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The Effect of Glycan Linked to Cationic Peanut Peroxidase on its Activity

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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ABSTRACT

The roles of heme-, calcium- and glycan moieties on peanut peroxidase enzyme activity have been previously studied. While the 3-D protein structure is known and some information about the glycan chains is known, due to the uneven distribution of the 3 glycans on the protein, only a partial understanding of the sugar groups of the 16 glycan chains that make up the three glycans is available. Now, having used gel-filtration column chromatography followed by lectin affinity column chromatography, I found that there is a rich array of galactose present in the glycan chain. Moreover, I determined that few sugars are removed during a 14-day culture period. Alternatively, I can also suggest that a large amount of peroxidase continues to be secreted into the culture medium even by the end of the 14-day culturing period. These late released proteins remain glycosylated; while those that were released at the beginning of the culture period had lost many sugars.

Keywords

Peroxides; peanut, N-linked glycans; Glycosylation; Lectin affinity chromatography.

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CHAPTER ONE

INTRODUCTION

1.1 General

Glycobiology is an interesting field that has continued to draw the attention of scientists over the past century (Mattaini and Vander Heiden, 2012; Sevice, 2012). The origin of glycobiology can be found in carbohydrate chemistry and biochemistry labs leading, where important discoveries such as lectins and hemagglutinins were made and influenced the field of glycobiology (Varki et al., 1999).

The term glycobiology refers to the study of glycans attached to other molecules. The term of glycan means a carbohydrate, which is a hydrate of carbon, in many derivations starting with the chemical formula $(CH_2O)_n$. Carbohydrates can be divided into monosaccharides, oligosaccharides and polysaccharides, in which the latter are polymers containing a large number of simple sugar monomers, usually between 20 to 2500. Oligosaccharidesare polymers containing a smaller number of simple sugar monomers, typically from 2 to 20 (Brooks et al., 2002). There are two types of glycoconjugates. The first type are glycoproteins, in which a glycan is linked to a protein. The second type are glycolipids, in which a glycan is linked to a lipid (Varki et al., 1999).

1.2 Classes of Glycoproteins

Glycoproteins are further classified into two categories: N-linked glycoproteins and O-linked glycoproteins. This classification depends on the glycan attachment site and the type of sugars in the glycan chains. In N-linked glycoproteins, the attachment

site is an asparagine amino acid, through a ß-N-glycosidic bond to N-acetylglucosamine (GlcNAC). In O-linked glycoproteins, the attachment site is through the hydroxyl of either a threonine or serine amino acid residue and N-acetylgalactosamine (GalNAC) (Brooks et al., 2002).

The glycans on peanut peroxidase are of the N-linked type, and are further described below. N-linked glycans are also called asparagine-linked glycans. These arefound in three distinct sub-structures types: high mannose, complex, and hybrid (Hounsell, 1998). A fourth type of N-glycan is paucimannosidic, which results from the elimination of terminal residues from the complex type of N-glycan (Figure 1)(Lerouge et al., 1998).

1.3 N-linked glycoprotein functions

N-linked glycans are involved in several biological functions. The carbohydrate groups are important for many physical properties, such as conformation, charge and water binding capacity (Paulson and Colley 1989), of the proteins on which they are found. They play an essential role in protein folding, oligomerization, sorting, and transport. N-linked glycans alsoserve as universal ligands for specific lectins (Helenius and Aebi 2001).

1.4 Complexity of glycoprotein studies

A comparison of the differences between the sequences of nucleic acids, proteins and glycan gives much information about the complexity of glycoproteins. To begin with, nucleic acids are derived from only four bases (adenine, cytosine, guanine and thymine) and are linear polymers. Proteins, while comprised of 20 different amino acids

are also linear polymers. On the other hand glycans can have either homogeneity of sugars or heterogeneity, and can be linked via either α or β linkages, that can link to other monosaccharides in various positions in a sugar chain or to other molecules; they can also form highly branched structures. Finally, glycan chains are in a constant state of flux, due to glycosidases, which can remove monosaccharides from the structure (Varki et al., 1999).

1.5 Lectins

Lectins are glycoproteins that bind to mono or oligosaccharides without altering their ligand structures (Urich ,1994). Plant lectins show a high degree of specificity for the carbohydrates to which they bind. Therefore, lectins have specific biological altering effects on carbohydrates. There are only two-research methods through which the biological effects of lectins can be examined, the biological and biometric methods. Plant lectins are known to be suitable for analyzing and isolating animal and human glycoconjucates. They are suitable for this function because the glycoconjucates are the natural target for most of the carbohydrate binding proteins in plants. Classically, in glycoconjucate research, plant lectins are used as analytical or preparative tools since their bioactive protein effects are suitable for inducing certain particular process in the target cells or organisms (Damme ,1998).

