OMB Approved 0524-0025
Expires 5/98

U.S. DEPARTMENT OF AGRICULTURE
SMALL BUSINESS INNOVATION RESEARCH
PHASE I AND PHASE II
PROJECT SUMMARY*

FOR USDA USE ONLY			
Program Office	Solicitation No.	Proposal No.	Topic No.

TO BE COMPLETED BY PROPOSER

Name and Address of Firm	Name and Title of Principal Investigator(s)
Protein Solutions, Inc. 6009 Highland Drive Salt Late City ,UT 84121	R. Van Wagenen Vice President Research and Development

Title of Project (80-character maximum)

Direct Reading Monosaccharide Sensors for Beverage Analysis

Technical Abstract (200-word limit)

We have developed a technology which spatially distributes the light emitted by the ATP-sensitive firefly luciferase bioluminescent reaction so that the <u>position</u> of the light is indicative of ATP concentration. The ATP-sensing device is analogous to a glowing "thermometer", with the length of the glow proportional to the ATP concentration.

Here we propose to employ a kinase as a front end enzyme to catalyze the phosphorylation of an unknown quantity of monosaccharide. ATP will be depleted in direct proportion to the monosaccharide consumed in the general reaction:

$$\text{monosaccharide} + \text{ATP} \xrightarrow{\text{kinase}} \text{phosphorylated monosaccharide} + \text{ADP}$$

The spatial bioluminescent detection system will detect the depletion in ATP concentration, thereby providing a measure of monosaccharide concentration. By using the <u>position</u> of the emitted light to indicate analyte concentration, we enable the human eye, which is very good at discriminating the position of a light source but poor at quantifying light intensity, to serve as the readout device. Appropriate photodetectors can also serve as detection and readout devices. In this SBIR Phase I Project we propose to stabilize the required enzymes, incorporate all reactants into a suitable prototype configuration, and evaluate the performance of the prototype monosaccharide biosensors.

Anticipated Results/Potential Commercial Applications of Research (100-word limit)

We anticipate demonstration of the feasibility of applying PSI's bioluminescent sensing technology to determine monosaccharide concentrations.

Rapid analysis of sugar and carbohydrate content is becoming increasingly important in the food, dairy, beverage, and biotechnology industries. The sensors developed will provide a means for rapid analyses of sugars in these industries, and provide the technical foundation for development of more complete panels of bioluminescent sensors in the future.

Keywords to Identify Technology/Research Thrust/Commercial Application (8-word maximum)

ATP, Luciferase, Bioluminescence, Biosensor, Monosaccharide, Sugar, Carbohydrate

*The Project Summary must be suitable for publication by USDA in the event of an award. Do not include proprietary information on this page.

C. Technical Content

1. Identification and Significance of the Opportunity.

There are two very special molecules that play unique and central roles in biology, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) and its phosphate form (NADPH). NADH is a ubiquitous electron donor and ATP is generally recognized as one of the key energy currencies in biology. The two molecules act in a cyclic manner and can be regenerated or recharged. They are the basic coupling agents of cellular metabolism. A very large number of biochemical enzyme processes involve one of these two molecules.

Two key bioluminescent processes dependent on these molecules are the firefly luciferase reaction which is specific to ATP and the bacterial luciferase reaction which is specific to NADPH. Both reactions efficiently produce photons in the presence of oxygen (1).

There is a large body of literature on the development of biosensors for ATP and ATP-dependent processes and for NADH and NADPH-dependent processes, using the firefly and bacterial luciferase enzymes, respectively. Such biosensors generally employ fiberoptic or other wave guided means for delivering the luminescence to a device which can accurately measure light intensities (1,2). Although one of the most portable and most sensitive photon detectors available to the scientist is his or her own eye, it is notoriously difficult to calibrate for accurate measurements of even relative light intensity. The human two dimensional photon detection system, however, can reliably and accurately measure changes in spatial position of light.

Using the direct reading luciferase based ATP sensors already developed at Protein Solutions, Inc., we propose to develop a quantitative, inexpensive, disposable, analytical device for the analysis of carbohydrates using ATP-dependent, kinase-based, phosphorylation reactions. Although the work proposed with this application is focused on the several carbohydrates of most immediate interest and importance to the fruit juice industry, it is important to note that there are literally hundreds of phosphorylation-dephosphorylation enzymes whose substrates and whose activity are of growing importance in food quality, nutrition, dairy, and related areas (3).

2. Background & Rationale

Bioluminescence is an enzyme-dependent chemical oxidation process which results in photon emission (1). The photoproteins involved in these processes, the luciferase series of oxidative enzymes, are now readily available in inexpensive form (4, 5). In collaboration with the University of Utah, PSI now has a recombinant process for producing luciferase at 1/100 of the commercial cost.

For the last 5 years, PSI has been involved in numerous scientific investigations and development projects covering a wide range of bioluminescent phenomena, including several studies dealing with applications of bioluminescent reactions for sensing applications. We funded a study at the Center for Biopolymers at Interfaces at the University of Utah to characterize the interfacial behavior of firefly luciferase, the photoprotein/enzyme responsible for catalyzing bioluminescence in the North American firefly, focusing on its adsorption at solid/liquid interfaces, air/water interfaces, and its denaturability or stability in solution (6). These studies, together with our commercial work on bioluminescent phytoplankton (the dinoflagellate *Pyrocystis lunula*) have provided a deep and diverse background in practical bioluminescence.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (1,7). Since the firefly luciferase reaction is dependent on an ATP co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Until very recently such applications were frustrated by the instability of luciferases and the difficulties encountered in attempts to incorporate them into commercial devices exhibiting required reliability, accuracy, and shelf life (8).

