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thesis entitled

DETERMINATION OF THE PROTEIN CONSTITUENT OF GUM ARABIC

presented by

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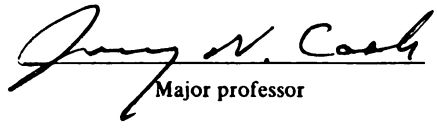
has been accepted towards fulfillment of the requirements for

M.S. degree in Food Science



Major professor

Date March 12, 1993

DETERMINATION OF THE PROTEIN CONSTITUENT OF GUM ARABIC

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science

1993

ABSTRACT

DETERMINATION OF THE PROTEIN CONSTITUENT OF GUM ARABIC

By

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Previous studies of the proposed models of gum arabic have been inconclusive regarding the protein content of this macromolecule. However, recently the structure of gum arabic (wound exudate from Acacia senegal (L.) Willd.) has been investigated and determined to be a linear molecule with a large peptide backbone of approximately 400 amino acid residues. This protein backbone, if existent, may be pertinent in providing gum arabic with its exceptional functional properties in food systems such as emulsification, etc.

The purpose of this project was to determine the amino acid compositions and sequences of the major peptides of gum arabic obtained from Acacia senegal (L.) Willd. Isolation and purification of the major peptides included: enzymatic digestion, deglycosylation, HPLC chromatography, amino acid analysis as well as sequencing. Results support the view of a protein backbone consisting of at least five peptides containing similar amino acid residues.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Derek Lamport and Dr. Marcia Kieliszewski for the opportunity to work on this project and the use of laboratory equipment and space. I look forward to participation as co-author with Dr. Derek Lamport and Dr. Marcia Kieliszewski on any future patent considerations. Also, I wish to extend my gratitude to Brad, Abdol, and Larry for their assistance and patience. Special thanks to Dr. Jerry Cash, Dr. Mark Uebersax, and Dr. Matt Zabik for being on my committee and offering advice. Finally, I would like to thank my family for their financial and emotional support and patience.

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LIST OF ABBREVIATIONS

Asx	Aspartate or aspartic acid
dGAGP	deglycosylated gum arabic glycoprotein
dH_2O	distilled water
GAGP	Gum arabic glycoprotein
Gly	Glycine
Glx	Glutamate or glutamic acid
HF	Anhydrous hydrogen fluoride
HILIC	Hydrophilic interaction chromatography
His	Histidine
HPLC	High pressure liquid chromatography
Hyp	Hydroxyproline
Leu	Leucine
PG_2H_1	Pronased peptide, gel filtration peak 2, Hamilton column desalt peak 1
PG_2H_2	Pronased peptide, gel filtration peak 2, Hamilton column desalt peak 2
$PG_1F_1H_1$	Pronased peptide, formic acid desalt peak 1, Hamilton column peak 1
$PG_1F_1H_2$	Pronased peptide, formic acid desalt peak 1, Hamilton column peak 2
PH_3G_2	Pronased peptide, Hamilton column peak 3, gel filtration peak 2
PolyLC	PolyHYDROXYETHYL Aspartamide
Pro	Proline
PRP	Polystyrene reverse-phase

List of Abbreviations....continued

SEC	Size exclusion chromatography
Ser	Serine
TFA	Trifluoroacetic acid
Thr	Threonine
Tyr	Tyrosine

INTRODUCTION

Gum arabic, or acacia, is a natural plant exudate secreted by various species of Acacia trees, primarily Acacia senegal (L.) Willd found chiefly in Africa. The gum is gathered by hand, sorted for quality and can be processed by several different methods, two of which include spray-drying and grinding. Over half of the supply of gum arabic is sold to the food industry where it is considered a GRAS food additive and utilized in numerous food products ranging from beverages to confections.

The advantages of gum arabic in food systems are its high solubility and low viscosity compared to other gums, making it especially useful for emulsification and spray drying applications. Other applications for gum arabic in the food industry include use as a thickener, stabilizer, surfactant, protective colloid, and flavor encapsulation. Due to its exceptional characteristics, gum arabic is the most widely used gum by not only the food trade but also the cosmetics industry.

The objectives of this research are to; 1) determine if gum arabic has a significant portion of protein and 2) if protein is indeed present, determine the amino acid composition or primary structure of this hydrocolloid. Knowledge of the amino acid composition would be beneficial in eventually understanding how and why gum arabic exhibits its functional properties, which may lead to better methods of

improving the capabilities of this food ingredient. Ultimately, gum arabic could be synthesized, promoting a steady cost and supply, and the structure modified to needed functions in food systems.

Included in the literature review are the origins of gum arabic, such as the countries involved in import, and the increasingly unstable cost and supply. The status of gum arabic as well as identifying physical and chemical attributes are addressed under the section; Characteristics of Gum Arabic. Finally, the original molecular structure and new, redefined structure of gum arabic are examined with special reference to the protein component.

I. Origin of Gum Arabic

Gummosis is the term commonly used to describe the process of gum exudation from a plant. The bark of the tree is usually wounded and subsequently scarred to force the plant to prevent tissue desiccation by producing gum in defense. Gum arabic is one of the natural plant gums which is exuded from the stems and branches of thorny Acacia senegal (L.) Willd. trees as well as other Acacia species (FCC III, 1981). Acacia senegal accounts for around 80% of the production of gum arabic with Acacia seyal, A. laeta, A. campylacantha, and A. drepanolobium supplying the remaining 20% (Anderson, 1977).

The primary production area of gum arabic is the so-called "gum belt" throughout the Sahelian regions of Africa. This area, which accommodates approximately 500 Acacia

species, expands east to west across Africa and includes such countries as Senegal, Nigeria, and Sudan. Sudan accounts for 90% of the world's supply of gum arabic followed by Senegal, Mauritania, and Nigeria (Adamson, 1974, Joseleau and Ullmann, 1990). Australia, India, Central and North America contain 600 species of Acacia with the Australian species yielding purer and whiter gum arabic (Glicksman, 1983). Also, a few of the Acacia trees of Australia allow more efficient and cheaper gum collection due to the reduction of thorns in these varieties.

The tedious method of gum arabic collection by local African natives involves piercing and stripping the bark of the trees then returning later to gather the dried, tear drop shaped, spherical balls that have formed. Unhealthy trees, whether from nutrient deficiency, environmental or physical damage produce more sizable amounts of gum. At any rate, the gum is harvested by hand and usually stored until enough is accumulated for transportation to the major central market of El Obeid, Sudan. Next, gum arabic is cleaned and hand-sorted into two distinct grades based on size and color; "hand-picked selected" and "cleaned amber sorts". The largest, colorless pieces (free of defects such as tannins) are considered the purest and assigned the grade of "hand-picked selected". This grade of gum is usually bland with no off-taste, whereas poor quality gum contains tannins which give the gum an unpleasant flavor and odor when used in food products. Both grades are packaged in burlap bags and exported to various suppliers and

distributors around the world for further processing.

Cleaning and processing of gums was introduced in the mid 70's in order to improve the dissolving quality of gum. While cleaning is required, processing is not. Cleaning is usually done mechanically with raw gum and the end product is either kibbled gum or a hammered powder which has a quicker dissolution time. Two methods; roller and spray drying are chiefly used to further process and purify gum in the kibbled state.

Roller-dried gum involves the flow of gum onto steamed rollers where water is evaporated and the gum scraped off continuously. Gum film thickness is controlled by adjusting roller gaps. The final product which is in the form of large flakes easily dissolves. This process is mainly utilized by the food industry where it provides satisfactory purity with low cost.

The spray-drying procedure is done by first dissolving the gum and sieving it through several different sized screens to remove impurities. Next, the gum is centrifuged and pasteurized for further decontamination. Spray-drying is the final step whereby the solution of gum is sprayed into droplets, water is evaporated and cyclones separate the dried powder of various sized particles. An important aspect of spray-dried gum is its excellent dispersibility in solvents. The pharmaceutical industry demands the utmost degree of purity and an extremely low microbial count which is acquired by spray-drying.

Quality control including sanitation is a very prominent area of gum processing. Samples are taken each hour and the gum is checked for purity by optical rotation, thin-layer chromatography or spectroscopy. Other tests on the samples may include emulsification which is tested by measurement of surface rheology and stabilization which is tested by yield stress. Microbial assays are also carried out to meet an adequate level of safety.

A stable supply of gum arabic is dependent on both the environmental and political conditions of a nation such as Sudan. A high yield of a commodity provides substinence for the natives and promotes economic development. However, drought or manual forest destruction can be significant factors in curtailing exudate production from year to year.

The world production of gum arabic is over 100,000 tons per year (Phillips, 1980; Meer, 1980). The Sahelian region experienced a severe drought from 1970 to 1973 which lowered gum production to 30,000 tons (Phillips, 1980). From that point on development programs were initiated within the Sahelian gum belt area with aid from the western world. These programs, funded by FAO and World Bank were designed to increase the supply of gum acacia by the establishment of plantations. These plantations are effective in several ways. First of all, they help to provide income to natives and deter migration. The trees provide firewood and building material. Finally, the seeds and seedlings can be regenerated naturally and the soil protected from erosion and nutrient deficiency.

Acacia senegal has root systems which fix nitrogen and help to retain moisture in the soil.

Australia has started plantations to increase employment and promote economic wealth of aborigines. This new farming operation has helped to increase the raw gum amount and end the monopoly of the market by Sudan. Despite the investment in plantations a drought in 1985 brought about severe shortages of gum acacia from Acacia senegal. Prices increased by 600% and other species were utilized for the exudate.

After 1985, Sudan made a marked effort to develop the yield of Acacia senegal in an attempt to continue gum arabic production at elevated levels compared to other countries. They have also increased funding with an emphasis on improving marketing and research. Sudan has since been the leader in gum arabic production with 90% of the market (Awouda, 1990). This is mainly due to the uniform exudate from one species, the quality which comes from experienced producers and the security of land tenure.

Three divisions were started in Sudan to improve the gum industry. In 1958, the Gum Research Division was created to focus on research of gum arabic but eventually concentrated on extension work to increase output. Marketing of the exudate was undertaken in 1969 by the Gum Arabic Exporting Company. In 1974 production dwindled, which led to the creation of the "Management and Services Division for Gum Arabic". All three areas of production, research, and marketing were covered with the main emphasis on sustaining the yield of gum for the

future.

Various other changes are being implemented over time, such as, pricing policies to stop the development of substitutes and to secure producer and consumer interest. Price increases are maintained by revising taxes and prices to suit need and creation of a stabilization fund to guarantee the producer a secure income. Other improvements include producer organizations so that producers participate as shareholders in the company and make decisions. Current efforts are underway to improve transportation facilities, which will further reduce production costs.

Sudan is reforming the areas of quality control and research to ensure a steady supply of gum arabic for the future. Field research techniques to improve methods of collecting the exudate are already available to plantations. Security against drought is becoming available by tissue culturing of drought-resistant strains. This will help to promote long term availability of gum acacia. A significant portion of research is the industrial research aiding Sudan in thwarting gum substitutes and investigating innovative uses of the hydrocolloid. Knowing the chemical structure could aid in uncovering and understanding potential functions of gum arabic in food products.

To ensure the quality of gum acacia, Good Manufacturing Practices (GMP) are followed. Certain guidelines are being met to assure a superior ingredient even before trees are planted. For example, high-yielding seeds are chosen from a

reputable supplier. Spacing of trees has to be optimum (4x4 m) to deter overcrowding or inaccessibility (Awouda, 1990). Plantations are supervised closely during the growth of trees to check for fire or stunted growth. Trees grown with the above regulations can yield 75% pure gum acacia before mechanical cleaning.

Future breakthroughs in the gum industry focus on utilizing modern technology such as tapping equipment to prevent adulteration of the exudate with bark and other contaminants. Timing of when to tap will most likely play a crucial role in quality and supply of the hydrocolloid. Lastly, actual plants are being built with quality control laboratories and automated equipment to clean and grade the gum. This rise in efficiency will inevitably boost the gum market for suppliers in the United States by lowering costs while providing a superior product.

II. Characteristics of Gum Arabic

The United States alone imports 12,500 tons of gum arabic from Sudan for use in the cosmetics and food industries (Phillips, 1980). Gum arabic has long been recognized as a soluble dietary fiber containing 90% carbohydrate, 4-6% protein and 4% ash (U.S. Pharmacopeia, 1980). In 1972, the Food and Drug Administration (FDA) reviewed the teratologic and mutagenic data on gum arabic and declared the exudate a GRAS food additive. The health report of gum acacia as a food ingredient by the Select Committee on GRAS substances was also

analyzed in order to delegate the GRAS status (FDA, 1973). Table 1 shows the permitted use levels of gum arabic in food products which was put into effect to ensure safety (Federal Register, 1976). Confections are allowed to have a higher percentage of the hydrocolloid as compared to beverages with only 2%.

In 1982, gum arabic was defined by the EEC, FCC III, and the FAO as the dried gummy exudate from stems and branches of Acacia senegal (L.) Willd. A test article or sample of the exudate was given the highest safety status of Acceptable Daily Intake (ADI) not specified by the Joint FAO/WHO Expert Committee on Food Additives (FAO, 1982). This status only applies to Acacia senegal gum and does not apply to gum exuded from other species of Acacia (Anderson and Morrison, 1989).

Authorities are mainly concerned with investigating the lowest allowable quantity of an additive in a food product before the additive becomes potentially hazardous to health (Anderson, 1988c). This causes difficulty in assessing the true safety of additives utilized in substantial quantities. Labelling regulations are currently in effect which require calorie and dietary fiber information on most food items. Gum arabic has a caloric value of 4.06 kcal/g as determined by bomb calorimetry (Anderson & Eastwood, 1989). The soluble fiber content on a dry basis is 95% and gum acacia is active in lowering serum cholesterol. Toxicological studies with humans and rats indicate that the hydrocolloid is digested in the large bowel with no toxic effects.