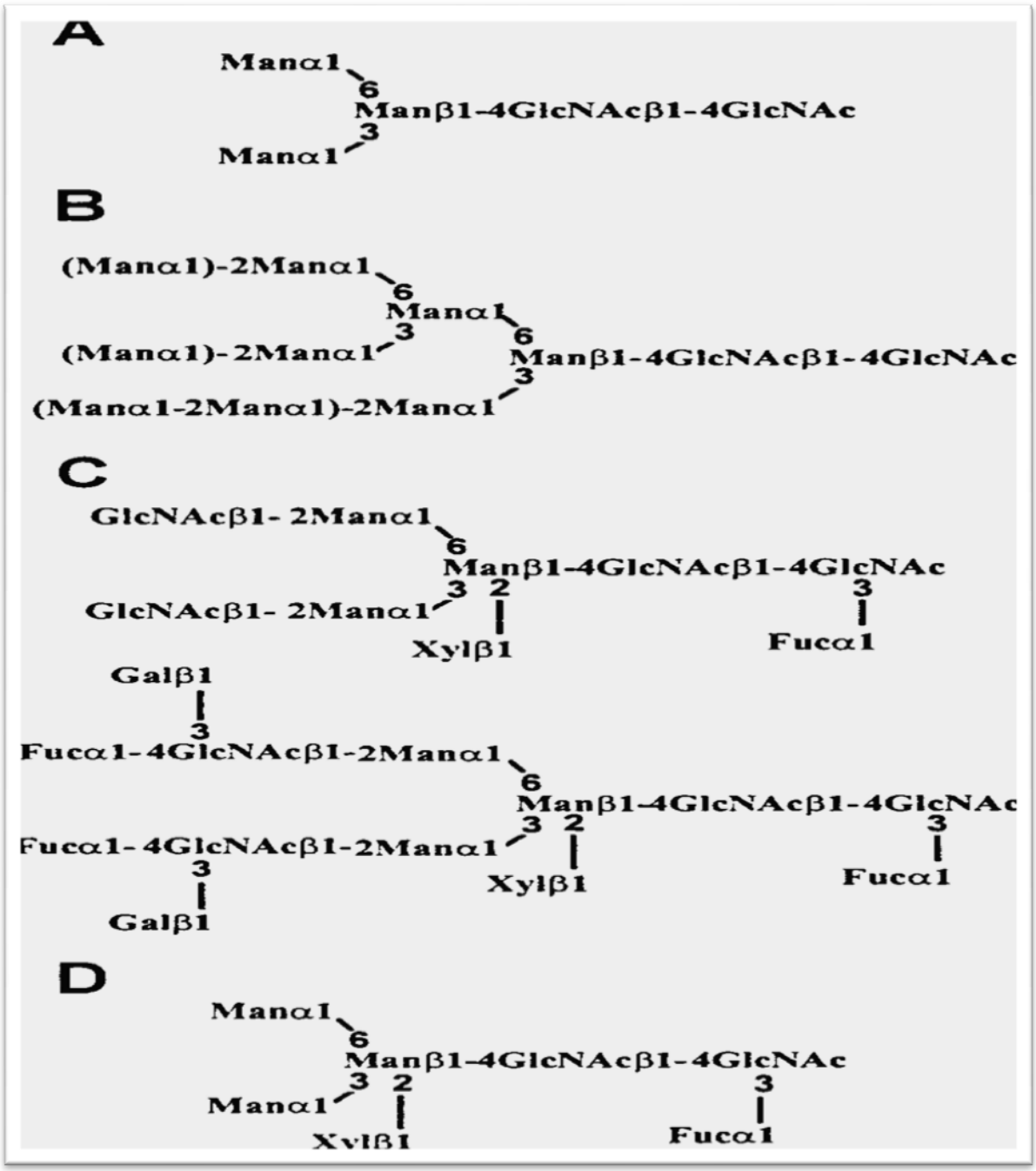


Figure. 1. Structure of plant N-linked glycans. (A) Common core of N-linked glycans, (B) high-mannose-type N-glycans Man5–9GlcNAc2, (C) complex-type N-glycans, and (D) paucimannosidic-type N-glycans. Adapted from Lerouge et al., 1998.

1.6 Peroxidases

Peroxidases (E.C.1.11.1.7) are a large family of heme contains enzymes that exist in microorganisms and plants that oxidize a hydrogen donor in a two-step reaction. The general reaction cycle is the following:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH^-$$

$$\text{Compound II} + AH_2 \rightarrow \text{Peroxidase} + AH + H_2O$$

$$2AH \rightarrow A + AH_2$$

Where AH_2 is a reduced organic substrate and AH- a radical product (Maranon et al., 1993).

Peroxidases can be divided into three classes: 1) prokaryotic organisms including yeast cytochrome c peroxidase, bacterial peroxidases, chloroplast and cytosol ascorbate peroxidases; 2) extracellular fungal peroxidises; 3) plant peroxidases (such as peanut peroxidase) (Welinder (cited in Esnault and van Huystee, 1994)).

1.7 Peanut peroxidase

The isolation of peroxidase from the spent medium of peanut cell cultures following a 14 day incubation period is much simpler than from cell extracts since fewer proteins are found in the spent medium compared to a cell extract. This has been confirmed with 2-D poly-acrylamide gel electrophoresis (2D-PAGE), where only about 27 peptides were seen on the gel of the spent medium (van Huystee and Tam, 1988). Peanut (*Arachis hypogaea* L.) peroxidase exists in three types as shown by separation on carboxy methyl sephadex chromatography. One of them is an anionic peroxidase (APRx) that carries a negative charge and washes from the column with other proteins

using 0.05 M Na-acetate pH 5. The second one is a major cationic peroxidase (CPRx) that carries a positive charge and elutes from the column using 0.07 M Na-acetate pH 5. CPRx elutes as the second fraction during ion exchange chromatography. The third peanut peroxidase is a minor cationic peroxidase, also carrying a positive charge, and which elutes from the column last using 5% NaCl (Sesto et al., 1989).

