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**PURIFICATION AND CHARACTERIZATION OF THE
OLIGOSACCHARYL TRANSFERASE**

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MASTER

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ABSTRACT

Oligosaccharyl transferase was characterized to be a glycoprotein with at least one saccharide unit that had a D-manno or D-glucopyranose configuration with unmodified hydroxy groups at C-3, C-4 and C-6, using a Concanavalin A affinity column. This afforded a 100 fold increase in the transferase purity in the solubilized microsomal sample and also removed over 90% of the microsomal proteins (the cytosolic ones being removed before solubilization). The detergent N,N-Dimethyldodecylamine N-oxide (LDAO) was used for solubilization and it yielded a system compatible with the assay and the purification steps. An efficient method for detergent extraction without dilution of sample or protein precipitation was also developed.

INTRODUCTION

Enzymatic modification of proteins during co- or post-translation is a ubiquitous means for diversifying existing protein structure and serves a variety of different purposes. One type of protein modification involves the co-translational glycosylation of the side chains of asparagine (N-linked glycosylation), threonine or serine (O-linked glycosylation). Asparagine derivatization is by far the major form of glycosylation and N-linked glycoproteins are universal features of all eukaryotes, from yeast to humans. The glycosyl moiety can serve several functions; some of which are:

1. It can be involved in cell adhesion- as in the neural cell adhesion molecule present in the brain cells.
2. It may have a role in coagulation of blood cells- prothrombin and bovine coagulation factors (1).
3. It may also be a recognition factor for the immune system- as in the blood group antigens.
4. Proteins with these moieties may form the extracellular matrix and may also be structural elements- as in bacterial cell walls.
5. In tumor cells, alterations in the normal carbohydrate structure of certain glycoproteins have been reported, though the reasons for these alterations are uncertain.

Given the wide range of functions of glycoproteins, it is not surprising that a great deal of research has been aimed at determining the steps involved in their biosynthesis.

N-LINKED GLYCOSYLATION

Almost all N-linked glycoproteins have a common 'core' saccharide structure. The 'core' oligosaccharide is built sequentially onto a

Scheme 1

N-ASPARAGINE-LINKED GLYCOSYLATION

lipid carrier, dolichol, through a pyrophosphate linkage, and can be transferred *en bloc* to an asparagine side chain of nascent protein by the membrane bound enzyme, dolichyldiphosphoryl-oligosaccharide protein oligosaccharyl transferase (also referred to as the oligosaccharyl transferase)-see Scheme 1. The glycosylated asparagine was proposed by Marshall (2), to be part of the 'marker sequence' -Asn-Xaa-Thr/Ser-, where Xaa was any naturally occurring L-amino acid. This reaction, which takes place in the Rough Endoplasmic Reticulum (RER), is mechanistically unusual since it occurs through the nucleophilic displacement of the pyrophosphate linkage by the amido nitrogen of the asparagine side chain, which is not normally a very nucleophilic entity.

Further processing of the newly synthesized glycoproteins takes place as they transverse the Golgi cisternae. The oligosaccharide functions as the signal from the time that it is transferred to the protein, and through the recognition by, and action of the different processing enzymes, the oligosaccharide targets the protein for compartmentalization, whether it is destined to be a lysosomal enzyme, or to be transferred to another organelle, or to be secreted.

THE OLIGOSACCHARYL TRANSFERASE

Universal features of the substrates involved in the transferase reaction have been shown to be recognized in studies of diverse biological systems, indicating that little change has occurred in the primary structure of the enzyme catalyzing the reaction throughout evolution.

Several differences have been reported in the properties of the

enzyme from yeast verses mammalian sources. The most striking difference is found in the reported molecular weight of the two transferases, by using an active-site directed photoaffinity label (4). Mammalian microsomes all gave labeled polypeptide at 60 kDa with SDS-PAGE. Yeast microsomes (from *Saccharomyces cerevisiae*), however, afforded a labeled band at 80kDa.

The difference in the pH optimum for the activity of the two transferases from yeast and mammalian sources has also been reported (5)(6). Also, Sharma *et al* (6) reported that the yeast enzyme was less labile than the one from mammalian sources.

