CHARACTERIZATION OF GLYCANS ROM TAMM-HORSFALL GLYCOPROTEIN USING SALT PRECIPITATION METHOD

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THIS DISSERTATION SUBMITTED TO FULFILL PART OF THE REQUIREMENTS IN OBTAINING THE BACHELOR DEGREE OF SCIENCE WITH HONOURS

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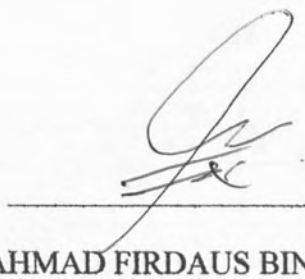

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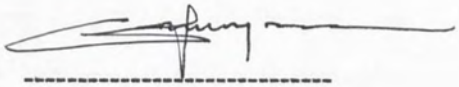

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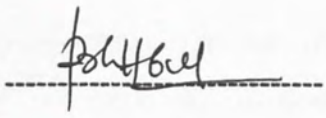

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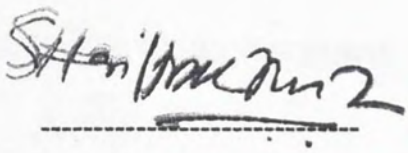

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ABSTRACT

Tamm-Horsfall glycoprotein (THP) is the most abundant protein exists in normal human urine. This glycoprotein can be isolated from the normal human urine by using the Tamm-Horsfall isolation method, which includes the salt-precipitation procedure. This mehod was applied because THP glycoprotein tends to aggregate with salt. The purity of THP glycoprotein was checked by using SDS-PAGE and the size of the protein can be compared with protein marker. From the SDS-PAGE analysis in the presence of reducing agent, the size of THP glycoprotein was in the range of 100 kDa to 120 kDa. The differences between concentration of THP glycoprotein isolated from male and female urine can be figured out by Bradford assay analysis. THP glycoprotein isolated from female urine is higher than THP glycoprotein isolated from male urine. In order to characterize the THP glycoprotein, several methods were used. The characterization of this protein can be achieved by releasing its N-glycans and O-glycans. The release of N-glycans and O-glycans by can be done by treating the THP glycoprotein with trypsin, and further the process with PNGaseF digestion. TLC (Thin Layer Chromatography) method used to determine the carbohydrate contained in both glycans. By doing this method, it showed that galactose existed in N-glycans. Besides, the interaction of THP glycoprotein with serum was also examined. THP glycoprotein showed no agglutination when treated with serum. The serums used in this research were mouse serum, Bovine Serum Albumin (BSA) and Foetal Bovine Serum (FBS).

ABSTRAK

Protein-gliko Tamm-Horsfall (THP) merupakan protein yang paling banyak kewujudannya di dalam air kencing manusia normal. Protein-gliko ini boleh diasingkan daripada air kencing manusia normal dengan menggunakan kaedah pengasingan Tamm dan Horsfall, di mana ia melibatkan prosedur pemendakan garam. Kaedah ini digunakan kerana protein-gliko THP mempunyai kcenderungan untuk menggumpal dengan garam. Ketulenan protein-gliko THP ini dapat ditentukan melalui SDS-PAGE dan saiz protein ini dibandingkan pula dengan penanda protein. Berdasarkan analisis SDS-PAGE dengan kehadiran agen penurunan, saiz protein-gliko THP ialah di antara 100 kDa hingga 120 kDa. Perbezaan kepekatan protein yang dipisahkan dari air kencing lelaki dan perempuan dapat dikenalpasti melalui kaedah pengujian Bradford. Protein-gliko THP yang diperoleh dari kaedah ini boleh digunakan dalam kajian pencirian glaikan. Untuk itu,beberapa kaedah telah digunakan. Pencirian glaikan protein-gliko ini boleh dicapai dengan melepaskan N-glaikan dan O-glaikannya. Pelepasan N-glaikan dan O-glaikan dapat dilakukan dengan menggunakan enzim tripsin, dan diteruskan pula dengan proses pemotongan PNGaseF. Kadeah TLC (Thin Layer Chromatography) digunakan untuk menentukan jenis karbohidrat yang terkandung dalam kedua-dua glaikan. Melalui kaedah ini, didapati galaktose wujud pada N-glaikan. Di samping itu, interaksi antara protein-gliko THP dengan serum juga telah diperiksa. Protein-gliko THP tidak menggumpal apabila diuji dengan serum. Antara serum-serum yang digunakan ialah serum tikus, Albumin Serum Lembu dan juga Serum Janin Lembu.