1.8 Peanut peroxidase structure

Heme

Cationic peanut peroxidase is a red peroxidase isozyme, which is a hemoglycoprotein. Removing the heme from the holoenzyme causes complete loss of enzyme activity (Chibbar and van Huystee, 1984). Radio-labelling studies determined that the heme metabolic pathway begins with glutamic acid (also a precursor for chlorophyll) instead of glycine as in animals (Chibbar and van Huystee, 1983), and the mass of the heme was found to be 616 Da as in hemoglobin (Chibbar and van Huystee, 1986).

Calcium

Analysis by atomic absorption spectroscopy of CPRx revealed that it contains two moles of calcium per mole of protein (Hu et al., 1987). Calcium is likely lost when peroxidase is dialyzed against water for a period of time (Maranon et al., 1993). Loss and gain of calcium resulted in the alteration of peroxidase structure and its heme site, observed using H^1NMR (Barber et al., 1995). Loss of calcium caused various amino acid moieties to modify the active site of the enzyme.

Glycans

The CPRx contains 20% carbohydrates by mass (Lige et al., 2000). The glycans linked to CPRx have been intensively studied (van Huystee 1990; Wan and van Huystee, 1993b), but the detailed functions of the glycan chains are not known yet. There are five potential N-glycosylation sites in the amino acid sequences of CPRx, but only three N-glycosylation sites (Asn60, Asn144, and Asn185) are glycosylated. The other two potential sites, Asn209 and Asn275 both have a proline residue within four amino acids at the C-terminal end following the asparagine. This prevents binding of glycan to the asparagine.

The length of glycan chains as well as alpha and beta linkages determined by H^1NMR, showed that there are 12 sugars in the glycan chain at site N-144. Fucose, xylose, galactose, mannose, and N-acetylglucosamine are the five types of sugar in the glycan chain as detected by high performance liquid chromatography (HPLC) (Sun et al., 1997) (Figure. 2). A recent study of the glycan chains linked to the major peanut peroxidase was done using electrospray ionization mass spectrometry (EI-MS), which showed the differences among the numbers of sugars in each of the three glycan chains. At N-185 carries 16 sugars, while the others have only 12 or 14 sugars. Moreover, the shortest glycan chains only showed 4 sugars (Zhang et al., 2004) (Figure. 3). From this it is clear that there is a lot of heterogeneity at each glycan attachment site, which it likely due to glycosidases that cleave sugars from mature glycans.

Hypothesis

I hypothesize that shortening of the glycan chains at three sites–Asn60, Asn144, and Asn185– (Wan and van Huystee, 1993b) will affect peroxidase activity.

Objectives

The main goal of my project is to establish whether shortening of the glycan chains at Asn60, Asn144, and Asn185 affects peroxidase activity. To achieve this objective, I will accomplish the following steps:

1) According to previous studies on peanut peroxidase, there are 27 proteins derived from the cultured peanut cells after 14 day culture, which means that it has other enzymes in addition to peroxidase. One of the 27 peptides is beta galactosidase (Wan et al., 1994a), so there may be other glycosidases in the spent medium. Since my aim is to look into the sugar released in spent medium by glycosidase action, I will confirm the time limit of glycosidase reaction with glycan chains linked to any peptides left in a limited volume of spent medium during an incubation time.
2) Loss of sugars from glycan chains during incubation times means that glycan chains get shorter, which leads to a decreased molecular weight. My second goal is to test the molecular weight of CPRx during various incubation times by using two methods. Using gel filtration chromatography I will use the sample after purifying CPRx.
3) Subsequently, I will test the specific enzyme activity by colorimetric assay and measure the absorbance at two different wavelengths, 280 nm for protein

quantification and 490 nm to measure the intensity of the peroxidase reaction with Guaiacol, to calculate the specific enzyme activity.

4) Lectins have a sugar specificity which allow them to interact with the N-linked glycan chain. Concanavalin-A and the effect of β-galactosidase have been used in previous CPRx studies (Wan et al., 1994b). My third goal is to see how a shorter glycan chain obtained by gel filtration affects binding to different types of lectin columns. This will allow me to determine whether a glycan chain is shorter or longer than the one that was found binding to Con-A at the residue of glycan chain is bind to other lectins will reveal the identity of exposed sugars. (Wan et al., 1994b).

