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TITLE: PURIFICATION OF THE ALPHA GLYCEROPHOSPHATE OXIDASE  
FROM AFRICAN TRYPAOSOMES

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## REPORT DOCUMENTATION PAGE

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| 19. ABSTRACT (Continue on reverse if necessary and identify by block number)<br>Affinity chromatography was employed as the most effective means to purify the glycerophosphate oxidase (GPO). The affinity gel matrix would be coupled with a competitive inhibitor of the dehydrogenase, such as suramin. The dehydrogenase component of the GPO would then bind to the inhibitor. After complete removal of all unattached enzymes, the GPO would be eluted with a buffer containing the substrate $\alpha$ -GP. Cyanogen bromide activated Sepharose 4B was coupled to the ligand trypan blue, an analogue of suramin. Trypan blue contains the $\text{SO}_3^-$ groups and $2\text{-CH}_3$ groups that appear to be essential to "suramin-like" drug activity against African trypanosomes. Unfortunately, suramin lacks the chemical side-groups to allow it to be attached to the Sepharose 4B affinity gel. Trypan blue does have suitable molecular side groups to permit its chemical linkage to the affinity gel. We envision that the glycerol-3-phosphate dehydrogenase component of GPO would bind to trypan blue and be released upon elution with a buffer containing 100 mM of $\alpha$ -GP. |       |                                                                                  |                                                                                                                                                     |                                                          |                                  |
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Trypan blue, an analogue of suramin, was an effective ligand. When experiments were performed using cyanogen bromide activated Sepharose 4B coupled to trypan blue, the dehydrogenase component of GPO was bound to the ligand and released upon elution with a buffer containing 100 mM of  $\alpha$ -GP. Unfortunately, in these experiments, trypan blue was eluted from the column and the enzyme was inhibited, preventing accurate assays for activity. These experiments were conducted with both fresh and frozen trypanosomes with similar results.

Experiments were carried out using epoxy activated Sepharose 6B with a twelve atom space arm coupled with the ligand trypan blue. This affinity gel matrix will couple the free amino and hydroxyl groups that are present in trypan blue to provide a stronger bond between the gel and ligand. With a 50mM borate buffer (pH 8.0), there was no leakage of the ligand but the GPO was not released from the column when eluted with 10mM  $\alpha$ -GP. Enzyme assays of the void volume indicated that approximately five percent of the enzyme was bound to the column.

Tests were also performed on different compounds and drugs to determine their efficiency as possible ligands in the purification of GPO. Epoxy activated Sepharose 6B was coupled with one of the following compounds: 8 amino-1-naphthol-5-sulfonic acid, o-toluidine (two major compounds found in trypan blue), trypan red, and ZP 57121 (from WRAIR). The drug, BJ-07673 (WRAIR), was coupled with AH Sepharose 4B.

Except for ZP-57121 (from WRAIR), all of these compounds proved to be in effective ligands. However, 98% of the enzyme was bound to the Epoxy activated sepharose 6B coupled with drug ZP 57121. Attempts to elute this enzyme with buffer containing either 10mM GP or 100mM GP and at various concentrations of NaCl (0.5M-1.5M) fail.

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## ABSTRACT

Affinity chromatography was employed as the most effective means to purify the glycerophosphate oxidase (GPO). The affinity gel matrix would be coupled with a competitive inhibitor of the dehydrogenase, such as suramin. The dehydrogenase component of the GPO would then bind to the inhibitor. After complete removal of all unattached enzymes, the GPO would be eluted with a buffer containing the substrate  $\alpha$ -GP. Cyanogen bromide activated Sepharose 4B was coupled to the ligand trypan blue, an analogue of suramin. Trypan blue contains the  $\text{SO}_3^-$  groups and 2- $\text{CH}_3$  groups that appear to be essential to "suramin-like" drug activity against African trypanosomes. Unfortunately, suramin lacks the chemical side-groups to allow it to be attached to the Sepharose 4B affinity gel. Trypan blue does have suitable molecular side groups to permit its chemical linkage to the affinity gel. We envision that the glycerol-3-phosphate dehydrogenase component of GPO would bind to trypan blue and be released upon elution with a buffer containing 100 mM  $\alpha$ -GP.

