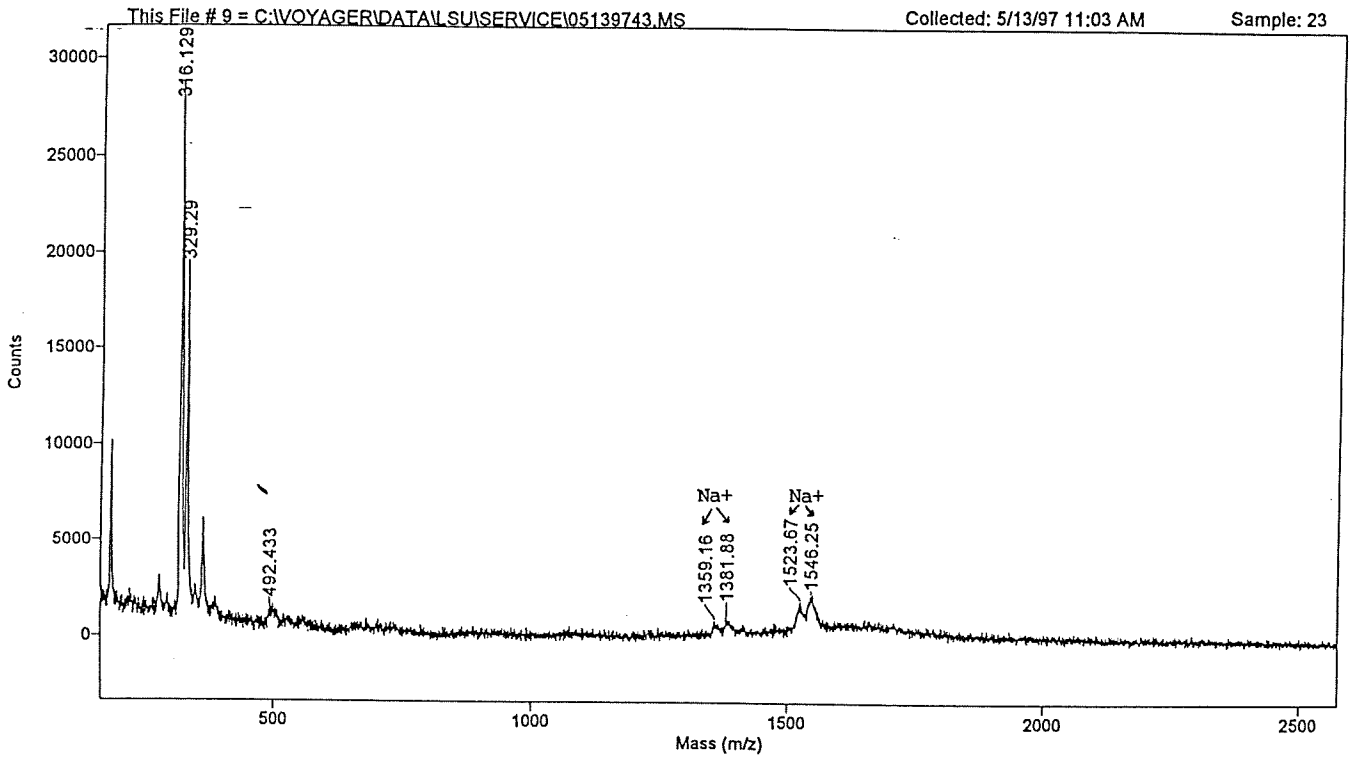


Figure 12. Mass spectrum of *T. acidophilum* isolated carbohydrates (Peak II) before hydrazinolysis

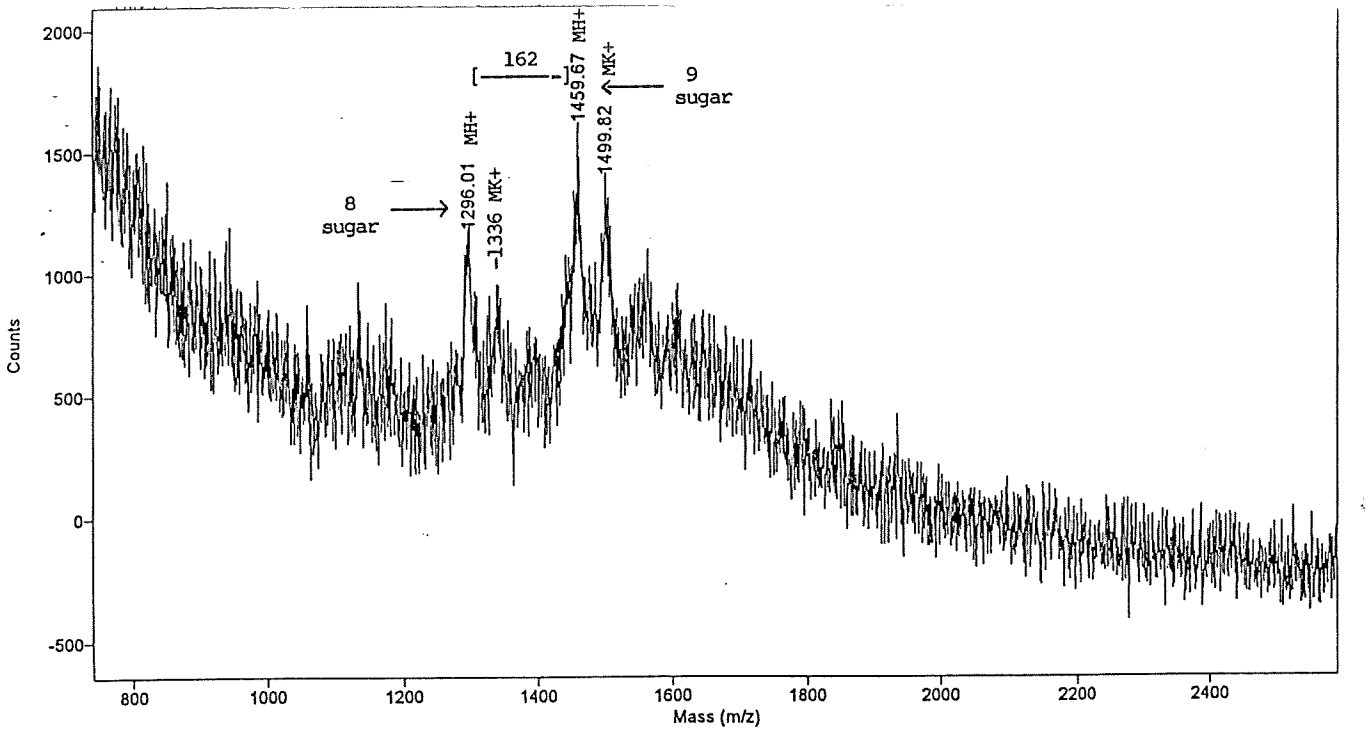


Figure 13. Mass spectrum of *T. acidophilum* isolated carbohydrates (Peak II) after hydrazinolysis

Figure 12 indicated the presence of two peaks of larger mass (1523/1546) and two peaks of smaller mass (1359/1381). The difference between either peaks was a sodium ion of mass 22.9. Figure 13 also showed two larger peaks of higher mass (1459/1499) and two smaller peaks of lower mass (1296/1336) in *T. acidophilum* oligosaccharides. However, the difference between the peaks here was a potassium ion K^+ of mass 40. (The potassium ion was probably a contamination in a vial from a previous run on MALDI-TOF). The mass of a linked monosaccharide is 180 g/mol less the mass of H_20 yielding 162 g/mol. The difference between the masses 1459 and 1296 was 163, approximately the mass of a monosaccharide. The 1459 mass derived from a nine-sugar oligosaccharide (1459/162 = 9), while the 1296 mass derived from an eight-sugar oligosaccharide (1296/162 = 8). The *T. acidophilum* isolated carbohydrates consisted of small 8 and 9-sugar oligosaccharides. The difference in mass of either peaks before and after hydrazinolysis was found to be approximately 63. Although this indicates that something was lost from *T. acidophilum* oligosaccharides after hydrazinolysis, the identity of the mass shift of 63 is still inconclusive.