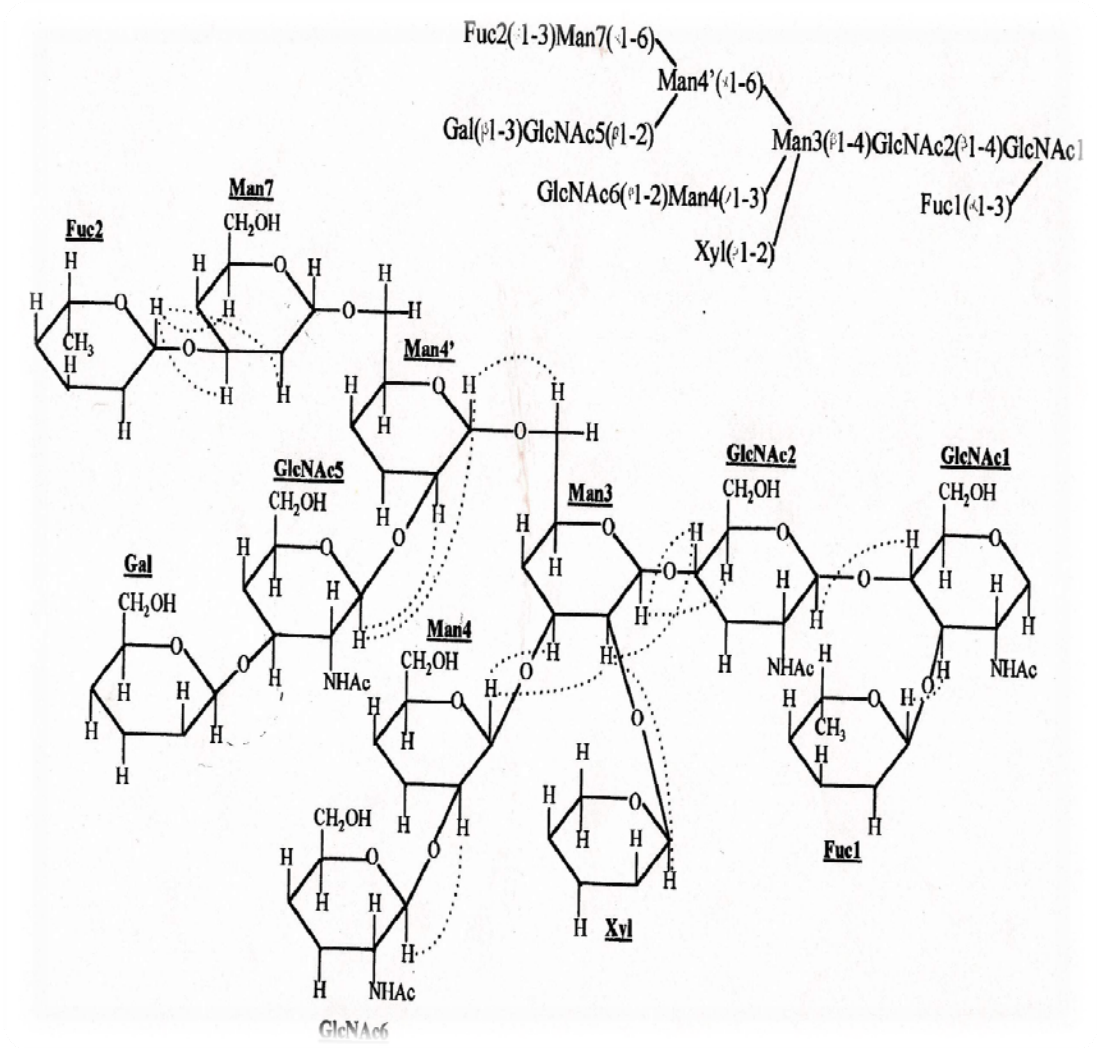


Figure. 2. The structure and sugar composition of the glycan chain at site N-144 (Sun et al., 1997).

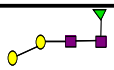
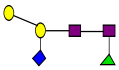
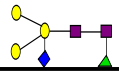
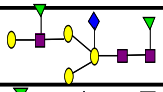
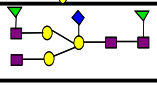
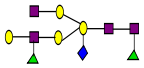
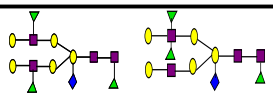
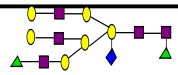
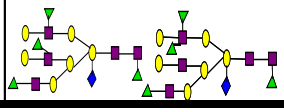
	Proposed glycan structure	Abbreviation	Glycan Mass Monoisotopic (Average)	Main species observed at m/z (charge states)		
				GPa	GPb	GPc
				m/z (CS) RI	m/z (CS) RI	m/z (CS) RI
I		N_2HF	714.27 (714.67)		958 (4+) ** 967(4+, K)	
II		N_2H_2F	876.32 (876.82)		998 (4+) * 1007(4+, K)	
III		N_2H_2FX	1008.36 (1008.93)		1031 (4+) **** 1374 (3+)	1390 (3+) **** 1397 (3+,Na)
IV		N_2H_3FX	1170.41 (1171.08)	1350 (2+) ***	1070 (4+) **	
V		N_2H_2NFX	1211.44 (1212.13)			1457(3+) 1465 (3+, Na) ****
VI		N_2H_3NFX	1373.49 (1374.27)	1451 (2+) * 1462 (2+,Na)	1122 (4+) * 1496 (3+)	1519 (3+, Na) *
VII		$N_2H_3NF_2X$	1519.54 (1520.41)			1568 (3+, Na) **
VIII		$N_2H_3NHF_2X$	1681.60 (1682.56)	1070 (3+) **** 1605 (2+)	1199 (4+) **** 1598 (3+)	
IX		$N_2H_3N_2F_2X$	1722.62 (1723.61)			1636 (3+, Na) **
X		$N_2H_3N_2HF_2X$	1884.68 (1885.75)	1139 (3+) **** 1707 (2+)	1251 (4+) *** 1666 (3+)	1689 (3+, Na) **
XI		$N_2H_3N_2H_2F_3X$	2192.79 (2194.04)	1241 (3+) ****	1327 (4+) **	1339 (4+) **** 1349 (4+, K) 1084 (5+, Na, K)
XII		$N_2H_3N_3H_3F_2X$	2411.86 (2413.23)	1326 (3+,K) *		
XIII		$N_2H_3N_3H_3F_4X$	2703.97 (2705.52)			1472 (4+) ** 1186 (5+, 3Na) 1963 (3+, Na)