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LIST OF SYMBOLS

%	percentage
μ	micro
Asn	Asparagine
Da	Dalton
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
ER	Endoplasm reticulum
GalNAc	N-acetylgalctosamine
GlcN	N-glucosamine
GlcNAc	N-acetylglucosamine
Glu	Glucose
GPI	Glycophosphatidylinositol
IAA	Iodoacetic acid
KDa	Kilo Dalton
L	Liter
M	molar
M	mili
MALDI	Matrix-assisted laser desorption ionization
Man	mannose

mg	miligram
°C	Celcius, unit of temperature
PNGaseF	Peptide N-glycosidase
rpm	rotation per minute
Ser/Thr	Serine or theorine residues
TEMED	N-, N'-tetramathylenediamine
THP	Tamm-Horsfall glycoprotein

CHAPTER 1

INTRODUCTION

1.1 Introduction

A normal 24-hour urine output contains about 60 grams of solid material. Urine is known as liquid waste excreted by the kidneys and eventually expelled from the body in a process known as urination. Urine flows through the following structures: the kidney, ureter, bladder, and finally the urethra and its produced by a process of filtration, reabsorption, and tubular secretion. About half of this is organic, consisting of substances like urea, uric acid, glycoprotein and creatinine. The inorganic portion will contain substances like sodium chloride, phosphates, sulfates, and ammonia. Normal urine should not contain any glucose or amino acids. Urine also has long been known as a rich source of diagnostic information, because of its physical properties and chemical composition. Previous attempts to use urinary protein profiles for diagnostic purposes resulted in some remarkably successful applications, such as pregnancy tests based on human chorionic gonadotropin excretion or base on the type of glycoprotein contained in it. The glycoprotein is normally known as Tamm-Horsfall glycoprotein (THP).

Many proteins carry covalently attached oligosaccharide or polysachharide chains. There are an astonishing variety of these modified proteins, which are known as glycoproteins, and they serve many different functions. The functions of glycoprotein depend on the glycan part. The capacity of glycans varies from one percent to 90 percent for different types of glycoproteins. This variation will cause the changes in their functions. Some significant biological roles of these glycans, or carbohydrate units include location of protein within the cell, protection of the protein against proteolytic attack, control of the lifetime of circulating cells and glycoproteins, induction and maintainance of the spatial conformation in a biologically active form, facilitation of the extracellular secretion and ultimate fate, direction and modulation of the immune response, and the provision of ligand structures for the cell recognition or interaction (Jolles and Jornvall, 2000).

As mentioned above, one of the substances contain in urine is glycoprotein. The earliest membrane of the glycoprotein family to be identified as a distinct class of biological compounds were mucins (so named in 1835 by Nicholas Theodore de Saussure), several of which were isolated in the second half of the nineteenth century. Glycoprotein has several important functions to our body. The glycoprotein includes immunoglobulins, hormone transport, lectins, THP glycoprotein and uromodulin. Uromodulin, an immunosuppressive glycoprotein isolated from human urine, can bind recombinant murine interleukin 1α with high affinity and this binding can be inhibited by addition of specific saccharides. (Muchmore and Decker, 1987).