Several years ago PSI researched another unique biological phenomenon -- the ability of certain plants and animals to survive almost total desiccation for extended periods and to spring back to life when rehydrated, a phenomenon called anhydrobiosis (9). We learned that many of these plants and animals depend on a unique disaccharide, trehalose, for their ability to withstand

severe desiccation stress without denaturation of their proteins and enzymes or disruption of their cell membranes (10). Sucrose and other osmolytes are also utilized by some species. Trehalose apparently serves two major functions. In high concentration it tends to (1) prevent phase separation and crystallization, and (2) substitute for the water normally hydrogen bonded to proteins and cell membranes, thereby stabilizing their structures when the last molecules of water are removed by desiccation or drought. Trehalose is now being widely applied to the stabilization of enzymes and antibodies and is being studied for the stabilization of air dried cells, tissues, and organisms (10).

PSI has a key group of personnel with extensive multi- and interdisciplinary bioengineering backgrounds as well as product development experience. Relevant background information for Drs. Joe Andrade, Rick Van Wagenen, Rob Scheer, and C.Y. Wang are summarized in section D.

PSI was recently awarded a STTR Phase II grant from NSF entitled, "Direct Reading Quantitative Biosensors for ATP" which focuses on enhancing the sensitivity of our existing bioluminescent spatial sensor by several orders of magnitude. We established the feasibility of spatial detection sensors via a National Science Foundation STTR Phase I grant which ended on August 15, 1995. Our current device is suitable for quantitatively determining ATP concentrations as low as 10^{-7} molar (10^{-9}g). Increasing device sensitivity by three orders of magnitude will enable it to be applied as a hygiene monitor to determine the bacterial load on surfaces in sensitive food, beverage, and pharmaceutical processing areas (12a). We therefore have the commitment and resources to fully develop the direct reading, quantitative ATP sensor.

We propose in this application to utilize the ATP sensor to assess the feasibility and practicality of using phosphorylating enzymes for common monosaccharides as the basis for direct reading dipstick type quantitative saccharide sensors. The carbohydrate analyses proposed in this application do not require enhanced sensitivity of PSI's luciferase-based ATP sensor. The current sensor is sensitive enough to be used for carbohydrate analysis. The goal of this scientific development project is to confer specificity for monosaccharides to PSI's spatial detection system by employing appropriate kinases as up front enzymes. Feasibility of employing the sensing system to accurately reflect monosaccharide concentrations will verify that the sensing concept is sound, and open up opportunities to develop assays for other substrates (vitamins, complex sugars, etc.) that are phosporylated by kinases at the expense of ATP.

3. Relation to Future R & D -- Phase II

In Phase I we propose to demonstrate the feasibility of developing sensors for the assay of mono saccharides based on specific kinase-catalyzed phosphorylation of the monosaccharide. Dephosporylation of ATP occurs in the reaction, resulting in a decreased ATP concentration which is detected through the ATP-specific firefly luminescence reaction using our unique spatial modulation technology. **(For a complete description of the spatial detection system, please refer to page 11).**

The major monosaccharide in many fruit juices is fructose, followed by glucose, sucrose, and sorbital (12b). Our Phase I feasibility study will focus on glucose and fructose using glycokinase and fructokinase, respectively. In Phase II we will utilize those two monosaccharide channels to develop a sensor for sucrose. The sorbital sensing problem will also be addressed in Phase II.

The selection of optimal enzyme concentrations begun in Phase I will be greatly aided by theoretical models and simulation which will be developed in Phase II. Models will include consideration of capillarity-based analyte delivery, and the diffusion of analyte through the gel and its interaction with enzymes. The various turnover numbers will be simulated. Existing enzyme kinetic models, diffusion models, and capillarity models will be used.

Admittedly, many of the carbohydrate kinases are not highly specific. The specificities of the various monosaccharide kinases do vary sufficiently such that a multi-channel and minimally multi-variate approach will indeed permit the determination of the individual monosaccharides. We will consider this in the Phase II work, with the hope and expectation of applying these sensors in the future to a much wider range of mono and disaccharides for a complete and comprehensive carbohydrate analysis of foods and related materials.

4. *Phase I Technical Objectives*

The overall research objective is to assess the feasibility of a direct reading sensor for the quantification of glucose and fructose employing PSI's spatial luminescent ATP detectors. The sensors have been shown capable of yielding a visible light output whose spatial position is indicative of ATP concentration. When simple sugars are phosphorylated by their respective kinases, they consume ATP. These changes in ATP concentrations will be proportional to the concentrations of the sugars present. Feasibility will be demonstrated if we can show that PSI's sensor is capable of reproducibly displaying a spatial distribution of light that can be correlated with the concentration of the simple sugars in sample solutions.

Objective 1. Prepare and Characterize Kinases

Various glucokinase (enzyme 2.7.1.2) and fructokinase (enzyme 2.7.1.4) enzymes will be obtained from commercial sources, characterized, purified and their respective stabilities, activities, and cross-reactivities determined.

Objective 2. In Solution, Confirm Ability Of Bioluminescent Reaction To Detect And Quantify ATP Consumption During Phosphorylation Of Simple Sugars By Kinases.

Before proceeding with studies incorporating the enzymes in gels, we will assess the ability of our sensor to detect changes in concentrations of sugars in a solution system. These preliminary experiments will be performed to obtain estimates of the concentrations of analytes and reactants that are required, and to identify any unanticipated interferences or incompatibilities of the system which can be addressed before proceeding to development of the gels described below.

Objective 3. Enhance Stability Of Enzymes and ATP by Incorporating Them Into Agarose Gels

Our present ATP sensing technology incorporates firefly luciferase, an ATP "consumase" (apyrase), and trehalose as a preservative and stabilizer, into agarose gels. The apyrase is used to modulate light as a function of position (11). The gels are dried and stored. Results of previous investigations have demonstrated that luciferase and apyrase maintain their activity when incorporated into the agarose gels (5). When rehydrated with an aqueous solution of an analyte sample containing ATP and luciferin, the preserved luciferase and apyrase are activated, yielding a light signal proportional to the concentration of ATP present in the sample (5).