Trypan blue, an analogue of suramin, was an effective ligand. When experiments were performed using cyanogen bromide activated Sepharose 4B coupled to trypan blue, the dehydrogenase component of GPO was bound to the ligand and released upon elution with a buffer containing 100 mM  $\alpha$ -GP. Unfortunately, in these experiments, trypan blue was eluted from the column and

the enzyme was inhibited, preventing accurate assays for activity. These experiments were conducted with both fresh and frozen trypanosomes with similar results.

Experiments were carried out using epoxy activated Sepharose 6B with a twelve atom space arm coupled with the ligand trypan blue. This affinity gel matrix will couple the free amino and hydroxyl groups that are present in trypan blue to provide a stronger bond between the gel and ligand. With a 50mM borate buffer (pH 8.0), there was no leakage of the ligand but the GPO was not released from the column when eluted with 10mM  $\alpha$ -GP. Enzyme assays of the void volume indicated that approximately five percent of the enzyme was bound to the column.

Tests were also performed on different compounds and drugs to determine their efficiency as possible ligands in the purification of GPO. Epoxy activated Sepharose 6B was coupled with one of the following compounds: 8 amino-1-naphthol-5-sulfonic acid, o-toluidine (two major compounds found in trypan blue), trypan red, and ZP 57121 (from WRAIR). The drug, BJ-07673 (WRAIR), was coupled with AH Sepharose 4B.

Except for ZP-57121 (from WRAIR), all of these compounds proved to be in effective ligands. However, 98% of the enzyme was bound to the Epoxy activated Sepharose 6B coupled with drug ZP 57121. Attempts to elute this enzyme with buffer containing

either 10mM GP or 100mM GP and at various concentrations of NaCl (0.5M-1.5M) fail.



## APPROACH TO THE PROBLEM

The electron transport system of African trypanosomes is a potential target for trypanocidal drugs. This is particularly true for bloodstream trypomastigotes that have a cyanide-insensitive  $\alpha$ -glycerophosphate oxidase (GPO). This enzyme has not been purified or characterized in detail. Inhibition of this enzyme coupled with inhibition of the anaerobic glycolytic pathway would destroy the parasites.

The mammalian stage of the life cycle of African trypanosomes is primarily dependent on glycolysis for ATP production. No cytochromes are present. The brucei subgroup, which includes those forms that infect man and several animals, is exclusively dependent on glycolysis. They produce pyruvate from glucose under aerobic conditions and pyruvate and glycerol in equimolar amounts under anaerobic conditions.

The GPO is a cyanide - insensitive oxidase which is inhibited by hydroxamic acids including salicylhydroxamic acid (SHAM) (1-4). Unfortunately, administration of SHAM had little or no chemotherapeutic effect due to the presence of an anaerobic glycolysis scheme (1, 5-7). A combination of SHAM and glycerol inhibits glycolysis in bloodstream trypomastigotes causing parasite destruction in vitro and in vitro. Further identification of the biochemical properties of the GPO could be valuable as a chemotherapeutic approach.

## BACKGROUND

Recent efforts in our laboratory have been devoted to the solubilization and initial enrichment of the GPO from Trypanosoma brucei (8).

Experiments during the previous years have been devoted to isolation and solubilization of the GPO, including the identification of an alternate electron donor. On solubilization with detergents, the GPO is separated from the alpha glycerophosphate dehydrogenase. In order to purify the GPO, it is necessary to have an artificial electron donor which will contribute electrons to this oxidase and facilitate its measurements. In addition, several solubilization procedures were investigated in order to identify an effective detergent which would allow retention of SHAM sensitive oxidase activity.

The GPO was solubilized from a trypanosome pellet using octylglucoside. Both octylglucoside and sodium deoxycholate proved to be effective in solubilizing the oxidase, whereupon octylglucoside was preferred due to ease of removal by dialysis. The optimal concentrations of detergent for approximately 20 mg protein were 2% octylglucoside or 1% deoxycholate, which resulted in 30-60% recovery of the activity in the supernatant. The presence of EDTA and 2-mercaptoethanol during the entire procedure and especially the addition of NaCl in the last step

increased the yield. The DNase step made the pellet less sticky and more manageable afterwards and remained in the procedure although it only slightly increased the yield. The stability of the solubilized enzyme was poor, the preparation losing 80% of its activity when stored overnight at 4°C.

