OMB No. 0925-0195
Expiration Date 4/30/01

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase II Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1a. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Quantitative, Direct Reading Phenylalanine Biosensor

1b. Phase I Grant No.
1R43HD36148-01

2. PRINCIPAL INVESTIGATOR ☐ New Investigator

2a. NAME *(Last, first, middle)*
Wang, C.-Y.

2b. DEGREE(S)
B.S. | Ph.D.

2c. SOCIAL SECURITY NO.
Provide on Personal Data Page.

2d. POSITION TITLE
Research Scientist and Principal Investigator

2e. MAILING ADDRESS *(Street, city, state, zip code)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093

2f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

BITNET/INTERNET Address:
biolight@concentric.net

3. HUMAN SUBJECTS	3a. If "yes," Exemption no. or IRB approval date	Full IRB or Expedited Review	3b. Assurance of compliance no.	4. VERTEBRATE ANIMALS	4a. If "Yes," IACUC approval date	4b. Animal welfare assurance no.
☒ NO ☐ YES		☐		☒ NO ☐ YES		

5. DATES OF ENTIRE PROPOSED PHASE II PERIOD	6. COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD		7. COSTS REQUESTED FOR ENTIRE PROPOSED PHASE II PERIOD	
	6a. Direct Costs	6b. Total Costs	7a. Direct Costs	7b. Total Costs
From: 04-01-99				
Through: 03-30-01	$ 265,068	$ 392,401	$ 494,136	$ 747,362

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 G Cipeta Way, Suite #320
Salt Lake City, Utah 84108

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093

10. ENTITY IDENTIFICATION NUMBER
Fed. Tax # 87-045-1813

Congressional District
2

11. INVENTIONS AND PATENTS
☒ NO ☐ YES If "Yes," ☐ Previously reported *OR* ☐ Not previously reported

12. SMALL BUSINESS CERTIFICATION
☒ Small Business Concern ☐ Women-owned ☐ Socially and Economically Disadvantaged

13. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages 16, 27-8, 31-4 of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

15. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J. D. Andrade
Title: President and CEO
Address: Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093

14. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☒ YES ☐ NO

Telephone: 801-583-9301
FAX: 801-583-4463
BITNET/INTERNET Address:

16. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 2a *(In ink. "Per" signature not acceptable.)*
C.-Y. Wang

DATE
8/13/98

17. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 15 *(In ink. "Per" signature not acceptable.)*
J. D. Andrade

DATE
8/13/98

Small Business Innovation Research Program
Phase II Grant Application

Table of Contents

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator at the top of each printed page and each continuation page.

Appendix *(Three sets. No page numbering necessary for Appendix.)*

Number of publications and manuscripts accepted for publication *(Not to exceed ten)*: 0

Other items *(list)*:

Principal Investigator *(Last, first, middle)*: Wang, C.-Y.

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, Utah 84158-0093 Phone: 801-583-9301

YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1988	5

TITLE OF APPLICATION

Quantitative, Direct Reading Phenylalanine Biosensor

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
C.-Y. Wang, Ph.D.	Protein Solutions, Inc.	Principal Investigator
R. Van Wagenen, Ph.D.	Protein Solutions, Inc.	Research Scientist
J. D. Andrade, Ph.D.	Protein Solutions, Inc.	Advisor

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded this description, as is, will become public information. ***Therefore, do not include proprietary or confidential information.*** DO NOT EXCEED 200 WORDS.

It is now generally recognized that phenylketonuric (PKU) patients should be on a phenylalanine controlled diet and be closely monitored for their entire lifetime. Although monitoring by the patient or family member in the home environment is now recommended, no suitable tests, kits, or devices are available for this purpose. The Phase I effort demonstrated the feasibility of an NADH based bioluminescence detection system in a simple, direct reading format for the determination of phenylalanine. Phase I showed that a simple measurement of the time for the bioluminescence to decay to a value below the visual detection limit directly correlated with Phenylalanine concentration. Phase II addresses the development of a direct reading Phenylalanine biosensor for use in the home by PKU patients or their care givers. The sensor will require less than 100 microliters of blood. All necessary reagents are incorporated into the device itself. The operator needs only to close the sensor at the appropriate time and start a timer. The operator then observes the luminescence visually until it disappears and stops the timer. The elapsed time correlates with the Phenylalanine concentration. The final sensor will also include a tyrosine channel for determination of tyrosine in the same sample.

Provide key words (8 maximum) to identify the research or technology.

PKU, phenylketonuria, biosensor, bioluminescence, phenylalanine, tyrosine, NADH

Provide a brief summary of the potential commercial applications of the research.

Phenylalanine self-monitoring and self-treatment of PKU in the home environment is essential for the 25,000-plus Americans that are afflicted with this metabolic disease. Monitoring by adults and adolescents needs to be done monthly to provide feedback on proper dietary compliance. Monitoring of children should be done weekly. Thus, there is a potential for roughly 600,000 tests annually at a cost of about $10 per test which results in a gross annual market of approximately six million dollars.

Financial: Fringe benefits are 30% of salary and indirect costs are 60% of total direct personnel salaries and benefits. No subcontracts, patient care costs or renovation expenses are requested for this project. A four percent (of total direct and indirect costs) fixed fee is requested for both years of this project. This fee will provide discretionary funds to address several key aspects of the project not directly allowed by direct and indirect costs, i.e., patent application preparation, etc.

Consultants: Two consultants are budgeted on this Phase II project. Their letters agreeing to consult are included in this proposal. **Dr. Russell Stewart** is an Assistant Professor of Bioengineering at the University of Utah. He is currently a consultant to our company and serves as the Principal Investigator on a NSF STTR Phase II subcontract which addresses recombinant firefly and bacterial luciferase. Dr. Russell will meet with us at least quarterly to advise us in areas of protein engineering and protein characterization. This will be particularly crucial as regards our proposed work to develop the in house protein engineering capability to engineer recombinant luciferases and related proteins in our own facility. **Dr. Vladimir Hlady** is an Associate Professor of Bioengineering at the University of Utah. He is an internationally recognized expert in the study of proteins at interfaces using a variety of techniques, i.e., atomic force microscopy, interfacial fluorescence spectroscopy, Langmuir films, etc. Dr. Hlady will also meet with us at least quarterly to assist us with any problems we may encounter regarding the use of the various proteins involved in our biosensor assay. His surface analysis lab is available to us on a minimal fee for service basis since the company is a member of the Center for Biopolymers at Interfaces, CBI (see below). While our experience to date indicates that surface adsorption and denaturation of proteins involved in this work is not a serious problem it should be stressed that we will be working with several new proteins and Dr. Hlady's resources and expertise will be a great asset.

Equipment: The only difference between the budgets for years one and two is due to the equipment funds requested in the first year. Funds for a lyophilizer are requested in order to provide proper preservation of the enzymes and proteins comprising our biosensor. Preliminary lyophilization work in Phase I showed that with proper cryo-protectants and drying protectants our proteins in the biosensor prototype could be preserved much more effectively via lyophilization. The remaining equipment (small incubator, refrigerated micro-centrifuge, and Class II biosafety hood) are the minimum essential pieces of equipment required to set up a small protein stabilization capability in our own laboratory. We have additional lab space allocated which will be dedicated for this work. The total equipment cost is estimated to be $45,000. The company will provide a 20 percent cost sharing from other sources to help cover the costs of this equipment. The total direct cost requested funds for the equipment in year one is then reduced to $36,000.

Supplies: Funds are requested for general lab supplies and biochemical supplies such as proteins, enzymes, buffers, analytical supplies, inorganic chemicals, plastic-ware, glass-ware, and supplies related to the protein engineering needs of the recombinant luciferases and oxidoreductases, etc.

Travel: Travel funds are requested to make four trips each year to conferences related to PKU and/or metabolic disease biochemistry and clinical chemistry such as the annual meeting of the American Association for Clinical Chemistry and the annual meeting of state health directors.

Other: Funds are requested for external clinical laboratory analyses of some samples. These analyses will be conducted by Associated Regional University Pathologists, Inc. (ARUP) a major clinical chemistry laboratory located here in Research Park and serving the western U.S. Funds are also requested for a partial annual membership fee for the Center for Biopolymers at Interfaces (CBI), a state-university-industry consortium at the University of Utah which provides a number of key benefits including: (1) fee-for-service access to many analytical services at the University of Utah at very low rates, (2) access to specialized laboratory equipment in the departments of Bioengineering (Optics Labs) and Chemistry (analytical biochemistry labs), and (3) access to faculty members who can provide expertise on a consulting basis.

Principal Investigator *(Last, first, middle):* Wang, C.-Y.

Biographical Sketch

Provide the following information for the key personnel listed on Page 2, beginning with the Principal Investigator. Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Chung - Yih Wang	P.I. and Research Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(If applicable)*	YEAR CONFERRED	FIELD OF STUDY
National Taiwan University - Taipei, Taiwan	B.S.	1985	Chemical Engineering
University of Utah - Salt Lake City, Utah	Ph.D.	1997	Bioengineering

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, authors, and complete references to those publications most pertinent to this application **DO NOT EXCEED TWO PAGES.**

Professional Experience

- Research Chemist, Taiwan Power Company, Taipei, Taiwan, 1987 - 1989.
- Research Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah 1989 - 1996.
- Teaching Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah, 1990 - 1992.
- Research Scientist, Protein Solutions, Inc. Salt Lake City, Utah 1996 - present.

Honors

- University of Utah Graduate Research Fellow, 1991-1992.
- Deluca Prize, 1996.