We will preserve the kinases by incorporating them into gels containing trehalose (10), sucrose, or raffinose as a preservative. The kinase concentrations in the gel will be optimized along with gel preparation conditions to obtain a dehydrated gel which can be rehydrated with maximal kinase activity.

In previous studies, ATP has been the analyte. In the proposed work, ATP concentration will be modulated by controlling its reaction with monosaccharides. Appropriate kinases will be used to catalyze the reaction. Therefore, ATP has to be provided in one of the device layers. We will incorporate ATP into the agarose gels and perform appropriate stability studies on the ATP gels. There is of course a concern that the kinases will also act on the preservatives trehalose, sucrose, or raffinose. This is addressed in section C, 5 *Phase I Work Plan*, Objective 3.

Objective 4. Configure Reactants so That the System Can Be Evaluated (Preliminary Prototype Development).

At this stage in the development of the devices we are focused on a design involving a support which serves to deliver the analyte as well as allow viewing. The geometry and viewing direction is very different from that of common typical dipstick type sensors such as urine dipsticks or pH paper.

Our present design concept is described below and shown in Figure 1, based on a dipstick type device. At the left we show the liquid sample solution containing the analyte of interest. That solution is drawn into the paper by capillarity. The system is designed so that the flow rate is uniform and the volume delivered and the residence time of the solution in each of the zones is optimized, based on substrate concentration, enzyme concentration, and enzyme activity. The analyte solution first enters a region of deposited ATP. As the analyte solution is drawn to the right (by capillarity), the ATP is solublized. The saccharide/ATP solution then moves into the second zone which contains the preserved kinase. The solution hydrates and activates the kinase and the phosphorylation reaction begins. The phosphorylation of the saccharide by its specific kinase results in a controlled depletion of the ATP concentration. We call the ATP leaving the kinase region the analyte ATP. Actually it is the ATP remaining after the phosphorylation of the saccharide. The solution then moves further to the right and enters the ATP sensing device, which indicates the ATP concentration. The first part of the ATP sensing device is a gradient of apyrase shown by the graded shading in the figure. This gradient produces a gradient in ATP concentration. As that ATP gradient enters the luciferin/luciferase region, light is produced with a spatial pattern representing the gradient in ATP concentration, thereby permitting direct reading. The spatial pattern of light is shown at the far right, viewed by the human operator. A second independent sensor ("channel") could be positioned parallel to and below the one shown in Figure 1 and is basically identical except that the kinase enzyme has been irreversibly inactivated so that the phosphorylation reaction cannot occur, and the ATP is therefore not depleted. This control channel allows direct measurement of the initial ATP concentration. The active kinase channel measures the decreased ATP concentration. The difference in ATP concentrations in the two channels directly relates to saccharide concentration in the sample. There would of course be other components and aspects to the sensor, including means to control flow, minimize channel cross talk or cross contamination, and related design and engineering issues.

The development and optimization of the ATP sensor shown in the right side of Figure1 is the subject of our existing NSF Phase II STTR grant. In order to fully preserve the activity of the ATP region and the kinase region and of course the activity of the ATP detection end of the sensor which involves luciferin (which must be protected from oxidation), the entire device would probably be sealed in a small polymer envelope which would be easily opened just prior to use. This envelope would include a barrier film to minimize oxygen and CO_2 transport, as is done routinely for packaged foods. The final device would include a volume indicator channel to be sure that the analyte solution did indeed have sufficient volume to be fully transported through the entire device. This is essentially identical to what is now done with common over the counter devices for the assay of pregnancy and cholesterol by other methods.

Objective 5. Establish Feasibility

The configurations developed to meet Objective 4 will undergo initial evaluation. Known concentrations of monosaccharide solutions will be introduced to the devices, and the light output determined. Studies will assess the sensitivity, reproducibility, and specificity of the system. It is anticipated that the developed configuration will perform sub-optimally; however, it is fully expected that the sensors will yield a light output whose position is indicative of monosaccharide concentration. Optimization would be a primary topic for Phase II.

Although these initial tests of a prototype device will utilize pure solutions, we will do a preliminary evaluation of real world kinds of samples. The pure solutions will enable us to assess in a device configuration, aspects of specificity and cross reactivity. It is also important to note that the kinase and indeed the luciferase enzymes involved have their own micro-environmental requirements, particularly pH and ionic strength. We are also fully aware that fruit juices tend to be highly acidic (12). A final sensor designed for direct application of the fruit juice or its concentrate would of course have to have a pH adjustment zone at the very entrance to the sensor, that is to the far left of Figure 1. As the analyte is drawn into the sensor, it would first enter a zone of deposited buffer which would serve to adjust the pH into an appropriate range required for the solution entering the ATP zone. It may also be necessary to include a spacer zone between the various enzyme zones. For example, such as between the kinase and the apyrase in Figure 1, not