Figure. 3. The cationic peanut peroxidase glycans investigated by Electrospray Ionization Mass Spectrometry, The symbols (③ Gal, Man,⑤ GlcNac,⑩ Fuc,❶ Xylose) are described in the reference (Zhang C. et al., 2004). Compare this with figure 3 for sugars in the hybrid glycan and variation among 3 chains. GPa refers to the glycan at Asn60; GPb refers to the glycan at Asn144; GPc refers to the glycan at Asn 185.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell Culture

A peanut cell culture for peroxidase isolation was obtained from seeds. Thin sections from sterile peanut (*Arachis hypogaea* L.) seeds of Virginia 56 R, were cultured on agar medium with nutrients, and then allowed to proliferate from a week to a month (Verma et al., 1970). The end result of this phase was masses of plant cells called peanut calli. Next, the best calli, which contained good cell growth were selected. The calli were sub-cultured onto new Petri dishes, which contained agar medium with nutrient (Table. 1). Other cells from the main dish are transferred into aqueous medium, of the same nutrient composition (except no agar) in a flask, and placed on a shaker (100-120 rpm) to establish cell suspension cultures. After 14 days, peroxidase enzyme presence in the spent medium was checked by testing a sample of the spent medium using a colorimetric assay, For this, 1 mL of spent medium was mixed with two drops of 0.3% H_2O_2 and two drops of 1.0% Guaiacol. A colour change from colourless to orange signified active peroxidase enzyme.

Table 1. The composition of the growth medium which support the growth of peanut cells in vitro (From Linsmaier et al., 1965). In the case of calli growth 7g/L agar was added.

Component	Concentration (mg/L)
Macro Elements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$ $2H_2O$	440
$MgSO_4$ $7H_2O$	370
KH_2PO4	170
Na_2EDTA	37.3
$FeSO_4$ $7H_2O$	27.8
Minor Elements	
H_3BO_3	6.2
$MnSO_4$ $4H_2O$	22.3
$ZnSO_4$ $7H_2O$	8.6
Na_2MoO_4 $2H_2O$	0.25
$CuSO_4$ $5H_2O$	0.025
$CoCl_2$ $6H_2O$	0.025
Organic Constituents	
Naphthalene Acetic Acid (NAA)	2.0
Kinetin	0.5
Thiamine-HCl	4.0
myo-Inositol	100
Ascorbic Acid	0.2
Carbohydrate Constituents	
Sucrose	30,000

The cells from the spent medium were sub cultured into new liquid medium and the rest of the culture filtrate (spent medium) was prepared for peroxidase purification. To purify peroxidase, 100% acetone was added to the filtrate to bring the composition to 70% acetone to precipitate proteins from the spent medium. The 70% acetone/medium solution was allowed to settle for a day, after which the excess acetone was siphoned away and the resulting mixture is centrifuged. The acetone pellet was re-suspended in 100 mL of 0.02 M sodium acetate (pH 5) and re-centrifuged at 13000 x g for 10 minutes. Then, the supernatant was brought to 80% ammonium sulphate to precipitate the proteins from the solution, and centrifuged at 13000 x g for 10 minutes. Subsequently, the ammonium sulfate pellet was re-suspended in 100 mL of 0.02 M Na-acetate buffer (pH 5), agitated by a magnetic stirrer for no less than 15 min., and dialized. After dialyzing against 4 liters of the same buffer with an added trace of calcium over two days with several changes to remove salts, insoluble material is removed by a final centrifugation (Figure. 4) (Maldonaldo et al., 1980; Sesto et al., 1989). Finally, the product of dialysis is ready for the ion exchange chromatography.