Other techniques using Thin Layer Chromatography for higher purification of the oligosaccharide peaks and MALDI- and FAB-MS will be used to later determine the monosaccharide composition of the glycoprotein oligosaccharides. Further investigation using

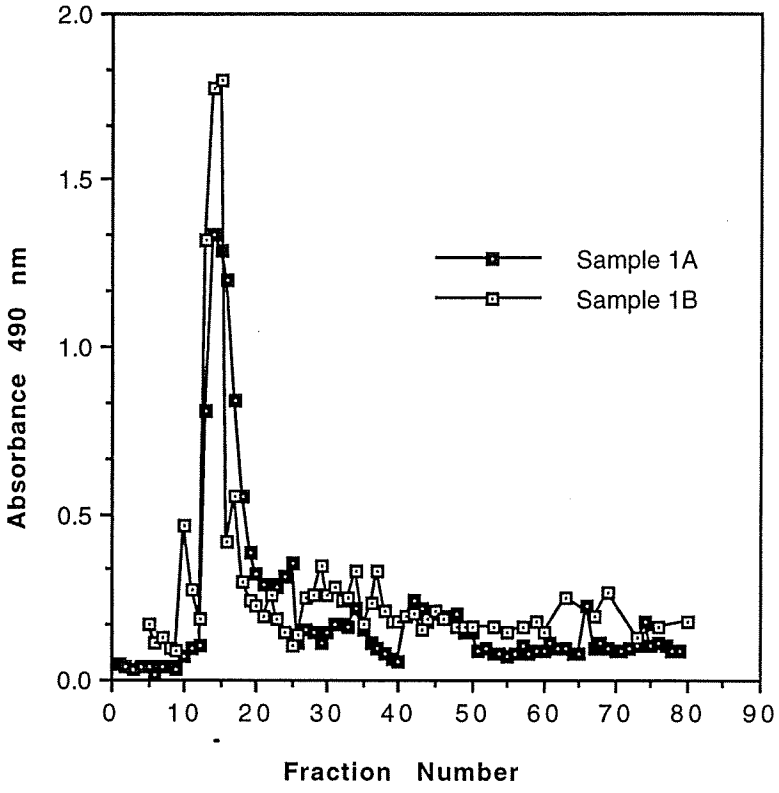
methylation, GC-Mass spectrometry, NMR techniques will characterize intersugar linkage positions. Anomeric configuration will be determined from enzymes on NMR.

3. Analytical Studies on the Cell Surface Layer of the Archae

3.1 Preparation and Fractionation

Preparation and fractionation of glycopeptides and oligosaccharides from *T. acidophilum* glycoprotein was adopted from Laine[8]. The plasma membrane along with the membrane-bound glycoproteins was isolated by ultracentrifugation (100,000 g). *T. aciodphilum* glycoprotein was exhaustively digested with Pronase (enzyme/substrate ratio of 1:10 w/w) under a toluene atmosphere for 72 hr at 45^{o}C in 0.01M Tris-HCl, pH 7.9, containing 2mM $CaCl_2$. Pronase (2%) was added twice more at 24-hr intervals. As the same time, the pH of the solution was checked and readjusted to pH 7.9 with 2 N NaOH. The resulting mixture was boiled at 100^{o}C for 5 min to destroy any residual proteolytic activity prior to centrifugation (10,000 g) to remove insoluble peptides.

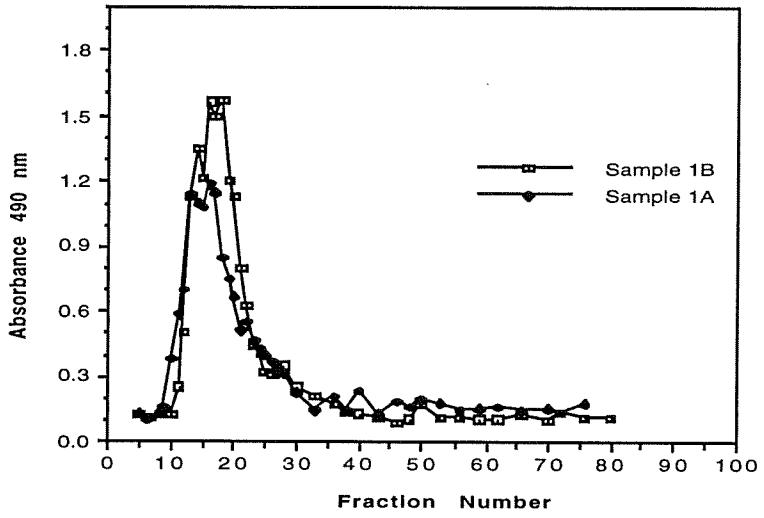
Figure 14. P-4 Chromotography of T. acidophilum glycopeptides



Estimation of molecular weights of oligosaccharides was carried out by size-exclusion chromatography. The supernatant was applied to a P-4 column (molecular weight up to 4,000) and eluted with water, to separate glycopeptides from free amino acids. Fractions of 250 drops were collected and monitored by a phenol-H_2SO_4 reaction for carbohydrate detection. The absorbance was measured at 490 nm using a spectrophotometer [Figure 14]. Sample 1A and Sample 1B are replicates of *T. acidophilum* glycoprotein. The excluded fractions which contain the glycopeptides were lyophilized for 24 hrs. *T. acidophilum* isolated carbohydrates were then dissolved in water. The presence of only one peak in Figure 14 indicated that the sample was not separated adequately. Intact amino acids or a high molecular weight prevented the separation of smaller oligosaccharides on the P-4 column. As a result, the above procedure was repeated using a P-10 column instead.

Figure 15 shows the absorbance spectra of *T. acidophilum* isolated carbohydrates obtained from fractionation on Bio-Gel P-10 column and detection with pheno-H_2SO_4.

Figure 15. T. acidophilum glycopeptides: Bio-Gel P-10 Profile



3.2 Hydrazinolysis

Extensive proteolysis and isolation of resulting glycopeptides by conventional fractionation techniques can be inadequate. Exhaustive protease digestion is usually incomplete, probably because of the steric effect of the carbohydrate moieties. The resulting glycopeptides are mixtures containing oligosaccharides and peptides. In this respect, hydrazinolysis of glycoproteins is a valuable technique which allows the isolation of peptide-free oligosaccharides from glycoproteins [9].