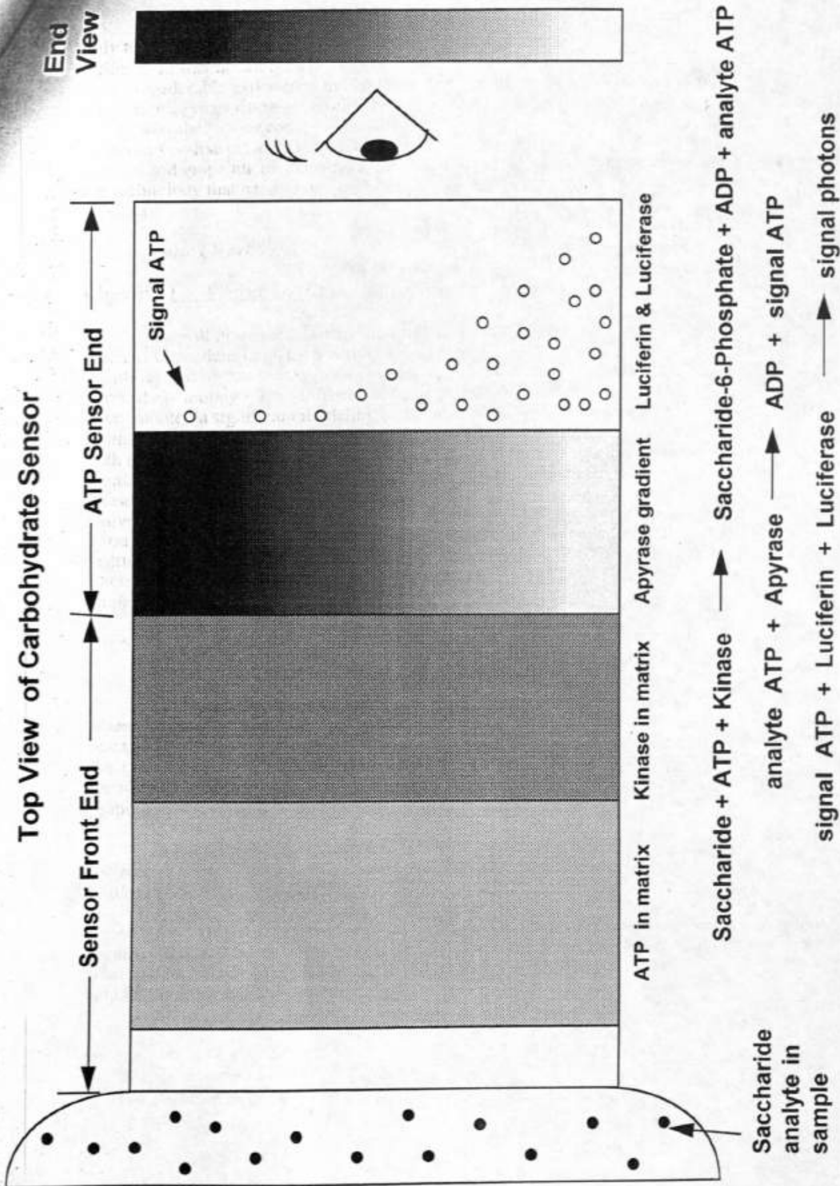

FIGURE 1 Carbohydrate luminescent sensor for monosaccharide and polysaccharide analysis.

only to allow for flow volume and kinetic control, but also to permit the placement of a second buffer to further adjust the pH to be near optimum for the next enzyme encountered. We already have considerable experience in the deposition and design of such zones for "intelligent" protein separation devices using ion exchange and displacement chromatography (18). We do not feel that any of the other major constituents in fruit juice are likely to provide any serious problem, although we will of course perform the evaluations in fresh reconstituted and frozen concentrate apple, orange, and grape juices. The evaluations will demonstrate feasibility and identify the elements of the technology that need to be optimized in Phase II studies in order to arrive at a marketable product.

5. *Phase I Work Plan*

Objective 1. Prepare and Characterize Kinases

We will obtain and screen existing commercially available hexo-, gluco-, and fructokinases, determine their activities, optimal pH, and kinetic constants as well as their stabilities and of course cost considerations. A screening of specificity and cross reactivity, particularly among glucose, fructose, sucrose, trehalose, and raffinose will be conducted. We have initiated a significant modeling and simulation effort on the existing Phase II grant in order to optimize the ATP detection end of the device. Those skills and expertise will be used here together with the information on the kinases to develop a preliminary simulation and to deduce the optimum kinase parameters to help direct and guide our screening and selection process. Our existing NSF Phase II STTR involves a major contract with Dr. Russell Stewart, Department of Bioengineering, University of Utah. In addition PSI is a member of the Center for Biopolymers at Interfaces, and through that membership has access to practically all of the specialized laboratory facilities and instruments within that center. In these labs the proteins will be characterized by polyacrylamide electrophoresis, and column chromatography. Their activity and kinetics will be assessed by employing them in our ATP/firefly luciferase detection system.

Objective 2. In Solution, Confirm Ability Of Bioluminescent Reaction To Detect And Quantify ATP Consumption During Phosphorylation Of Simple Sugars By Kinases

We have devised a series of simple liquid-phase experiments that enable us to rapidly assess the feasibility of our sensing methods. They consist of preparing a series of dilutions of the reactants and evaluating their performance in all combinations to arrive at optimal concentrations. We react the proteins with the firefly luciferase system in microtitration test wells, and determine the relative light output from the wells using a CCD camera. The light profiles give us a very graphic record of which combinations are successful (see Figure 2), and also yield information about the sensitivity of the assay.

In this case the test matrix will be rather large, including variations in ATP, kinase, luciferase, luciferin, and apyrase concentrations. We will also determine if any incompatibilities and/or cross-reactivities exist.

The use of multi-well plate, multi-channel pipettes, and a CCD camera with wide dynamic range allows very rapid screening of the many variables described earlier. We are of course well aware of the limitations of the human eye for direct visual detection depending on the application. The human eye can of course be enhanced by the use of high speed photographic film detection, and photo diode CCD detection.