Representative Patents and Publications

- **C.-Y. Wang** and J.D. Andrade, "Denaturation of Firefly Luciferase" *in Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and E. Kricka, eds. Wiley, p. 427, 1991.
- **C. - Y. Wang** and J. D. Andrade, "Interfacial Behavior of Firefly Luciferase" in *Bioluminescence and Chemiluminescence: Status Report*, A.A. Szalay, ed., Wiley, pp.99-103, 1993.
- **C. - Y. Wang** 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, pp. 494497, 1995.
- D.J. Min, **C.-Y. Wang** and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes" in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds, Wiley, pp. 494-497, 1995.
- **C.-Y. Wang**, S. Hitz, J.D. Andrade, and R. Stewart, "Biotinylation of Firefly Luciferase in vivo: A recombinant Protein with a Special Immobilization Site" submitted to Anal. Biochem., 1996.
- J.D. Andrade, **C.-Y. Wang**, D.-J. Min, C. Eu, R. Van Wagenen, R. Scheer, "Toward Dollar Devices for Measuring Metabolic Biochemistry", in *Anti-Microbial, Anti-Infective Materials*, S.P. Sawan and G. Manivannan, eds., Technomic Publishing, 1998, in press.

ALL CURRENTLY ACTIVE SUPPORT

Source: NSF Phase II STTR Grant
Title: Direct Reading, Quantitative Biosensors for ATP Dependent Processes
Grant #: DMI-9531303
Project Dates: October 1, 1996 - September 30, 1998
Summary of Objectives: The project goal is to develop an inexpensive, direct-reading, disposable ATP biosensor which does not require expensive or sophisticated instrumentation for analysis of analyte ATP. The quantification of the ATP concentration is determined via the spatial distribution of luminescence which is created by controlling the ATP analyte concentration via an ATP filter, i.e., a second ATP consuming enzyme which is spatially distributed on or in the biosensor. The empirical goal is 1×10^{15} moles of ATP with visual detection in the dark.
Total Direct Costs: $270,410
Percent Effort: C.-Y. Wang = 40 R. Van Wagenen = 50 R. Scheer = 40 J.D. Andrade = 10

Source: NIH Phase I SBIR Grant
Title: Quantitative, Direct Reading Phenylalanine Biosensor
Grant #: 1 R43 HD36148-01
Project Dates: 04/01/98 to 09/30/98
Summary of Objectives: Develop and evaluate a prototype biosensor employing an NADH based bioluminescence system with the goal of creating an inexpensive, direct-reading device for the diagnosis of hyperphenylalanemia and management of disorders related to phenylalanine metabolism.
Total Direct Costs: $70,970
Percent Effort: C.-Y. Wang = 40 R. Van Wagenen = 15 R. Scheer = 10 J.D. Andrade = 0

Source: NIH Phase I SBIR Grant
Title: CCD-Based Analyzer for Multi-channel Biosensor
Grant #: 1 R43 RR13087-01
Project Dates: 07/01/98 to 12/31/98
Summary of Objectives: Assess the feasibility of developing a small, relatively inexpensive, CCD-based analyzer for the direct determination of low molecular weight biochemicals related to ATP metabolism. The device would be used to analyze a multi-channel biosensor card simultaneously for a panel of clinically related analytes necessary for a correct medical diagnosis.
Total Direct Costs: $69,290
Percent Effort: C.-Y. Wang = 10 R. Van Wagenen = 60 R. Scheer = 20 J.D. Andrade = 10

Principal Investigator *(Last, first, middle):* Wang, C.-Y.

Biographical Sketch

Provide the following information for the key personnel listed on Page 2, beginning with the Principal Investigator. Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Richard A. Van Wagenen	VP for R & D and Research Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(If applicable)*	YEAR CONFERRED	FIELD OF STUDY
University of Utah - Salt Lake City, Utah	B.S.	1971	Mechanical Engineering
University of Utah - Salt Lake City, Utah	Ph.D.	1976	Materials Science
University of British Columbia - Vancouver, B.C.	Post Doc.	1976-1977	Cell Surface Research

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, authors, and complete references to those publications most pertinent to this application. **DO NOT EXCEED TWO PAGES.**

Professional Experience

- Post Doctoral Fellow, University of British Columbia, Vancouver, B.C. 1976-1977.
- Research Assistant Professor, Department of Bioengineering, University of UT, Salt Lake City, UT 1978-1985.
- Director of Instrumentation, Biomaterials International, Inc., Salt Lake City, UT 1981-1987.
- Vice President Research for Development, Albion Instruments, Salt Lake City, UT
- Director of Research and Development, OHMEDA Medical Systems, Salt Lake City, UT 1990-1994.
- RVW Consulting, Salt Lake City, UT 1995-1996.
- Vice President for Research and Development, Protein Solutions, Inc., Salt Lake City, UT 1996 - present.

Honors

- Member of Pi Tau Sigma, National Honorary Mechanical Engineering fraternity.
- Winner of the NASA Innovation Award, 1981.
- Recipient of the R & D 100 Award for Innovative Design and Development of the RASCAL II, a respiratory/anesthetic gas monitor based upon Raman light scattering spectroscopy, 1993.

Representative Patents and Publications:

- **R.A. Van Wagenen**, D.R. Westenskow, R.E. Benner, D.E. Gregonis, and D.L. Coleman, "Dedicated Monitoring of Anesthetic and Respiratory Gases by Raman Scattering", J. Clinical Monitoring, 2 215 (1986).
- D.R. Westenskow, D.L. Coleman, D.E. Gregonis, K.W. Smith and **R.A. Van Wagenen**, "Laboratory and Clinical Evaluation of a Raman-Scattering, Multiple Gas Analyzer", J. Clinical Monitoring, 3 312 (1987).
- **R. A. Van Wagenen**, J.D. Geisler, D.E. Gregonis and D.L. Coleman, "Multi-Channel Molecular Gas Analysis by Laser-Activated Raman Light Scattering", November 15, 1988, United States Patent 4,784,486.
- D.R. Westenskow, K.W. Smith, D.L. Coleman, D.E. Gregonis, and **R.A. Van Wagenen**, "Clinical Evaluation of a Raman Scattering Multiple Gas Analyzer for the Operating Room", Anesthesiology, 70 350 (1989).
- D. Gregonis, **R. Van Wagenen**, D. Coleman, and J. Mitchell, "A Commercial Anesthetic Respiratory Gas Monitor Utilizing Raman Spectroscopy", J. SPIE 1336 247 (1990).
- J.D. Andrade, C.-Y. Wang, D.-J. Min, C. Eu, **R. Van Wagenen**, and R. Scheer, "Toward Dollar Devices for Measuring Metabolic Biochemistry", in *Anti-Microbial, Anti-Infective Materials*, S.P. Sawan and G. Manivannan, eds., Technomic Publishing, 1998, in press.

ALL PENDING FUTURE SUPPORT (Applications & Proposals Pending Review or Funding)

Source: NIH Phase I SBIR
Title: A Novel Luminescent Biosensor to Measure Serum Folate
Grant #: 1 R43 AG16173-01
Project Dates: 10/01/98 to 3/30/99
Summary of Objectives: Feasibility assessment of a serum folate sensor which is a small, inexpensive, disposable biosensor based on ATP-luciferase-luciferin bioluminescence.
Direct Costs: $70,935
Percent Effort: C.-Y. Wang = 50 R. Van Wagenen = 33 R. Scheer = 50 J.D. Andrade = 10
Priority Score: 317

Source: NIH Phase I SBIR
Title: A Novel Enzyme Based Homocysteine Biosensor
Grant #: 1 R43 HL61099-01
Project Dates: 10/01/98 to 3/30/99
Summary of Objectives: Assess the feasibility of developing a simple, specific, accurate and inexpensive, biosensor for the analysis of serum homocysteine. The technology is a multi-enzyme based bioluminescent assay with the final stage being the ATP-luciferase-luciferin reaction to produce light.
Direct Costs: $70,935
Percent Effort: C.-Y. Wang = 50 R. Van Wagenen = 33 R. Scheer = 50 J.D. Andrade = 10
Priority Score 291

Source: NIH Phase I SBIR
Title: Quantitative, Rapid Sensor for Glycosylated Hemoglobins
Grant #: 1 R43 DK54580-01
Project Dates: 10/01/98 to 3/30/99
Summary of Objectives: The focus of this work is to develop and evaluate a thin layer chromatography based device to permit high resolution separation, visualization, and quantification of glycosylated hemoglobin (gHb) without the need for instruments or expensive detectors. Such a device would be disposable, inexpensive, and easy to use and would permit high resolution separation coupled with direct visualization and quantification of gHb.
Direct Costs: $70,037
Percent Effort: C.-Y. Wang = 25 R. Van Wagenen = 33 R. Scheer = 25 J.D. Andrade = 10
Priority Score 344

Source:	NIH Phase I SBIR Grant			
Title:	Creatinine Biosensor for Renal Transplant Monitoring			
Grant #:	1 R43 DK55426-01			
Project Dates:	01/01/99 to 06/30/99			
Summary of Objectives:	Evaluate the feasibility of developing an easy to use, low cost personal biosensor for serum creatinine which is a critical indicator of renal function of a transplanted kidney in the home environment. The biochemistry involves the conversion of creatinine to creatine. The later is then phosphorylated by the enzyme creatine kinase with the simultaneous consumption of ATP. Unused ATP is then quantified via the firefly-luciferase bioluminescent reaction which produces light measured with a CCD based luminometer. The two channel biosensor measures both creatinine and creatine and will be applicable to both blood and urine samples.			
Direct Costs:	$71,062			
Percent Effort:	C.-Y. Wang = 50	R. Van Wagenen = 33	R. Scheer = 50	J.D. Andrade = 10
Priority Score:	289			

Source:	NIH Phase II SBIR Grant			
Title:	Biosensor for Rapid Screening of Galactosemia			
Grant #:	1 R43 MH57591-01			
Project Dates:	01/01/99 to 12/31/00			
Summary of Objectives:	Develop an ATP-luciferase based bioluminescent dip-stick biosensor for the rapid, point of care screening of newborns for galactosemia.			
Direct Costs:	$494,136			
Percent Effort:	C.-Y. Wang = 80	R. Van Wagenen = 40	R. Scheer = 50	J.D. Andrade = 10
Priority Score:	312			

THERE IS NO OTHER ACTIVE OR PENDING SUPPORT FOR WORK RELATED TO THIS PHASE II NIH PROPOSAL WITH THE EXCEPTION OF SOME RESIDUAL FUNDS LEFT IN THE PHASE I PROJECT ACCOUNT.