Degradation of protein backbone is one of the process in order to characterize the glycosylation sites in a glycoprotein. Selective mild proteolytic digestion followed by the fractionation of the formed glycopeptides is applied for this purpose. The location of the glycosylation sites in the polypeptide chain can be determined by the analysis of the amino acid sequence around each glycan attachment site of the glycopeptide and comparison of this sequence with the known amino acid sequence of the protein (Jolles and Jornvall, 2000).

There are several methods that are widely used in order to isolate and determine the glycoprotein of urine such as salt precipitation (McKenzie *et.al.*,1964), or by using Diatomaceous Earth (DE) Filter (Serafini-Cessi *et al.*, 1989) and electroimmunoassay (Bichler et al., 1977 ; Wieslander *et al.*,1977 ; Samuel, 1978).

In this research, THP glycoprotein will be isolated from male and non-pregnant woman by using salt-precipitation method, described by Tamm and Horsfall in 1950. After that, the glycoprotein will be run through a series of steps that separated the compound based on its characteristic size and biochemistry. Identification of THP can be done by comparing the bands that result from SDS-PAGE technique with the protein marker. The THP glycoprotein then will undergo several method in order to characterize it such as Bradford assay analysis, interaction THP glycoprotein with serum and the migration of N-glycans and O-glycans by TLC (Thin Layer Chromatography) method.

1.1 Objectives

In this research, there are several objectives need to be achieved such as :

1. To isolate THP glycoprotein by using salt-precipitation method.
2. To determine the purity of isolated THP glycoprotein.
3. To know the characteristic of Tamm-Horsfall glycoprotein.
4. To investigate the differences of THP glycoprotein isolated from male and female urine.

CHAPTER 2

LITERATURE REVIEW

2.1 Glycoprotein

Glycoprotein is defined as protein chain attaches to glycans, or also known as saccharide chain, or polysaccharide. Glycoprotein in other word also known as protein containing oligosaccharide covalently attached to selected acid amino residue (Hughes, 1983). What is oligosaccharide itself? Oligosaccharide is defined as carbohydrate polymer comprised from 2-10 monosaccharide residue and the attachment to glycoprotein may assist in their proper folding, help to protect the mature proteins from proteolysis, and in some cases participate in cell-cell adhesion (Hughes, 1983). Glycans is usually attached to the protein in a posttranslational modification, either at asparagines, hydroxylysine, hydroxyproline, serine, or threonine. Possible carbohydrates or glycans include glucose, glucosamine, galactose, galactosamine, mannose, fructose, and sialic acid.

2.1.1 Uses of glycoprotein

There are several important uses of glycoproteins in our body. Glycoproteins are often used in proteins that are at least in part located in extracellular space or outside the cell. Glycoproteins are important for immune cell recognition, especially in mammals. Glycoproteins enable antibodies or immunoglobulin to interact directly with antigens. Besides, molecules of the major histocompatibility complex or MHC , which are expressed on the surface of cells and interact with T-cells as part of the adaptive immune response.

Besides that, the attachment of glycan, or carbohydrate to glycoprotein may give several effects such as help the protein to fold in the proper geometry and stabilize the protein. The attachment also can affect physical properties such as solubility or viscosity and helps it to familiarize correctly in a membrane, or make it recognizable to another biochemical or cell. These structures occur in many life forms and they are common and very important in mammalian tissues.

2.1.2 Glycosylation

Glycosylation is one of the most common post-translational modifications of proteins in eukaryotic cells. Glycosylation is defined as a process of sugar attachment such as saccharides, monosaccharides and polysaccharides to protein or lipid to form glycoproteins. In other word, glycosylation is an addition of glycosyl groups to a protein

to form a glycoprotein. The polypeptide chains of glycoproteins are synthesized under genetic control. The carbohydrate chains are generated enzymatically and covalently linked to the polypeptides. The glycoproteins have variable carbohydrate compositions. Glycan binds to protein in 3 ways, by (1) N-linked glycan, that attach to the amide nitrogen of asparagines side chain, (2) O-linked glycan, the glycosylation that attach to the hydroxy oxygen of serine and threonine side chains and (3) glycosyl-phosphatidylinositol, (GPI-) anchors, proteins that are attached at their carboxy-terminus through a phosphodiester linkage of phosphoethanolamine to a trimannosyl glucosamine core structure.