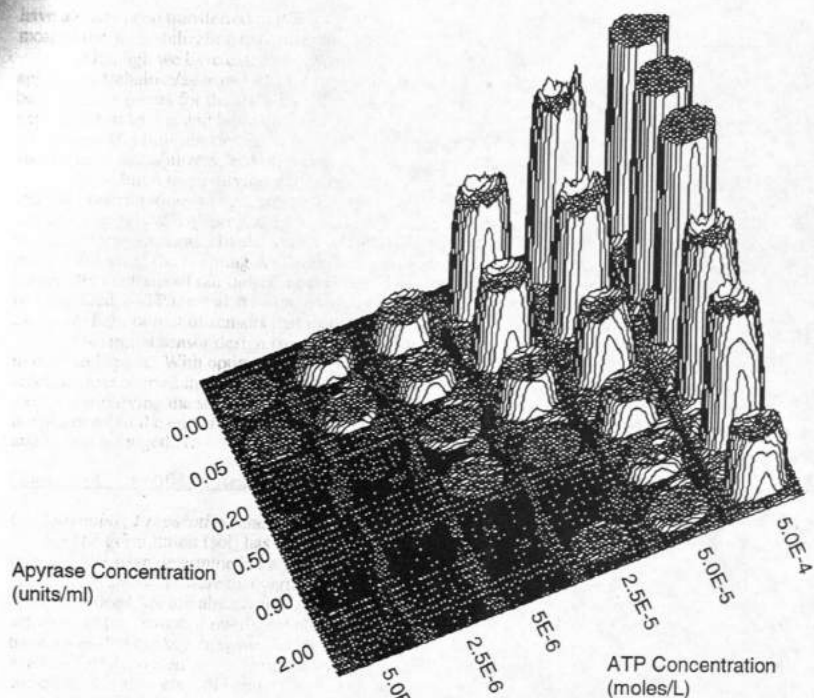

Figure 2 Typical CCD 3-D profile of light intensity for a six by six ATP detector array.

Objective 3. Enhance Stability of Enzymes and ATP by Incorporating Them Into Agarose Gels

Trehalose has been successfully employed as a stabilizer for both luciferase and apyrase in agarose gels, permitting them to be dehydrated and stored, and then rehydrated at a later date to full enzyme activity (5). Optimum conditions for the preparation of trehalose/agarose gels containing the kinases and ATP in the desired concentrations will be explored.

In addition, the ability of sucrose to stabilize the proteins will be assessed. Sucrose is more readily available and less expensive than trehalose, and it has been shown to be a fairly effective protein preservative. Raffinose (17) has also been shown to be highly effective in dehydration applications as noted earlier. It has also been shown that some amino acids in particular proline are also highly useful in this regard. If our studies and objectives I and II indicate that there really are serious cross reactivity problems which cannot be alleviated by the careful selection of these specific kinases, then we will of course seriously examine non-saccharide dehydration preservatives. That would clearly be a major activity in Phase II. However, we are quite confident that this potential problem can be solved by careful selection of a kinase and preservative.

Under a contact between Protein Solutions, Inc. and the University of Utah, Dr. C.Y. Wang has been working on the trehalose stabilization of firefly luciferase for the past five years. Rights to the stabilization procedures he developed at the University in a project funded by PSI

have already been transferred to PSI for firefly luciferase applications. Dr. Wang will perform most of the gel stabilization experiments.

Although we have extensive experience with the stabilization of firefly luciferase and apyrase in trehalose/agarose gels, we have little experience with kinases. Trehalose has, however, been used by others for the stabilization of a variety of enzymes, including kinases (19). We expect that trehalose will be an effective additive for stabilizing the proteins in polysaccharide gels, i.e. agaroses, celluloses, dextrans, etc.. Likewise, we anticipate that sucrose and raffinose can also be used as stabilizers, and offer cost and availability advantages over trehalose.

In addition to modifying stabilizer concentration, we will modify gel density (by varying agarose concentration) and gel composition in order to effectively immobilize the reactants in the device. The gels will be prepared with different enzyme/preservative ratios. The enzyme activity will be directly measured by delivering reference amounts of ATP, luciferin and sugar to the kinase gel and detecting the resulting ATP diffusion into a luciferase gel by measuring light output, essentially the basis of our biosensor concept. The performance of the various preservatives will be compared. ATP must also be included in the test system. Its stability will be evaluated by assessing light output of sensors that incorporate different ATP gels as a function of storage time.

Our initial sensor design (see Figure 1) shows the various reactions involved as separated in time and space. With optimal enzyme activities and concentrations, the kinetic constants and reaction time courses may allow for the combination of a number of the steps into a region thereby greatly simplifying the sensor. We have already shown in the case of glucose analysis with hexokinase that the enzyme parameters are such that we can actually mix the hexokinase, apyrase, and luciferase together.

Objective 4. Configure Reactants so that the System Can Be Evaluated.

Gel Spreading, Preparation, and Stability.

The gel solution (sol) has to be spread on suitable supports in a uniform and homogeneous manner. We have determined that suitable support materials include polyester film and cellulose papers. We are well aware that various fiber surfaces adsorb and immobilize proteins and other solutes. Indeed, we are already using our long experience in protein adsorption and interfacial activities in the design of intelligent planar devices for protein separation (18, 20). That is one of the reasons that the key enzymes involved in the sensor were actually immobilized in the very thin agarose gels deposited on the support. Although this is yet to be studied and optimized, we anticipate that the gels will be in a range of 10 to 100 microns in thickness, thereby serving as a minimal diffusion barrier for solute transfer. We are now using a low melting agarose whose transition temperature and hysteresis are such that it is prepared in the solid form at 30 - 40 °C, well within the stability regime for the enzymes of interest. It can be prepared as a relatively low viscosity sol under those conditions and then coded, dip-sprayed or otherwise processed for optimum support where upon it cools forming a semi-rigid gel which is stable at room temperature. The deposition of the various bands of enzyme and gel as indicated by the various regions in Figure 1 is accomplished by use of an air spray or an ink jet printing process. Indeed, by simply taking an ink jet printer with chambers for black and the three primary colors one can directly print the zones of interest at any relative concentration one likes using the four solution reservoirs. The process is fundamentally no different than an ink jet printing process used to produce the gradients drawn in Figure 1.

Capillarity: Sample, Delivery, and Transport.