POTENTIAL OVERLAPS (SCIENTIFIC, BUDGETARY, AND LEVEL OF COMMITMENT) OF THIS PHASE II PROPOSAL VERSUS OTHER PROJECTS FUNDED OR PENDING:

All currently active SBIR grant support will end by December 31, 1998. In terms of pending future support there is very little realistic possibility that any of the pending proposals will be funded during 1999. This is based on the priority scores received to date and conversations with program management personnel regarding the possibility of funding.

There are no scientific overlaps anticipated between this Phase II proposal and current or future work.

Specific Aims

The feasibility of a direct reading phenylalanine assay was established in the Phase I study. As suggested in the final report, a quantitative biosensor in time mode has strong potential for commercialization because it is instrument-free and can be used in both point of care and home environments. The major goal of the phase II proposal is the design and validation of a prototype phenylalanine biosensor (Figure 1). The specific aims include:

1. Design and prototype the phenylalanine direct reading sensor with intensity-time profiles suitable for direct visual detection. Calibration of the operator's visual detection thresholds will be developed.

2. Design and prototype the sample zone. This is the first part of the biosensor. The whole blood sample will be separated into cells and plasma. The materials will be selected based on the recovery of plasma.

3. Design and prototype the phenylalanine conversion zone (Reaction zone 1). The pH of the plasma is adjusted to pH 10.5, required for optimum phenylalanine dehydrogenase activity. Phenylalanine dehydrogenase and NAD^+ will be pre-deposited on the matrix. The plasma sample will be incubated in this zone until all the phenylalanine has been reacted. We will determine the optimum reaction time (1-5 min). The NADH produced will flow into the signal transduction zone when the two piece sensor is closed.

4. Design and prototype the signal transduction zone (Reaction zone 2). This zone contains the dual enzyme system that converts NADH to photons. The bacteria luciferase and oxidoreductase, together with FMN and aldehyde, will be deposited on the matrix and the concentration of each component will be optimized.

5. Design and prototype a tyrosine channel. Because the PKU condition prevents the normal conversion of phenylalanine to tyrosine, measurement of tyrosine is needed to monitor the nutritional status of the patient. There is a phenylalanine dehydrogenase (from Bacillus sphaericus) that is specific for both phenylalanine and tyrosine allowing us to measure the total concentration of phenylalanine and tyrosine. The difference between the summation channel and the phenylalanine channel provide the concentration of tyrosine.

6. Assemble, calibrate, validate, and study the biosensor prototype and its long-term stability. The prototype biosensor will be calibrated with known phenylalanine concentrations in plasma. The linearity, sensitivity and reproducibility in the clinical range of phenylanine (50 μM to 1000 μM) will be studied. The prototype biosensor will be validated with the commercial Quantase phenylalanine assay (EG&G Wallac) and by HPLC methods.

Principal Investigator *(Last, first, middle):* Wang, C.-Y.

Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. (The research to be performed by the applicant small business concern and its collaborators must be in facilities that are available to and under the control of each party for the conduct of each party's portion of the proposed project.) Indicate the facilities at the applicant small business concern and any other performance site listed on the FACE PAGE where the facilities are located and describe their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as secretarial, machine shop, electronics shop, and the extent to which they will be available to the project. Use continuation page(s) if necessary.

Laboratory: Chemistry lab (480 sq.ft.), Biology lab (150 sq.ft.), Optics lab (150 sq.ft), and an option on a Class II Biosafety lab (200 sq.ft) if the Phase II effort is funded.

Clinical: There are no clinical facilities available and no clinical work is planned for the Phase II effort.

Animal: There are no animal research facilities available and no animal experiments are proposed for the Phase II effort.

Computer: Computer resources include two Pentium PC machines and two Pentium lab top machines.

Office: There are two offices with a combined space of 650 sq. ft. There is one 600 sq.ft comference room

Other: Office and secretarial resources are standard and adequate to address the administratve aspects of the proposed project, i.e., computers, laser printers, copy machine, FAX machine, etc. The company has licensed technology from the University of Utah in the area of bioluminescence for biosensor applications. The company is also a member of The Center for Biopolymers at Interfaces (CBI), a state-university-industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) low fee for service access to many analytical services at the University of Utah, e.g., the SEM/TEM facility, the Surface Analytical facility (XPS, SIMS, etc.), (2) access to specialized laboratory equipment in the departments of Bioengineering (Optics Lab) and Chemistry (analytical biochemistry labs), and (3) access to faculty members who can provide expertise on a consulting basis.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Basic Laboratory equipment at Protein Solutions, Inc. includes analytical balances, stirrers, hot plates, a fume hood, oven, pH meter, water baths, HEPA filtered laminar flow air work stations, steam autoclave, reverse osmosis - filtered water, light microscopes, Turner Designs luminometer, cameras, vacuum system, gel electrophoresis system, and a -85 C freezer. Charge coupled device (CCD) array cameras and associated computers and controllers are also available for the recording and quantification of luminescence experiments to summplement the PMT based luminometer. A Beckman UV-Vis spectrophotometer is available for spectral absorbance analysis of proteins. If the proposed equipment in the Phase II budget is approved the equipment resources will be adequate to accomplish all of the specific aims of the project.

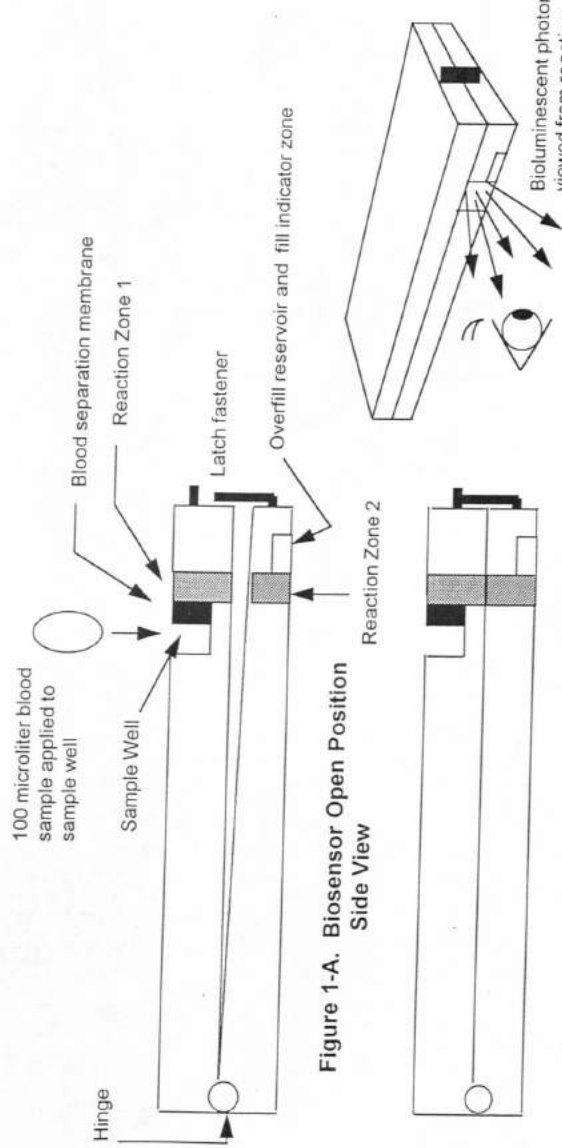

Figure 1-A. Biosensor Open Position Side View

Figure 1-B. Biosensor Closed Position Side View

Figure 1-C. Biosensor Viewing Orientation

FIGURE 1. Preliminary design of the phenylalanine biosensor. A 100 microliter blood sample is applied to the sample well which includes a commercial blood separation membrane to retain formed cells from the plasma sample. The plasma volume is wicked by capillary forces to reaction zone 1 where the phenylalanine dehydrogenase reaction produces NADH. (1-A) This reaction requires a fixed completion time of less than five minutes. After that time the operator snaps the sensor device closed via the hinged mechanism, thereby making reaction zone 1 contact reaction zone 2 (1-B). Zone 2 contains the NADH reaction reagents. Zone 2 fills rapidly, i.e., about ten seconds, and bioluminescence commences. Timing begins with the closure of the biosensor device. The operator views the bioluminescence light from the side and records the time at which the light emission disappears (1-C). This time is proportional to the phenylalanine in the blood sample.

Significance

PKU is an inborn error of metabolism of moderate incidence (~1 in 10,000). About 99% of PKU patients have a recessively inherited deficiency of phenylalanine hydroxylase, an enzyme important in the conversion of phenylalanine (Phe) to tyrosine (Smith, 1993). The defect results in excessive concentrations of Phe (normal range is about 100 microM), ranging to 800 microM or even higher. Such high Phe levels lead to severe mental and growth retardation. Fortunately, most states and developed nations now routinely screen infants for PKU within a few days of birth. PKU patients are placed on a Phe-restricted diet (Smith, 1994). If the diet is started early and maintained for life, treated patients can lead normal lives with no evident mental or physical problems.

There has been growing awareness that PKU requires regular self-monitoring to ensure dietary compliance and facilitate diet adjustments (Wendel and Langenbeck, 1996). The management and treatment of PKU has many similarities to diabetes. The problem is that there are no simple, inexpensive means to measure Phe in the home environment. The availability of such a device would greatly improve the monitoring and management of PKU. The best way to verify this statement is to quote recent key papers.

From Smith, 1994:

"...Methods currently used for measurement of blood phenylalanine concentrations will need to be improved and the frequency of monitoring will need to be increased."

"The management of phenylketonuria is going to be even more taxing for the patients and their families than in the past."

"Diagnostic investigation should include an assessment of protein intake, quantitative measurement of plasma amino acids."

"Blood phenylalanine concentrations taken at a standard time (ideally early morning when concentrations are likely to be at a peak) should be monitored at least weekly once intake has stabilized, aiming to keep phenylalanine concentrations between 120-360 microM."

"Biochemical monitoring should continue on a weekly basis up to at least 4 years of age. After 4 years and up to 10 years the frequency of monitoring can be reduced to fortnightly and, thereafter, monthly."