Table 1. Permitted Usage Levels for Gum Arabic[†]

Food product	Percent
Confections	85.0
Nut products	8.3
Oils and Fats	1.5
Dairy products	1.3
Beverages	2.0

[†]Glicksman, 1983

Gum arabic is a complex polysaccharide containing magnesium, calcium and potassium ions. The pH of gum arabic is 4.5-5.5 and it is considered a strong monobasic acid (Taft, 1931). The exudate consists of 4-6% protein and six carbohydrate moieties; galactose, rhamnose, arabinopyranose, and glucuronic acid. Gum arabic has unique functional properties when compared to other hydrocolloids which justifies its use in a wide variety of food products. These properties are discussed below.

Solubility of gum acacia is superior to other gums because it dissolves well in either hot or cold water, although it is relatively insoluble in oil and organic solvents. While other exudates are limited to a 5% solution because of their excessive viscosity, gum arabic can be dissolved in solutions of at least 55%, forming a starch type gel.

One of the most distinctive properties of gum arabic is its ability to achieve a wide range of viscosities depending on concentration and other variables. Gum arabic when combined with other insoluble ingredients can stabilize and emulsify at excessive concentrations too. Thomas and Murray in 1928 determined that viscosity rises with an increase in pH to a pH of about 6 then levels off at pH 12. At concentrations up to 40% this hydrocolloid exhibits Newtonian behavior and above 40%, with an increase in shear stress, the gum becomes pseudoplastic (Araujo, 1966). A loss of viscosity can occur with ultraviolet light, and microbial growth, as well as the

addition of electrolytes. Depolymerization of the exudate can also be caused by ultrasonic or radiation treatment.

Gum acacia is a practical hydrocolloid for emulsification in food products at almost any pH range without need for a second stabilizing agent. The hydrocolloid is compatible with almost all gums, starches, carbohydrates, and proteins. However, it is not compatible with sodium alginate or gelatin where it forms a cloud. Parameters such as pH and temperature can be used to overcome these disadvantages. It is an essential component in most oil-in-water food formulations because of its protective film forming capability. This is believed to be due to a protein interface on the molecule. The current hypothesis of emulsification is that gum arabic allows dispersion by diminution of the diameters of the oil globules which prevents coalescence (Schaub, 1958). A stabilizing layer is formed in most oil-in-water emulsions whose size varies with the oil type (dispersed phase). The volume fraction and the emulsion viscosity are both affected by the size and thickness of this stabilizing layer (Glicksman, 1983).

In 1880, gum arabic was first utilized as a food ingredient. Now the food industry uses half of the total amount of hydrocolloid imported. Why use gums in food formulations? Gums are added to food formulations to prohibit undesirable physical characteristics that occur with transportation or storage. These characteristics may include mechanical disaggregation, flocculation, sedimentation or

crystallization. They can also be added to create a more aesthetically pleasing product to the consumer. At any rate, Table 2 displays the wide range of functional properties of gum arabic, which allows its application in a diverse array of food items. These items extend from bakery products where the exudate acts as a thickener and stabilizer to meat where it is utilized as a thickener.

Dry packaged food like boxed desserts, soup bases and seasonings rely on the ability of gum acacia to fixate flavors, thus prolonging shelf-life and product quality. Gum arabic is spray-dried along with the flavor mixture where it encapsulates the flavor particles with a thin film. The flavor is then stable until dissolved in water for final food preparation. The usual formulation for flavor fixation is 4 parts gum arabic to one part flavor oil. If more protection is needed against volitization or oxidation, the hydrocolloid may be increased to a 9 to 1 ratio (Wenneis, 1956; Broderick, 1954). In 1957, Wellner demonstrated the effectiveness of flavor retention by coating vanillin with gum acacia. The vanillin which normally loses 80% of its flavor during 50 days of storage at 70^0F remained stable for 50 days at 113^0F. In a similar study by Stoll, the volatile flavor chemical diacetyl was successfully coated with gum arabic and retained 18.3% of its activity whereas the control lost all the diacetyl (1952). Although gum arabic is the most effective gum in flavor fixation, cost and supply problems deter its use, thereby favoring modified starches which are cheaper and

Table 2. Certain Functions of Gum Arabic in Food Industry[†]

Food product	Function
Meat	Competitive interaction
Beverages	Stabilizer, emulsifier
Baked goods	Thickener, stabilizer
Confections	Adhesive, encapsulator
Flavorings	Encapsulator, emulsifier
Icings	Inhibitor of crystallization

[†]Stephen, 1990

in excess.

The confection industry uses half the supply of gum arabic to promote emulsification and retard sugar crystallization especially in high sugar, low moisture candy. The concentration of the hydrocolloid in these products is usually 40% or more (Fogarty, 1988). To prevent a greasy, oxidizable film on high fat candies like toffee and caramel, gum acacia is added to emulsify the lipid establishing a creamy mouth feel. In lozenge production, the exudate forms a paste base and binds the ingredients together to allow stiffening quality for stamping. A few other candies which frequently contain gum arabic are marshmallows and nougats where it is a stabilizing and softening agent. Blends of gum acacia with other substances including different acacia gums have shown promise in certain food applications.

Thirty percent of the total gum supply finds its use as stabilizers and emulsifiers in the beverage industry (Fogarty, 1988). For example, several popular brands of soft drinks mainly Mountain Dew and Faygo Moon Mist include gum arabic in their ingredient list. In many beverages such as beer and soft drinks, the hydrocolloid acts as a stabilizer of foam and is responsible for the "lace curtain" effect on the sides of a glass (Bavisotto, 1968). Wine and other alcoholic beverages can also be clarified by minute quantities of gum arabic (Bruno, 1954). In 1974, Naarden discovered that in sugar containing, non-alcoholic beverages, gum arabic inhibits turbidity. However, analyzing spray-dried beverage mixes

containing an emulsion of gum arabic and vegetable oil, Common et. al. learned that the hydrocolloid causes the formation of a cloud when dissolved in water. This cloud was similar to the cloud present in many citrus juices. It is thought that the hydrophobic amino acid content of gum arabic aids in emulsification by trapping or binding hydrophobic flavor particles.

Gum acacia is a useful additive in the baking industry due to its cold water solubility and high viscosity. The adhesive characteristics and stability are helpful in both retention and flexibility of glazes. Staling may also be inhibited by the addition of this exudate which binds water and preserves softness. Many cooking oils may be encapsulated fruitfully with gum arabic for prolonged periods under extreme temperature conditions without becoming rancid or developing an off-taste.

Finally, with meats, vitamins and grains, gum arabic exhibits much of the same functional properties listed above. An innovative use for gum arabic has been in dietitic foods. Here, the hydrocolloid can replace the bulk and texture of sucrose, thus giving the food the same sensory qualities obtained with sugar but with fewer calories. This bulking effect of the gum has also found useful application in diabetic foods.

Some factors to consider when selecting a gum for a food formulation are what particular problem needs to be solved such as stabilization, etc., will other ingredients interfere

with the behaviour of the hydrocolloid, and will the gum leave an off-flavor or aroma. The amount of time the gum takes to hydrate or disperse may also be of interest to the manufacturer. Lastly, other elements to contemplate are texture effects, microbe content, supply and most importantly, cost.

III. Protein Structure of Gum Arabic

Many studies have been undertaken on the composition of the gum arabic molecule. Knowledge of the structure of this hydrocolloid is of critical importance to the food industry in order to discern why and how it operates in various food systems. If the structure of gum arabic is uncovered, improvements as well as substitutes can be generated to enhance its usefulness in food products. Data is revealing that gum arabic may have a significant protein component which may be responsible for certain functional properties such as emulsification (Anderson, 1976). However, in the past gum acacia was thought to be composed entirely of carbohydrate with little or no protein.

In 1962 and 1966, Mukherjee, Deb, and Warburton analyzed the hydrocolloid and found it to be a short, stiff spiral with a length of 1050Å to 2400Å depending on charge. At the time, the molecular weight was determined to be between 250,000 and 1,000,000 and varied with the procedure. For instance, one study disclosed a molecular weight of 350,000 (Anderson, 1976).

Hirst in 1966 completely hydrolyzed Acacia senegal gum into four carbohydrates; L-arabinose, L-rhamnose, D-glucuronic acid, and D-galactose. These four sugar constituents were present in other Acacia species as well but in differing proportions.

Anderson and Stoddart investigated the possibility of gel filtration techniques in determining the molecular size of gum arabic polysaccharides (1966). Initial analyses indicated that the exudate had a moisture content of 11.0%, a nitrogen content of 0.33% and a protein content of 2.1%. The sugars present were rhamnose 12-14%, arabinose 25-28%, and galactose 34-39%. In their experiment, the gum (after initial analysis) was then precipitated fractionally with sodium sulphate and autohydrolyzed on a boiling-water bath. Molecular sieve chromatography was carried out on a Bio-Gel P300 column with an exclusion limit of less than 300,000. Results demonstrated successful separation of polysaccharides by molecular size with different degrees of branching and the heterogeneity of gum acacia due to the differing amounts of galactose to arabinose. In 1956, Heidelberger et al. had obtained similar results of heterogeneity of the molecule. At any rate, Anderson and Stoddart observed that gum arabic is composed of six carbohydrates including galactose, arabinopyranose, arabinofuranose, rhamnose, glucuronic acid, and 4-O-methylglucuronic acid. The galactose units were conjectured to be reducing end groups. Evidence was shown by their studies for the presence of 6-O-(4-O-methyl-β-D-

glucopyranosyluronic acid)-D-galactose in gum arabic. No analysis of the protein was indicated in their research.

In 1969 and then again in 1975, gum arabic was discovered to be a main chain of β-1,3-galactopyranose units with side chains of 1,6 galactopyranose units ending in glucuronic acid or 4-O-methyl-glucuronic acid residues (Anderson et al.). The galactose side chains also contained additional polysaccharide group. Re-examination of the gum arabic structure was done by Churms et al. in 1983 by Smith degradation. Their results were very similar to Anderson and Stoddart's observations in 1966. The molecular weight was 340,000, the galactose 44%, arabinose 35%, and the rhamnose 9%. They hypothesized that the A. senegal polysaccharide includes uniform sugar sub-units like other arabinogalactans (AGs).

Arabinogalactan-proteins are abundant in plant tissues. Gum arabic is considered a Type II arabinogalactan because it contains a 1,3-β-galactopyranosyl backbone substituted by 1,6-β-galactopyranosyl side chains as well as protein linkages. One assay to detect an arabinogalactan is the precipitation of the proteoglycan with Yariv's (synthetic carbohydrate) antigen. Binding is thought to occur in a hydroxyproline-rich site (Jermyn and Yeow, 1975 and Gleeson and Jermyn, 1979).

In 1982, Akiyama, Eda and Kato were able to precipitate gum arabic and tobacco arabinogalactan-proteins (AGPs) with β-galactosyl Yariv antigen during gel diffusion. On the basis of this characteristic, they predicted gum arabic to be an

arabinogalactan-protein. Akiyama et al. conducted various experiments to further confirm their hypothesis that gum acacia was similar to AGP (1984). Their initial analysis of gum arabic resulted in 2.0% protein, arabinose 38%, rhamnose 17%, and galactose 45%. These observations correlated well with Anderson and Stoddart's (1966) and Churm et al. (1983) results. Purification of the hydrocolloid was done by the use of DEAE-Sephadex A-25. Amino acid analysis revealed that intact gum arabic contained a high level of hydroxyproline, proline, and serine but was relatively deficient in methionine and arginine.

These same authors decided to investigate the gum's sugar-protein linkages. They hydrolyzed the exudate with saturated barium hydroxide in order to uncover the protein-carbohydrate linkages (Lamport and Miller, 1971) and fractionated it on a gel filtration column. The void volume contained 23% of the hydroxyproline content which proved to be similar to the original analysis of the gum, that of highly branched polysaccharides. Sixty-four percent of the hydroxyproline eluted before free hydroxyproline and contained arabinose indicative of hydroxyproline oligoarabinoside.

β-elimination was done to determine if there was a connection between serine or threonine and the sugar substituent. Gum arabic was again alkaline hydrolyzed and analyzed for the amino acids present. Serine content was reduced and alanine increased while threonine remained constant suggestive of a serine-carbohydrate bond.

Gum acacia was deglycosylated by Akiyama and Kato (1984) with hydrogen fluoride in pyridine and reacted with Yariv's antigen after pronase digestion. The reaction was positive and this convinced the authors' of a hydroxyproline-rich region in the gum arabic molecule which is not affected by enzyme cleavage.

The structure of the carbohydrate portion of the Acacia senegal gum was studied in 1986 by Defaye and Wong as well as Aspinall and Knebl. Their conclusions, similar to previous researchers, suggest a 1,3-β-D-galactan core with β-D-galactopyranose residues as side chains. Outer chains at positions 3 and 6 may contain D-glucuronic acid, L-arabinose L-rhamnose or other variations of these carbohydrates.

Research has been lacking on the protein component of gum arabic. The studies which have been performed have demonstrated the presence of a few amino acids, mainly hydroxyproline, proline and serine. One scientist in the forefront of exudate protein assay is Dr. D.M.W. Anderson, who has written several research papers on the composition of amino acids from gum arabic.