The sample containing the analyte will be deposited on a prototype sensor and spread across the appropriate portions by capillarity and rehydration processes. This technology is already applied in existing dry reagent chemistry diagnostic kits. Capillarity is readily controlled via the porosity of the paper or support medium and its contact angle or wetting characteristics. The vendors which supply the dipstick dry reagent diagnostic industry, have a wide repertoire of papers and support from which to choose. We will of course characterize these papers and the individual fibers from which they are constituted by standard capillarity measurements using a Wilhelmy plate device operated in a fiber mode. Such a wicking test is quite standard in the textile

fiber community and Dr. Andrade has considerable experience with such methods. We also have substantial experience with surface modification and characterization (20). In the unlikely event that we cannot find a suitable commercial paper, we can easily modify the surface to produce the wetting characteristics desired. These studies, of course, will be closely correlated with the enzyme characterization and analysis work and with the reaction modeling and simulation work. We are fully aware that these sensors are entirely based on kinetic processes which are in part controlled by the capillarity characteristics of the device, via the effect of capillarity on solute flow and transport and of course on enzyme rehydration rates.

The sensor concept is based on the fact that, for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a detectable light output. Above that concentration, light is visible; below that ATP concentration, no light is detected. By filtering or consuming the ATP before it reacts with the luciferase we control the intensity of the light at each position along the sensor. Refer to Figure 1 end view. A high concentration of ATP will still produce light even at the higher apyrase concentrations because enough ATP remains after consumption to produce a measurable light output. A low concentration of ATP will produce light only at the lowest apyrase concentrations because at higher apyrase concentrations all of the ATP is consumed before it reacts with the luciferase.

To measure an ATP concentration, a sample containing ATP is distributed to the luciferase/apyrase gel at which time the ATP is consumed by the two competing enzymes. Because the turnover rate of apyrase is two orders of magnitude higher than that of luciferase, the apyrase quickly moderates the ATP concentration. The amount of ATP which reacts with the luciferase to produce light depends on both the initial ATP concentration and the concentration of apyrase.

To demonstrate and test the dependence of light output on both of these concentrations, we produced two dimensional matrices with gradients of ATP concentration ($5x10^{-4}$ to $5x10^{-7}$ mol/l ATP) versus apyrase concentration (2 units/ml to 0 units/ml). The gradients were produced as serial dilutions of ATP and apyrase, and pipetted in the appropriate patterns into test wells. At the bottom of the test wells we had fabricated gels containing luciferase. A positive surface profile whose height is directly proportional to light intensity was made immediately after ATP was applied to the test wells. The image was made with a CCD camera and appears as Figure 2.

With high initial concentrations of ATP, a large concentration of apyrase is required to eliminate emission of a detectable light signal. With low initial concentrations of ATP, only small concentrations of apyrase are required to prevent emission of light at a detectable signal. The goal of modulating the light signal with apyrase is to generate a specific light cut off point indicative of a specific concentration of ATP in the analyte solution.

In the final ATP sensor configuration whose development in the basis of our existing Phase II NSF grant. The apyrase gradient would likely be continuous as indicated schematically in Figure 1, rather than discontinuous as indicated in the example in Figure 2. Although it is certainly possible that there may be applications where a discontinuous approach is preferred such as a sensor that indicates whether a particular saccharide is in excess of or below a particular preset level.

In this proposal the intent is not to determine ATP concentration, but rather to use its concentration as an indicator of the concentration of monosaccharide. This is accomplished by reacting the ATP with the saccharide in the presence kinase. The phosphorylation of the sugar and the dephosphorylation of the ATP occur before the ATP reaches the apyrase/luciferase gel layer where light emission is initiated. Thus, the kinases serve as a mediator of ATP concentration by catalyzing its reaction with the monosaccharides.

The depleted ATP "produces" a light signal inversely proportional to the concentration of monosaccharide in the test sample. The light is focused or waveguided onto photographic film for detection, or detected by the unaided eye. Comparison with an empirically derived table correlating monosaccharide concentration with obtained light pattern will allow for a rapid estimation of the concentration of monosaccharide in the sample. The final sensor will include a scale along the light output zone which will permit the operator to read out the monosaccharide concentration by correlating the scale with a sharp inflection region between light and no light. In Phase II we would optimize the gradients to produce optimally sharp inflections. Although, we have focused

here on the fruit juice analysis, clearly the technique can be used for dairy food and even veterinary and medical arenas. With proper concern for the dynamic range, sensitivity, and cross reactivity one could even design the sensor with a gradient in kinase concentration permitting it to be essentially auto ranging although we do not propose to do that here.

The stability of luciferin is such that it may not be feasible to incorporate it into the device. We will investigate the use of metallized polyester film packaging materials to preserve it in a dry powdered form incorporated in the gels.

Objective 5. Evaluate Feasibility.

The prototype devices will be tested by introducing a range of monosaccharide concentrations of commercial interest (12) to the multi-layer test strips and assessing the ability of the strips to reproducibly and predictably generate a pattern of light which can be correlated with sugar concentration. Light emission will be deOptical Imaging Laboratory at the Center for Biopolymers at Interfaces. In addition, short term storage, dehydration, stability, and experiments to assess the reproducibility of the assay will be performed.

6. Related Research and Development

On August 15, 1995 we completed an NSF Phase I STTR contract titled "Direct Reading Quantitative Biosensors for ATP-dependent processes." Under this contract we clearly established the feasibility of combining the ATP-dependent firefly luciferase bioluminescent reaction with a method of modulating ATP concentration to make a system capable of emitting photons in proportion to ATP concentrations. ATP concentration is modulated with an apyrase gradient. The spatial pattern is directly related to ATP concentration. See Figure 2.

We have just learned that our Phase II NSF STTR to extend the sensitivity of ATP detection by three orders of magnitude will be funded effective October 1, 1996, thus we now have the resources to fully develop the ATP detection side of this sensor concept. This is represented on the right side of Figure 1. The concentration of monosaccharides in food and beverage applications is such that our existing sensor technology is adequate. High sensitivities are not required. Thus none of the studies proposed in this phase I application are dependent on the results of the Phase II STTR work. Nevertheless, the Phase II work is highly complimentary to the studies proposed herein and once feasibility is established in this Phase it will greatly aid in moving the work forward to practical monosaccharide sensors.