Some properties of the solubilized oxidase were investigated using a Clark-type oxygen electrode. Glycerophosphate could no longer be used as substrate once the holoenzyme had been in contact with the detergent, no matter whether the detergent was added during the assay or in the solubilization procedure. However, the successful assay for the enzyme was ubiquinol oxidase. Activity of the oxidase component could be measured with ubiquinol analogs such as CoQ 1, with its isoprenoic side chain, or the 6-nonyl (NB) and 6-decyl (DB) derivatives with their saturated straight chain alkyl groups. NB was preferentially used because it is more stable than CoQ 1, and can be synthesized easily. Probably due to their poor solubility, CoQ 7, and 10 showed negligible activity, even after the addition of several types of detergents. To investigate whether the ubiquinol-oxidase activity is indeed part of the GPO complex, cross reactions with the two substrates were performed. When the enzyme activity of the supernatant was measured before treatment with detergent, the addition of ubiquinol analogs alone to the assay had no effect, whereas the addition of glycerophosphate to the oxidase assay with ubiquinol analogs resulted in an increase

in the rate of oxygen consumption. This indicates that the oxidation of glycerophosphate and ubiquinol analogs proceeds through a terminal oxidase step that represents a single oxidation process for these two substrates.

The pH optimum of the oxidase was found to be higher than 7.4-8.0 of the holoenzyme complex, but the relative insolubility and autoxidation of the artificial substrates at higher pH values prevented an accurate determination of the pH optimum and  $K_m$ . The activity of the solubilized oxidase could be stimulated with bovine serum albumin and this stimulation had the same optimum of 3.0 mg/ml as the holoenzyme. The solubilized oxidase was fully inhibited by SHAM (0.5 mM), but not by cyanide (5 mM), whereas suramin (0.1 mM) completely inhibited the holoenzyme but did not prevent the oxidation of the artificial substrates. The oxidase activity disappeared completely after treatment with perchloric acid (10%), acetone (25%, 30 min) or trypsin (1.5 mg), and also after heating to 100 C for 2 min. This strongly suggests that a protein is involved, and that the oxidase activity cannot be solely explained by a fatty acid peroxy radical scheme was proposed for the cyanide-insensitive pathway of plant mitochondria.

Thus as previously reported from our laboratory, the ubiquinol oxidase of trypanosomes can be solubilized and studied with artificial substrates. Its instability has so far precluded

a further purification and study.

## **RESULTS AND DISCUSSION**

The bloodstream forms of African trypanosomes are completely dependent on glycolysis for their energy supply and utilize a unique shuttle which includes a terminal oxidase, to reoxidize the glycolytically-produced NADH (4). This terminal oxidase which is located in the mitochondrial membrane (9), is cytochrome independent, not inhibited by classical inhibitors of the mammalian respiratory chain and not coupled to ATP productions. The holoenzyme complex has been partially purified and characterized (10). The oxidase component, however deserves further investigation as it is the enzyme which is absent in the mammalian host and is specifically inhibited by salicylhydroxamic acid (SHAM).

#### Harvesting of bloodstream trypanosomes

Male Wistar rats infected with Trypanosoma brucei brucei EATRO 110 were anesthetized with ether and bled to death by cardiac puncture. A buffy coat was prepared and the trypanosomes were separated from the blood on a DEAE- cellulose column that was eluted with phosphate buffered saline with glucose and heparin (PBSGH). The trypanosomes were centrifuged, pelleted and either stored at -70°C or used fresh for experiment to be described.

### Synthesis of an ubiquinol analog ND

The synthesis of the ubiquinol analog for use as a substrate to measure the activity of the oxidase component was as follows: to 0.91g of 2,3-dimethoxy-5-methyl-1,4 benzoquinone (Sigma and Aldrich Co) was dissolved in 25 ml glacial acetic acid and heated to 95°C under N<sub>2</sub> (12). Then a mixture of diundecanoyl peroxide (3.6g) in 5 ml of acetic acid was added drop-wise over a 2 hour period. The reaction mixture was allowed to stir for another 20 hr at 95°C and then evaporated to a dark orange oil under vacuum. The residue was then applied to a silica gel column and eluted with a mixture of hexane and ether. The dark orange fraction was collected and dried under vacuum. For use as a substrate in the ubiquinol oxidase assay, the ubiquinol analog was reduced according to procedure described by Trumpower and Edwards (11). Although these procedures are relatively simple, the yield has been considerably low with no improvement when the synthesis was increased. The other problem with ubiquinol is that it will reoxidize to the hydroquinone very easily and become unusable.

