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Project Report

- I. Contract Number: N00014-90-C-0042**
- II. Project Title: An Endotoxin Binding Protein for Treatment of Septic Shock**
- III. Grantee: Norman R. Wainwright, Associates of Cape Cod, Inc.**
- IV. Grant Period: 16 OCT 1989 to 15 OCT 1992**
- V. Period Covered by this Report: 15 OCT 1990 to 15 FEB 1991**
- VI. Summary of Progress to Date:**

A. Native Endotoxin Neutralizing Protein nENP

Native ENP has been extracted from the 1990 blood collection season. The extract has been purified over the first purification column (ion exchange) and 10% has been processed through the final purification. Estimated total product from this year is 25 grams.

B. Product Application

Flat sheet membrane filters employing covalently coupled ENP have been evaluated for their ability to remove endotoxin from a variety of pharmaceutically important solutions. Larger volume fluids can be depyrogenated using hollow fiber devices. Table 1 lists the results of endotoxin clean-up procedures on a variety of solutions.

Solutions	Initial ng/ml	Final ng/ml	Endotoxin Removed %
Serum Albumin	100	0.5	99.95
IgG	2	0.003	99.85
Vaccine(peptide)	2000	0.9	99.95
Lymphokines	125	1.0	99.20
Hemoglobin	0.4	0.005	99.75
Culture Medium	8.0	undetected	>99.90

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C. Recombinant Endotoxin Neutralizing Protein (rENP)

1. Fermentation / Secretion into Medium

Fermentation has been scaled from 7 liters to 30 liters. The major improvement in this period has been in the down stream processing. Our extraction/purification procedure has required incubation of the yeast fermentation broth with 3 molar urea to dissociate complexes formed between ENP and uncharacterized medium or cellular components. It was found that changing the medium pH from 5.5 to 6.5 results in both increased secretion of ENP into the broth and elimination of a urea pre-incubation. The omission of urea, however, has created a new need to process the broth faster. Presumably, urea was acting to dissociate complex as well as denature proteases. To avoid protease digestion, the clarified yeast broth is loaded immediately onto the ion exchange column for rapid purification.

2. Clarification

The removal of cells from the broth has been achieved by centrifugation in 250 ml. bottles. For small volumes of a few liters this is adequate, but for the 30 liter volumes and above, we have investigated another method. A hollow fiber cartridge having a 0.45 μ pore size was evaluated. Five liters of medium was processed in approximately 50 minutes with a one square foot surface area membrane. We will evaluate a larger membrane to collect the broth from an entire 30 liter run. If the total time to collect is the same, this technology will be acceptable.

3. Antigenicity

Rabbits receiving only rENP at 5 and 10 mg/kg were maintained for 30 days post injection. Serum was analyzed for the presence of antibodies generated to rENP. The method was to immobilize rENP onto a membrane surface and capture any anti-ENP in the sample. After thorough washing, the bound antibody was detected with anti-rabbit IgG conjugated to the enzyme, peroxidase. A chromogenic substrate indicates a color change in positive samples. Using serial dilution of the serum, the anti-ENP titer was found to be between 1,000 and 10,000. Future toxicity studies must address the effect of this degree of antigenicity. It must also be determined what role the glycosylation by mannose plays in this effect.


D. Changes in project schedules

1. In order to investigate whether the protein structure can be modified to eliminate undesirable side effects, such as antigenicity or localized tissue accumulation or toxicity, we are trying to determine the active site. Three dimensional structure determination is being attempted through a collaboration with Dr. Alex Rich's laboratory at MIT. To date, his group has successfully crystallized recombinant ENP. Although a complete structure determination could 1-2 years, attainment of a crystal is a first large step.

2. We are approaching the active site determination by another method. Small synthetic peptides are being constructed to determine if less than the whole molecule of ENP is necessary for biological activity.

VII. Projections for Next Period

1. Complete purification of nENP from this years production.
2. Develop clarification methods for larger scale fermentation and purification under compliance to FDA regulations for producing human clinical trial material.
3. Assay synthetic peptide fragments for bioactivity.


Norman R. Wainwright, Ph.D.
Scientific Officer

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Date

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