Anderson and McDougall in 1987 examined the protein structure of gum arabic and its relationship to the sugars present by utilizing four sequential Smith-degradations (0.34, 0.56, 0.87, 0.90) and amino acid analysis. The Acacia senegal (L.) Willd. exudate which initially had a 31:1 molar polysaccharide/protein ratio was degraded to a 11:1 molar polysaccharide/protein ratio after the fourth Smith-

degradation. This last product eliminated 85% of the protein and 94% of the carbohydrate and consisted of proline, hydroxyproline, serine, leucine and threonine as part of a branched galactan core which is consistent with glycoproteins. The Smith-degradation reactions continuously removed large amounts of sugars and only small portions of the amino acids making the molecule more proteinaceous than the original gum. For instance, the first stage removed 78% of the arabinose, 97% of the rhamnose, and 61% of the galactose but only a small percentage of the amino acids. The authors hypothesize that the amino acids may be in the interior of the molecule associated with arabinose and galactose units away from the carbohydrate exterior.

Anderson and McDougall (1987) determined that hydroxyproline was the major amino acid left after all degradation reactions. Arginine, lysine, tyrosine, and methionine were eliminated immediately after the first degradation while alanine, glutamic acid, phenylalanine, and valine were gradually reduced after each degradation. Many amino acids such as leucine, proline, serine, histidine, and threonine increased after each degradation sequence. Anderson and McDougall predicted that the gum arabic structure consists of a protein interior which cannot not be cleaved by enzymes or chemicals unless the whole molecule is totally damaged. They attribute their previous failures to eliminate nitrogen and to detect protein to this fact of internal amino acid residues. Lastly, surface activity and other functional

properties such as emulsification may be lost when deproteinization is attempted.

Other experiments on the amino acid composition of the hydrocolloid were carried out by Anderson and McDougall in 1987. Data included results of three hydrolysis reactions on gum arabic solutions. To begin with, a sample of gum arabic was dissolved in water and subjected to sieving and centrifugation. Autohydrolysis was undertaken in a boiling water bath for 48 hours and then centrifuged and dialyzed (recovery: 82%, protein: 1.73%). Sulfuric acid was used to acid hydrolyze gum arabic in a boiling water bath (recovery: 47%, protein: 3.38%). Finally, the sample was subjected to ultraviolet radiation (recovery: 84%, protein: 1.52%). The diffusate, degraded gum and the insoluble material were analyzed. Results showed that both the insoluble material and the diffusate contain carbohydrate and amino acids. The diffusate contained serine, glycine, and glutamic acid while the insoluble material contained mostly alanine, glycine, leucin and valine. Degraded gum consisted of hydroxyproline, serine, proline, and threonine.

Anderson and McDougall heated gum arabic at 90^{0}C for 4-6 hours in another experiment which resulted in the release of arabinose, rhamnose and amino acids. They advise that heat treatments be reduced to prevent protein denaturation and degradation which may affect the hydrocolloid's functional properties.

Fenyo et al. in 1985, 1987, and 1988 purified gum arabic

into two separate components. The first component (~30%) was a high molecular mass arabinogalactan-protein while the second was a protein deficient low molecular mass fraction. Their hypothesis of the gum's structure was that of a "wattle blossom" model. This spherical model has a main polypeptide backbone (1600 amino acid residues) with attached carbohydrates of approximately 200,000 molecular weight. The authors suggest that the protein interior assists in emulsification by binding hyrophobic substances such as oil, while the exterior carbohydrates bind hydrophilic medium such as water.

In 1989, Randall et al. used hydrophobic affinity chromatography to separate gum arabic into three parts. These fractions are an arabinogalactan (AG) 88.4%, a glycoprotein (Gl) 1.2%, and an arabinogalactan-protein (AGP) 10.4% which can be enzymatically digested by pronases. The percentage of protein in the three compared to intact gum (2.24%) was arabinogalactan (.35%), arabinogalactan-protein (11.8%), and glycoprotein (48%). Their data coincides with that of Fenyo et al. (1985, 1987, and 1988) except for the identification of a glycoprotein constituent.

Significant research in the area of gum arabic carbohydrate and protein chemistry was carried out by Qi et al. in 1991. The scientists propose a new model for the gum arabic glycoprotein structure based on their data. To begin with, a Superose-6 preparative column was used to separate the exudate into two distinct components. These two components

consisted of a heterogeneous low molecular weight polysaccharide and a higher molecular weight glycoprotein (GAGP). The gum arabic glycoprotein (GAGP) was further analyzed and determined to contain approximately 90% sugar. Anhydrous hydrogen fluoride treatments at different time periods and temperatures were used to remove the carbohydrate portion of the Superose-6 gel purified gum arabic glycoprotein. The most successful treatment was for 2 hours at 0^0C as indicated by the pure peak of dGAGP eluted from an analytical Superose-6 column. From this data it was concluded that the gum arabic glycoprotein is 93% (determined by weight loss from deglycosylation).

The authors discovered that the gum arabic glycoprotein reacted positively with Yariv's reagent correlating with Akiyama et al. results (1984). A Sephadex G-25 column was used to determine gum arabic glycoprotein linkages after alkaline hydrolysis. Data showed a low molecular weight compound which was hypothesized to be Hyp arabinosides. A Hyp glycoside profile of crude gum arabic was obtained by use of a Chromobeads column where the researchers observed 12.1% monoglycosylated Hyp, 63.5% oligoglycosylated Hyp, and 24.3% polyglycosylated Hyp. A further hydrolysis of Hyp-polysaccharide on Chromobeads portrayed four peaks eluting. Two were identified as free Hyp and two were O-galactosylhydroxyproline.

Calculations on the molecular size of the gum arabic glycoprotein were carried out and it was determined to be 220

kD with a ~400 residue peptide backbone. From the Hyp/Pro content it was concluded that the molecule was not spherical as suggested by Fenyo et al. but rod shaped.

Amino acid composition results on crude gum arabic, GAGP, and dGAGP indicate the presence of seven major amino acids. These are hydroxyproline (~30 mol%), serine, threonine, proline, glycine, leucine, and histidine. The authors predicted from this information a 10-12 residue repetitive peptide backbone with empirical formula; Hyp_4 Ser_2 Thr Pro Gly Leu His for gum arabic glycoprotein with polyglycosylated Hyp at every twelth residue. The model postulated resembled a "twisted hairy rope". The structure consists of a main protein core with Hyp-arabinosides and attached galactan backbones containing rhamnose, arabinose, and glucuronic acid sidechains. They feel that hydrogen bonding would be increased by the Hyp-arabinosides and the B-galactan backbone thus, forming a double helix which could entrap flavors. Lastly, electron microscopy results confirmed for Qi et al. that the gum arabic glycoprotein is not a "wattle blossom" suggested by Fenyo et al. but a "twisted hairy rope" which could help explain its ease of exudation through the Acacia senegal cell wall.

IV. Purpose of Research

The objective of this research was to determine if gum arabic contained a significant interior protein backbone as hypothesized. If so, then to analyze the composition and sequence of amino acids present, using the following steps:

1. Separate and purify the protein component of gum arabic (GAGP) by gel filtration.
2. Deglycosylate the gum arabic glycoprotein to yield the highest percentage of protein.
3. Cleave the gum arabic protein into several low molecular weight peptides with an appropriate enzyme.
4. Use various HPLC columns to purify the individual peptides and determine molecular size.
5. Determine the amino acid composition of the gum arabic protein backbone by amino acid analysis.
6. Obtain the sequences of the homogeneous peptides by Edman degradation.

MATERIALS AND METHODS

I. Initial Procedures to Isolate and Purify Protein of Gum Arabic

A. Source of Gum Arabic

Gum arabic (Acacia senegal) nodules were a obtained from Pepsico, Inc. A Tekmar A-10 mill was used to grind the nodules for two minutes into a fine, white powder.

B. Isolation and Purification of Gum Arabic Glycoprotein

Gum arabic was isolated and purified with a Pharmacia Superose-6 preparative column (1.6 x 50 cm; 30 µm particle; Superose buffer = 0.2 M phosphate, pH 7.0). Two hundred milligrams of gum arabic were dissolved in 2 ml distilled H_2O and the solution was centrifuged for 10 minutes x max speed in a microfuge. The whole 200 mg sample was injected onto the column. The flow rate of the column was 1 ml/min. and the eluate was monitored at 220 nm. The data were recorded using PE Nelson Turbochrom software run on a Compaq 386. The first peak was collected with an elution time of 48 minutes corresponding to high molecular weight gum arabic glycoprotein (see Figure 1). The collected fractions were then dialyzed for 72 hours at 4^0C, freeze-dried, and desiccated overnight (over P_2O_5). A 10% yield was obtained from each run (~20 mg GAGP/200 mg gum arabic) so material was pooled from 20 runs to get enough dried material (400 mg) for HF deglycosylation.

C. Anhydrous HF Deglycosylation

Gum arabic glycoprotein (~400 mg) was deglycosylated 3 hr

at 0^0C using 10 ml anhydrous HF (10% v/v dry methanol) per 400 mg material (Sanger & Lamport, 1983). The reaction was quenched using a 10:1 ratio of cold dH_2O (100 ml) to hydrogen fluoride. The resultant solution (100 ml) was dialyzed for 72 hr at 4^0C against dH_2O and then freeze-dried. An 8% yield (~30 mg) of deglycosylated gum arabic glycoprotein was obtained. Gum arabic glycoprotein was checked for deglycosylation by injecting 3.8 mg/ml dH_2O on an analytical Superose-6 gel filtration column (1.0 x 30 cm; 14 µm particle). The elution time of dGAGP was compared to the elution time of gum arabic glycoprotein and the later elution time of 27.61 minutes as compared to 20.17 minutes indicated a lower molecular weight compound because of deglycosylation (see Figure 3).

II. Pronase Digestion of dGAGP

Ten milligrams of deglycosylated gum arabic glycoprotein per 1 ml dH_2O and 10 mM $CaCl_2$ were denatured by boiling for 5 minutes and cooling in ice. The pH of the sample was then brought to 8 with NaOH and a Streptomyces griseus protease enzyme (Calbiochem) added at an enzyme:substrate ratio 1:100. The dGAGP was digested overnight in a pH Stat (Radiometer - Copenhagen, Denmark).

The digestion was monitored every 15 minutes by injection of 2 µl on a PolyHYDROXYETHYL Aspartamide column (PolyLC; 9.4 mm I.D. x 200 mm) in gel filtration mode until complete to determine the size of peptides. Elution was done isocratically with a pH 3.0 buffer containing 0.2 M Na_2SO_4, 5

mM KH_2PO_4 and 25% MeCN and the run was monitored at 220 nm with a diode array detector.

III. Peptide Mapping, Isolation and Purification of the Various Gum Arabic Peptides

A. Purification of Peptide 1 (PH_3G_2) before Amino Acid Analysis

Three hundred micrograms of the pronase digest were centrifuged (10 minutes x max speed in microfuge). The supernatant was loaded onto a reverse-phase HPLC Hamilton PRP-1 (4.1 mm x 150 mm) column at a flow rate of .5 ml/min. and monitored at 220 nm. Two buffers were used: A) 0.1% TFA, and B) 0.1% TFA/80% MeCN. The following gradient was utilized for elution with buffer A and buffer B above:

PH_3G_2 gradient on PRP-1 column:

Time (min.)	Flow	%A	%B
0	0.5	100	0
100	0.5	100	0
105	0.5	75	25
110	0.5	75	25
130	0.5	100	0
200	0.05	100	0

The major homogeneous peptide was collected (peak 3) with an elution time of 30 minutes and the solvent was blown off with nitrogen.

1. Further Purification of PH_3G_2 by SEC

PH_3G_2 (peak 3) was then loaded onto a PolyLC column for

further size purification. The peptide of interest eluted as peak 2 at 514 seconds and was collected and the solvent blown down with nitrogen (see Figure 4).

To double check that the peptide eluting at 514s was the same peptide which eluted at 30 min. on the reverse-phase column the collected peak was loaded back onto the PRP-1 column to verify elution time.

2. Desalting of PH_3G_2 on Hamilton reverse-phase

After being blown to dryness with nitrogen, peak 2 (~514s) from PolyLC column was injected back onto the PRP-1 reverse-phase column for desalting. The salt voided the column while the peptide eluted at ~30 min (see Figure 5). Several runs were pooled together to get enough protein (10 µg) for amino acid analysis.

B. Purification of Peptide 2 (PG_2H_1) and Peptide 3 (PG_2H_2) before Amino Acid Analysis

Several runs were completed with the pronase digest on the PolyLC column and the second peaks (with elution times of 472s) were collected for each run and pooled together (see Figure 4). The buffer was then blown down with nitrogen.

1. Desalting of PG_2H_1 and PG_2H_2 on Hamilton reverse-phase

The pool of 470s peaks collected from the PolyLC were injected onto the PRP-1 reverse-phase column where they eluted as two separate peaks, one at 31 min. and the other at 39 min. (refer to Figure 9). These two peaks were collected separately and blown to dryness with nitrogen in preparation

for amino acid analysis.

Again, these two peaks were individually loaded back onto the PolyLC column to make sure they were not impurities and that they both eluted at 470s.

C. Purification of Peptide 4 ($PG_1F_1H_1$) and Peptide 5 ($PG_1F_1H_2$) before Amino Acid Analysis

The pronase digest was loaded onto the PolyLC column and peak 1 (420s) was collected this time (refer to Figure 4). Several runs were pooled together.

1. Desalting of $PG_1F_1H_1$ with Formic Acid

The PolyLC column was used in the hydrophilic interaction mode with a buffer of 50 mM of formic acid. The salt voided the column and the resulting peak of interest was collected and blown to dryness (see Figure 10).

2. Further Purification of $PG_1F_1H_1$ by reverse-phase HPLC

$PG_1F_1H_1$ was injected onto the PRP-1 column where the peptide eluted as two heterogeneous peaks (~35 min. and 50 min.) indicative of two different peptides (refer to Figure 12). These were both collected separately after several runs and blown dry for amino acid analysis preparation.