Procedure for the hydrazinolysis of *T.acidophilum* carbohydrates was adapted from Kobata [9] and Fukada [10]. The hydrazinolysis procedure was applied to *T. acidophilum* glycoproteins (100,000 g) (TAGP) and the *T. acidophilum* glycopeptides (TAP) which were subjected to protease digestion and fractionation on the P-4 and P-10 columns. The samples were dried over sulfuric acid in a vacuum dessicator overnight. The dried samples were heated at 105° for 4 hr with anhydrous hydrazine in a sealed tube. The amount of hydrazine was the amount necessary to dissolve the samples, approximately 500mg-1g per mL of hydrazine. Immediately

after opening the tube, the reaction mixture was dried under nitrogen with frequent additions of toluene to evaporate the excess hydrazine, then the residue was lyophilized overnight. The dried samples were dissolved in water and subjected to size-exclusion chromatography on a Bio-Gel P-10 column with water. Fractions were monitored by a phenol-H_2SO_4 reaction. Samples obtained were determined as N-deacetylated oligosaccharides. Samples were then lyophilized overnight. Figures 16 and 17 show the absorbance spectra of N-deacetylated oligosaccharides following hydrazinolysis. Results for TAP and TAGP indicated that separation on the size-exclusion matrix was very similar and did not result in any greater separation of TAP oligosaccharides.

3.3 Re-acetylation

N-Acetylation of N-deacetylated oligosaccharides was followed as described by Spiro [11] and Fukuda [10]. Samples were re-acetylated with 500 *u*L methanol, 100 *u*L acetic anhydride, and 100 *u*L pyridine. Samples were sonicated for 3 hrs and dried under nitrogen. Immediately afterwards, 100 *u*L of 10% methanol-4.5 M sodium acetate was added to a concentration of about 3 *u*mols/mL and reaction was allowed to take place overnight. Because of the presence of a small precipitate in the samples, 400 *u*L of water and 200 *u*L of 4.5 M sodium acetate was added. Samples were then applied to a Bio-Gel P-4 column and carbohydrates were detected with a phenol-H_2SO_4 reaction. Figure 18 shows the elution profile of TAP and TAGP at 490 nm using a spectrophotometer following hydrazinolysis, re-acetylation, and detection with a phenol-H_2SO_4 assay. Results from TAGP were inconclusive.

Figure 16. P-10 Gel Permeation Profile of TAGP Following Hydrazinolysis

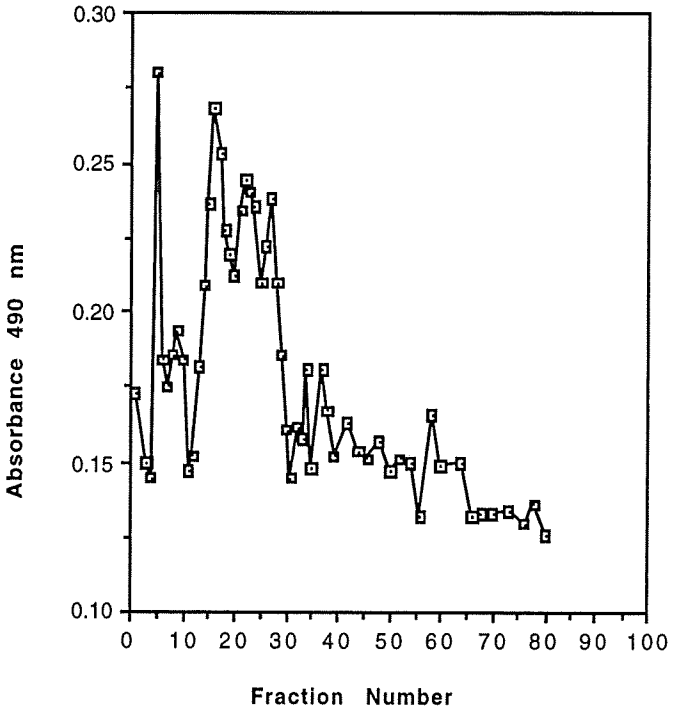


Figure 16 above shows the P-10 Gel Permeation Profile of TAGP (*T. acidophilum* glycoproteins 100,000 xg) following hydrazinolysis. Samples were not subjected to re-acetylation and were designated as N-deacetylated oligosaccharides.

Figure 17. N-deacetylated TAP Gel Permeation Profile

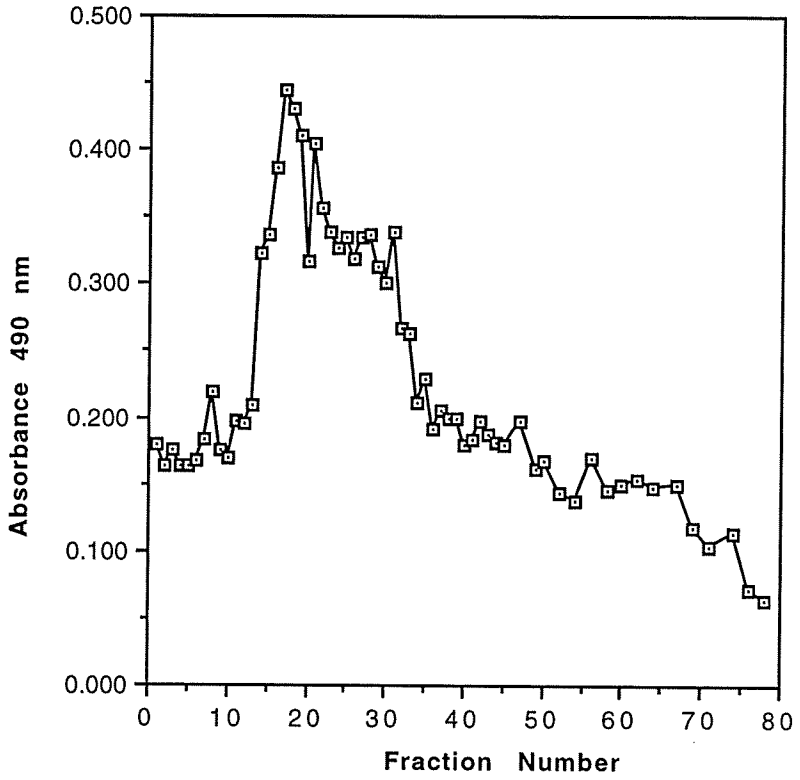


Figure 17 above shows the P-10 Gel Permeation Profile of TAP (*T. acidophilum* glycoproteins 100,000 xg treated with pronase) following hydrazinolysis. The samples were not yet re-acetylated and were designated as N-deacetylated oligosaccharides.