Das and Heath (7) reported a 2000-fold purification of the enzyme solubilized from hen oviduct while the yeast enzyme has not been purified beyond solubilization. The Das and Heath method was later said to yield unstable enzyme, making mechanistic studies difficult (8). This later report involved a method to solubilize mammalian transferase while still maintaining its activity. This was accomplished with phosphatidyl choline which was added to the detergent solubilized enzyme, *reportedly mimicking its natural environment*.

The reported difference in molecular mass of the two enzymes has not been confirmed by any method other than the active-site directed labeling described. Problems with this method include non-specific labeling, the resultant enzyme is inactivated and cannot be tested for biological activity, and also that the detergent interaction with the enzyme during SDS-PAGE can often yield misleading results. The researchers projected the use of polyclonal

antibody to obtain purification of the enzyme. However, in this case the antibody is generated from the inactive enzyme and may not bind to the active form. No further results were published.

The above studies show that the transferase is very unstable when removed from its membranous environment. Also, the precise molecular weight is uncertain. Since the yeast enzyme is considered to be less labile than that from mammalian sources, it was decided to use yeast as the source for the oligosaccharyl transferase. With the aim to keep the enzyme active through out the purification and characterization, all steps were carried out so that the transferase was not removed from its membrane environment, or from an analogous environment mimicking it. The assay for the enzyme was used to probe for the transferase through each purification step.

RESULTS AND DISCUSSION

Yeast Cell Harvest

Saccharomyces cerevisiae (wild type) were transferred in late log phase to a growth media (20g/l dextrose, 20g/l peptone, 10g/l yeast extract) and the cells were incubated at 30°C for 18 hrs before the cells were spun down at 2400g for 10 min at 2°C. The cells were then resuspended in 5 vols of Homogenizing Buffer (Hb) (50mM Tris HCL Buffer at pH 7.5, 0.15mM MgCl₂, 0.04% β-Mercaptoethanol) and again spun down at 2400g for 10 min at 2°C. It has been found that the cells could, at this point, be stored at -30°C for over 10 days without any appreciable loss of the transferase activity.

Typical Yield: 1 l of growth media gave 8.2g of cells after 18 hrs of incubation.

Microsomal Preparation

The frozen cells were completely resuspended in a 1:1 vol of Homogenizing Buffer (Hb). The suspension was transferred to a homogenizing bead beater to obtain a 1:1 ratio of glass beads:cell suspension. The suspension was now pulse homogenized for 20s, eight times, with 1 min cooling intervals. The homogenization was carried out at the temperature of a freezing solution of ice and NaCl. The homogenate was then spun down at 2400g for 10 min at 2°C to obtain a clearer supernatant. The supernatant was again spun down at 2400g for 10 min at 2°C. Having rid the supernatant of the beads and cell debris, the supernatant was spun down at 48000g for 90 min at 2°C. The pellet obtained was the microsomal pellet. The

entire work was done keeping the suspensions and homogenate below 4°C.

The supernatant obtained was assayed for activity. No activity was found. This confirmed the absence of transferase in a soluble form in the cytosol.

Solubilization of Microsomes

The pellet was completely resuspended in Hb containing detergent (see below). The smallest possible volume of the detergent solution was used so that the protein concentration was as high as possible. The solution was spun down at 35000g for 60 min at 2°C, to get rid of the insoluble lipid and protein matter.

<u>Detergent used:</u>	<u>Nature</u>	<u>% used</u>	<u>CMC(mM)*(9)</u>	<u>Activity**</u>
Nonidet	non ionic	0.6% in Hb.	0.11	0.396

N,N-dimethyl
dodecylamine

N-oxide(LDAO)	zwitterionic	0.5% in Hb.	0.14	0.375
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(*Critical Micellization Concentration)

(** pmol/hr/mg)

No appreciable difference was found in the transferase activity using the different detergents. However it was found that Nonidet interfered with the ammonium sulfate fractionation step while LDAO did not.