IV. Amino Acid Analysis and Peptide Sequencing

A. Amino Acid Analysis of all Peptides

1. Acid hydrolysis of Peptides

All individual peptides were dried and hydrolyzed with 200 µl of 6N constant boiling HCl in a closed tube

for 18 hr at 110^{0}C.

2. Amino Acid Analysis using Cation Exchange

Amino acid analysis involved a Pickering High Speed Sodium Cation Exchange column (3 x 150 mm) with buffers A, B, and C (Buffer A: Na+ eluent, pH 3.15, Buffer B: Na+ eluent, pH 7.4, [Na+] = 1.0 N, C: Na+ Regenerant, [Na+] = 2.0 N). NaOCl oxidation followed by OPA coupling was used to detect secondary amino acids, Hyp and Pro by post-column derivatization (Yokotsuka & Kushida, 1983). A 22.7 mM solution of N,N-dimethyl-B-mercaptoethylamine HCL was used as the reductant for this reaction (Frister et al., 1988). A Gilson 3301 Spectra/Glo fluorometer (360 nm excitation, 455 nm emission) was available to monitor the eluate. A Compaq 386 and P.E. Nelson Turbochrom II software were utilized to collect data.

B. Peptide Sequencing

Peptides were sequenced via Edman Degradation (Edman, 1970) on a 477A Applied Biosystems, Inc. gas phase sequencer at the Michigan State University Macromolecular Facility by Joe Leykam.

RESULTS & DISCUSSION

I. Initial Procedures to Isolate and Purify Protein of Gum Arabic

A. Elution and Purification of Gum Arabic Glycoprotein

Gum arabic when injected onto the Superose-6 preparative column and purified by gel filtration eluted into two separate peaks (Figure 1). The first peak coincided with a homogeneous, high molecular weight gum arabic glycoprotein (GAGP) which contained about 10% hydroxyproline-rich protein and 90% carbohydrate. The second heterogeneous peak contained mainly glucouronic acid and an insignificant amount of protein deficient in hydroxyproline which was termed gum arabic polysaccharide. Gum arabic glycoprotein accounted for 10% of the total mass of gum arabic while gum arabic polysaccharide accounted for 90% of the total mass. Only the GAGP (peak 1) was collected after several runs and pooled together until enough was available to be hydrogen fluoride deglycosylated.

A small portion of gum arabic glycoprotein was loaded onto an analytical Superose-6 column to certify that when collected it was indeed homogeneous and pure (Figure 2). This proved to be the case due to the steepness of the peak with an elution time of 20.13 minutes.

B. Deglycosylation of Gum Arabic Glycoprotein

Gum arabic glycoprotein was deglycosylated by

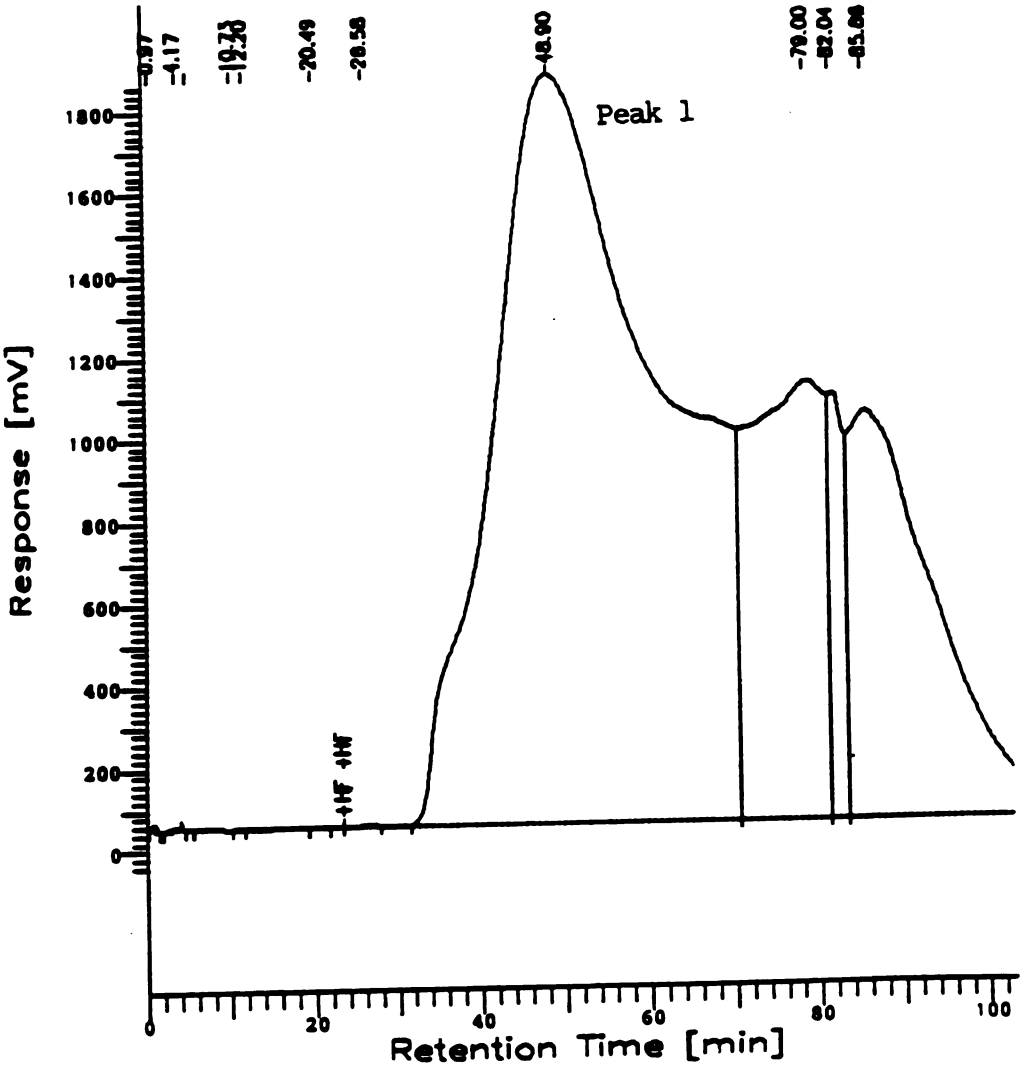

Figure 1. Gum arabic on Superose-6 Prep Column

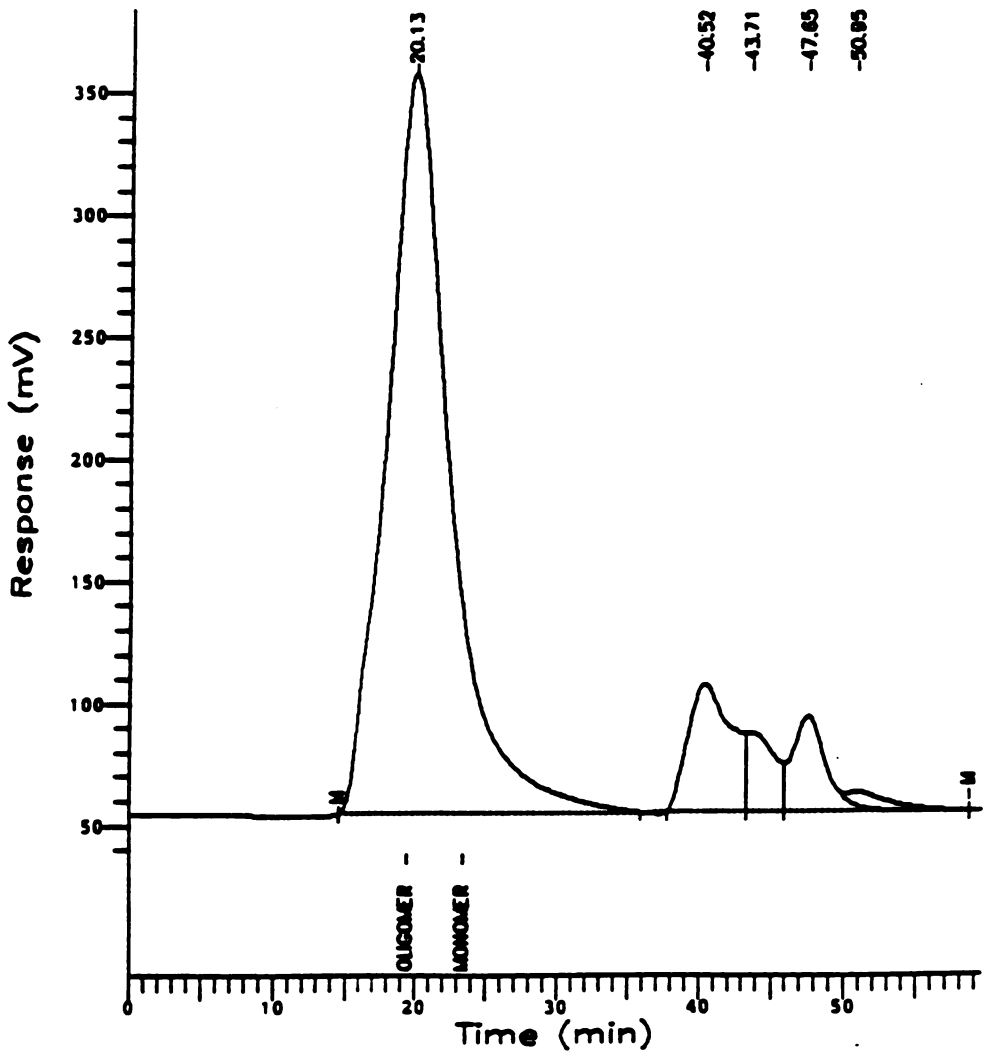

Figure 2. Gum Arabic Glycoprotein on Superose-6 Column

anhydrous hydrogen fluoride when ~400 mg was accumulated. This procedure removed all carbohydrate and resulted in a yield of about 7-8% protein or ~30 mg (dry weight) of deglycosylated gum arabic glycoprotein (dGAGP) signifying an immense amount of sugar in GAGP. Gel filtration was employed to determine the accuracy of deglycosylation by utilizing an analytical Superose-6 column. Completeness of deglycosylation was determined by comparing the elution time of dGAGP with that of GAGP on the column (Figures 2 and 3). A later elution time by dGAGP of 27.61 minutes indicated a decrease in molecular weight corresponding to carbohydrate loss.

II. Enzymatic Digestion of dGAGP

After deglycosylation, enzymatic digestion of dGAGP was undertaken in a pH stat by the use of a non-specific pronase. The time course of the digest was monitored every 15 minutes by gel filtration on a PolyLC column until digestion was complete (18 hours). Figure 4 shows a peptide map of an 18 hour overnight digestion on the PolyLC column. Three major peptides (elution times of 422s, 472s, and 507s) with varying numbers of amino acid residues resulted from cleavage of dGAGP (Table 3). Amino acid residue numbers were estimated by the use of molecular weight markers injected on the PolyLC column under the same conditions as the sample digest. Peak 3 or the peptide eluting at 507s proved to be the smallest peptide with only about 12 residues while the 422s peptide had the largest molecular weight and the most residues with about 36 amino