The Phase II NSF STTR will permit dramatic progress to be made in our fundamental understanding of the ATP-dependent firefly luciferase bioluminescent reaction. Everything we learn about improving the sensitivity of this technology so that it can be used for microbial ATP detection simply enhances the practicality and feasibility of its application for monosaccharide-and eventually disaccharide-specific detection.

D. Key Personel & Bibliography

1. Key Personnel/Vitas

Dr. R. Van Wagenen, Ph.D., Principal Investigator and Vice President of R and D, is a bioengineer with considerable product research, design, and development experience in the medical device industry. Before joining PSI in 1996, he spent ten years working on biomedical instrumentation as VP of R & D and Director of R & D for Albion Instruments and then Ohmeda Medical Devices, respectively. During this time, Rick and his co-workers developed a unique Raman spectroscopy respiratory/anesthesia gas monitor. His background in Materials Science, and his earlier research work dealing with the characterization of surfaces for biomedical applications are also directly relevant particularly to the design and characterization of the gels and the supports for the sensors. Dr. Van Wagenen will serve as principle investigator and have primary responsibility for the design and conduct of the project. His product development

background will enable this concept to be effectively developed into commercial products. His bio-sketch is included.

Dr. Robert Scheer received his Ph.D. in Materials Science and Engineering in 1993 and was Principal Investigator of PSI's NSF-STTR Phase I grant on the development of ATP-based biosensors using firefly luciferase. He has had considerable experience with the handling of native firefly luciferase and its stabilization in agarose gels and fiber matrices. He has worked and will continue to work closely with Dr. C.Y. Wang. Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. His biosketch is included.

Dr. Joseph Andrade is founder, President, and CEO of PSI. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. He will provide much of the interfacial biochemistry, bioluminescence, and biosensor expertise required.

Dr. C.Y. Wang recently completed his Ph.D.. studies under Joe Andrade's supervision at the University of Utah. Dr. Wang has worked on the Firefly luciferase system for five years. He is an expert on the trehalose stabilization of firefly luciferase in agarus gels. Such gels can be desiccated and successfully rehydrated with full enzymatic activity. He is now working as a Research Scientist at PSI and will conduct the firefly luciferase-based studies. He will play a key role in transferring his comprehensive expertise on this system to the others involved in the project.

2. Bibliography

1. Brolin S, Wettermark G, editors. *Bioluminescence Analysis*. New York, VCH, 1992.
2. Blum LJ, Gautier SM, Couplet PR. "Fiber-optic biosensors bases on luminometric detection." In: Wagner G, Guilbault G, editors. *Food Biosensor Analysis*. New York: Marcel Dekker, Inc., 1994: 101-21
3. Werner, Baltis, ed., *Rapid Methods for Analysis of Food and Food Raw Material*, Technomic Publishing Company, 1989, Chapter 15, "Enzymatic, Rapid Methods" by G. Henniger.
4. Wood, K., Ph.D. Dissertation, Department of Chemistry, University of California, San Diego, 1989.
5. Wang, C.Y., Ph.D. Dissertation, in preparation, "Firefly Luciferase at Model Interfaces: Activity, stability, and Sensor Aplications," September 1996.
6. Wang, C.Y. and J.D. Andrade, "Purification and Preservation of Firefly Luciferase," in *Bioluminescence and Chemiluminescence:" Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka, and P.E. Stanley, eds. Wiley, 423-426 (1994).
7. Kricka, L.J., "Chemiluminescence and Bioluminescence," *Analytical Chemistry* **67** (1995) 499R.
8. Roda, A., et al., "Coupled Reactions for the Determination of Analytes and Enzymes Based on ... Luminescence," *J. Biolum. Chemilum.* **4** (1989) 423-435.
9. Crowe, J.H., et al., "Anhydrobiosis," *Ann. Rev. Physiol.* **54** (1992) 579.
10. Colaco, C., et al., "Extraordinary Stability of Enzymes Dried in Trehalose," *BioTech.* **10** (1992) 1007.
11. Andrade, J.D., Hlady, V., Scheer, R., Triolo, P., and Wang, C.Y., "Method of Measuring Chemical Concentration and/or Light Intensity Based on Spatial Separation and Resolution," U.S. Provisional Patent Application filed 8/2/95.

12a. Fung, D.Y.C., "Rapid Methods and Automation in Food Microbiology: A Review," *Food Reviews International* **10** (1994) 357-375.

12b. Elkins, E.R., et al., "Characterization of Commercially Produced Apple Juice Concentrate," *J. Food Composition and Analysis* **9** (1996) 43.

13. Kress-Rogers, E., "Chemosensors, Biosensors and Immunosensors" in Instrumentation and Sensors for the Food Industry, Kress-Rogers, E. And Noltingk, B.E. eds., Butterworth Heinemann, Oxford (1993), pp. 581-669.
14. Perez, S. and Colon, L.A., "Determination of Carbohydrates as their Dansylhydrazine Derivates by Capillary Electrophoresis with Laser-Induced Fluorescence Detection" *Electrophoresis* **17** (1996)352.
15. Stewart, K.L., "Carbohydrate Contents of Foods: A tower of Babel and Confusion", J. Food Composition and Analysis **9** (1996) 98.
16. Moro, J. and Burlingame, B., "Carbohydrates and Related Food Components: IN FOODS, Tagnames, Meanings, and Uses." *J. Food Composition and Anlysis* **9** (1996 110.
17. SaLeki-Garhardt, A., et al., "Hydration and Dehydration of Crystalline and Amorphous Forms of Raffinose," *J. Pharmaceutical Sciences*, **84** (1995) 318.
18. Luo, Q. Ph.D. dissertation University of Utah, in progress

