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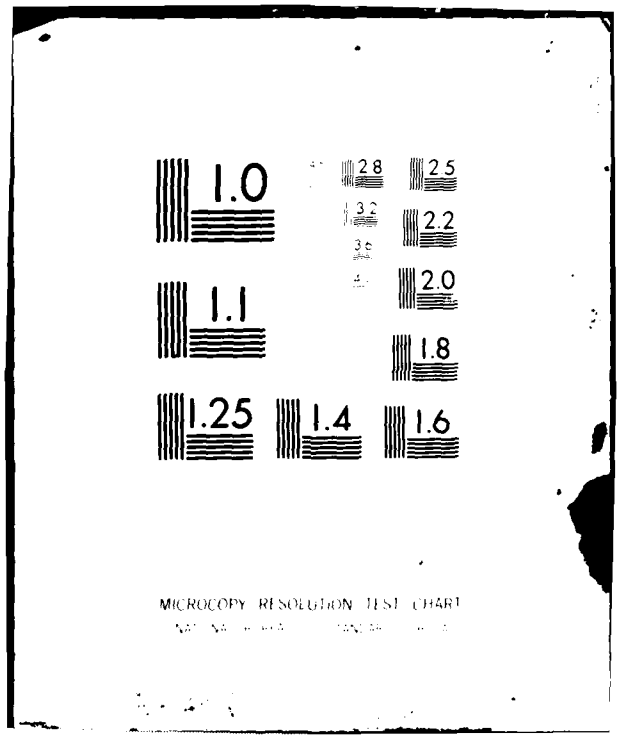
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Transfusion, Universal Donor Red Cells, Blood Groups, Enteric Bacteria, Glycosidases

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The aims of this research contract were: 1) to isolate in pure culture strains of human enteric bacteria with the specialized ability to produce strong activities of extracellular glycosidases that convert blood type A or B erythrocytes to universal donor blood type O erythrocytes; 2) to purify the blood type B-degrading enzyme produced by a fecal strain of Ruminococcus AB;

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3) to determine whether human type B red cells could be safely converted by this glycosidase to universal donor type O red cells for use on blood transfusion.

Results. Aim 1 was accomplished with the isolation of 2 strains that produce strong blood group A-degrading activity but no B-degrading activity. These complement our previous isolation of the strain of Ruminococcus AB that produces B-degrading but no A-degrading activity.

Aim 2, purification of the B-degrading enzyme in culture supernates of Ruminococcus AB, resisted a wide variety of classical protein separation methods until the last 2 months of the contract. As detailed in the report, major purification appears to have been achieved by digestion with papain followed by gel exclusion of chromatography on Sephadex G-200 in 3M NaCl. With successful completion of Aim 2 it will now be possible to meet the objectives of Aim 3.

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To : Program Director,
Biophysics/Biochemistry Program, Code 444
Office of Naval Research
Arlington, VA 22217

Report Date: 23 Oct, 1981

From : Lansing C. Hoskins, M.D.
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10701 East Blvd.
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Subject: Final Technical Report on ONR Contract # N00014-79C0034, File Code NR 207-177, "Enzymatic Production of Universal Donor Erythrocytes"
Principal Investigator: Lansing C. Hoskins.

A. Research Accomplished.

The overall aims of this research contract were:
1) to isolate in pure culture strains of human enteric bacteria with the specialized ability to produce strong activities of extracellular glycosidases that convert blood type A or B erythrocytes to universal donor blood type O erythrocytes;
2) to purify the blood type A- or B-degrading glycosidases produced by these bacteria in culture (specifically the B-degrading enzyme produced by a fecal strain of Ruminococcus AB);
3) to determine whether human type A or B red cells could be safely converted by these glycosidases to universal donor type O red cells for use in blood transfusion.