"Strategies aimed at getting children to be responsible for their own diet and blood tests by school age need to be much more actively promoted than in the past."

"It is the common experience that it becomes increasingly difficult to maintain strict phenylalanine control in older children.."

"Adults and adolescents with phenylketonuria require continued delivery of services in an appropriate setting. Services must include facilities for frequent biochemical monitoring and dietetic advice (by post and telephone) and specialist medical adult services for both outpatient follow up and inpatient care. Adult physicians with a special interest in metabolic disease need to be linked to existing regional services."

"Phenylalanine concentrations in pregnancy need to be at least as strictly (and probably more strictly) controlled as in infancy. Due to the positive amino acid gradient across the placenta the fetus is exposed to even higher phenylalanine

Another use...would be to provide information for the maintenance of metabolic control in pregnant women with PKU... maintain strict metabolic control throughout the pregnancy."

The "Quantase" technology, used in the study by Wendel and Lagenbeck, 1996, has not been developed for direct home use. Perhaps the reason for the lack of commercial interest is the relatively low incidence of PKU (roughly 20,000 patients and perhaps 500,000 tests per year in the USA). One could, therefore, call a Phe dipstick device for PKU an "orphan diagnostic" (an analogy to "orphan" drugs). There is a market, but it is not a large one.

Tyrosine (Tyr) is also of vital importance in the diagnosis and management of PKU, thus direct Tyr measurement is also of major interest. We therefore plan to include a Tyr channel in the final sensor.

Our Phe and Tyr sensor will serve as a model for other amino acid sensors. Our experience with Phe, and the technology generated via this Phase I/II SBIR grant, will enable us to develop other specific amino acid sensors, eventually producing a sensor which measures each of the essential amino acids. Such a multi-channel sensor will have a much larger market, including the nutrition, parenterals, and gerontology communities.

Phase I final report

SPIB Phase I grant NO. 1 R43 HD36148-01
Project period: from 4/1/1998 through 9/30/1998

Table 1. The key persons and working effort

Title	Name	Dates of service	% FTE	% Effort
Principal Investigator	Chung-Yih Wang, Ph.D.	4/1/98-9/30/98	100	50
Research Scientist	Robert J. Scheer, Ph.D.	4/1/98-8/10/98	33	50
Research Scientist	Rick Van Wagenen, Ph.D.	4/1/98-9/30/98	100	20
Technical Advisor	Joseph D. Andrade, Ph.D.	4/1/98-9/30/98	25	10
Lab Technician	Mara Hammer	4/1/98-9/30/98	50	50

Phase I Abstract

The management of diseases based on inborn errors of metabolism could be significantly improved if simple, low cost means for the measurement of the biochemical abnormality were readily available to patients in the home environment. Although all states and most developed nations have effective screening programs for newborns, the management of patients diagnosed with PKU and related diseases can be significantly improved.

We propose to apply a unique NADH based bioluminescence based detection system in a simple, direct reading format to the determination of phenylalanine in plasma and urine. We avoid the problem of the need for instrumentation to measure the intensity of light output, by application of a proprietary technology where the spatial position of the light is directly related to the concentration of the substrate of interest. The spatial approach has greatly simplified the detection problem and even permits direct visual detection. We propose to demonstrate the feasibility of this approach to phenylalanine

concentrations than the mother. Biochemical monitoring should be undertaken twice a week, both in the period before conception and during pregnancy, aiming at values of 60-250 microM. Effective contraception should be continued until control has been achieved. Pregnancies require careful monitoring."

From Wendel and Langenbeck (1996):

"...A satisfactory diet compliance with ideally low blood phenylalanine (Phe) concentrations can only be obtained if the principle of frequent monitoring the metabolic control (blood Phe) by the patient himself (self-monitoring) is realized."

"Frequent self monitoring of the blood Phe concentration is important in enabling a patient to arrive at an exact knowledge of the individual variables of Phe metabolism in everyday life and at an efficient way of self treatment. In this respect the patient himself can determine how to maintain the metabolic balance between Phe intake and blood Phe levels during accelerated protein catabolism as seen in fasting and illness and how to reduce a high blood Phe level in a practical way."

"Another important aspect of self-monitoring blood Phe might be its motivational effect... participants of a PKU camp... during a 1-week period... experienced that they could influence Phe levels directly by changing Phe intake."

"Self-monitoring and self-treatment in PKU is feasible and should be instituted as early as possible in this disorder. In principle, PKU patients are able to close the feedback loop in metabolic control of their disorder. Self-monitoring might have a motivational effect for the patient and might encourage him or her to be responsible for their own diet and blood tests, and in this way might improve dietary compliance. Self-monitoring might also allow greater flexibility in food intake."

And from Wendel in 1994:

"...There is a recommendation for a continued low-phenylalanine diet in juveniles and adults. Apparently, a satisfactory dietary compliance with ideally low plasma phenylalanine concentrations cannot be expected in this age group until the principle of monitoring the metabolic control by the patient himself is realized, such as is practiced in diabetes mellitus since long. Self-monitoring requires assaying the phenylalanine concentration in capillary blood."

"The actual phenylalanine value... can guide the patient's adjustment of dietary phenylalanine intake provided he or she were informed in detail about their disease and trained in practical diet competence."

Although McCabe and co-workers (Peterson, et al., 1988) proposed a home device for Phe "estimation" some ten years ago, they were disappointed that there was no commercial/industrial interest in manufacturing and selling such a device. McCabe noted that:

"A simple, portable monitoring system would provide families and their local physicians with an estimate of the blood phenylalanine concentration within an hour of obtaining the specimen. This might be especially useful in attempting to moderate rising phenylalanine concentrations during intercurrent illnesses. Home or office monitoring should not replace the clinical laboratory, but would supplement the traditional process by providing more rapid and frequent phenylalanine estimates.

measurement for the diagnosis of hyperphenylalanemia and for the management of diseases related to disorders in phenylalanine metabolism.

Specific Aims of Phase I proposal

1. To evaluate the stability and reaction kinetics of available L-phenylalanine dehydrogenase [PheDH] enzymes (E.C. 1.4.1._) as a function of pH, temperature, ionic strength, buffer type and substrate concentration.
2. To apply the NADH/FMNH2 oxidoreductase (OR)-bacteria luciferase (BL) reaction in the quantitative spatial mode to permit instrument-less detection.
3. To spatially separate the sample PheDH rection from the NADH bioluminescence reaction so as to facilitate optimum enzyme environments.
4. To demonstrate response of sensing conditions to phenylalanine in clinically relevant ranges (100-1000 micro molar with < 5% accuracy); to compare phenylalanine sensor response with existing kits and methods; to assess feasibility of producing a true dry dipstick device.

Results

1. Evaluation of phenylalanine dehydrogenase

Phenylalanine dehydrogenase is the enzyme that catalyzes the interconversion of phenylalanine and phenylpyruvate (reaction 1). We have reviewed six different phenylalanine dehydrogenases (Table 2). Although the optimum pH of the reaction is species specific, in general the deamination of phenylalanine requires a high pH to ensure the forward reaction. The amination of phenylpyruvate dominates at lower pH. Phenylalanine dehydrogenase also oxidizes other amino acids and such interferences must be considered in the calibration of the biosensor.

$$\textbf{Phenylalanine} + NAD^+ + H_2O \xrightarrow{\text{Phenylalanine dehydrogenase}} \text{Phenylpyruvate} + \textbf{NADH} + NH_4^+ \quad (1)$$

$$\textbf{NADH} + FMN + H^+ \xrightarrow{\text{Oxidoreductase}} NAD^+ + \textbf{FMNH}_2 \quad (2)$$

$$\textbf{FMNH}_2 + RCHO + O_2 \xrightarrow{\text{Bacteria luciferase}} FMN + RCOOH + H_2O + \textbf{Light} \quad (3)$$

The phenylalanine dehydrogenases reviewed are all stable at room temperature and can be used in the assay. Two commercially available phenylalanine dehydrogenases, Sporosarcina species from Sigma and Rhodococcus Sp from Calbiochem, were chosen for further study. Phenylalanine dehydrogenase from Sporosarcina species has a lower km for phenylalanine, and thus is expected to bind stronger to phenylalanine in the equilibrium state.

Table 2. Review of phenylalanine from different species

Species	Molecular weight	Km	Specificity (Phenylalanine= 100%)	Optimum pH and Stability	References
Sporosarcina ureae	305,000 (octamer)	For phenylalanine: 0.096 mM For NAD: 0.14 mM For phenulpyruvate: 0.16 mM	Tyrosine: 5.4% Leucine: 2.3% Methionine: 4.1% Tryptophan: 5.0%	For oxidative deamination: 10.5 For reductive amination: 9.0 Thermostability: 40 °C	Y. Asano et al., J. Biol. Chem., 262, 10346-10354, 1987
Rhodococcus Sp	69,000	For phenylalanine: 0.16 mM For NAD: 0.12 mM For phenulpyruvate: 0.16 mM	Tyrosine: - Leucine: - Methionine: - Tryptophan: -	For oxidative deamination: >10 For reductive amination: 9.25 Thermostability: -	W. Hummel et al., Home-monitoring and screening of phenylketonuria. S. Girotti et al., Talanya, 40, 425-430 (1992).
Rhodococcus maris	70,000 (dimer, 35,000/subunit)	For phenylalanine: 3.8 mM For NAD: 0.25 mM For phenulpyruvate: 0.5 mM	Tyrosine: 2.0% Leucine: 2.0% Methionine: 5.4% Tryptophan: 7.5%	For oxidative deamination: 10.8 For reductive amination: 9.8 Thermostability: 35 °C	H. Misono et. al., J. Bacteriology, 171, 30-36, 1989
Bacillus badius	310,000 (octamer)	For phenylalanine: 0.088 mM For NAD: 0.15 mM For phenulpyruvate: 0.11 mM	Tyrosine: 9.0% Leucine: 3.0% Methionine: 8.0% Tryptophan: 4.0%	For oxidative deamination: 10.4 For reductive amination: 9.4 Thermostability: 65 °C	Y. Asano et al., Eur. J. Biochem., 168, 153-159, 1987
Bacillus sphaericus	340,000 (octamer)	For phenylalanine: 0.22 mM For NAD: 0.17 mM For phenulpyruvate: 0.4 mM	Tyrosine: 72% Leucine: 1.3% Methionine: 3.0% Tryptophan: 1.2%	For oxidative deamination: 11.3 For reductive amination: 10.3 Thermostability: 55 °C	Y. Asano et al., J. Biol. Chem., 262, 10346-10354, 1987
Thermoactinomyces intermedius	270,000 (hexamer, 41,000/subunit)	For phenylalanine: 0.078 mM For NAD: 0.045 mM For phenulpyruvate: 0.045 mM	Tyrosine: 0% Leucine: 3.9% Methionine: 0% Tryptophan: 0%	For oxidative deamination: 11 For reductive amination: 9.2 Thermostability: 70 °C	T. Ohshima et. Al. J. Bacteriology, 173, 3943-3948, 1991