E. Facilities and Equipment

Protein Solutions occupies 1400 square feet of office and lab space in the Research Park adjacent to the University of Utah campus, Salt Lake City, UT. This space includes a chemical lab, a biology/biotechnology lab, a small manufacturing and production area, and two offices. Basic laboratory equipment includes scales, stirrers, ovens, cell culture facilities, a fume hood, computer, and appropriate optics and light measuring equipment. A CCD camera to be used in this work and the protein characterization facilities are available to PSI through its membership in the Center for Biopolymers at Interfaces in the University of Utah. Surface analytical facilities at the University of Utah will also be utilized on a fee for service basis. PSI's Technology Transfer Agreement with the University of Utah allows for transfer of jointly developed technologies and is relevant to all technology covered in this application. Although no equipment was budgeted in this proposal, PSI is committed to obtaining the necessary equipment and facilities required to carry out its research and development missions. Some of this equipment will be obtained via funds from the NSF Phase II STTR.

F. Consultant

Dr. Bart Weimer, Assistant Professor in the Department of Food Science at Utah State University in Logan, Utah, will serve as consultant and advisor. Dr. Weimer is heavily involved in analytical methods for food and milk applications, including capillary electrophoreses and a range of spectroscopy techniques. His strong biosensor and analytical chemistry interest will provide perspective insight and direction. Utah State University has extensive programs and activities in food, dairy, veterinary, and agriculture. Dr. Weimer has agreed to consult with us at a rate of $400/day. Utah State University is a 90 minute ride from the University of Utah. Dr. Weimer frequently travels to Salt Lake City for meetings and other activities and the PSI staff frequently travels to Logan, Utah for discussions with Dr. Weimer and his colleagues. His letter of interest and two paged vita are enclosed.

G. Commercialization

There is rapidly growing interest in the food, dairy, and beverage industries in the more detailed chemical analysis of their products with particular interest in developing rapid methods for carbohydrate analyis. Rapid sugar detection is of major importance for effective monitoring of the fermentation-based processes. Although there has been some interest in enzyme-based electro-chemical reactions for the development of sensors and sensing systems for carbohydrate analysis, high pressure liquid chromatography is still the most common method, although FTIR is rapidly being applied (13). There are also rapidly evolving capillary electrophoresis (14) and other modern techniques for carbohydrate separation analysis. Although such methods are well suited to laboratory and large volume process applications, they are at present far too expensive and

complicated for the small producer or processor, or for the retail consumer. There is also some concern that many of the methods for carbohydrate analysis of foods are not specific, and therefore analysis by different methods are not directly correlateable. This was pointed out in the current issue of the *Journal of Food Composition and Analysis* (15,16). Such concerns argue for an enzyme specific approach to carbohydrate analysis.

We have made preliminary inquiry to a number of firms that sell sensors for carbohydrate analysis and a range of firms which sell equipment and reagents for ATP analysis, generally for bacterial hygiene monitoring. All are very interested in rapid, specific methods for carbohydrate quantitative analysis. There is a strong commercial potential for such sensors, not only in the food, dairy, and beverage process industries, but also in the general consumer market where consumers are taking a greater interested in the chemical composition of what they eat and drink.

H. Current and Pending Support.

Dr. Rick Van Wagenen is now supported on internal corporate funds and will be partially supported on the NSF Phase II STTR grant. Dr. Robert Scheer is a member of the teaching faculty in the Department of Materials Science and Engineering at the University of Utah, and spends one quarter of his time with Protein Solutions, Inc. He will also have some minimal support from the Phase II STTR.

Dr. J. Andrade is 3/4 professor of Bioengineering and Materials Science at the University of Utah, and spends 1/4 of his time with Protein Solutions, Inc.. Although he is not budgeted and receives no salary from PSI he is a major stock holder and spends a full 1/4 of his time on technical and administrative activities with Protein Solutions. No funds are budgeted for his services.

I. Budget Justification.

Dr.s Van Wagenen and Scheer are PSI's key technical staff members. They, along with the post-doctoral fellow, Dr. C.Y. Wang, will be responsible for the completion of the technical aspect of the project.

Most of the budget is self explanatory. Dr. Van Wagenen is budgeted at 3 months, that is half time during this Phase I. Dr. Scheer is budgeted at 1/2 month, that is 1/12 time for this Phase I. Dr. Wang, is budgeted at 3 months, or half time. In addition, a graduate student is budgeted to assisted Drs. Wang, Van Wagenen, and Scheer in the technical work.

The materials and supplies items include proteins, enzymes, gels, reference solutes, luciferin, ATP, and related general laboratory supplies.

The consultants entry is the payment for Dr. Bart Weimer described above. Other direct costs, budged at $3400, are primarily for outside analytical services. $1000 is for analytical services through the University of Utah's Surface Analysis Laboratory. This includes the capillarity wicking instrument time and x-ray photoelectron spectroscopy analysis to perform surface characterization of the various supports and substrates, also $1000 is budgeted for protein/enzyme analytical surfaces within the Center for Biopolymers at Interfaces. This includes high-pressure liquid chromatography, high-resolution gel electrophoreses, and amino acid analysis. The remaining $1400 is budgeted for outside analytical services through commercial laboratories. These will be primarily carbohydrate analyses to test and validate our own measurements and the newly developed sensors. PSI's indirect cost rate is 60% of salaries, wages, and benefits. Although, we did not put an entry into the cost sharing category, there is substantial cost sharing. Dr. J. D. Andrade, Chief Scientific Officer of PSI and one of its founders, will not receive any compensation for the significant portion of his time he will devote to this project.

J. Multiple Phase II Awards.

PSI recently received its first Phase II award from the National Science Foundation, "Direct Reading, Quantitative Biosensors for ATP." There is no direct overlap between that proposal and this SBIR Phase I application.