Figure 18. P-4 Column: Gel Permeation Profile

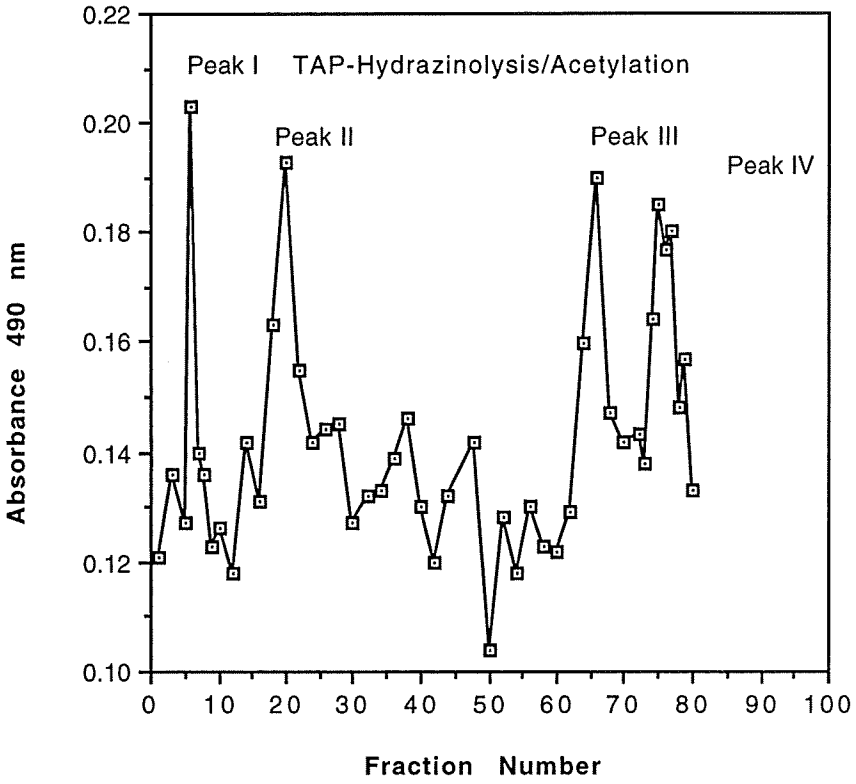


Figure 18. P-4 Gel Permeation Profile of TAP following hydrazinolysis and re-acetylation. Molecular weight of separated oligosaccharides on size-exclusion matrix is inversely proportional to elution volume or increasing fraction number. Peak III and Peak IV are small 1 or 2-sugar saccharides, most likely monosaccharides and disaccharides; Peak II is an 8-or 9-sugar oligosaccharide as was found in MALDI-TOF [Figure 13]. The area under Peak I is very small compared to the others; it is of minor composition and probably could not be detected on MALDI-TOF [Figure 13].

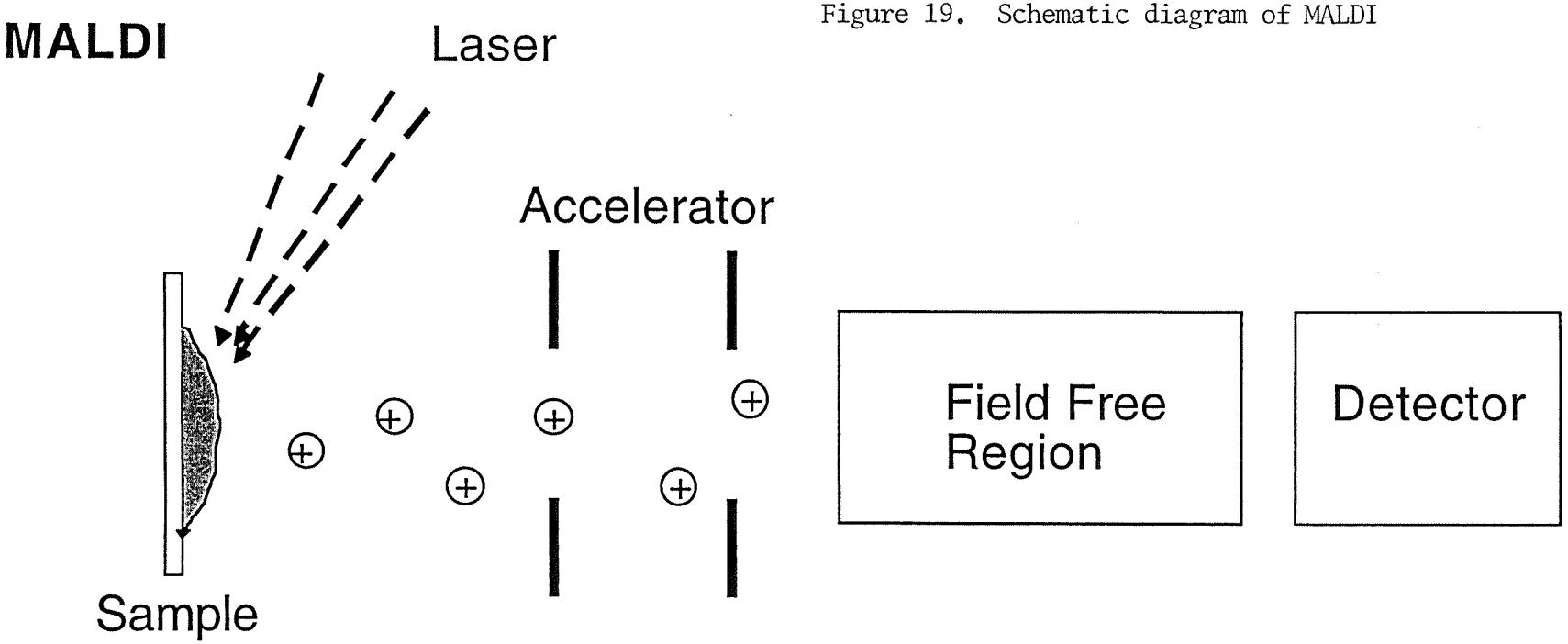
3.4 MALDI

Using Matrix Assisted Assisted Laser Desorption Ionization (MALDI) mass spectrometry **[Figure 19]**, we determined the approximate average molecular weight of Peak III **[Figure 20]**.

3.5 Methylation

Carbohydrates can be subjected to methylation in order to gain information on linkage sequences and anomeric configurations. Methylation assay was adopted from Hakomori [12], Lindberg [13], and Phillips [14]. Lactose (beta-1,4 linkage of galactose and glucose) was used as a standard for the methylation procedure. To approximately 5 mg of lactose, 0.5mL of methylsulfonyl carbanion was added under nitrogen and sonicated for 30 min on ice and the reaction was allowed to proceed overnight. A test with triphenylmethane powder was used to monitor the reaction. A red color indicates a positive reaction. Under nitrogen, 0.5 mL of CH_3I was added and the sample was sonicated for 30 minutes. A water-chloroform (1: 1 v/v) extraction was used to separate the upper phase (aqueous) from the lower phase. Water was added and the extraction was repeated five times using 3 mL water and 3 mL chloroform. To the lower phase, 1 mL of absolute ethanol was added and the sample was dried under nitrogen. Two ml of 1M methanolic HCl was added and the reaction was hydrolyzed for 3 hours at 100°C. In an N-acetylation step, 500 *u*L of methanol, 100 *u*L of acetic anhydride, and 100 *u*L of pyridine were added to the sample, sonicated for 30 minutes, and dried under nitrogen. A 1 mL of 2 N trifluoroacetic acid was added and the reaction hydrolyzed at 120°C for 3h. After drying the sample under nitrogen, 0.5 mL of 1M sodium borodeuteride in 1 N NH_4OH was added and sonicated for 30 minutes. After drop by drop addition of acetic acid until cessation of bubble formation, sample was again dried under nitrogen with subsequent addition of methanol to remove borate. A 1 mL of acetic anhydride/pyridine (1:1) was added and the reaction was incubated at 100°C for 1 hour. After drying under nitrogen, the sample was subjected to a water-chloroform (1:1) extraction 3 times with water, saving the lower phase. Sample was then dried under nitrogen and dissolved in 50 *u*L of ethyl acetate for GC-MS. Refer to **Figure 21-Figure 25**.