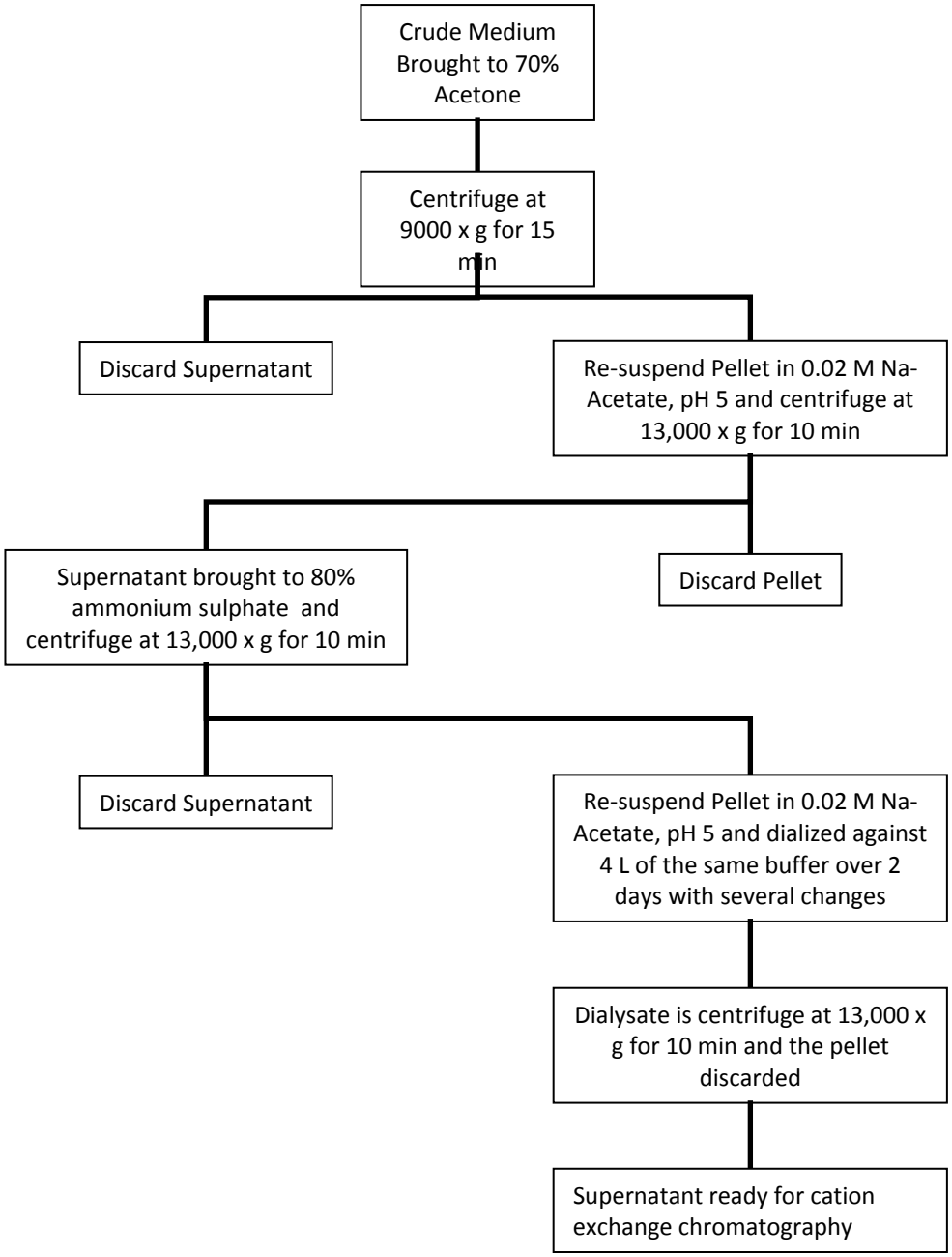


Figure 4. Isolation and purification of CPRx. Based on protocols by Maldonaldo et al., (1980) and Sesto et al., (1989)

2.2 Using a *Molmol* to obtain a sterilization culture

Studies have been done (El-Sherbiny and El-Sherbiny, 2011) on how the *Commiphora molmol* affects inflammation in humans or other animals. There is no information on plant response to “Mur” because the research of this compound has only been done on animals. Molmol is traditionally used as anti-inflammatory agent by taking a fresh dose orally. In the Middle East; it is known as “ Mur” or “Myrrh” but its components have not been studied. However, in 1985 a published article showed that a molmol suspension produced a significant anti-inflammatory and antipyretic activity (Tariq et al., 1985). A 0.30 g grounded molmol (*Commiphora molmol* from the public market in Jeddah, Saudi Arabia) per 100 mL culture medium was used. Molmol suspension is done as common practice. The common practice is adding the grounded molmol to the hot water at 80°C and wait for few hours to solubilize. In this experiment, it is solubilized overnight in a 100 mL of distilled H_2O. A two mL suspension of Myrrh was add to the liquid medium where the peanut suspension was transferred to after 14 days.

2.3 Peroxidase purification using Ion Exchange Chromatography

CM- Sephadex ion exchange chromatography was used to purify three types of peanut peroxidase, anionic, major cationic, and minor cationic. The ion exchange chromatography column was prepared using methods employed by Maldonaldo et al., (1980) and Sesto et al., (1989). The column size normally used was 5.08 cm x 10.16 cm packed with Carboxymethylcellulose-Sephadex (CM-Sephadex C-50, Pharmacia) in 0.02 M Sodium acetate pH 5. A gradient maker was used to create a gradient of

increasing Na-acetate pH 5 concentration from 0.02 M to 0.10 M, using 100 mL of each of 0.02 M to 0.10 M buffer (Maldonaldo and van Huystee, 1980). The eluted sample is ready for the specific enzyme activity test by meauring Optical Density (OD) or RZ at 490 over 280 nm (Lobarzewski and van Huystee, 1982) .

2.4 Enzyme activity measurement

The enzyme activity was measured by mixing 0.5 mL of 0.05 M Na-Phosphate pH 7, 0.005 ml of peroxidase sample and 0.166 mL 3% Guiacol, 0.333 mL of 1% H_2O_2. The mixture (medium and reagents) in quartz cuvette and agitated, the absorbance at 470 nm was measured after 5 minutes (van Huystee and Lobarzewski, 1982). The specific enzyme activity was measured by dividing the enzyme activity by the amount of protein as determined from the measurement at 280 nm, A standard graph for HRP was used to determine the quantity of protein at 280 nm . The presence of sugars was determined at 490 nm in each fraction by adding 0.625 mL of concentrated H_2SO_4 and 0.250 mL of 5% phenol 0.125 mL of fraction (Dubois et al. 1956). All the absorbances were measured by using Varian Cary 50Bio UV- Visible Spectophotometer.

2.5 Separation of CPRxs' glycoforms using Gel Filtration

A gel filtration column (10 cm x 0.3 cm) made of Sephadex G-75, (Pharmacia) was allowed to settle 24 hours before the experiment. The column volume was 2 mL. The column was washed with 10 mL of 0.02 M Na-acetate buffer pH 5. Then 20 μL of pure CPRx containing 2.1 μg protein was loaded. Twelve 1 mL fractions were collected using 10 mL of 0.02 M Na- acetate buffer pH.5 and 2 mL of 0.05 M of

$CaCl_2$. The absorbance at 280 nm for protein quantification and 403 nm for the heme was determine for each fraction.