### Effect of the detergent octylglucoside on the release of GPO

To determine the effect of octylglucoside on the release of GPO, fresh bloodstream trypanosomes were harvested (minimum of  $9 \times 10^9$  cells) as previously described and exposed to a lysis buffer (3.3 mM Tris, 0.7 mM EDTA, 1.7 mM 2-mercaptoethanol and

0.5% bovine serum albumin, pH 8.0) for 30 min at 0°C and then passed through a 26 G 1/2" needle at 80 psi. The lysate was then exposed to various concentrations (5-30 mM) of octylglucoside for 30 min at 0°C. Then enzyme assays for the oxidase and dehydrogenase were performed, a summary of these results are given in figure 1. Additional experiments were designed to examine the amount of enzyme removed from the membranes and to determine the optimum detergent concentration. Lyzed cells were exposed to various concentrations of octylglucoside (0-30 mM) for 30 min at 0°C, then centrifuged at 48,000 x g for 40 min and the supernatant was assayed for enzyme activity. A summary of the results are shown in figure 2, however it appears that the highest amount of enzyme activity was found with the concentration of 25 mM octylglucoside.

As demonstrated in figure 2, the optimum concentration of octylglucoside appears to be 25mM. In order to increase the enzyme activity of GPO, an ammonium sulphate precipitation procedure was attempted. Lyzed cells were exposed to 25 mM octylglucoside for 30 min, centrifuged at 48,000g for 40 min, the supernatant removed and ammonium sulphate was gradually added until a final concentration of 50% was also obtained. Afterwards, the ammonium sulphate solutions was centrifuged for the previously described time and enzyme assays were performed on both the supernatant and pellet, results of which are presented in figure 3. Since the only activity detected was for the



oxidase the supernatant and pellet were dialyzed against 1 liter of lysis buffer. Samples were taken at different times and assayed for enzyme activity, and similar negative results were observed. Apparently the high salt concentration inhibited the dehydrogenase activity. This experiment was repeated using a final concentration of 25% ammonium sulphate instead of 50% but was not successful.

#### Affinity Chromatography of GPO

Affinity chromatography was employed as the most effective means to purify the GPO. The affinity gel matrix was coupled with a competitive inhibitor of the dehydrogenase such as suramin. The dehydrogenase component of the GPO would then bind to the inhibitor. After complete removal of all unattached enzymes, the GPO would be eluted with a buffer containing the substrate glycerol-3-phosphate (GP).

Suramin, a specific inhibitor of GPO, would have been the ideal ligand. However, this compound lacks the chemical side groups needed for attachment. Trypan blue an analog of suramin contains the  $\text{SO}_3^-$  and  $2\text{-CH}_3$  groups, that appear to be essential to suramin - like drug activity against GPO. Trypan blue also has suitable chemical side groups required for binding to the affinity gel matrix. The major problem with using trypan blue is that it is unstable as a ligand, for leakage would occur when the

matrix was eluted with different buffers during the elution of the attached enzyme. This resulted in an eluted inactivated enzyme due to the presence of the inhibitor.

Epoxy activated Sepharose 6B with a twelve atom spacer arm coupled with the ligand trypan blue was used. This affinity gel matrix coupled the free amino and hydroxyl groups that are present in trypan blue to provide a stronger bond between the gel and ligand and thereby prevent leakage of the ligand. In order to use the epoxy activated Sepharose 6B properly, the procedure for extracting and purifying GPO from trypanosomes (figure 4) was modified slightly in order to desalt the sample and exchange buffers before the final pellet was applied to the affinity column. When this procedure was first attempted using a 50mM phosphate buffer (pH 8.0) it failed due to the formation of crystals which prevented the flow of the buffers. When a 50mM borate buffer (pH 8.0) was substituted for the phosphate buffer, the samples were free flowing and no leakage of the ligand occurred. But assays of the void volumes indicated that only five percent of the enzyme had bound to the column. However, attempts were made to find the proper condition for the release of the enzyme GPO. A pH gradient (8.0-10.5) contain either 10mM or 100mM GP was applied to the column, however the enzyme remained bound to the column, indicating that neither a high pH or a high substrate level would aid in the release of the enzyme.