Figure 19. Schematic diagram of MALDI



The sample is co-crystallized with a matrix that absorbs at or near the wavelength of the laser source. Gas-phase ions are produced by a pulse of laser light absorbed by the matrix/sample. The ions are accelerated simultaneously into the analyzer, commonly a time of flight mass analyzer. The ions drift through a field free region; lighter ions drift faster than the heavier ions and thus reach the detector faster.

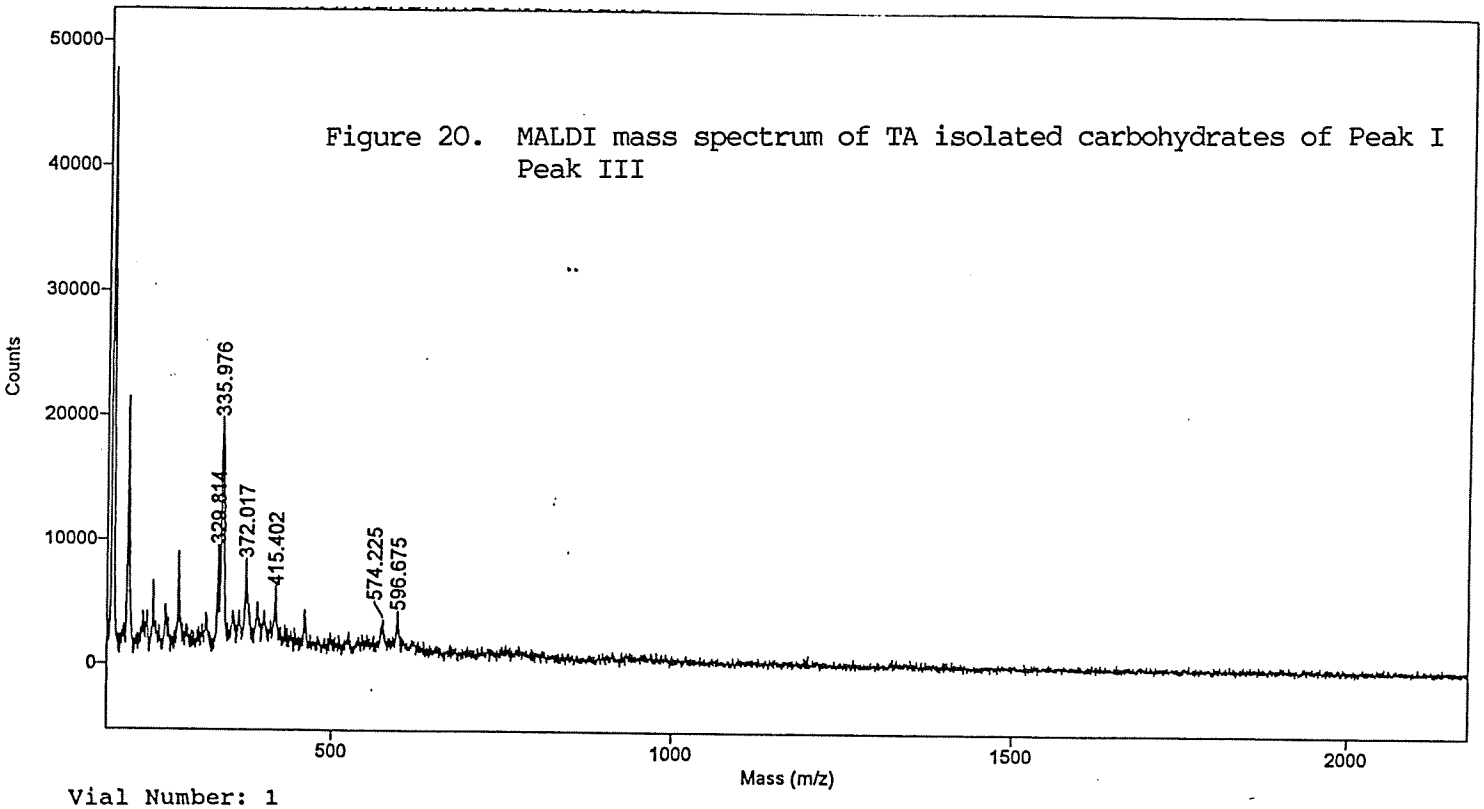


Figure 20. MALDI mass spectrum of TA isolated carbohydrates of Peak I Peak III

Vial Number: 1

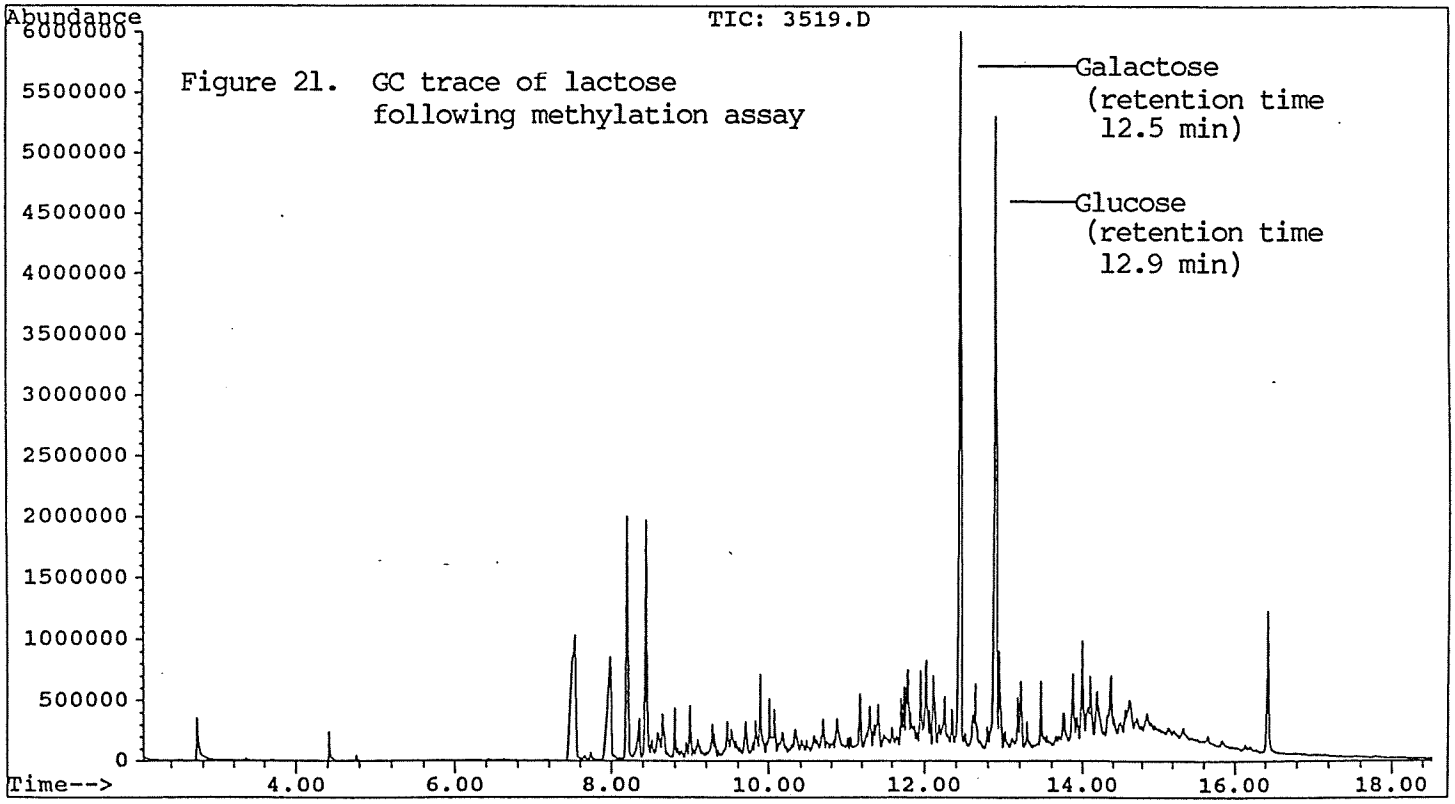


Figure 21. GC trace of lactose following methylation assay

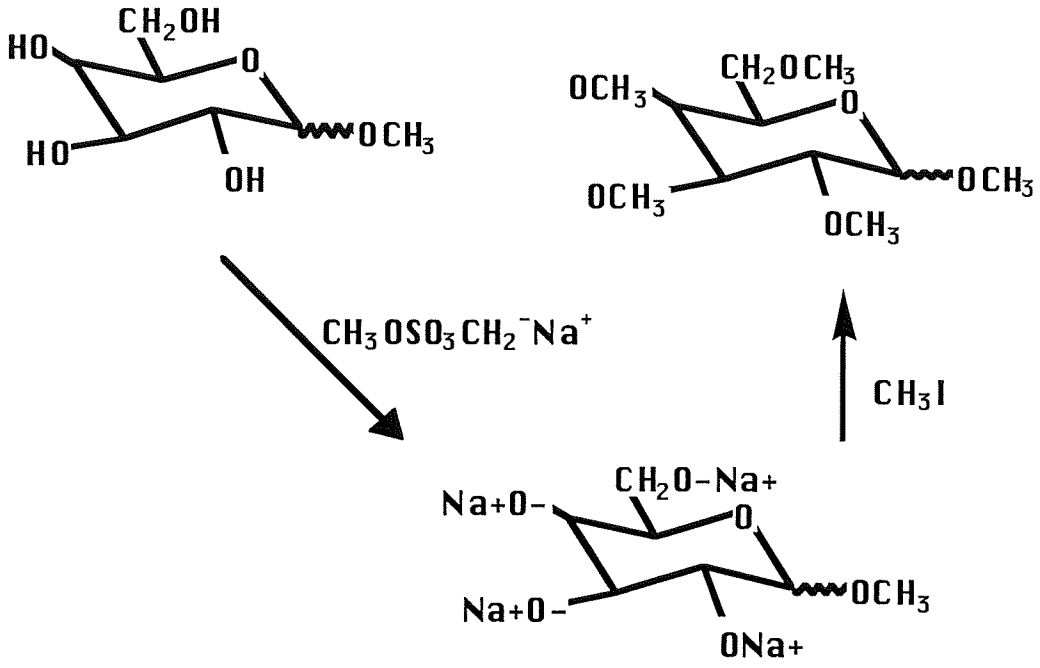
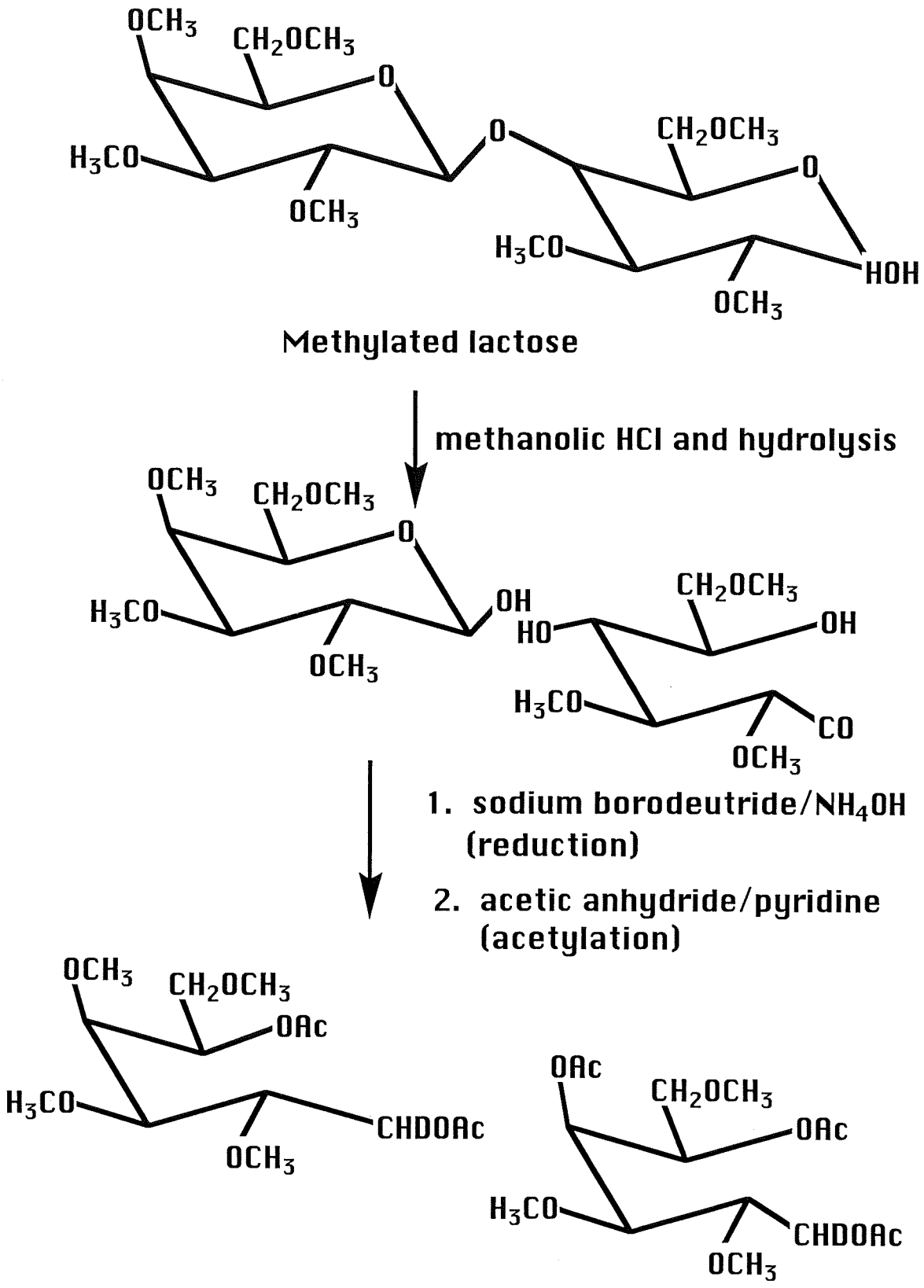