Aim 1 was satisfactorily accomplished. Accomplishment of Aim 2 has been the major effort of the contract. We met unexpected difficulty in purifying the B-degrading enzyme produced by Ruminococcus AB from other contaminant glycosidases; successful purification may now have been achieved through a novel approach developed in the last month of the contract period as summarized below. Aim 3 could not be pursued until Aim 2 had been achieved,

Specific Research accomplishments are as follows:
Aim 1. Isolation of bacterial strains producing glycosidases that degrade ABO blood group antigens. Two strains that produce a blood group A-degrading glycosidase have been isolated and partially characterized. One of these was described in the first annual report; the other was recently isolated, Both are gram-positive obligate anaerobes that produce the A-degrading enzyme constitutively and extracellularly. We are currently attempting to isolate others more that produce either A-degrading or B-degrading activity. Isolation of these strains is partly for the purpose of using their purified enzymes in transfusion research and partly to study how bacteria degrade the mucous coat lining the human gut mucosa. Equipment purchased under this contract helped us demonstrate that bacteria with mucin-and blood group-degrading properties exist as normal subpopulations in the human colon; they are distinguished from other enteric bacteria by their ability to produce the requisite glycosidases as extracellular enzymes (refs. 1 and 2 in index of publications listed below).

Aim 2. Purification of the blood group B-degrading glycosidase produced during growth of a fecal strain of Ruminococcus AB. This strain produces large amounts of an extracellular B-degrading α-galactosidase during growth in culture. Enzyme production and tne culture medium used are defined in the initial contract application, but in studies done during the contract period it was found that controlled infusion of glucose during culture incubation improved enzyme yield by 133% and the specific activity 4-fold.

Although other glycosidases produced by this strain had weaker activities, their separation from the much larger amounts of B-degrading activity proved to be very difficult. In particular, we sought to remove traces of contaminant blood group H-degrading activity; yet this remained closely associated with the B-degrading enzyme throughout use of classical protein separation techniques that included fractionation with salts and various organic solvents, affinity, gel exclusion, ion exchange, and hydrophobic chromatography. Disc gel electrophoresis revealed that B-degrading activity corresponded to 7 protein staining bands and that these also contained small amounts of H-degrading activity.

The multiple electrophoretic forms of the B-degrading activity together with inability to separate it from small amounts of H-degrading activity suggested that these enzymes may have been secreted from the bacterial surface variably linked to fragments of cell surface components, specifically to lipoteichoic acid polymers. These are highly charged, polar molecules; successful enzyme purification would require cleaving any linkages between the enzymes and the polymer fragments before the enzymes could be separated and purified. Treatment with sulfhydryl reducing agents, 4M urea, 3M NaCl and detergents failed to affect separation, suggesting that the putative linkages between enzymes and cell surface polymers might be covalent. But ammonolysis to cleave acylester residues from lipoteichoic acids, and periodic acid oxidation to degrade glyceryl teichoic acid polymers inactivated the enzymes.

As a third approach digestion of the crude enzymes with proteases was attempted to determine if such treatment separated the B-degrading enzyme from contaminating enzyme activities. This approach was based on our observation that the B-degrading glycosidase was resistant to pancreatic proteases normally present in the colon lumen. Of 7 proteases tested only papain successfully cleaved contaminating H-degrading activity from the B-degrading glycosidase without appreciably degrading the latter. Following papain treatment residual H-degrading activity was found in small fragments migrating with the tracking dye during disk gel electrophoresis while B-degrading activity was now associated with 3 protein bands whose electrophoretic mobility was altered only slightly. Despite their apparent small size, fragments of H-degrading activity re-associated with B-degrading activity so that both co-eluted during gel exclusion chromatography on G-200 Sephadex under ordinary elution conditions. But by performing this step in the presence of high ionic strength (3 M NaCl), these molcular re-associations were apparently prevented so that most of the H-degrading activity eluted as smaller molecules in a partially retarded protein peak distinct from the front-running protein peak containing B-degrading activity. The small amounts of H-degrading activity remaining associated with B-degrading activity was successfully removed from the latter duting anion exchange chromatography on DEAE cellulose using ionic strength gradient elution. The B-degrading fraction from DEAE cellulose has 3 strong bands and 3 weaker ones which correspond to B-degrading activity on gel slices. There are 2 faster - migrating bands of inactive

protein. Protease activity from the papain treatment and α-glucosidase activity present in the original culture supernate were removed during purification, but a small amount of pnp-α-galactosidase is associated with the B-degrading activity. I plan to try affinity chromatography on p-amino-pheny- α-D-galactoside - linked agarose to separate active enzyme from these faster migrating components. SDS-disc gel electrophoresis of this latest purified fraction is pending.