Tests were also performed on different compounds and drugs

to determine their efficiency as possible ligands: 8-amino-1-naphthol-5-sulfonic acid, O-toluidine (two major compounds found in trypan blue), trypan red, and ZP 57121 (from WRAIR) were coupled with epoxy activated Sepharose 6B. The drug, BJ-07673 (WRAIR), was coupled with AH Sepharose 4B. For control experiments, the affinity gel matrix epoxy activated sepharose 6B and AH were exposed to the same coupling conditions but without the ligand.

All of these compounds except for ZP-57121 (form WRAIR) proved to be ineffective ligands. However, 98% of the enzyme was bound to the Epoxy activated Sepharose 6B coupled with the drug ZP-57121. Attempts to elute this enzyme with buffer containing either 10mM or 100mM GP and at various concentrations of NaCl (0.5M-1.5M) fail.

Additional experiments were carried out using the affinity agarose gel with the fabricated skeletal support from Sterling Organic. The epoxy sites of this gel was first activated and then the ligand trypan blue was bound to the matrix. After the affinity gel matrix was activated, it was immediately exposed to the enzyme enriched sample. However, there was no binding of the enzyme and leakage of the ligand was observed.

These experiments have been fruitful in identifying possible analogues, affinity gel matrix, and detergent concentration which may be effective in the purification scheme.

## CONCLUSIONS

1. Optimum release of the enzyme occurred with the addition of 25 mM octylglucoside.
2. The addition of 25 and 50% ammonium sulphate did not increase the amount of GPO released.
3. When used as ligands, trypan red, 8-amino-1-naphthol-5-sulfonic acid, O-toluidine and BJ-07673 (from WRAIR) did not bind the enzyme GPO.
4. When used as a ligand, the drug ZP 57121 bound 98% of the enzyme GPO. However, attempts to elute the enzyme from the column when it was washed with high salt (0.5-1.5 M) and with high concentrations of the substrate  $\alpha$ -GP (10-100 mM) failed.

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## EXPLANATION OF FIGURES

Figure 1 - The effect of various concentrations of the detergent octylglucoside on the release of GPO after 30 min incubation at 0°C.

Figure 2 - The effect of the detergent octylglucoside at various concentrations on the release of GPO after 30 min incubation at 0°C and centrifugation at 48,000 x g for 40 min. Results given are enzyme assays of the supernatant.

Figure 3 - The effect of a 50% concentration of ammonium sulfate plus a 25 mM concentration of octylglucoside the on release of the enzyme GPO. Enzyme assays were performed on the supernatant after a 48,000 x g, 40 min centrifugation.

Figure 4 - Outline of the purification scheme of GPO from trypanosomes.