Figure 22. A methylation reaction

Figure 23. Summary of methylation assay



Ionization Fragments obtained from lactose standard

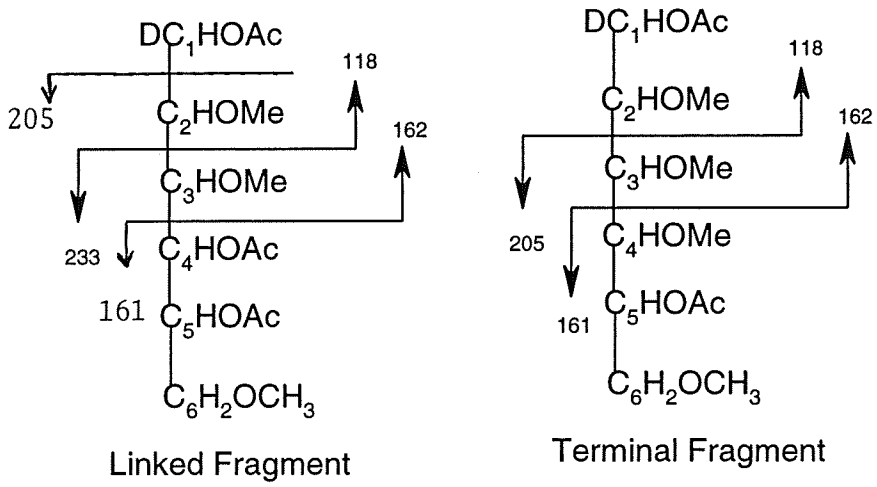
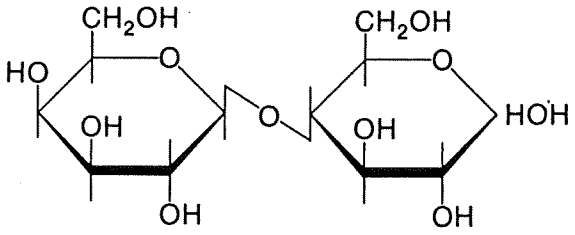


Figure 24. ABOVE- Lactose and ionization fragments. Also note that there will not be a break between C-4 and C-5 due to charge retention of the acetyl (Ac) group.

BELOW- Unmethylated lactose



Lactose: galactose-β-1,4-glucose

Library Searched : C:\DATABASE\WILEY.L
Quality : 83
ID : GALACTITOL-1-D-2,3,4,6-TETRAMETHYL-1,5-DIACETATE

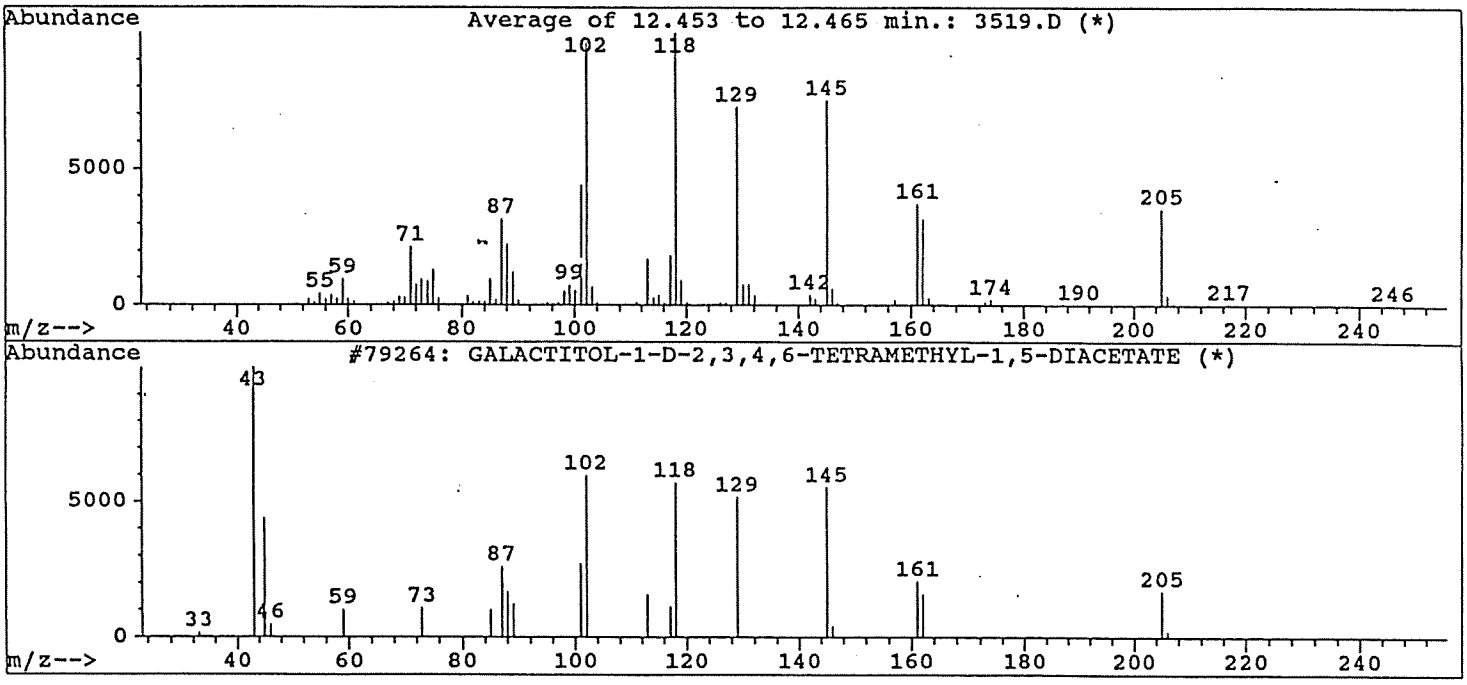


Figure 25B. Mass spectrum of terminal fragment of methylated lactose; retention time 12.5 min

Library Searched : C:\DATABASE\WILEY.L
Quality : 16
ID : D-Glucose, 2,3,6-tri-O-methyl-