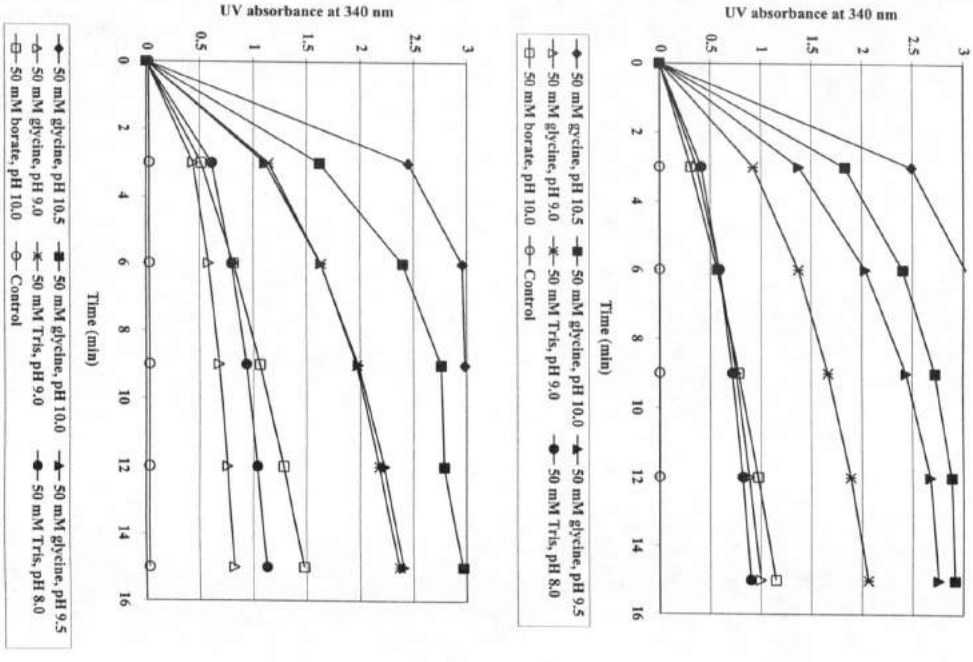

Figure 2. The influence of buffer and pH on the activity of commercially available phenylalanine dehydrogenase. (Top) Sigma phenylalanine dehydrogenase. (Bottom) CatBiochem phenylalanine dehydrogenase

The purity of two phenylalanine dehydrogenases was determined by SDS-polyacrylamide gel electrophoresis. The enzyme prepared by Sigma showed a major band at 40 kD which corresponds to one subunit of the enzyme. The enzyme prepared by Calbiochem showed a very light band at 69 kD and many impurity bands.

The specific activity and stability of both enzymes were determined by measuring NADH absorbance at 340 nm. Both enzymes showed about one third of activity after being reconstituted in water (Table 3). The Calbiochem enzyme quickly deteriorated in solution and retained only 22% activity after 1 week storage. The Sigma enzyme exibited good stability in solution and retained full activity after 1 week storage.

Buffer type and pH

Both phenylalanine dehydrogenases showed similar response to the change of buffer and pH. The highest enzyme activity for the oxidation of phenylalanine was in 50 mM glycine buffer, pH 10.5 (Figure 2). The enzyme activity decreases with decreasing pH in glycine buffer, as expected. The enzyme showed better activity in 50 mM Tris buffer than 50 mM glycine buffer at pH 9.0, suggesting Tris buffer may shift reaction equilibrium in favor of oxidation of phenylalanine. Enzyme in 50 mM borate buffer at pH 10.0 showed poor initial reaction rate (0-3 min). However, after 15 min reaction, the NADH produced in borate buffer exceeded that produced in glycine pH 9.0 and Tris pH 8.0. It suggested that borate buffer inhibited enzyme activity and the reaction equilibrium developed slowly in borate buffer. From these results we concluded that 50 mM glycine at pH 10.5 should be used to prepare the phenylalanine assay. 50 mM glycine at pH 10.0 will also be used to study enzyme properties.

Table 3. Comparison of two commercially available phenylalanine dehydrogenases

Sources	Claimed activity	Tested activity	1day storage in solution, 4 °C	1week storage in solution, 4 °C
Sigma (from Sporosarcina species)	14.9 units/mg solid	4.7 units/mg solid (31.4%)	100% activity	100% activity
Calbiochem (from Rhodococcus Sp. M4)	0.33 units/mg solid	0.10 units/mg solid (30.6%)	76% activity	22% activity

Tested conditions: for claimed activity: 50 mM glycine buffer, pH 10.5, 30 °C; for specific activity: 50 mM glycine buffer, pH 10.5 and room temperature; for storage stability: 50 mM glycine buffer, pH 10.0 and room temperature

Lyophilization

It is well known that the long-term stability of proteins and enzymes in liquid formulations is difficult to achieve relative to the stability of proteins and enzymes in the dry (lyophilized) state. Because we intend to market this assay as a dry reagent type device, use of liquid reagents (other than the sample itself) is prohibitive. Therefore, we

have developed a formulation and protocol for freeze-drying (lyophilizing) our assay reagents on a common support.

Because firefly luciferase catalyzes a bioluminescent reaction, it is easy to measure the enzyme activity. We used firefly luciferase as a model protein to evaluate the performance of the wicking materials (Table 4). The protectants used in the lyophilization include BSA/DTT (final concentration: 0.6 mg/ml, 0.6 mM), PEG 8000 (90 mg/ml) and trehalose (280 mg/ml). The results suggested that polyester "Transorb™" wicks from Filtrona Richmond was a suitable material with which to prepare the dry reagent. We further used this material as the substrate for lyophilization of phenylalanine dehydrogenase.

Sigma phenylalanine dehydrogenase, which retains better stability in solution, was used in the lyophilization study. The wicking materials were cellulose nitrate membrane (from Sartorius) and polyester wick (from Filtrona Richmond). Phenylalanine dehydrogenase was deposited on the wicking materials and lyophilized with protectants. The activity of phenylalanine dehydrogenase was determined by measurement of the NADH produced and measured with an UV spectrophotometer (at 340 nm). For the frozen samples, the enzyme activity recovered was 70 % for the membrane and 100 % for the wicking stick. For the lyophilized samples, the enzyme activity recovered was 89 % for the membrane and 97 % for the wicking stick. Similar to our experience with firefly luciferase, phenylalanine dehydrogenase lyophilized on the polyester wicking stick retains better activity. The phenylalanine dehydrogenase was also lyophilized in the wells of multiwell plates and stored for 25 days. There was no loss of enzyme activity during 25 day storage at 4 °C.

Table 4. Materials evaluated

Material	Manufacturer Location	Performance
8 μm pores, cellulose nitrate	Sartorius (Edgewood, NY)	fair
"Hemasep V™"	Gelman/Pall (Port Washington, NY)	fair
"Cellulosic"	Micron Separations (Westborough, MA)	fair
"Magna™ Nylon"	Micron Separations	good
"Separation Membrane"	Spectral (Toronto, ON, Canada)	good
polyester "Transorb™" wicks	Filtrona Richmond (Richmond, VA)	excellent

Ionic strength

The influence of ionic strength on the phenylalanine dehydrogenase activity was determined in 50 mM glycine, pH 10.0. The ionic strength was adjusted by adding 50, 100, 150, 200, 300, and 400 mM NaCl (final concentration) into the phenylalanine assay. The absorbance at 340 nm was measured. There were no significant (variation within 10%) differences for enzyme activity in the range of ionic strength studied.

Reaction rate of phenylalanine dehydrogenase

The conversion rate of NAD^+ to NADH, catalyzed by phenylalanine dehydrogenase, was determined at phenylalanine concentrations of 0.1 mM to 1 mM. The NADH produced was monitored by UV absorbance at 340 nm. An equilibrium was reached in 6 min for phenylalanine concentrations below 0.4 mM (Figure 3). The conversion was over 99% of the phenylalanine added. There is no absorbance increase from 15 min to 30 min for those samples. The absorbance increases 3.1%, 4.7% and 6.0% from 15 min to 30 min for 0.6 mM, 0.8 mM and 1.0 mM phenylalanine, respectively. It is concluded that the phenylalanine dehydrogenase catalyzed reaction can be considered complete after 30 min incubation. This reaction time can be reduced by increasing the enzyme concentration. Such optimization work will be performed in the Phase II project.

Conclusion

Although both enzymes showed similar activity when freshly reconstituted, we selected Sigma phenylalanine dehydrogenase for further study because of its higher purity and stability.