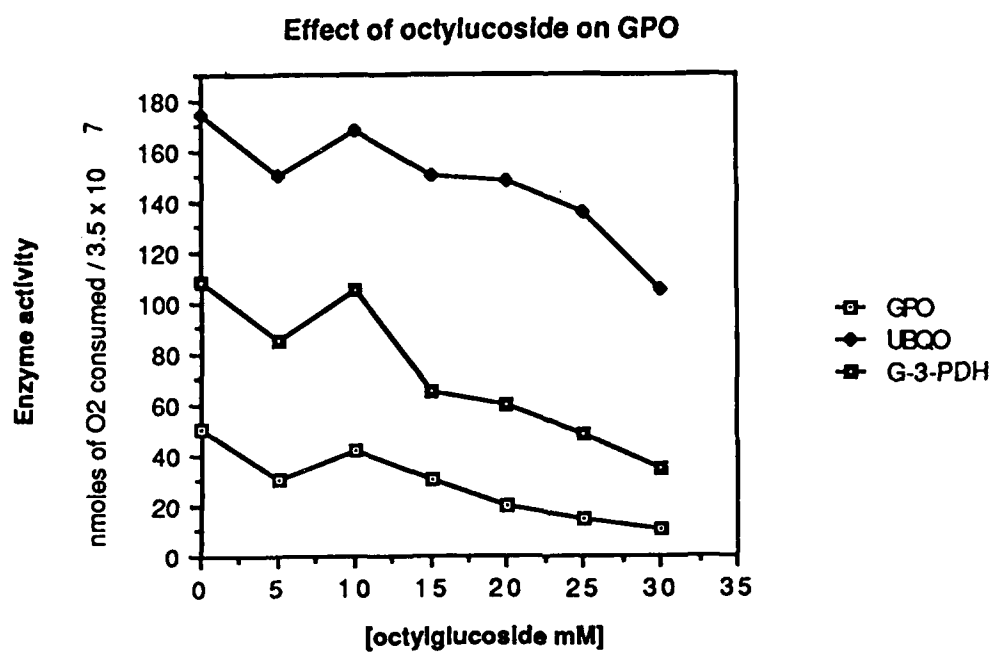


Figure 1



Effect of octylglucoside on GPO from  
bloodstream T.brucei (EATRO II0)