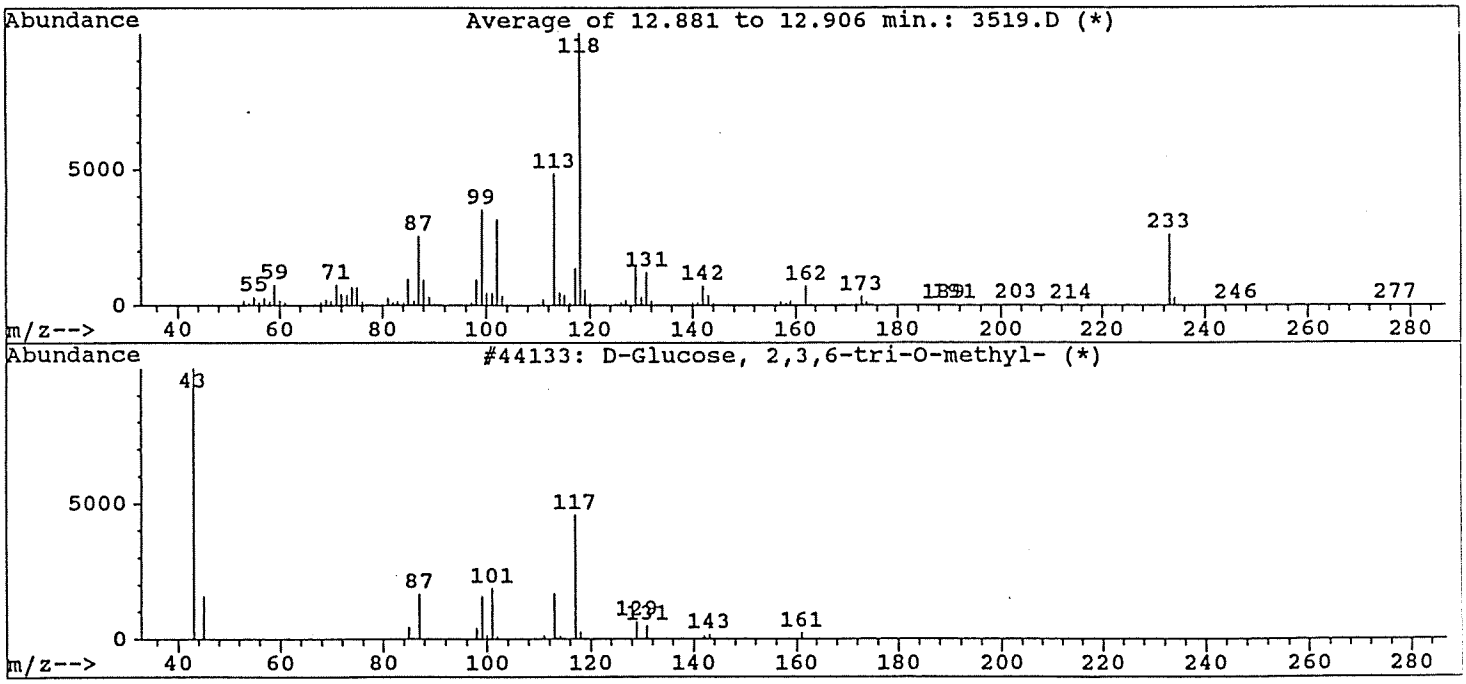


Figure 25B. Mass spectrum of 4-linked fragment of methylated lactose; retention time 12.9 min

3.6 Discussion and Conclusions

Inadequate protease digestion and/or a fairly large carbohydrate moiety may be accountable for the presence of one large peak at relatively low fraction numbers as seen in Figure 14 and Figure 15. As a result, hydrazinolysis had to be carried out to cleave remaining amino acids attached to the carbohydrates. Hydrazinolysis produced some separation of *T. acidophilum* carbohydrates due to a broad absorbance spectra noted in Figure 16 and Figure 17. Re-N-acetylation of N-deacetylated oligosaccharides resulted in a successful separation of protease-digested *T. acidophilum* glycoprotein (TAP). However, re-N-acetylation of N-deacetylated oligosaccharides which were not subjected to protease-digestion (TAGP) resulted in only partial separation on the Bio-Gel P-4 column. Four distinct peaks were found for TAP. Peak I and Peak II contain fractions of fairly large molecular weight, too large for gas chromatography and mass spectrometry analysis.

MALDI mass spectrometry was used to determined the average molecular weight of Peak III (Figure 20). The average molecular weight of Peak II was found to be 574.23. The difference between a molecular weight of 574.23 and that of 596.68 as shown in Figure 20 is a sodium ion of molecular weight 22.9. The methylation assay **[Figure 22 and Figure 23]** performed using lactose as a standard confirmed the beta-1,4 glucose-galactose linkage in lactose. Two peaks were found on the GC trace of methylated lactose. The first peak represents galactose at a retention time of 12.5 min and the second peak represents glucose at a retention time of 12.9 min **[Figure 21]**. **Figure 24** shows the ionization fragments of methylated lactose. The mass spectrum of methylated lactose **[Figure 25A and Figure 25B]** showed the 118 peak characteristic of a methyl in the carbon-2 position; 162 is characteristic of methyl on C-3 position; 233 is characteristic of methyl-acetyl-methyl-methyl substitution on carbons 3, 4, 5, 6 respectively. All together, this data indicates a 4-substitution. Refer to Figure 21A, Figure 21B, and Figure 22 which shows the lactose ionization fragments. Methylation of *T. acidophilum* carbohydrates will provide valuable linkage sequences further in the study.

4. Dolichyl-phosphomannose synthase from the Yeast *S. cerevisiae*

4.1 Cell Culture

S. cerevisiae cells were grown in YPED media (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose, 2% Bacto-agar) at 37°C. Cells were cultured aerobically and were grown to the stationary phase at A_{540}~1.5-2.0.

4.2 Membrane preparation and solubilization

The procedure was adapted from **Zhu and Laine, 1996**. Wet cells were prepared by centrifuging at 6,000 x *g* for approximately 5 minutes. All preparation steps were carried out at 0-4°C. Wet cells (50 g) were lysed with 300 mL 0.2 M Tris-HCl buffer, pH 8.0, 1 mg Dnase and PMSF (1 mM final concentration) were added, and the mixture was stirred for 60 minutes. The lysate was centrifuged at 10,000 x *g* for 30 minutes. The pellet was discarded, and the supernatant was centrifuged at 98,000 x *g* for 90 minutes. Membranes were collected and solubilized with 20 mM phosphate buffer pH 6.0 with 1.0% polidocanol (polyoxyethylene 9 lauryl ether), 10 mM $MgCl_2$ for 2 hours and centrifuged at 98,000 x *g* for 90 minutes. The pellet was discarded and the supernatant containing the solubilized dol-P-man synthase was used for further purification. The solubilized enzyme was concentrated with Amicon P-10 membrane and kept frozen at -20°C.

Although this phase of the study was not completed, the aim of this particular aspect of the research was to investigate the substrate specificity of the *Archae* dolichyl-phosphomannose synthase with the eukaryotic homologue isolated from the yeast *S. cerevisiae*. It would be interesting to study the yeast homologue and compare it to the *Archae* dolichyl-phosphomannose synthase.

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