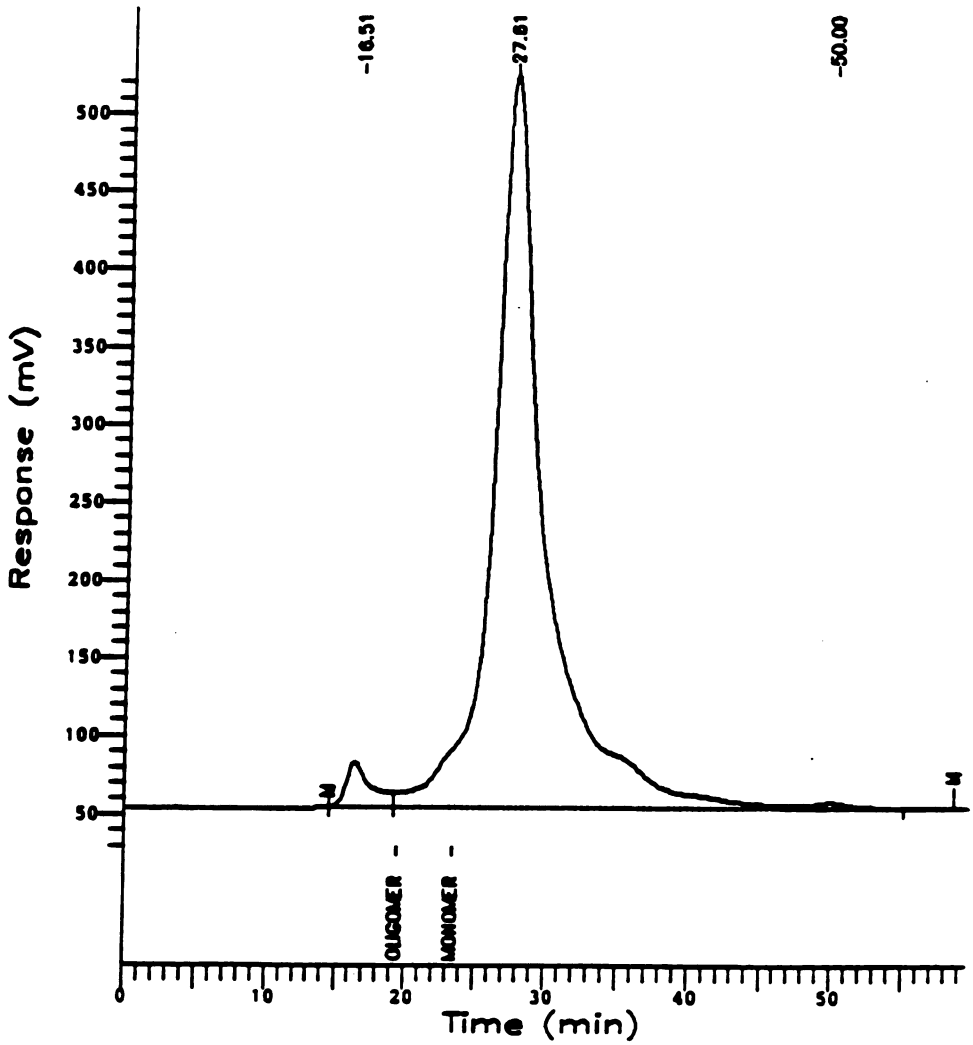

Figure 3. Deglycosylated Gum Arabic Glycoprotein on Superose-6 Column

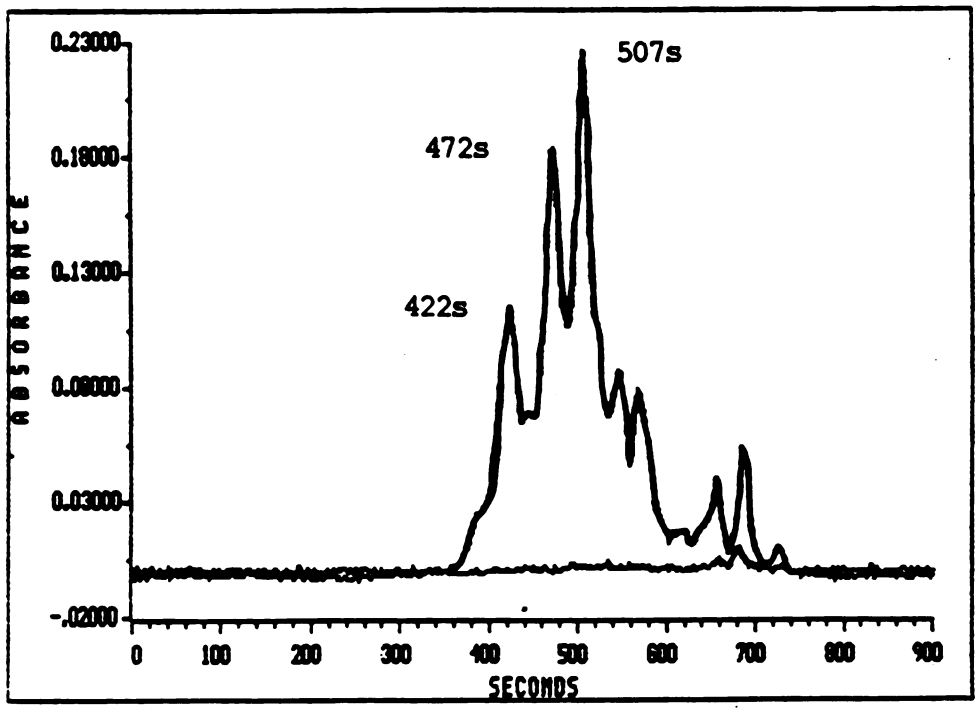

Figure 4. Pronased Peptides on PolyHYDROXYETHYL Aspartamide

Table 3. Elution times of gum arabic pronased peptides on PolyLC[*] and corresponding amino acid residues

Elution time (seconds)	Amino acid residues
422	~100
472	~36
507	~12

[*]PolyHYDROXYETHYL Aspartamide

acid residues. The pronase digestion resulted in a simple, uncomplicated peptide map with three clean peaks. dGAGP is demonstrated to be a high molecular weight protein with at least 100 amino acid residues.

III. Characterization of Gum Arabic Peptides

Each of the three peptides from cleavage of dGAGP were purified and analyzed using varying sequences of different chromatographic columns. The peptides were named according to the order of columns from which they were eluted.

A. Analysis of Peptide 1 (PH_3G_1)

1. Purification Steps

Twenty-five micrograms of the pronase digest was loaded onto a Hamilton reverse-phase column and a peptide map obtained based on the polarity of the peptides. One major peak (H_3) resolved well and appeared larger and purer than the other four (Figure 5). Since this peptide was cleaner in regard to the other peptides it was collected and blown dry with nitrogen for further purification.

The next step involved determining the size or residue number of the peptide by gel filtration utilizing the PolyLC column. This was undertaken in order to determine if it corresponded to the size of one of the three major peptides eluted on the PolyLC after enzymatic cleavage. Peak 3 from the Hamilton column was dissolved in the column buffer and injected. Figure 6 depicts the chromatogram obtained and the resolution of a small size peptide (peak G_1) with an elution

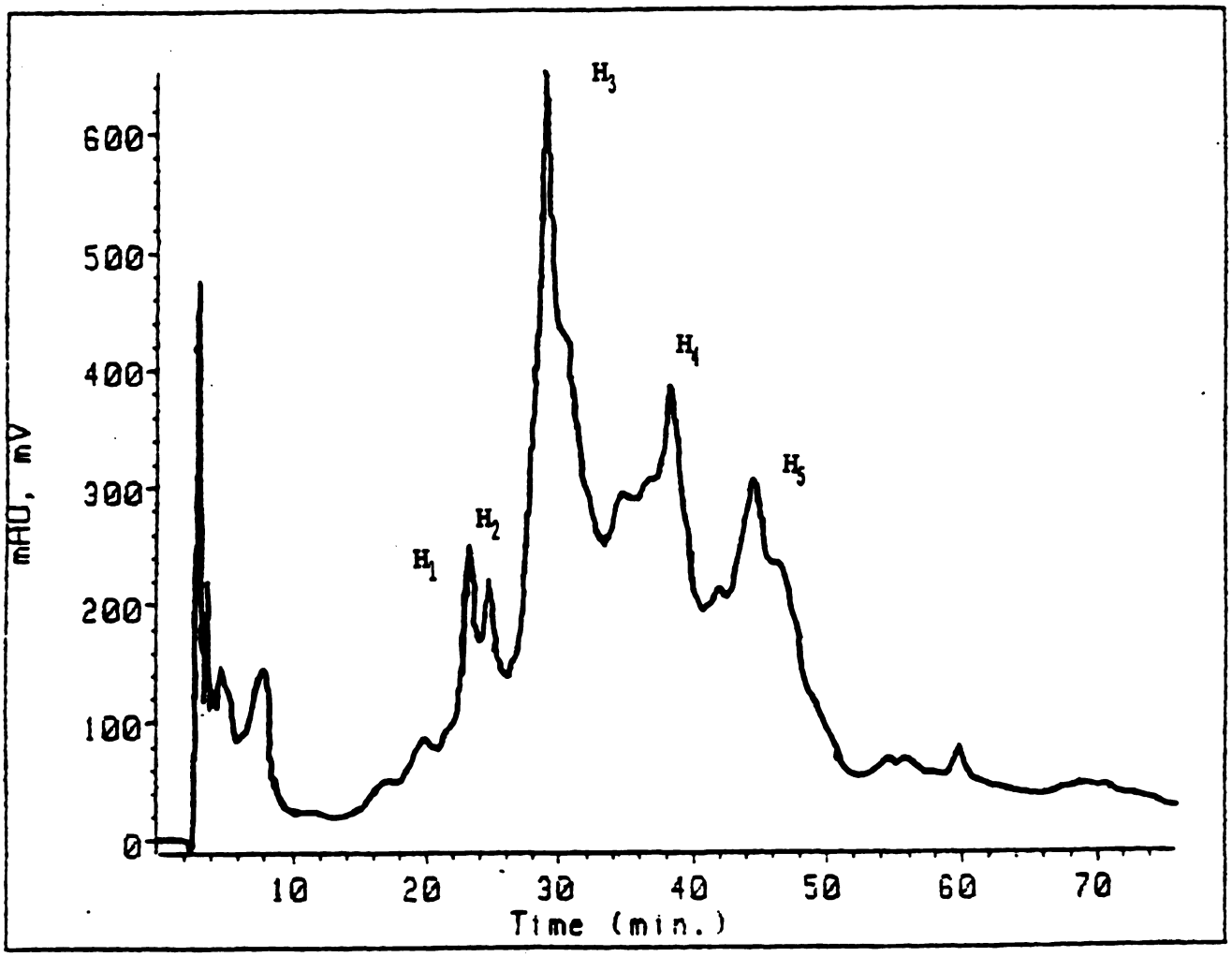

Figure 5. Pronased Peptides (H) on reverse-phase column

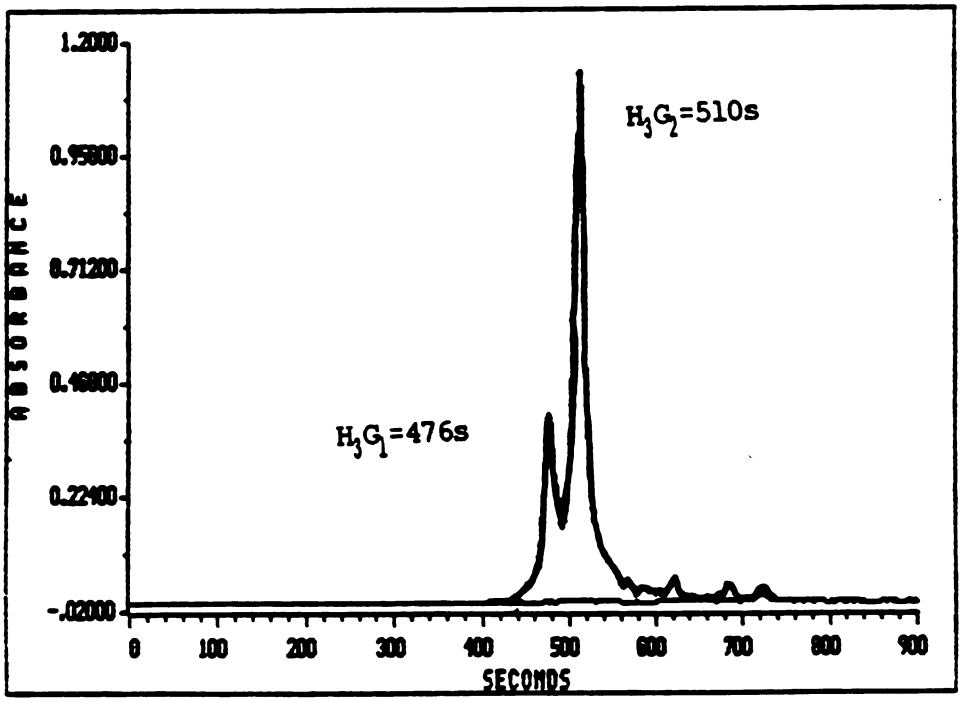

Figure 6. Peptide H_3 on PolyHYDROXYETHYL Aspartamide

time of ~510s and a somewhat larger peptide (peak G_2) with an elution time of 476s. The second peak at 510s was of the most interest since it corresponded with the results of the original peptide map of the pronase digest. This was the smallest of the three major peptides with 12 amino acid residues. Also, there was a greater amount of this second peak in comparison to the first peak which would allow for easier collection and more protein material to work with. At any rate, peak 2 was collected during several runs, pooled together and blown dry with nitrogen.

Final clean-up of the peptide before amino acid analysis consisted of desalting to remove buffer salts as well as additional purification on the PRP column. When injected on the PRP column, the salt voided the column while the peptide eluted at 37 minutes as a well-defined peak (Figure 7). A small fraction of this peak was run on the PolyLC column to certify that it was indeed the 12 amino acid residue peptide and did not contain any contaminants.

2. Amino Acid Analysis

Approximately 10 µg of the peptide PH_3G_2 were dissolved in 0.1M acetic acid and 50% retained for sequencing while the remaining half was acid hydrolyzed. Acid hydrolysis or cleavage of the protein into individual amino acids consisted of blowing dry the acetic acid with nitrogen, adding 200 µl of 6N constant boiling HCl, and heating at 110^{0}C for 18 hours in a sealed tube. When complete, the mixture was blown to dryness and 20 µl of column buffer (Pickering pH 2.2 sodium