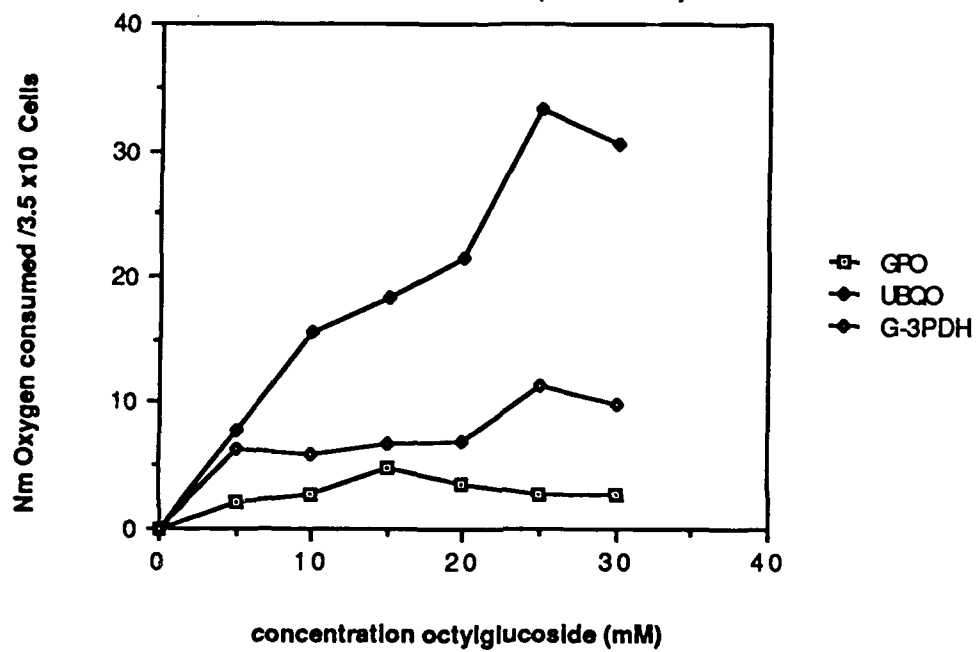


Figure 2

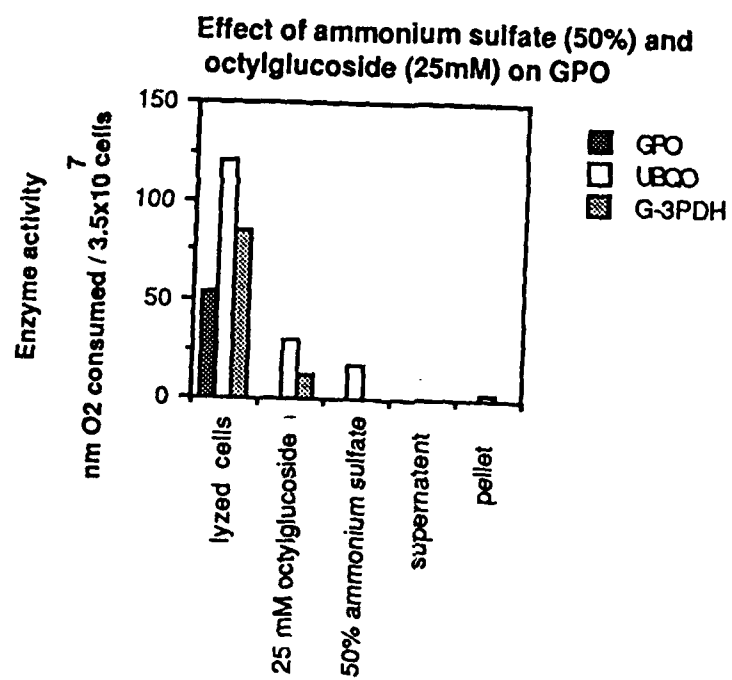


Figure 3

## PURIFICATION OF GPO

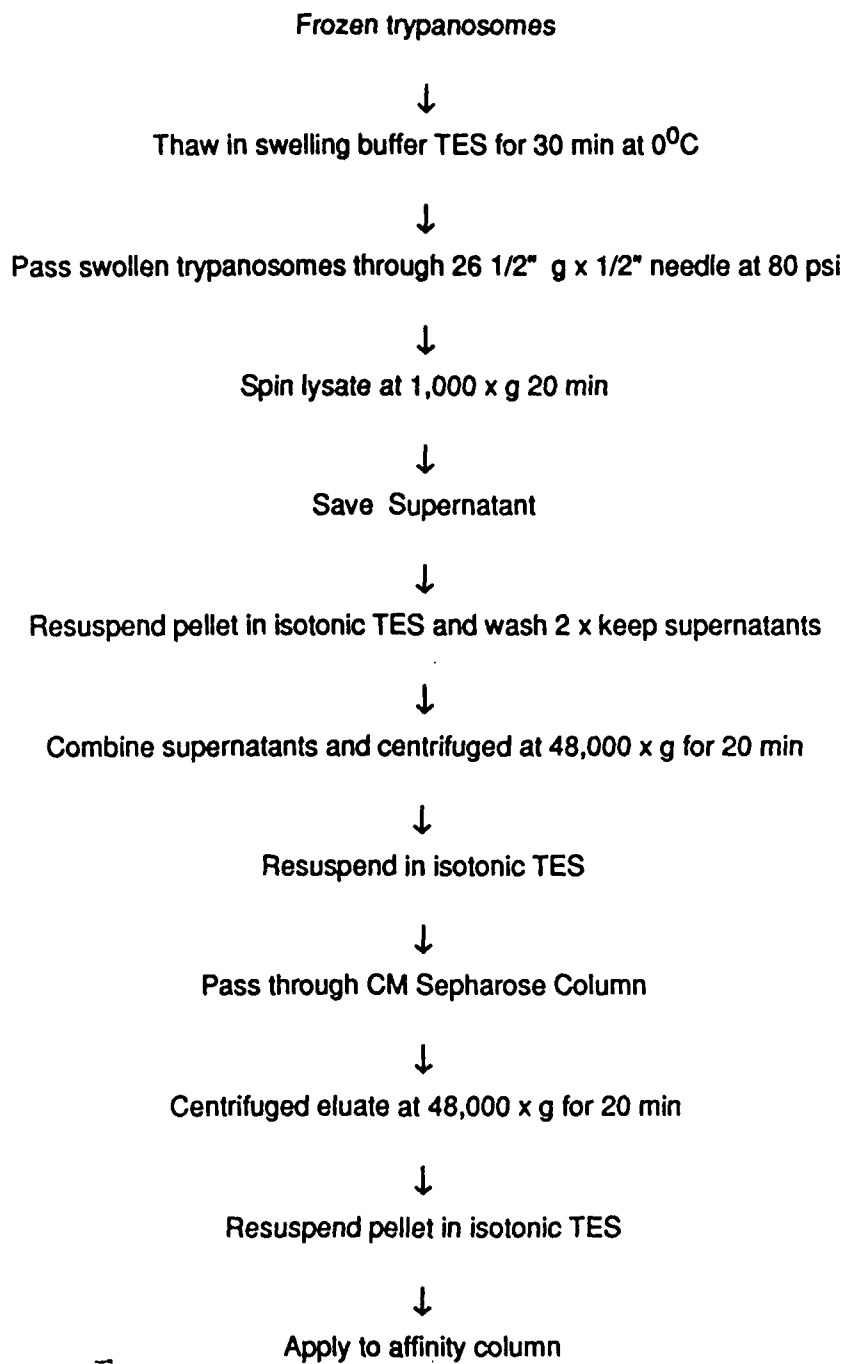


Figure 4