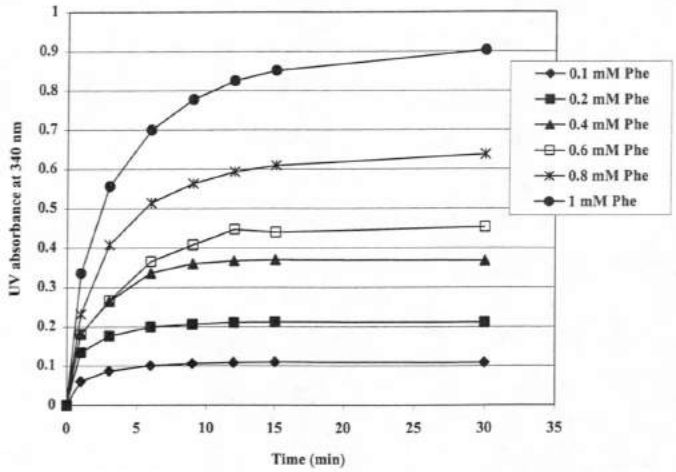

Figure 3. Evaluation of the completeness of the phenylalanine dehydrogenase catalyzed reaction. Phenylalanine concentrations shown here are the original concentrations in samples. The final concentration of phenylalanine is one tenth of the original concentration.

NADH concentrations to test our assay: 0.2 mM, corresponding to a normal phenylalanine concentration, 0.3 and 0.4 mM corresponding to suspected elevated values of phenylalanine; and 0.7 mM, corresponding to highly elevated phenylalanine concentration. In principle, a quantitative biosensor with numerous channels or a continuous enzyme gradient can be made to measura a highly precise phenylalanine concentration. Our experience with these experiments suggested that a time mode could provide a simpler, more quantitative sensor.

The quantitative NADH assay in time mode

Optimum conditions for NADH assay in the time mode were studied. The bacterial luciferase concentration, the ratio of bacterial luciferase/oxidoreductase, and the FMN concentration were adjusted to provide maximum bioluminescent intensity. The results suggested that the best assay should include 20 µl bacteria luciferase (Sigma, 5 mg protein/ml), 5 µl NADH:FMN oxidoreductase (0.8 unit/ml), 20 µl FMN (10 µM in 0.1 M phosphate pH 7), 20 µl dodecanal (1% in methanol), 135 µl phosphate (0.1 M, pH 7), and 50 µl NADH (in 50 mM glycine, pH 10.5). The CCD camera was set in half size image and auto-grab modes. The integration time was 30 sec. Figure 5 (top) shows that the bioluminescence develops almost immediately. It is a high value for NADH concentration > 0.4 mM and is maintained for up to 10 minutes at high NADH (1.2 mM). The decay of bioluminescence is rapid when the residual NADH concentration fell in the range of 0.1 to 0.2 mM. The maximum decay rate is about 800 counts/min, and the bioluminescence becomes invisible at 2 min for low NADH initial concentrations. Figure 5 (top) also shows the visual detection line. At intensities below 500 counts, the light is not visible to the average, non-dark adapted eye. We thus define this point as the visual detection threshold. Figure 5 (bottom) is the plot of the time for bioluminescence decay to below the visual threshold as a function of NADH concentration, showing that the time measure is linear with NADH concentration.

3. Coupling the phenylalanine conversion reaction reaction with NADH assay

To determine the phenylalanine concentration in the sample, we studied the two reactions using multi-well plates. The first reaction was at pH 10.5 and used phenylalanine dehydrogenase to convert phenylalanine to phenylpyruvate and NADH in the presence of NAD^+. After 15 min incubation, the NADH concentration was maximal and the solution was transferred to the second well. The phosphate buffer in the well adjusted the pH to 7.3, which is suitable for the bacterial bioluminescent assay. The time for bioluminescence to disappear was recorded. The NADH concentration in the sample is determined from the calibration chart (Figure 5 bottom). Figure 6 shows the decrease of bioluminescence in the coupled assay. The bioluminescence quickly fell below the visual threshold for phenylalanine concentrations smaller than 0.6 mM. Because the assay is in wells in aqueous solution, the dilution (dilute 2.5 fold) decreases the sensitivity of this assay. In the phase II project, a dry reagent will be prepared, eliminating the need for dilution and thus enhancing the sensitivity of assay.

2. To prepare the oxidoreductase-bacteria luciferase assay in quantitative spatial mode.

Why abandon NADH oxidase ?

In the original proposal we suggested using NADH oxidase to consume excess NADH. The NADH oxidase was to be used as a filter to build up spatial resolution in the phenylalanine biosensor. The enzyme required as part of the bioluminescent assay, NADH:FMN oxidoreductase, can perform the same function. Excess NADH would be converted to $FMNH_2$ and oxidized via an auto-oxidation pathway. To simplify the design of the phenylalanine sensor, we decide to drop the use of NADH oxidase. Instead, we used a proper ratio of NADH:FMN oxidoreductase to bacteria luciferase to create the spatial resolution as proposed for the Phase I project.

The semi-quantitative NADH assay in spatial mode

The bioluminescent assay to spatially resolve the NADH concentration was designed according to different enzyme combinations (bacteria luciferase/oxidoreductase). The number of bright spots which remained after 5 min incubation was dependent on the NADH concentration (Figure 4). We selected four

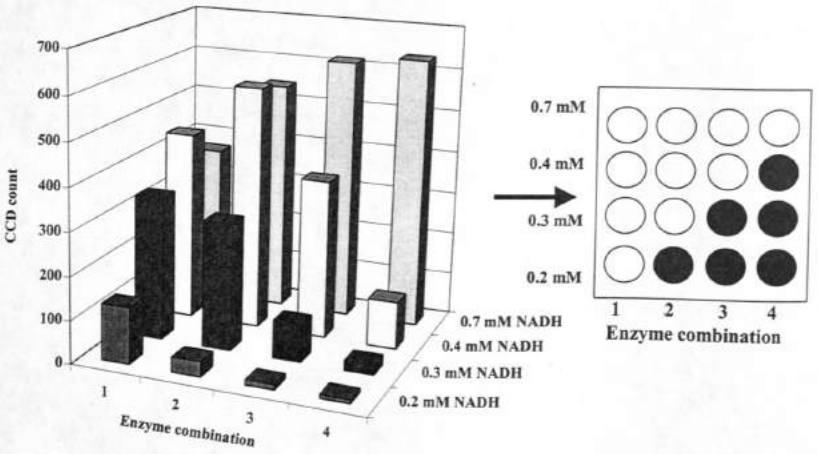

Figure 4. The phenylalanine biosensor with spatial resolution. Data was recorded with a CCD camera 5 min after the reaction was initiated. The figure at right was filtered using a 100 counts cutoff subtraction. CCD counts below 100 are invisible by eye and were assigned a black spot. The white spot indicates those wells visible by eye. Enzyme final concentrations of bacteria luciferase to oxidoreductase, respectively: (1) 5.2 μM, 1 μM; (2) 5.2 μM, 1.33 μM; (3) 5.2 μM, 1.67 μM; (4) 5.2 μM, 2 μM.

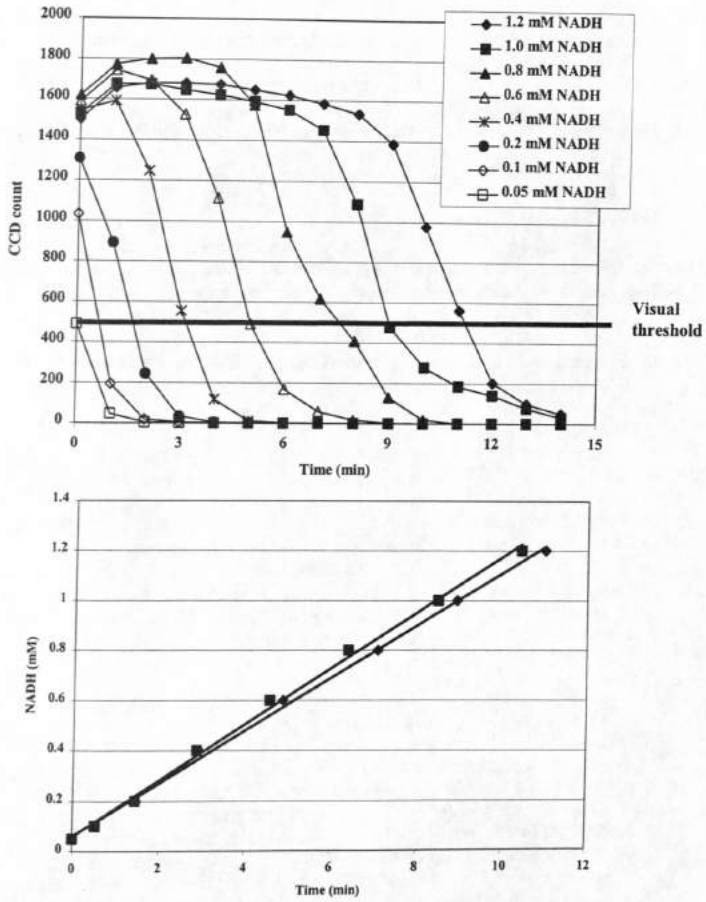

Figure 5. Feasibility of direct reading bioluminescent based sensor in time mode. (Top) Bioluminescent intensity recorded with a CCD camera. (Bottom) The time for bioluminescence to reach the inflection point (—■—) and to disappear (—◆—).

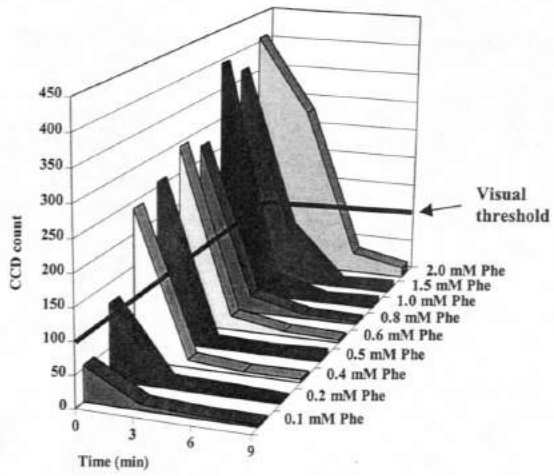

Figure 6. Direct measurement of phenylalanine concentration with coupled assay. The original concentration of phenylalanine in the sample is shown on the axis. Note that the bioluminescence is present almost immediately upon mixing, followed by a time-dependent drop to below the visual threshold. The vertical axis here is a different scale than in Figure 5 due to different dilution and to a shorter CCD integration time. The data do indicate the feasibility of measuring phenylalanine via the bioluminescence decay time.