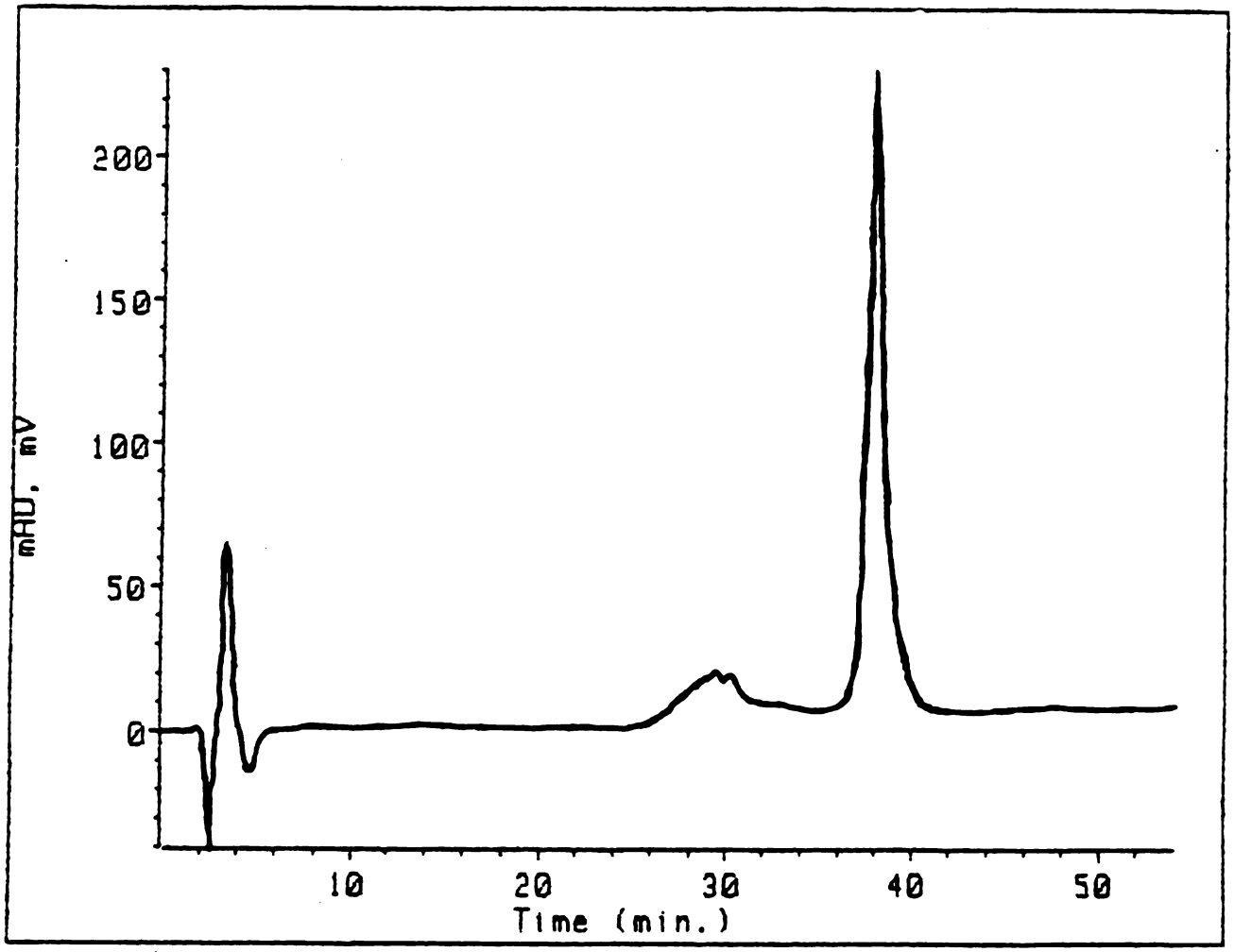

Figure 7. Desalting of Peptide H_3G_2 on reverse-phase column

diluent) added and filtered.

A cation exchange column was utilized for amino acid analysis where the most basic amino acids (strong positive charge) eluted last at a pH of 3.1. A Turbochrom computer software program was employed for analysis of peak areas and presentation of data via chromatograms. In addition, calculations of the peak areas into mole% amino acids were accomplished by using Lotus 1-2-3. Table 4 indicates the results of the amino acid analyses for crude gum arabic, GAGP, dGAGP (Qi et al. 1991) and peptide PH_3G_2. Hydroxyproline, at 46 mole%, is the amino acid found in the largest concentration followed by serine, threonine, glycine and finally proline. Therefore, the simplest composition postulated would be a twelve residue peptide with Hyp_6 Ser_2 Thr_2 Pro Gly.

3. Sequence Data

The remaining 5 µg of PH_3G_2 dissolved in .1M acetic acid were taken to the Michigan State University Macromolecular Facility for sequencing via Edman Degradation. The final sequence of the peptide correlated closely to the compositional data obtained through amino acid analysis. The sequence of peptide PH_3G_2 was Ser-Hyp-Ser-Hyp-Thr-Hyp-Thr-Hyp-Hyp-Hyp-Gly-Pro-(Pro). The last proline was ambiguous, possibly due to some sugar contamination. Gum arabic glycoprotein has carbohydrate which makes complete deglycosylation difficult.

Nevertheless, the sequence of this initial gum

Table 4. Amino acid composition (mole%) Crude gum arabic, GAGP[1], dGAGP[2] (Qi et al. 1991) and peptide PH_3G_2[3]

Amino acid	Crude Gum Arabic	GAGP	dGAGP	PH_3G_2
Hydroxyproline	32.7	36.9	30.4	46.0
Aspartate[4]	3.9	1.6	3.4	0.0
Threonine	7.0	8.8	8.8	11.5
Serine	16.3	19.4	21.0	14.8
Glutamate[4]	2.9	1.9	4.0	2.6
Proline	7.6	6.8	6.2	7.3
Glycine	6.9	6.4	7.5	9.1
Alanine	1.9	1.3	2.2	1.3
Valine	2.3	0.8	1.0	0.0
Isoleucine	1.2	0.4	0.7	0.0
Leucine	6.8	6.4	5.4	0.7
Tyrosine	0.6	0.3	0.7	1.1
Phenylalanine	1.8	0.9	1.0	2.9
Lysine	2.7	1.0	1.2	0.3
Histidine	5.8	7.1	6.4	1.5
Arginine	0.0	0.0	0.3	0.0
Protein (%w/w)	4.0	7.0		

[1] Gum Arabic Glycoprotein
[2] Deglycosylated Gum Arabic Glycoprotein
[3] pronased, Hamilton (pk. 3), gel filtration (pk. 2)
[4] could be aspartic acid or glutamic acid

arabic peptide represents an attempt to uncover the primary protein structure of this exudate. Further work would need to be done to analyze the protein's secondary structure and how this structure determines the functional properties of gum arabic in food systems. Modifications of the structure could then be made which would facilitate more practical applications of the gum. Also, cloning of the gum arabic glycoprotein could be undertaken with eventual synthesis of gum arabic.

B. Analysis of Peptide 2 (PG_2H_1) and Peptide 3 (PG_2H_2)

1. Purification Steps

Figure 8 is a chromatogram of the peptide map from the pronase digest. This time the second peptide at 472s (G_2) was of interest because it has the least number of amino acid residues of the two peptides remaining. The 472s peak was collected after several runs on the PolyLC column and the peaks pooled together and blown dry with nitrogen.

Next, the peptide was reconstituted in column buffer and injected onto the Hamilton reverse-phase column for desalting and final clean-up. Figure 9 represents this last purification step on the PRP column. As shown, the 472s (G_2) peak eluted as two distinct, broad peptides by polarity separation. The earliest peak eluted at 32 minutes (H_1) while the later peak at 39 minutes (H_2). Both peaks appeared homogeneous and uncontaminated because of the absence of shoulders. At any rate, both peaks from each run were individually collected and blown dry for acid hydrolysis and

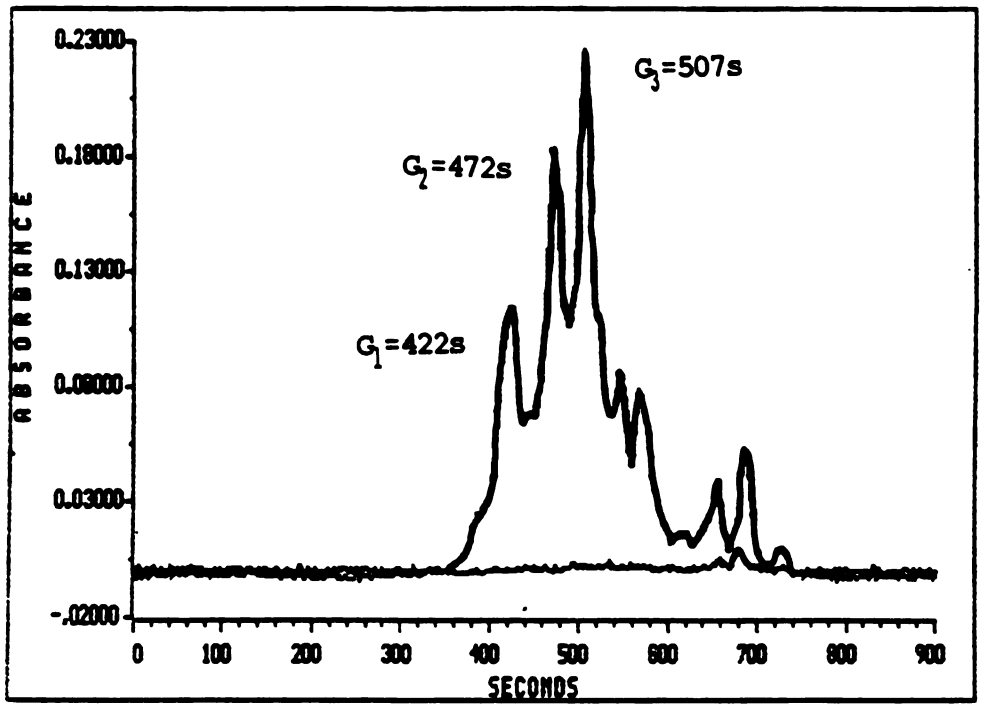

Figure 8. Pronased Peptides on PolyHYDROXYETHYL Aspartamide

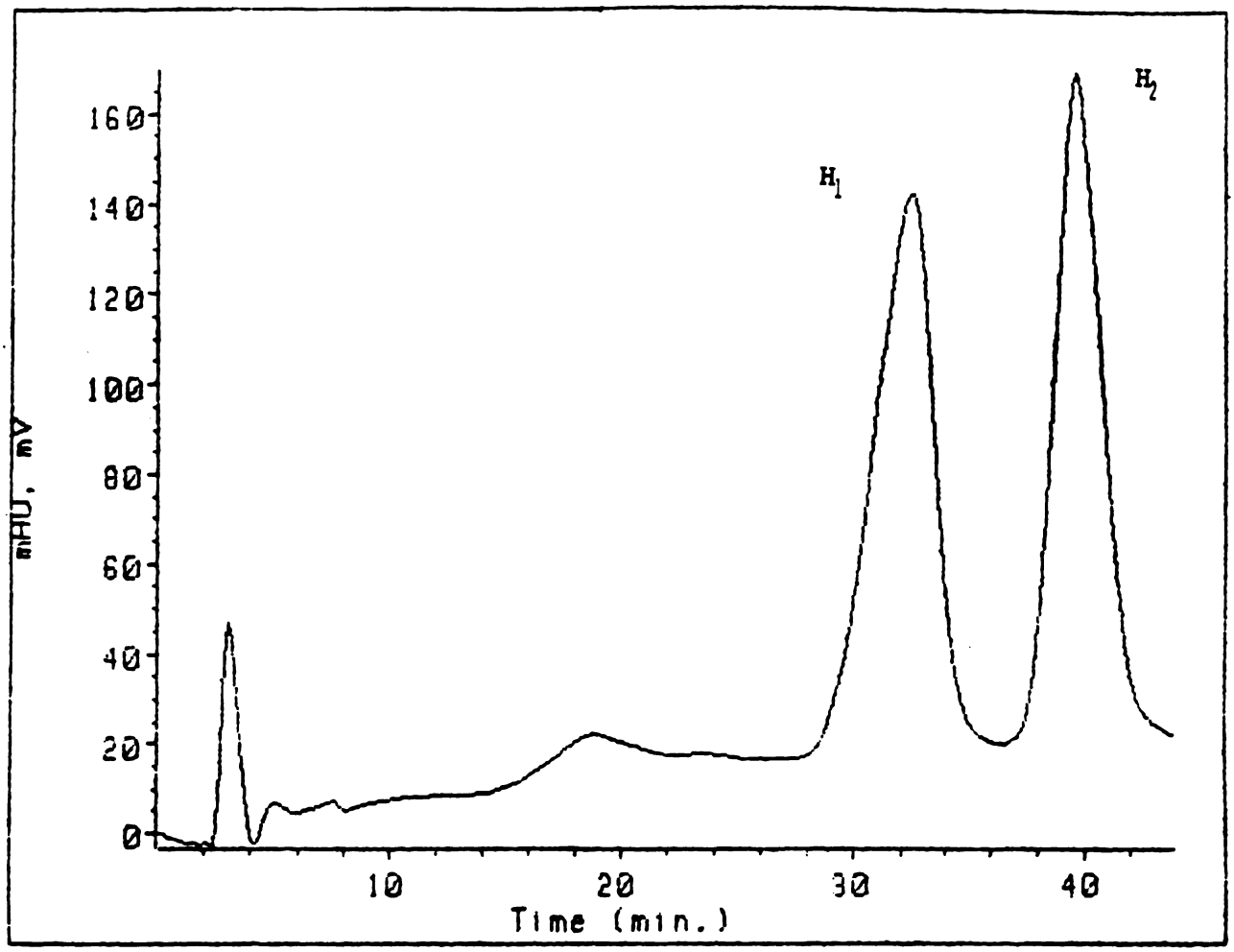

Figure 9. Desalting of Peptide G_2 on reverse-phase column

sequencing.

2. Results of Amino Acid Analysis for PG_2H_1 and PG_2H_2

Peptides PG_2H_1 and PG_2H_2 were acid hydrolyzed as stated above with constant boiling 6N HCl. Approximately 25 µg (PG_2H_1) and 18 µg (PG_2H_2) were loaded onto the sodium cation exchange column for analysis of amino acids by ion-exchange chromatography.

The amino acid content of all peptides were similar with hydroxyproline accounting for the major portion of protein at ~27 mole% for PG_2H_1 and ~23.7 mole% for PG_2H_2, and serine succeeding (Table 5). Nevertheless, PG_2H_1 was figured to have an empirical composition of Hyp_5 Ser_5 Gly_2 Thr_2 Pro His (around 16 residues) and PG_2H_2 had a composition of Hyp_5 Ser_4 Thr_2 Gly Pro His Leu Glu (16 residues). His, Glu and Leu were the three new amino acids that were not components of PH_3G_2.

3. Sequence Data

Both PG_2H_1 and PG_2H_2 were sequenced by Edman Degradation at the Michigan State University Macromolecular Facility. However, data was witheld due to pending patent consideration with myself as co-author.