The protein solution obtained was made to 25% Glycerol and

stored at -80°C. No appreciable loss of activity of the solubilized transferase in this crude preparation kept at -80°C was observed after 5 days:

<u>Time</u>	<u>Activity (pmol/hr/mg)</u>
0.5 days	0.415
5 days	0.365

However, the transferase did not maintain its activity when stored in its solubilized form in LDAO or Nonidet, after being kept for a few hours at -80°C.

<u>Detergent</u>	<u>Activity(pmol/hr/mg)</u>
LDAO	0.050
Nonidet	0.075

Typical Yield: 1 g of cells yielded 0.54 mg of active, solubilized microsomal protein.

The ASSAY

The oligosaccharyl transferase was assayed using a procedure modified from Bause *et al* (10) (and had been previously used in the laboratory for assaying the pig liver transferase). Dol-PP-GlcNac-[³H]GlcNac was obtained from the lab. 30,000 disintegrations per min. (dpm) of a specific activity of 18 Ci/mmol of this dry substrate was kept in a 1 ml Eppendorf. To this M µl of a batch solution of 50 mM Tris HCl buffer, pH 7.5; 10 mM MnCl₂; 1.2% Triton X-100; and 5 µl of 20 mM Bz-Asn-Leu-Thr-NMe tripeptide. The reaction was started when L µl of protein solution was added to the eppendorf so that M+L=200µl. The assay was shaken at room

temperature for a fixed time. At desired times T1, T2, T3 50 μ l of the assay was quenched into a vial containing 0.8 ml 3:2 chloroform:methanol and 0.2 ml of 4 mM $MgCl_2$ with immediate vortexing. Typical values of T1, T2, T3 are 1 min, 5 min, 10 min respectively. The vials were allowed to sit until a clear phase separation was seen. The upper phase was removed. To the lower phase, 0.6 ml of theoretical upper phase(TUP)(3:48:47:0.168 chloroform:methanol:water: 1M $MgCl_2$) was added and vortexed. Again the upper phase was removed and to the lower phase another 0.6 ml of TUP was added, vortexed and extracted on complete phase separation. The 3 extracts corresponding to each time point, were pooled, to which 200 μ l of chloroform was added and the mixture vortexed. After letting the phases separate, The chloroform was carefully back-extracted. The upper phase constitutes the amount of radioactive label transferred from the organic layer to the aqueous layer and with the subtraction of any background, the amount of sugar transferred from the lipid donor to the acceptor peptide. The disintegrations per minute (dpms) were then counted for each time point. The slope at T=0 of the plot of dpms vs. time was used to calculate the specific activity of the crude protein for each experiment.

Ammonium Sulfate Fractionation

An attempt was made to fractionate the several solubilized proteins present in the microsomal preparation by varying ammonium sulfate saturation in the microsomal solution. Ammonium sulfate has a very high solubility in water and is also the mildest- having negligible denaturing effect on proteins- of the

salts commonly used in crystallizing proteins. It precipitates proteins by competing for the water molecule hydrating the proteins. Different proteins have different affinities for the hydrating water molecule and are precipitated at different saturations.

The solubilized microsomal solution was brought to 20% saturation of ammonium sulfate and the solution was gently stirred at 4°C for 45 min. When the ammonium sulfate was added to the microsomes solubilized in Nonidet, a phase separation was observed and it was feared that a clean and well defined precipitation would not be obtained. The Zwitterionic detergent LDAO, however, gave no such precipitation and also did not interfere with the assay or the transferase activity. The LDAO solubilized microsomal preparation was thus used. The 20% saturated solution was then spun down at 10,000g for 30 min. The pellet obtained was then solubilized in 0.5% LDAO in Hb (call this 20%ppt). The supernatant (call this 20%sup) was assayed and found active- see below. The supernatant, 20%sup, was then made to 40% Ammonium Sulfate by addition of salt and was gently stirred at 4°C for 45 min. The solution was then spun down, at 9600g for 30 min at 2°C to obtain a pellet (call it 40%ppt) that was again redissolved in 0.5% LDAO in Hb. The supernatant (call it 40%sup) was found to be inactive and further precipitation was not carried out. The ammonium sulfate had to be dialyzed out in buffer containing 0.5% LDAO, since the activity of the transferase falls rapidly if the detergent concentration falls below the CMC value.