4. The status of commercial kits

There are four well-estabilished methods for phenylalanine measurement. The first one is Guthrie bacteria inhibition assay, which grows Bacillus subtilis in the presence of phenylalanine. The second assay is an enzymatic assay, which uses phenylalanine dehydrogenase to oxidize phenylalanine in the presence of NAD^+. The product, NADH, is then measured colorimetrically using a tetrazolium (colorless)/formazan (colored) system. This assay requires a spectrophotometer, is not specific, and suffers from sensitivity problems. The third method is a fluorometric method involving a ninhydrin reaction, which is enhanced by L-leucine-L-alanine. All three assays were evaluated by Wang et al. in Canada. They concluded that all three perform adequately for newborn screening. The fourth method uses HPLC for amino acid analysis and can be automated, but requires expensive equipment (Dhondt, 1993). We select the third assay (distributed by Sigma) to compare with our results. (Table 5).

1. Design of prototype

The analysis mode for the Phenylalanine biosensor is direct visual observation of the emitted bioluminescence. This approach was successfully demonstrated in the Phase I feasibility work using a total 250 microliter volume of sample plus reagents. The use of a 250 microliter reaction/analysis volume was primarily for convenience during Phase I. We were able to demonstrate that 15 microliters of the original 250 microliter volume was visually detectable in the dark with only minimal dark adaptation of the observer, i.e. less than two minutes. The 15 microliter sample was in the form of a 3mm diameter droplet on a white, hydrophobic Teflon background. This volume is the basis of the proposed sensor design shown in Figure 1. A 36 microliter portion of the 100 microliter blood sample is carried by capillarity into Zone 1 and then, after the sensor is closed, 18 microliters is pulled from Zone 1 into Reaction Zone 2 and the indicator zone by capillarity. This process provides about 15 microliters in Zone 2 for direct visual observation of the bioluminescence. The dimensions of Zone 2 will be approximately 2 mm high x 4 mm wide x 2 mm deep. The light emission from Zone 2 will be viewed from the side of the biosensor as depicted in Figure 1-C. Because the wicking material in Zone 2 becomes largely transparent when wet, a considerable fraction of the luminescence is able to escape from the 2 mm depth of Zone 2 and reach the observer.

The success of this phenylalanine biosensor design depends on the ability of all observers to both see the bioluminescence from Zone 2 and to determine when the light ceases. The measured time between closing the biosensor and determining when the luminescence ends is proportional to the phenylalanine in the blood sample. A necessary condition for success is that all potential users would respond in the same way for the same concentration of phenylalanine. A number of factors affect human visual dark sensitivity, i.e., age, health, nutritional status, and most importantly, prior exposure to ambient light and length of time to dark adaptation. There are two distinct phases of dark adaptation. The first phase requires about five minutes and is primarily a response of the iris to lower light flux. The second phase requires another 20-30 minutes and is dominated by changes in the rods and cones of the retina.

Funding from a Phase II NSF STTR project allowed us to determine the visual response of volunteers to both varying degrees of dark adaptation and a wide range of photon flux which was provided by well quantified luminescence standards from Biolink, Ltd. England. The NSF STTR addressed the development of an ATP sensor based on luciferase catalyzed bioluminescence from luciferin. Our studies determined that prior ambient light exposure was the dominant factor in the detection threshold for various luminescence standards. However, given five minutes of dark adaptation all of the volunteers were able to see standards having a flux of 8×10^9 photons/second/sterradian or greater.

Our approach to this Phe biosensor includes a design wherein the observer places the sample on the sample well and allows the reaction in Zone 1 to proceed for up to five minutes while the observer sits a low ambient light level environment. The device is then snapped shut and the timer is started as the reaction begins in Zone 2. Luminescence begins virtually immediately and the observer continues to watch in the dark until the light ceases at which time the timer is stopped. Based upon our findings in Phase 1, this prior dark adaptation of about 5 minutes is sufficient to see the

The sensitivity of the Sigma phenylalanine kit (fluorometric method) is reported to be 0.5 mg/dl. The reproducibility is from 7.1% to 12.8% and the recovery of added phenylalanine ranges from 80% to 105%. It also requires a 2 hr incubation at 60 °C and requires a fluorometer. It is clear that this method does not generate very accurate results.

Table 5. The results of Sigma phenylalanine kit. The data are the average of three tests.

Standard phenylalanine	Phenylalanine concentration determined with Sigma kit	Error
0.8 mg/dl (0.05 mM)	0.71 mg/dl	11%
8 mg/dl (0.5 mM)	6.9 mg/dl	13%
20 mg/dl (1.2 mM)	15.2 mg/dl	24%

Recommendation from phase I final report:

The feasibility of a bioluminescent based direct reading biosensor for phenylalanine measurement is established. The sensor can be in spatial mode or in time mode. We will focus on the final design of sensor, the study of the dry reagents needed, and prototype sensor construction and evaluation in the Phase II project. The bioluminescent assay should be optimized to enhance the intensity and sharpen the decay rate of bioluminescence, which will enhance direct visual detection.

Experimental Design and Methods

During the phase I study, we finished most of the evaluation work on the chemical reactions in solution phase. The experiments in Phase II will focus on the design of the prototype biosensor (Figure 1) and the optimization of dry reagents. The proposed schedule for each Specific Aim is shown in Table 6.

Table 6. Proposed time frame for completion of specific aims

	First year (quarter)				Second year (quarter)			
Specific Aims	1	2	3	4	1	2	3	4
1. Design of prototype	→							
2. Design and prototype the sample zone	→	→						
3. Design and prototype the phenylalanine conversion zone		→	→					
4. Design and prototype the signal transduction zone			→	→				
5. Design and prototype a tyrosine channel				→	→			
6. Assemble, calibrate, validate, and study the prototype and its long-term stability					→	→	→	→
Final report								→

bioluminescence which commences immediately after snapping shut the device, initiating the reaction in Zone 2.

Part of the work in Specific Aims 3 and 4 includes enhancing the light intensity and making the light decay curve steeper to enhance visual detection sensitivity (Figure 5).

2. Design and prototype the sample zone.

The spot for blood loading is designed to hold a 100 µl blood sample. A cells/plasma separation membrane is placed on the open-end of the holder allowing whole blood to enter the membrane. The maximum available plasma volume is about 60 µl. We will evaluate two cells/plasma separation membrane, PlasmaSep Ls from Whatman and Hemasep L from Pall Gelman Science. Both materials are designed to separate plasma from whole blood in the lateral direction. The distance that clear plasma travels will be measured. Reaction zone 1 (see Specific Aim 3) will be placed on the point where the clear plasma starts. The plasma yield from the cells/plasma separation membrane will be estimated. According to the data provided by the vendor (Whatman), a 60% recovery from available plasma or 36 µl plasma from 100 µl of blood sample is possible. Although the capillary force is enough to wick the membrane, we will also study the influence of gravity on the separation speed by changing the angle of wicking plane.

3. Design and prototype the phenylalanine conversion zone (Reaction zone 1)

Reaction zone 1 includes two parts: a buffer adjusting area and a phenylalanine conversion area. The total wicking volume in this zone is designed as 18 µl. An excess of plasma will be retained in the sample zone and used as the driving force to wick the plasma into Reaction zone 2 (see Specific Aim 4).

Because plasma is buffered at about pH 7.4 in the human body, a higher concentration of glycine buffer at pH 10.6 will be used to guarantee the required pH of 10.5. We will monitor the pH change during the mixing of plasma and glycine buffer. The glycine buffer is then applied to the wicking material. The materials for reagent deposition were studied in the phase I project (see final report, Table 3). Polyester wicks (by Filtrona Richmond) coated with surfactant (Triton X-100 or Tween 20) showed the best result for reagent and enzyme stability. We will use this material to deposit the glycine buffer.

Phenylalanine dehydrogenase is the only enzyme used in this zone. Because this reaction (reaction 2) requires NAD^+ as a substrate, we will mix phenylalanine dehydrogenase with an excess of NAD^+ (10 mM or higher) to push the equilibrium of the reaction toward the direction of producing NADH. A constant volume of enzyme-substrate mixture will then be applied to the wicking material. The same material used for buffer deposition will be pre-treated in a 1 mg/ml solution of bovine serum albumin to reduce the detrimental effects of the surface-enzyme reactions. The deposited phenylalanine dehydrogenase and NAD^+ will then be lyophilized (see below). The phenylalanine dehydrogenase activity will be determined by adding phenylalanine and measuring the absorbance at 340 nm.

Immobilization of phenylalanine dehydrogenase on the matrix will also be considered in the design of the phenylalanine conversion zone. The benefit of immobilizing the enzyme is that it will not flow with the plasma when the biosensor is closed, preventing possible interference in the signal transduction zone. A pre-activated immobilization membrane, Immunodyne ABC (from Pall Gelman Sciences) will be used to immobilize phenylalanine dehydrogenase. A pre-cut membrane will be incubated in borate buffer (pH 10.0) at room temperature. Amine groups of phenylalanine dehydrogenase will be covalently linked to the membrane at the high pH. The microporous structure of this membrane provides a large surface area for enzyme immobilization (300 cm^2 for each cm^2 of planar membrane). Assuming the area occupied by one enzyme is 10^{-14} cm^2, up to 50 nM of enzyme can be immobilized on 1 cm^2 membrane, which can provide enough enzyme activity for the reactions (e.g. convert more than 1 micromoles reactants per min). The enzyme activity will be determined by monitoring the NADH produced.

A primary concern regarding the practicality of our biosensor design is the long term stability of the governing enzymes and substrates. In this case we are most concerned with the storage stability of phenylalanine dehydrogenase and NAD^+. Preliminary studies using a lyophilization process and several preservatives have shown positive results (Phase I report). It now appears that our lyophilization process, as described below, will effectively preserve the viability of the governing enzymes and substrates for long term storage at room temperature under desiccated conditions.