C. Analysis of Peptide 4 ($PG_1F_1H_1$) and Peptide 5 ($PG_1F_1H_2$)

A numerous succession of columns were manipulated in an attempt to purify the heterogeneous peptides $P_1F_1H_1$ and $P_1F_1H_2$. The following purification scheme did not yield consistent results after every column run with regard to

Table 5. Amino acid composition (mole%) for Peptides PG_2H_1[1] and PG_2H_2[2]

Amino acid	PG_2H_1	PG_2H_2
Hydroxyproline	27.2	23.7
Aspartate*	1.8	3.4
Threonine	13.1	8.8
Serine	26.8	21.9
Glutamate*	3.1	4.5
Proline	6.0	5.5
Glycine	8.9	12.1
Alanine	1.3	2.5
Valine	0.7	0.9
Isoleucine	0.5	0.6
Leucine	1.5	4.8
Tyrosine	1.0	3.0
Phenylalanine	0.53	0.5
Lysine	0.94	1.5
Histidine	4.9	4.1
Arginine	0.4	0.7

[1]pronased, gel filtration (pk. 2), Hamilton (pk. 1)
[2]pronased, gel filtration (pk. 2), Hamilton (pk. 2)
*could be aspartic acid or glutamic acid

elution times and peak definition, but was continued regardless, all the way through to amino acid analysis. This inconsistency could be due to impurities such as carbohydrate which may be present.

1. Purification Steps

The beginning stage of purification was identical to that of the last two peptides (PG_2H_1 and PG_2H_2). First of all, the pronase digest material was injected onto the PolyLC column in the size exclusion mode (SEC) and the same peptide map was obtained as before with three major peptides eluting (see Figure 8). This time, however, the largest peptide (36 residues) was collected corresponding to peak G_1 (422s). Many runs were completed and the peaks combined into one aliquot and blown dry.

Desalting was the subsequent procedure in order to remove the salts contained in the previous column buffer. This was accomplished by converting the PolyLC column to the hydrophilic interaction mode (HILIC) and eluting with 50 mM formic acid at a wavelength of 220 nm. Figure 10 shows the outcome of 200 ul of peptide $PG_1F_1H_1$, which emerged as a predominant peak (F_1) at 398s. The additional and extremely sizable peak at 690s was low molecular weight contaminants. At any rate, peak F_1 was collected and blown dry with nitrogen.

Further refinement of PG_1F_1 on the reverse-phase column (PRP) was essential at this time to assure purity of the peptide, and to determine if the elution time corresponded

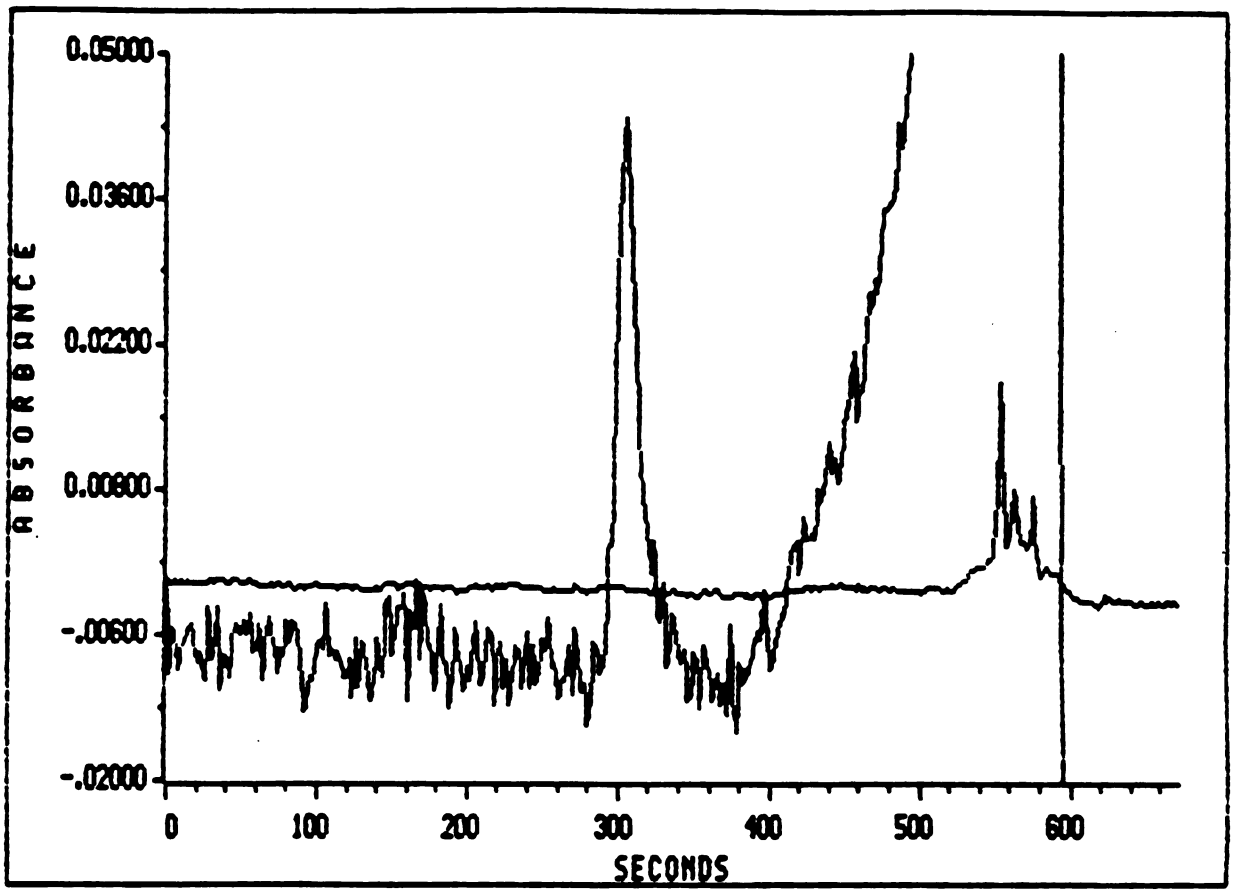

Figure 10. Desalting of Peptide $PG_1F_1H_1$ on PolyHYDROXYETHYL Aspartamide column (HILIC mode)

with the previous peptide map of the pronase digest on the PRP column (see Figure 5). Also, it was important to ascertain whether or not peak F_1 would elute as a single or double peak indicative of two distinct peptides. Various aliquots of the sample (peak F_1) were injected onto the column and a series of separate chromatograms acquired, one of which is shown (Figure 11). For each chromatogram, two heterogeneous peaks (H_1 and H_2) always materialized with varying elution times. Nevertheless, both peaks were collected separately after each run and pooled together for consequent amino acid analysis. None of the diagrams were consistent, perphaps due to either carbohydrate impurities or a mixture of several peptides which would not separate thoroughly with this particular column or buffers. Because of this inconsistency, elution times could not be compared to the prior pronase digest peptide map on the reverse-phase column.

2. Amino Acid Analysis

Each peak (H_1 and H_2) was acid hydrolyzed after the buffer was removed and evaluated for amino acids according to the previous methods listed. Table 6 specifies the mole% of amino acids for $PG_1F_1H_1$ and $PG_1F_1H_2$. The outcomes of $PG_1F_1H_1$ and $PG_1F_1H_2$ were similar to that of the other three peptides with the exception of Asx previously not detected (Table 7). $PG_1F_1H_1$ possessed an empirical composition of 16 residues; Hyp_5 Ser_3 Gly_2 His_2 Pro Thr Glu Asx and $PG_1F_1H_2$ 13 residues; Hyp_5 Ser_3 Gly_2 Pro Thr Asx.

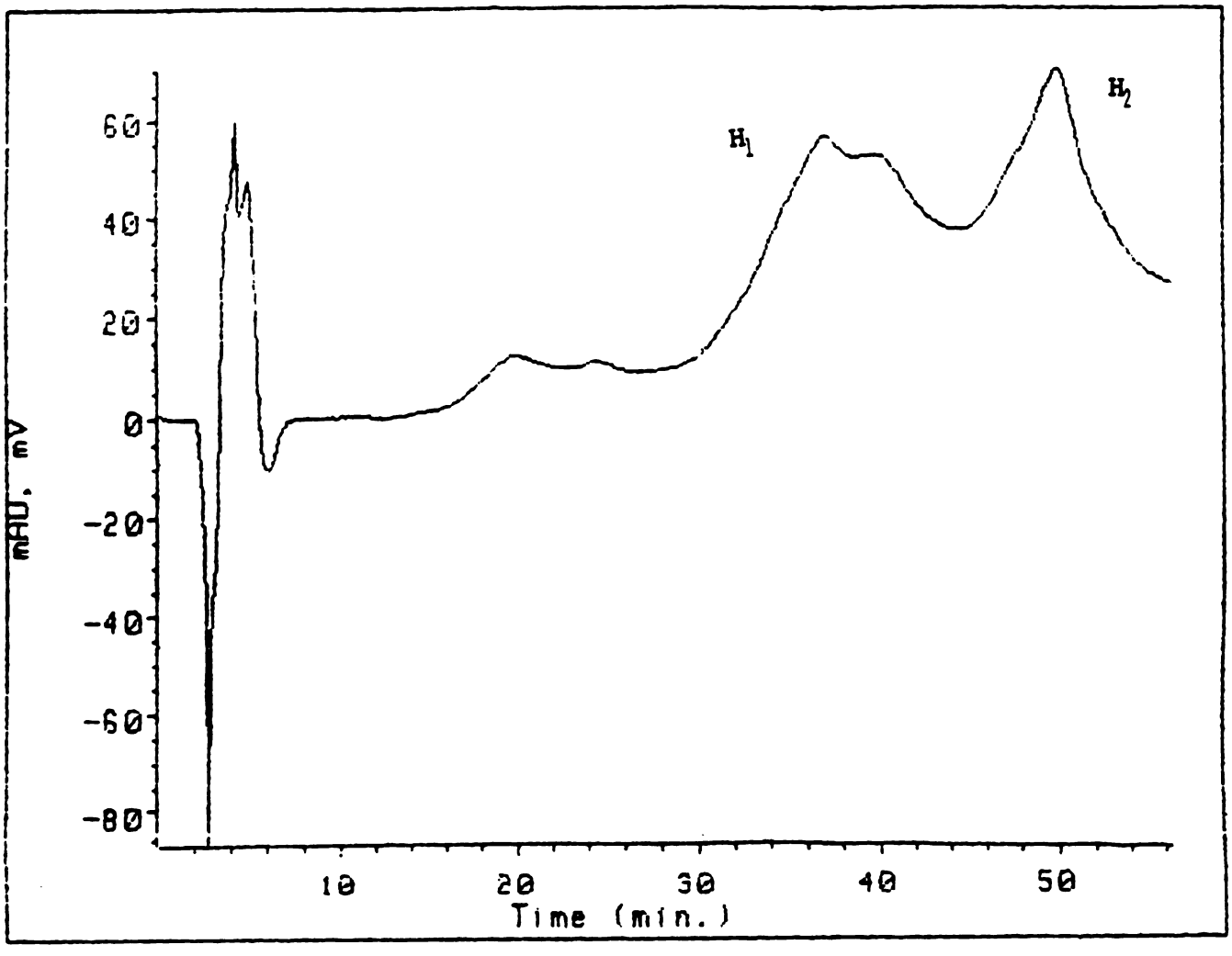

Figure 11. Final Purification of PG_1F_1 on reverse-phase

Table 6. Amino acid composition (mole%) for Peptides $PG_1F_1H_1$[1] and $PG_1F_1H_2$[2]

Amino acid	$PG_1F_1H_1$	$PG_1F_1H_2$
Hydroxyproline	25.7	26.6
Aspartate[3]	4.9	5.0
Threonine	6.5	7.0
Serine	13.9	15.4
Glutamate[3]	5.0	3.8
Proline	5.4	5.6
Glycine	8.7	9.0
Alanine	4.0	1.9
Valine	2.5	0.6
Isoleucine	1.2	0.4
Leucine	2.4	3.0
Tyrosine	2.6	0.7
Phenylalanine	1.4	0.0
Lysine	2.9	13.8
Histidine	7.9	3.0
Arginine	1.9	1.1

[1] pronased,gel filt.(pk.1),formic(pk.1),Hamilton(pk.1)
[2] pronased,gel filt.(pk.1),formic(pk.1),Hamilton (pk.2)
[3] could be aspartic acid or glutamic acid

Table 7. Amino acid composition for all five gum arabic peptides

Peptide	Amino acid composition
PH_3G_2[1]	Hyp_6 Ser_2 Thr_2 Pro Gly - - - - -
PG_2H_1[2]	Hyp_5 Ser_5 Thr_2 Pro Gly_2 His - Leu -
PG_2H_2[3]	Hyp_5 Ser_4 Thr_2 Pro Gly His Glu Leu - Tyr
$PG_1F_1H_1$[4]	Hyp_5 Ser_3 Thr Pro Gly_2 His_2 Glu - Asp -
$PG_1F_1H_2$[5]	Hyp_5 Ser_3 Thr Pro Gly_2 - - - Asp -

[1]pronased, Hamilton (pk. 3), gel filtration (pk. 2)
[2]pronased, gel filtration (pk. 2), Hamilton (pk. 1)
[3]pronased, gel filtration (pk. 2), Hamilton (pk. 2)
[4]pronased, gel filt.(pk.1),formic(pk.1),Hamilton(pk.1)
[5]pronased, gel filt.(pk.1),formic(pk.1),Hamilton(pk.2)

CONCLUSION

It can be concluded that the gum arabic glycoprotein does contain a significant protein backbone as postulated by Qi et al. This protein is able to be cleaved by pronase into distinct peptides which contain very similar amino acids such as hydroxyproline, serine, proline, threonine, and glycine. However, further sequencing data has to be acquired to determine if there is a 10-12 residue repetitive motif common to all peptides.