The protein concentration was then determined using a modified Lowry protein quantization procedure with precipitation.

Typical fractionation data:

24.47 g of cells gave 5.01 ml of solubilized microsomal protein prep.

<u>Fraction</u>	<u>Protein Conc(mg/ml)</u>	<u>Abs. Prot(mg)</u>	<u>Activity(pmol/hr/mg)</u>
Crude extract	10.33	51.5	0.404
20%ppt	13.33	26.66	0.017
40%ppt	7.16	8.96	0.353
40%sup	6.16	30.89	0.108

Assume, as the limiting case, that all the protein obtained at 40%ppt is the transferase, then, in 1 mg of the crude extract, there is $(7.16/51.5)=0.14$ mg of transferase. If this were the case, then the activity at 40P should be $(1+0.14)=1.14$ times that of the crude. But this is not the case. Over 85% of the activity of the transferase has been lost in this mild step.

Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

Assuming that the apparent molecular weight of the transferase to be 80 kDal, a 7.5% acrylamide;2.7% bisacrylamide gel was prepared following the procedure due to Sapiro *et al* (10a), so as to be able to obtain maximum resolution in this molecular weight region. The gel run with samples solubilized in LDAO showed no resolution whatsoever. This was believed to be due to the effect of the zwitterionic detergent with its low CMC value, not being displaced

by even 1.0% SDS.

Extraction of detergent:

An Extracti-gel (a very hydrophobic gel having a very high affinity for other hydrophobic molecules or hydrophobic parts of molecules) column was used so as to remove LDAO in the samples obtained after the fractionation, so that resolution might be obtained during SDS-PAGE. The actual results and success of the fractionation would only be known after a completely resolved gel showing the different protein bands corresponding to each fraction is obtained.

0.5 ml of the gel suspended in Hb was loaded onto a 1 cm by 10 cm column. The pore size of the column was less than 20 μm . The column was run under gravity, and three 0.5 ml fractions were collected in Eppendorfs containing 50 μl of 10% SDS. All the fractions from each fractionation sample was run separately on the gel. This time however, resolution was seen but, because of the dilution due to the Extracti-Column run, no bands were seen in the 40%ppt and 20%ppt lanes.

Detergent Extraction without dilution:

A procedure was developed, modified from Horigome *et al* (11), through which the detergent extraction was possible without any significant dilution or any detectable protein precipitation. A hole was pierced at the bottom of an Eppendorf using a 18.5 gauge syringe. The hole was blocked using siliconized glass-wool. 0.5 ml of Extracti-Gel was equilibrated with the Buffer in which the microsomes were solubilized. The sample was placed- typically 100-150 μl - in the Eppendorf and left open for 5 min. The

Eppendorf lids were then shut and they were placed in appropriate test-tubes containing 10-15 μ l of 10% SDS to obtain 1% conc in the solution. The Eppendorf sat on the mouth of the test tube and together they were spun down at 200g for 2 min at room temperature. There was negligible change in the protein concentration after the extraction. A Lowry Protein assay with precipitation was carried out to determine the success of the procedure. The results of the assay for the microsomal preparation were:

Concentration of Sample Before Extraction :	2.40 mg/ml
Concentration of Sample After Extraction :	2.17 mg/ml

Results of SDS-PAGE:

The samples from the Ammonium Sulfate Fractionation were loaded onto a 7.5% gel, after the removal of LDAO. The gel showed that even though there was only 17.40% of protein by weight, in the 40%ppt fraction, the number of different proteins and/or their subunits thereof, was considerably high. This meant that another step of fractionation must follow. But over 85% of the transferase activity had been lost and thus, to carry on another step implied the use of a very large quantity of the Crude would have to be used before the Ammonium Sulfate Fractionation Step.

However, on assaying the 20%sup fraction, it was found that while this step removed 51.77% of the inactive protein, the loss of transferase activity was negligible.

Activity of 20%sup :	0.843 pmol/hr/mg
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As a subsequent step after the 20% ammonium sulfate 'Cut off', a

Concanavalin A affinity column was considered.