We will next scale-up the lyophilization process and study the parameters that influence the stability of the assay. We will use a pilot plant freeze-dryer (Virtis model Genesis 12EL) with shelf temperature control capability and a minimum condenser temperature of -70°C. It will be use to evaluate both freeze rate effects and drying schedule effects. Drying schedules play a large role in determining how much water is removed from the formulation. Several are mentioned in the literature (Carpenter (1988, 1997), Fagain). Evaluation of the glass transition temperature (Tg) will be performed after the drying step to determine effectiveness of the drying and formulation. It is imperative that the Tg should be above the expected storage/transportation temperatures.

As part of the lyophilization process, we will include several preservatives. Polyethylene glycol (8000 M.W.) will be included as a freezing protectant (50 mg/ml) (Carpenter, 1993). Trehalose will be included as a dehydration protectant (120 mg/ml) (Crowe, 1993). Bovine serum albumin (0.3 mg/ml) will be included as a denaturation protectant. After mixing the preservatives with the phenylalanine dehydrogenase and NAD^+, the solution is applied to BSA treated wicking material for preservation. There may be some weak binding of phenylalanine to BSA- this possibility will be evaluated. The sample is frozen at -70°C for at least one hour. The final step in the preservation process involves removing the water from the reagent solution at high vacuum and various temperatures. The moisture removal begins with temperatures near -30°C and ends near room temperature. The drying process lasts approximately 24 hours. Once the samples are dry, they will be stored at room temperature in dry air for extended periods. The performance of the assay will be tested.

tyrosine (70%) (Asano, 1987). We will design a second channel that uses this enzyme to determine the Phe plus tyrosine total concentration. This is similar to Shen's assay which uses a single enzyme, L-phenylalanine ammonia-lyase, to simultaneously determine the concentration of phenylalanine and tyrosine (Shen, 1977).

The only difference between this channel and the phenylalanine channel is that the enzyme used in the Reaction zone 1 has a different specificity. The same sample zone and Reaction 2 zone design can be used in this channel. In this specific aim, we will focus on the characterization of phenylalanine dehydrogenase from Bacillus sphaericus. The response of this enzyme to phenylalanine and tyrosine will be monitored by the production of NADH as in Phase I study. The photon signal produced by bioluminescent assay will correspond to the summation of phenylalanine and tyrosine concentration. A subtraction of the phenylalanine signal from the summation signal provides the tyrosine concentration. The calibration of this channel will be given in specific aim 6.

6. Assemble, calibrate, validate, and study the prototype and its long-term stability

The biosensor housing will be molded with a hole for sample loading and for signal output (Figure 1). The wicking materials will be adhered by heat sealing. A foil or hydrophobic membrane will be used to cover the bottom of Piece 1 and the top of Piece 2, preventing the evaporation of sample and the contamination of the surfaces. Both membranes will be removed just before the two pieces of the biosensor are snapped closed.

The linearity of prototype biosensor to phenylalanine will be studied. Whole blood samples will be purchased from Vital Products, Inc. The whole blood will be applied to the sample zone of the prototype biosensor. The phenylalanine in plasma will be determined by incubation for an appropriate time (determined in Aim 2) and then closure of the two piece biosensor, which initiates the bioluminescent reaction. The time required for the disappearance of bioluminescence will be measured. The biosensor will be calibrated by adding known amounts of phenylalanine to the whole blood sample.

The detection limit of this bioluminescence based phenylalanine assay at this time is 0.1 mM phenylalanine (Phase I final report). The dry reagent used in the prototype biosensor requires no dilution of the phenylalanine/blood sample (compared to a 2.5 fold dilution used in the Phase I study), thus a lower detection limit should be achieved.

Reproducibility of the prototype biosensor will be studied by people of different ages and visual threshold characteristics. The reproducibility for the same person will be reported. The variation of results obtained from people in the same group and from different age groups will be studied. The personal accomodation method developed in Aim 1 will be used to assess the variation from person to person. Compared with the precision of well-established methods (see below), a 10% error should be acceptable.

The prototype biosensor will be validated using well-established methods. The common Guthrie microbiological assay is a semi-quantitative assay that is not suitable to validate our quantitative biosensor (Guthrie, 1963). The HPLC method for amino acid determination (Campbell, 1992) and Quantase enzymatic assay (Dhondt, 1993), using phenylalanine dehydrogenase and detecting NADH by reduction of tetrazolium salt, will be used to validate the prototype biosensor. We will send the reference whole blood

4. Design and prototype the signal transduction zone (Reaction zone 2)

This zone includes three parts. The first part is the buffering area. Because the optimum pH for bacteria luciferase is 7, the pH of NADH solution (pH=10.5) needs to be adjusted in this area. The second part is loaded with bacteria luciferase, oxidoreductase, aldehyde, and FMN. The NADH is "converted" to photons in this area. The third part includes a pH indicator, which shows the signal transduction zone is completely filled and the pH is correct. This indicates to the user that the sensor has been filled completely. The total volume in the zone should be smaller than the difference of plasma volume and the volume of Reaction zone 1 to ensure complete loading.

Phosphate buffer at pH 6.0 will be deposited onto the wicking material and lyophilized as in Aim 3. The buffer capacity should be enough to make a solution with pH=7.

The ratio of enzymes used in the assay is very important because $FMNH_2$ produced by oxidoreductase quickly binds to bacteria luciferase and stays until aldehyde binds and the production of light starts (Meighen, 1971). This mechanism prevents a fast non-enzymatic oxidation of $FMNH_2$ ($t_{1/2}$=0.1 S). Hence, bacteria luciferase should always be in excess to oxidoreductase. According to the Phase I study, the optimum ratio of bacterial luciferase to oxidoreductase in solution is 4 mg/ml to 0.4 mg/ml (a molar ratio of 3.3 to 1). We will use this enzyme combination to prepare the dry reagents. A mixture of enzymes and FMN (final concentration 1 µl) will be deposited onto the wicking material and then lyophilized.

A long chain aldehyde (C-8 to C-12) is a key reactant in the bacteria luciferase reaction. Such aldehydes are relatively insoluble and volatile. Development of a dry dipstick sensor requires incorporation of the aldehyde reagent, together with FMN, bacteria luciferase and oxidoreductase, directly into the sensor material. Fortunately the aldehyde can be stabilized in solid form as a tablet, pellet or powder. Indeed, decanal tablets are available for use in bacteria luciferase assays. After the enzyme reagents are deposited and lyophilized onto the sensor material, the powdered decanal formulation will be deposited in the appropriate zone. The response of this dry reagent to NADH will be studied with a CCD camera. The enzyme ratio and the substrate concentration should be further optimized to get the maximum light intensity. The entire sensor will of course be sealed in a foil pouch in a fully dessicated (and if needed, oxygen-free) environment.

Hydrion pH pencil (PGC Scientifics) will be used to mark the wicking material. When the wicking material is moistened, the color will turn yellow-green, indicating the reaction zone is completely filled and the pH is 7. If the color turns blue (pH>10), it suggests the buffering zone has failed to adjust the pH to the correct value and the result in the signal window is incorrect.

5. Design and prototype a tyrosine channel

Tyrosine is also important for the management of PKU. The use of a phenylalanine/tyrosine ratio in PKU screening improves the test reliability by reducing false positive and false negative results (Souza, 1998). The phenylalanine dehydrogenase from Bacillus sphaericus showed specific activity to both phenylalanine (100%) and

samples (number of sample>30) to Associated Regional University Pathologists, Inc. (Utah) for HPLC analysis. We will use the Quantase phenylalanine kit to measure the same samples. The data obtained with our prototype biosensor will be plotted against the data obtained from HPLC and the Quantase kit. The data will be analyzed by normal statistical methods.

The long term stability of prototype biosensor and each of its components will be evaluated. The dry components prepared by lyophilization will be stored in desiccators without the light at 4 °C and room temperature. Although the design of sensor may be modified during the storage period, long-term stability (up to 1 year) of the original design will be determined.

Phase III and commercial potential

The markets for this blood phenylalanine biosensor include in-hospital newborn screening and the monitoring of dietary compliance for PKU patients. This instrument-free biosensor will be a low-cost device which is designed for neo-natal nurses, pediatricians and family practitioners. This biosensor will be the first product in our amino acid analysis series. The complete amino acid analysis line will increase the acceptance of this biosensor in the market.

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THE UNIVERSITY OF UTAH

August 8,1998

Dr. C.Y. Wang
Protein Solutions Inc.
PO Box 58093
Salt Lake City, UT 84158-0093

Dear Dr. Wang:

I am writing to confirm my willingness to serve as a consultant to Protein Solutions Inc. on matters regarding genetic manipulation, production, and application of recombinant luciferases and oxidoreductase proteins. A recent c.v. is enclosed to illustrate my background and qualifications in the area of genetic engineering of proteins. Beyond my formal graduate education in Biochemistry and Molecular Biology I have worked for the past 10 years on the structure and function of cytoskeletal proteins using the techniques of molecular biology and protein chemistry. I understand that you will have in your budget funds to reimburse me $3000 per year for each of the two years of your proposed grant, "Quantitative, Direct Reading Phenylalanine Biosensors."

Sincerely,

Russell Stewart

Department of Bioengineering
2480 Merrill Engineering Building
Salt Lake City, Utah 84112
(801) 581-8528
FAX: (801) 585-5361

THE UNIVERSITY OF UTAH

August 10, 1998

Dr. C.-Y. Wang
Protein Solution Inc.
P.O.Box 58093
Salt Lake City, UT 84158-0093

Dear Dr. Wang,

I am pleased to learn that Protein Solutions, Inc. is submitting a phase II proposal "Quantitative, Direct Reading Phenylalanine Biosensor". I am writing to confirm my willingness to serve as a consultant to Protein Solution Inc. on matters regarding optical sensor design, light detection, proteins and interfaces, and interfacial characterization as these relate to your proposed work. A recent CV is enclosed with this letter to illustrate my background and qualification in these areas.

I understand that you will budget consulting fees of $3,000 for each year of the two years of the proposed grant.

Sincerely,

Vladimir Hlady

Vladimir Hlady, D.Sc.
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