2.6 Loss of sugars from the glycans in limited volume with time

The first objective was to confirm the time limit of releasing sugar by glycosidase from glycan chains of CPRx in a minimal incubation volume. To accomplish this, the sample should not be purified because applying the sample to the ion exchange chromatography will separate the glycosidase from CPRx. For that reason, I took the crude pellet (the spent plant cell culture medium collected by acetone precipitation (Figure. 4) in the purification steps, and then re-suspended it with 3 mL of 0.05 M sodium phosphate buffer, pH 5. I determined the amount of protein in the solution by measuring its absorbance at 280 nm. Next, I centrifuged the sample at 13000 X g for 10 minutes in 5°C to inactivate enzyme activity. I took the supernatant and kept it under 4°C. Then I started free sugar and protein assays. Subsequently, I took samples starting from 5 minutes until 5 hours at a room temperature. Samples were then tested using a phenol-sulphuric acid reagent (Dubois et al. 1956). In each 3 mL tube I added 1 mL of concentrated (98.09%) sulphuric acid and 200 µL of 5% phenol and then added 20 µL of the sample. Following the above protocol I added the sample, waited 5 minutes, then agitated it. Following 30 minutes the amount of sugar released was determined by measuring absorbance at 490 nm in a spectrophotometer (Beckman).

2.7 Determination of sugars exposed over time of incubation by using Affinity Column (Lectins)

Four different types of lectin columns were purchased from EY laboratories, Inc. Purified *Concanavalin A* (ConA) has an affinity to mannose, purified *Ricinus communis* (RCA) has an affinity to galactose, purified *Triticum vulgare* (WGA) has an affinity to N-Acetylglucoseamine, and purified *Ulex europaeus* (UEA-I) has an affinity to fucose .

2.7.1 *Concanavalin A* (ConA) affinity chromatography

The column volume was 2 mL while the size was 2.5 cm x 0.5 cm. The column was equilibrated with 10 mL of TCM-Saline buffer (0.01 M Tris-HCL (pH 7.5) containing 0.5 M NaCl and 1 mM of each $CaCl_2$, $MgCl_2$, and $MnCl_2$) (Wan et al., 1994). The elution sugar used was 0.2 M Mannose. A 2 µL of protein sample from the pooled first three gel filtration fractions contained 0.35 ng of protein and was loaded onto the column after a pre wash with 10 mL of TCM-saline buffer. Fractions were eluted with 0.2 M mannose in TCM-saline buffer pH 7.5 (EY laboratories. Inc) and the absorbance of each fraction was measured at 280 nm,.

2.7.2 *Ricinus communis* (RCA) affinity chromatography

The column volume was 2 mL while the size was 2.5 cm x 0.5 cm. The method is as described above in section 2.7.1. However, the elution sugar was 0.2 M Galactose in TCM-saline buffer pH 7.5. A 2 µL sample of protein from the pooled first three fractions of gel filtration that contained 0.35 ng was loaded.

2.7.3 *Triticum vulgare* (WGA) affinity chromatography

The column volume was 2 mL while the size was 2.5 cm x 0.5 cm. The method is as described above in section 2.7.1. However, the elution sugar was 0.1 M N-acetylglucosamine in TCM-saline buffer pH 7.5. A 2 µL sample of protein from fraction number five from the gel filtration column, containing 0.16 ng of protein was loaded.

2.7.4 *Ulex europaeus* (UEA-I) affinity chromatography

The column volume was 2 mL while the size was 2.5 cm x 0.5 cm. The method is as described above in section 2.7.1. However, the elution sugar was 0.05 M α-Fucose in TCM-saline buffer pH 7.5. A 2 µL sample of protein from fraction number five from the gel filtration column, containing 0.16 ng of protein was loaded..

CHAPTER THREE

RESULTS

3.1 The effect of molmol "Myrrh" on the peanut cell growth

One of the biggest challenges in collecting enough protein was contamination. To overcome microbial contamination Myrrh (*Commiphora molmol)* (El-Sherbiny et al., 2011) was included in the medium.

A 14-day cell culture without any addition of Myrrh was compared to the one with Myrrh. After 14 days, the number of cells did not increase anymore; however, the volume of the cells in the medium with no added Myrrah grew from 25 mL to 200 mL (packed cell volume) in the medium over 14 days. By contrast the 200 mL of autoclaved medium containing Myrrh, inoculated with 25 mL of cells, grew to 175 mL packed cell volume. This result suggested that the Myrrh may inhibit the plant cell growth. However, the cultures were free of contamination.

3.2 Ion Exchange Chromatography

Carboxymethylcelulose (CM) Sephadex was used to isolate the major cationic peroxidase, from the 27 peptides found in the spent medium of peanut cell cultures (van Huystee and Tam, 1988). I obtained one fraction with both high protein and high heme peak measurements (not shown). Due to charge affinity, the anionic peroxidase with many other proteins were removed by washing with 0.02 M Na-acetate buffer. All the fractions' absorbances were measured at 280 nm for protein and at 403 nm for heme moiety quantification. Approximately 42 μg of pure CPRx sample eluted with

0.7 M Na-acetate buffer pH 5, and collected from this column. The data were very similar to those of Maldonaldo et al. (1980).