Con A Affinity Column:

Concanavalin A, the lectin obtained from Jack beans (*Canavalia ensiformis*), binds, in its tetrameric form, at pH 7.0, and in the presence of one manganese ion and one calcium ion for each Con A monomer, to saccharides and oligosaccharides with D-manno- or D-glucopyranose configuration and unmodified hydroxyl groups at C-3, C-4 and C-6(12). The lectin on an agarose support has also been reported to have stability in the presence of several detergents, including LDAO (no effect on saccharide binding at 0.25% LDAO(13)).

1 ml of the Gel obtained from Bio-Rad, was equilibrated using Hepes Buffer (HeB) (50 mM Hepes, 1M NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 1mM $MnCl_2$ at pH 7.0), and loaded onto a pipette column. Knowing that dilution will be a definite consequence of running the column, as concentrated a sample (of the microsomal preparation) as possible would have to be loaded onto the column.

A concentration procedure, with minimal protein loss or precipitation was found. The dilute sample was placed in a dialysis bag in Polyethylene Glycol (Peg) at 4°C. After a time T, decided on by visual inspection, the sample was retrieved in a concentrated form (A six fold concentration was observed after T=2 hrs(Approx.)).

Results of a 4-fold concentration of a dilute 'Crude' sample using Peg:

Activity before: 0.061 nmol/hr/mg

Activity after : 0.287 nmol/hr/mg

A negligible loss of transferase activity or concentration is apparent.

6 ml of Crude was concentrated to 1 ml and dialyzed in HeB (containing 0.25% LDAO). It was then loaded onto the column. The column was carefully run under gravity, collecting 0.5 ml fractions. After collecting eight fractions (elution Buffer was HeB with 0.25% LDAO), the column was bumped using 25 mM Mannose Pyroniside in Heb containing 0.25% LDAO. 6 more fractions were collected. The protein concentration was determined by the modified Lowry method (with precipitation). The activity of the fractions was determined to trace the transferase.

Results:

As the graphs show, the transferase was retained by the lectin. This implies that the oligosaccharyl transferase is certainly a glycoprotein with at least one saccharide unit that contains D-manno or D-glucopyranose configuration with unmodified hydroxyl groups at C-3, C-4, C-6. While the activity of the crude is amplified by at least 100 times, the SDS-PAGE data after detergent extraction showed this purification to be even more significant. Comparing the lanes of Fraction-5 with the 'Crude' lane, it is found that at least 90% of the different proteins can be seen in both the lanes- implying that these are very unlikely to have been retained by the lectin. The concentration of Fraction-10 and 11 was too low to show any bands even after silver staining.

CON A AFFINTY COLUMN

CON A AFFINTY COLUMN

CONCLUSION

The above results show that the activity of the transferase is dependent on its environment- removal of detergent or lipid membrane drops the activity by over 85%. The detergent LDAO, being a 'small amphiphile', better mimics the membrane environment, and appears to be a good detergent to be used for subsequent work. The fact that the transferase bound to the lectin-Con A, it is very likely to be a glycoprotein with at least one saccharide unit having a D-manno or D-glucopyranose configuration with unmodified hydroxy groups at C-3, C-4 and C-6. The transferase will probably have an asymmetric orientation in the RER membrane, with the saccharide units facing the luminal side.

Using the fact that the microsomal preparation and solubilization can be stopped at convenient points, a large preparation is now feasible. Further characterization and purification of the transferase can now be carried out by first first doing the 20% Ammonium Sulfate Precipitation and following this by a concentration step and then Gel Filtration- a step that will allow a fractionation on the bases of molecular weight. If need be, a final purification step using the Con A Affinity Column will be carried out. All the steps will be carried out using an LDAO solubilized microsomal preparation and at temperatures below 4°C. The transferase will be followed through each step by assaying for its activity.

The N-terminus of the purified enzyme could then be sequenced. This would allow the development of an oligonucleotide probe. This

probe would enable the screening of transformants of a DNA library and the cloning of the gene corresponding to the transferase. Identification of the gene encoding the oligosaccharyl transferase would allow for a significant advance in understanding this crucial enzyme involved in glycoprotein biosynthesis.

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