The structures of these three glycans are totally different, and different sugar residues are usually found in each type. The different of these structures reflect differences in their biosynthesis. For example O-linked sugars are added at one at a time, and each sugar transfered is catalyzed by different glycosyltranferase enzymes. For N-linked glycan, it begins with the addition of a large preformed oligosaccharide, containing 14 sugar residues subsequently certain sugar residues are removed and others are added one in a time.

Endoplasmic Reticulum (ER) and the Golgi complex are the places that protein glycosylation occurs. Both organelles play central roles in protein trafficking. As we know, these glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation and pathogenicity. There are three types of protein glycosylation such as N-linked glycosylation that attach

to the amide nitrogen of asparagines side chain, O-linked glycosylation that attach to the hydroxy oxygen of serine and threonine side chains and lastly glycosyl-phosphatidylinositol, (GPI-) anchors, proteins that are attached at their carboxy-terminus through a phosphodiester linkage of phosphoethanolamine to a trimannosyl glucosamine core structure.

An asparagines residue can only accept an oligosaccharide if the residue is part of an Asn-X-Ser or Asn-X-Thr sequence, in which X can be any residue. Thus, potential glycosylation site can be detected within amino acid sequences. Glycosylation can lead the protein to fold correctly and to confer the protein stability on some secreted glycoprotein. This is because there is some proteins do not fold correctly unless they are glycosylated first. The polysaccharides that linked at the amide nitrogen of asparagine in the protein will give the stability. Otherwise, glycosylation also play a role in cell-cell adhesion, a mechanism employed by cells of the immune system.

2.1.3 N-Linked Glycans

Protein glycosylation of N-linked glycans is actually a co-translational event, occurring during protein synthesis. Glycosylation occurs most often when this consensus sequence occurs in a loop in the peptide. The consensus sequence that required is Asn-X-Ser/Thr, where X can be any amino acid except proline (Pro) (Gates *et al.*, 2004; Rosenberg, 1996). N-linked glycans binds through N acetylglucosamine or N-acetylgalactosamine to the side chain amino group in an asparagine residue. An asparagines residue can only

accept an oliogosaccharide if the residues is part of an Asn-X-Ser or Asn-X-Thr sequences. These olgosaccharides are important in the proper processing, correct membrane targeting, intrinsic biological activity, or protein stability (Rosenberg , 1996).

There are many assortments exist in N-linked glycans. This diverse of assortments are based on the common core of pentasaccharide, $Man_3GlcNAc_2$ (Man: Mannose, GlcNAc: N-acetylglucosamine). There are three types of N-linked glycans. There high mannose, complex and hybrid glycans (Gates *et al*., 2004).

In high-mannose glycan, it consist of alpha-(1-3) and alpha-(1-6) mannose residues in addition to the core pentasaccharide structure. There exists clearly a general binding function such as interaction with human epithelial cells. If these both alpha-mannosyl residues in the core have GlcNAc attached, the glycan will be known as complex glycan. Complex glycans are so named because they can contain almost any number of the other types of saccharides, including more than the original two *N*-acetylglucosamines. This type of N-glycans occurs in erythrocyte membranes and human lactotransferin. The branching structures derived from the GlcNAc residues is referred as antennae. The complexity of these multi-branched glycans components increases through tri- and tetra- to penta-antenarry types. In hybrid glycan, it has the structural features of both high-mannose-type and complex-type glycan. The alpha-(1-6)-linked core mannose has only mannose residues attach to it, while the alpha-(1-3)- linked core mannose has one or more GlcNAc-initiated antennae attach to it.

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