Gum arabic is one of four natural plant exudates used most often in the food industry. The reason for gum arabic's high demand is its excellent functional properties including the ability of the hydrocolloid to form citrus emulsions without the need of a secondary stabilizing agent and the encapsulation of flavors prolonging shelf-life.

The recent focus of gum research in the food industry has been to improve the general quality, including texture, appearance, and stability as well as processing performance of the finished product. Future research should concentrate on examination and analysis of the other gum acacia peptides as well as further studies on the carbohydrate-protein linkages of the molecule using various modern assays. Molecular modeling using computer programs such as Silicon Graphics should be carried out to determine the secondary, tertiary, and quaternary structure of the molecule. Understanding the conformation of gum arabic can aid in learning how its

structure and properties, such as emulsification, relate in multicomponent food systems including low or high moisture foods.

Since the supply of gum arabic is often unstable, this drives up cost and makes the gum unacceptable in some food applications. Substitutes are then used which lower overall product quality. Thus, it would be very beneficial to clone the gum arabic protein either by PCR amplification or a cDNA library of Acacia senegal. Investigation of the mechanism behind formation of gum in the plant can lead to eventual production of gum arabic glycoprotein by tissue cultures or microorganisms.

Modification of the Acacia senegal gum structure could occur by enzymes or chemicals in order to change its properties to suit specific needs. Regulatory agencies would have to approve these changes and stringent quality control standards and tests would need to be developed. At any rate, desired attributes would be a gum that could be used as a bodying agent in low calorie foods, a gum with improved soluble-fiber characteristics or able to replace lipids. Also, enhancement of gum arabic to create superior gels or create temperature stability. Future knowledge gained about the protein composition of gum arabic as well as the associations between molecular structure and the functional qualities exhibited will ultimately allow the food scientist to select a gum perfectly correlated to their desired food system.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adamson, A.D. and Bell, J-M.,K. 1974. The Market for Gum Arabic. Publication G-87. Tropical Products Institute, London, England.

Akiyama, Y., Eda, S., and Kato, K. 1982. Agric. Biol. Chem. 46: 1395.

Akiyama, Y., Eda, S., and Kato, K. 1984. Gum arabic is a kind of arabinogalactan-protein. Agric. Biol. Chem. 48: 235.

Anderson, D.M.W. 1976. Analytical methods for the identification of gum exudates from different Acacia species. In Gums and Hydrosoluble Natural Vegetal Colloids, 4th Int. Symp., S.A. Iranex (Ed.). p. 105. Paris, France.

Anderson, D.M.W. 1977. Water-soluble plant gum exudates-Part I: gum arabic. Proc. Biochem. 12: 24.

Anderson, D.M.W. 1988c. Exudate and other gums as forms of soluble dietary fibre. In Nutritional and Toxicological Aspects of Food Processing, R. Walker, and E. Quattrucci (Ed.), p. 257. Taylor and Francis, London.

Anderson, D.M.W. and Andon, S.A. 1988. Water-soluble food gums and their role in product development. American Association of Cereal Chemists 33: 844.

Anderson, D.M.W. and Dea, I.C.M. 1969. Chemotaxonomic aspects of the chemistry of Acacia gum exudates. Phytochemistry 8: 167.

Anderson, D.M.W. and Eastwood, M.A. 1989. The safety of gum arabic as a food additive and its energy value as an ingredient: a brief review. J. Human Nutr. and Diet. 2: 137.

Anderson, D.M.W. and Gill, M.C.L. 1975. The composition of Acacia gum exudates from species of the subseries Juliflorae. Phytochemistry 14: 739.

Anderson, D.M.W. and McDougall, F.J. 1987. The amino acid composition and quantitative sugar-amino acid relationships in sequential Smith-degradation products from gum arabic (Acacia senegal (L.) Willd.). Food Additives and Contaminants 4: 125.

Anderson, D.M.W. and McDougall, F.J. 1987. Degradative studies of gum arabic (Acacia senegal (L.) Willd.) with special reference to the fate of the amino acids present. Food

Additives and Contaminants 4: 247.

Anderson, D.M.W., and Morrison, N.M. 1989. Some Acacia gums which are not permitted food additives. Food Hydrocolloids. 3: 57.

Anderson, D.M.W. and Stoddart, J.F. 1966. Studies on uronic acid materials. Carbohydrate Res. 2: 104.

Araujo, O.E. 1966. Effect of certain preservatives on the aging characteristics of Acacia. J. Pharm. Sci. 55: 636.

Aspinall, G.O. 1970. Plant gums. In The Carbohydrates 2B. W. Pigman, D. Horton (Ed.) p. 522. Academic Press, New York.

Aspinall, G.O. and Knebl, M.C. 1986. The location of -D-galactopyranose residues in gum arabic. Carbohydrate Res. 157: 257.

Awouda, E.H.M. 1990. Indicators for present and future supply of gum arabic. In Gums and Stabilisers for the Food Industry 5, G.O. Phillips, D.J. Wedlock, and P.A. Williams (Ed.), p. 45. Oxford University Press, New York.

Bavisotto, V.S. and Hansen, G.L. 1968. Solubilization and stabilization of hop extracts and products containing the extracts. C.A. 69, P9746e.

Broderick, J.J. 1954. Superior powdered flavors. Food Eng. 26: 83.

Bruno, P.J. 1954. Substitute for animal albumins. Chem. Abstr. 49: 13550i.

Churms, S.C., Merrifield, E.H., and Stephen, A.M. 1983. Some new aspects of the molecular structure of Acacia senegal gum (gum arabic). Carbohydrate Res. 123: 267.

Clarke, A.E., Anderson, R.L., and Stone, B.A. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. Phytochemistry 18: 521.

Connolly, S., Fenyo, J.C., and Vandevelde, M.C. 1987. Food Hydrocolloids 1: 477.

Connolly, S., Fenyo, J.C., and Vandevelde, M.C. 1987. C.R. Soc. Biol. Fr. 181: 683.

Connolly, S., Fenyo, J.C., and Vandevelde, M.C. 1988. Effect of a proteinase on the macromolecular distribution of Acacia senegal gum. Carbohydrate Polymers 8: 23.

Defaye, J. and Wong, E. 1986. Structural studies of gum arabic, the exudate polysaccharide from Acacia senegal.

Carbohydrate Res. 150: 221.

Dziezak, J.D. 1991. A focus on gums. Food Technol. 45: 115.

FAO. 1982. Specification of Identity and Purity for Gum Arabic. Food and Nutrition Paper, #25, Rome, Italy.

FDA. 1972. Study of Mutagenic of Gum Arabic (FDA #71-15), PB 221-821, NTIS, U.S. Department of Commmerce, Washington, D.C.

FDA. 1972. Teratologic Evaluation of FDA 71-15 (Gum Arabic), PB 221-796, NTIS, U.S. Department of Commerce, Washington, D.C.

FDA. 1973. Evaluation of the Health Aspects of Gum Arabic as a Food Ingredient, PB 234-904, NTIS, U.S. Department of Commerce, Washington, D.C.

Federal Register. 1976. Gum Arabic (Acacia), FR 41, 236, 53609.

Fenyo, J.C. and Vandevelde, M.C. 1990. Physico-chemical properties of gum arabic in relation to structure. In Gums and Stabilisers for the Food Industry 5. G.O. Phillips, D.J. Wedlock, and P.A. Williams (Ed.). p. 17. Oxford University Press, New York.

Fincher, G.B., Stone, B.A., and Clarke, A.E. 1983. Arabinogalactan-proteins: structure, biosynthesis, and function. Ann. Rev. Plant Physiol. 34: 47.

Fogarty, D. 1988. No longer a raw deal. Food, Flvr., Ingred., Proc., and Pkg. 10: 25.

Food Chemicals Codex III. 1981. Acacia (Gum arabic), National Academy Press, Washington D.C.

Food Drug Cosmetic Law Reports. 1980. Acacia (gum arabic), #57,914.3, 21 CFR 184.1330.

Gleeson, P.A. and Jermyn, M.A. 1979. Aust. J. Plant Physiol. 6: 25.

Glicksman, M. 1969. Gum Technology in the Food Industry, Academic Press, Inc., New York.

Glicksman, M. 1983. Food Hydrocolloids Vol. I, CRC Press, Inc., Florida.

Glicksman, M. 1983. Food Hydrocolloids Vol. II, CRC Press, Inc., Florida.

Heidelberger, M., Adams, J., and Dische, Z. 1956. J. Am. Chem.

Soc. 78: 2853.

Hirst, E.L. 1966. Review of the chemistry of water soluble gums. In The Chemistry and Rheology of Water Soluble Gums and Colloids; and Soc. Chem. Ind. (London) Monograph Vol. 24. p. 3.

Jermyn, M.A. and Yeow, Y.M. 1975. Aust. J. Plant Physiol. 2: 501.

Joseleau, J. and Ullmann, G. 1990. Biochemical evidence for the site of formation of gum arabic in Acacia senegal. Phytochemistry 29: 3401.

Lamport, D.T.A. and Miller, D.H. 1971. Hydroxyproline arabinosides in the plant kingdom. Plant Physiol. 48: 454.

Meer, W. 1980. Gum arabic. Ch. 8. In Handbook of Water-Soluble Gums and Resins, R.L. Davidson (Ed.). McGraw-Hill, New York.

Mukherjee, S.N. and Deb, S.K. 1962. J. Indian Chem. Soc. 39: 823.

Naarden Int. N.V. 1974. Stabilization of sugar containing, nonalcoholic drinks. South African Patent 7400-648.

Phillips, G.O., Pass, G., Jeffries, M., and Morley, R.G. 1980. Use and technology of exudate gums from tropical sources. In Gelling and Thickening Agents in Foods, H. Neukom, and W. Pilnik (Ed.), p. 135. Forster-Verlag AG, Zurich.

Qi, W., Fong, C., and Lamport, D.T.A. 1991. Gum arabic glycoprotein is a twisted hairy rope. Plant Physiol. 96: 848.

Randall, R.C., Phillips, G.O., and Williams, P.A. 1988. The role of the proteinaceous component on the emulsifying properties of gum arabic. Food Hydrocolloids 2: 131.

Randall, R.C., Phillips, G.O., and Williams, P.A. 1989. Food Hydrocolloids 3: 65.

Randall, R.C., Phillips, G.O., and Williams, P.A. 1989. Effect of heat on the emulsifying properties of gum arabic. In Food Colloids, R.D. Bee, P. Richmond and J. Mingins (Ed.), p. 386. Royal Society of Chemistry, Great Britain.

Ross, A.H.M., Eastwood, M.A., Brydon, W.G., Busuttil, A., and McKay, L.F. 1984. A study of the effects of dietary gum arabic in the rat. British Journal of Nutrition 51: 47.

Ross, A.H.M., Eastwood, M.A., Brydon, W.G., Anderson, J.R., and Anderson, D.M.W. 1983. A study of the effects of dietary gum arabic in humans. The Amer. J. of Clin. Nutr. 37: 368.

Sanger, M.P. and Lamport, D.T.A. 1983. A micro-apparatus for liquid hydrogen fluoride solvolysis: sugar and amino sugar composition of Erysiphe graminis and Triticum aestivum cell walls. Anal. Biochem. 128: 66.

Schaub, K. 1958. Rheological standardization of tragacanth and the evaluation of the emulsifying powers of Acacia. Pharm. Acta Helv. 33: 797.

Showalter, A.M. and Varner, J.E. 1989. Plant hydroxyproline-rich glycoproteins. Ch. 12. In The Biochemistry of Plants Vol. 15. p. 485. Academic Press, Inc., New York.

Smith, F. 1939. The constitution of arabic acid. Part I. The isolation of 3-d-galactosido-l-arabinose. J. Chem. Soc. 744.

Stephen, A.M., Churms, S.C., and Vogt, D.C. 1990. Exudate gums. Ch. 14. In Methods in Plant Biochemistry Vol. 2. p. 483. Academic Press Ltd., New York.

Stoll, A.C. 1952. A study of the effect of various substances on the volatibility of diacetyl at an elevated temperature. Food Res. 17: 278.

Street, C.A. and Anderson, D.M.W. 1983. Refinement of structures previously proposed for gum arabic and other Acacia gum exudates. Talanta 30: 887.

Strobel, S., Ferguson, A., and Anderson, D.M.W. 1982. Immunogenicity of foods and food additives-in vivo testing of gums arabic, karaya and tragacanth. Toxicology Letters 14: 247.

Stryer, L. 1988. Biochemistry. W.H. Freeman and Company, New York.

Taft, R. and Malm, L.E. 1931. Some physico-chemical properties of gum arabic-water solutions and their interpretation. J. Phys. Chem. 35: 874.

Thomas, A.W. and Murray, H.A. 1928. Gum arabic. J. Phys. Chem. 32: 676.

U.S. Pharmacopeia. 1980. Acacia. XX/National Formulary XV, p. 1205.

Vandevelde, M.C. and Fenyo, J.C. 1985. Macromolecular distribution of Acacia senegal gum (gum arabic) by size-

exclusion chromatography. Carbohydrate Polymers 5: 251.

Voet, D. and Voet, J.G. 1990. Biochemistry. John Wiley and Sons, New York.

Warburton, B. 1966. The rheology and physical chemistry of some Acacia systems. In The Chemistry and Rheology of Water Soluble Gums and Colloids. Soc. of Chem. Ind. London Monograph 24: 118.

Wellner, G. 1957. Stabilizes imitation vanilla. Food Process. 18: 44.

Wenneis, J.M. 1956. Spray-dried flavors. Proc. Flavoring Extract Manuf. Assoc. U.S. 47: 91.