The successful use of papain together with gel exclusion chromatography at high ionic strength is a break-through in our attempts to utilize the B-degrading glycosidase from Ruminococcus AB. These steps may also be applicable to purification of A-degrading activity from our isolated strains producing this activity. To date we have used these steps in the purification of B-degrading enzyme from a single 10 liter batch of Ruminococcus culture supernate. The steps are summarized in Table 1. The final product has the highest specific activity of any B-degrading prep we have made heretofore. Whereas the ratio of B- to H-degrading activity in culture supernates is 15:1, the amount of H-degrading activity in the final product was insignificantly small; the ratio of B- to H-degrading activity was 400, 000:1.

B. Index of Technical Reports Issued Under the Contract, None.

C. Index of Publications Issued Under the Contract,

1. Hoskins LC, Boulding ET, Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations that produce glycosidases as extracellular enzymes. J. Clin. Invest. 67:163-172, 1981.

2. Miller RS, Hoskins LC, Mucin degradation in human colon ecosystems. Fecal population density of mucin -degrading bacteria measured by a "most probable number" method. Gastroenterology 81: 759-765, 1981.

Others are in preparation.

D. Conclusions Derived from the Research and Their Significance

1. We have isolated in pure culture species among normal human enteric bacteria which secrete glycosidases that degrade the human ABH (O) blood group antigens. It is possible to use these isolates to produce large amounts of ABH (O) blood group antigen-degrading enzymes and other glycosidases.

2. As with other glycosidases produced by gram-positive bacteria, those that degrade blood group antigens are difficult to purify from one another. Successful purification of the B-degrading enzyme appears to have been achieved using papain digestion followed by gel exclusion chromatography at high ionic strength. This novel approach should greatly facilitate large scale production of B-degrading enzyme for preparation of universal donor red cells. The approach should also be applicable to the purification of the A-degrading enzyme from our fecal isolates as well as other enzymes produced by gram-positive bacteria and would be of general importance.

E. List of Major Accomplishments,

1. Isolation of hitherto uncharacterized human fecal bacteria that produce strong extracellular glycosidase activities. These glycosidases would be very useful in current cell membrane research as well as in production of universal donor erythrocytes.
2. We have contributed original information about degradation of mucin glycoproteins by man's indigenous gut bacteria.

3. The use of papain to purify the blood group B-degrading glycosidase produced by Ruminococcus AB represents a novel approach that may also be applicable to purifying a wide variety of potentially useful bacterial glycosidases.

TABLE 1. Preparation RH-III-130. Purification of the B-Degrading Glycosidase from 12 Liters of Ruminococcus AB Culture Supernate.

STEP	TOTAL PROTEIN, mg	TOTAL ACTIVITY, units*	SPECIFIC ACTIVITY, U/mg prot.
12L Culture Supernate	NA (not assayed)	>168,000	
↓			
1. ULTRAFILTRATION PM-10, 1.3L	NA	510,000	
↓			
2. 2.0-5.4M Am_2SO_4 PPT	106	315,000	4860
↓			
3. PAPAIN DIGESTION 2%w/w, 16hr, 37° C.			
↓			
4. 0-3.5M Am_2SO_4 PPT	15	> 27,000	
↓			
5. SEPHACRYL G 200-3M NaCl CHROMATOGRAPHY Pool A	15	76,500	5100
↓			
6. DEAE CELLULOSE CHROMATOGRAPHY Pool B	1.3	87,000	67,000

* 1 Unit= that amount of enzyme causing a 50% decrease in antigen concentration, measured by 2-fold hemagglutination inhibition titers, in 30 min at 37° C, pH 6.4.

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