3.3 Gel filtration Chromatography

Sephadex G-75 was used to separate the various forms of CPRx, according to their molecular weights, which differed due to variation in their glycan chains lengths. The modification of glycan lengths is thought to be due to the presence of ß-galactosidase in the spent medium (Wan et al., 1994). The gel filtration column fractions were measured at 280 nm for protein (not shown) and at 403 nm for the heme moiety (not shown) quantification. The gel filtration column is able to separate the 33-40 kDa protein as shown by O'Donnell et al. (1992). Thus, CPRx with larger glycan chains will be eluted at the beginning followed by the CPRx with shorter glycan chains.

3.4 Specific Enzyme Activity

The absorbances for all the gel filtration fractions were also measured at 490 nm after using the phenol-sulphuric acid assay, which is used for measuring sugars including hexoses in solution (Dubois et al. 1956) (Figure. 5). The various peaks in the 490 nm chromatogram (Figure 5) relate to the different types variations of CPRx that result when sugars present in the glycan chains linked to the CPRx are modified, as shown by Zhang et al. (2004).

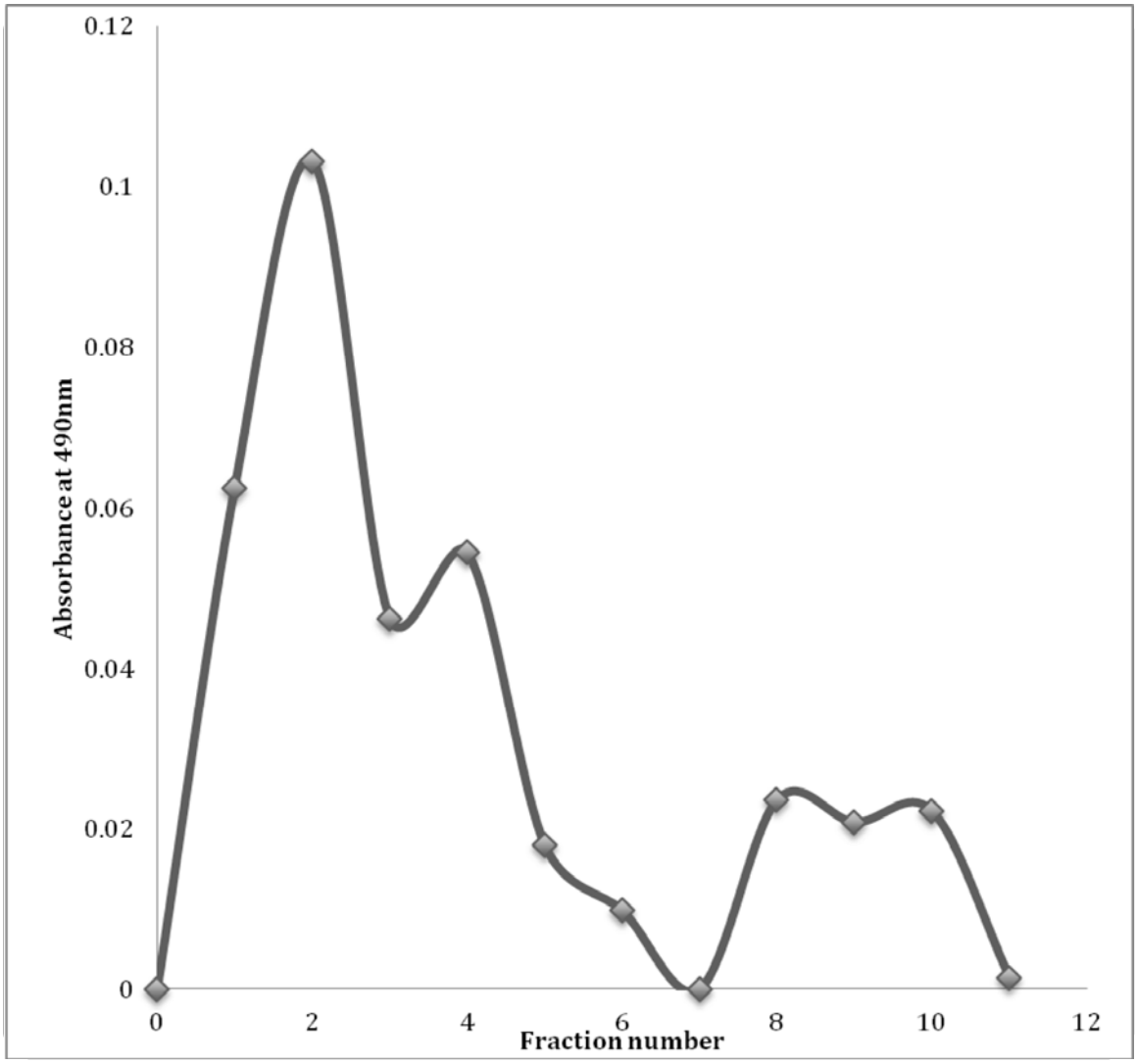


Figure 5. The absorbance at 490 nm of the gel filtration (Sephadex-G75) fractions to measure general sugars by using the Sulphuric acid- phenol assay, by adding 0.625 ml of concentrated H_2SO_4 and 0.250 ml of 5% phenol to 0.125 ml of the fractions, fraction volume is 0.6 ml (Dubois et al. 1956).

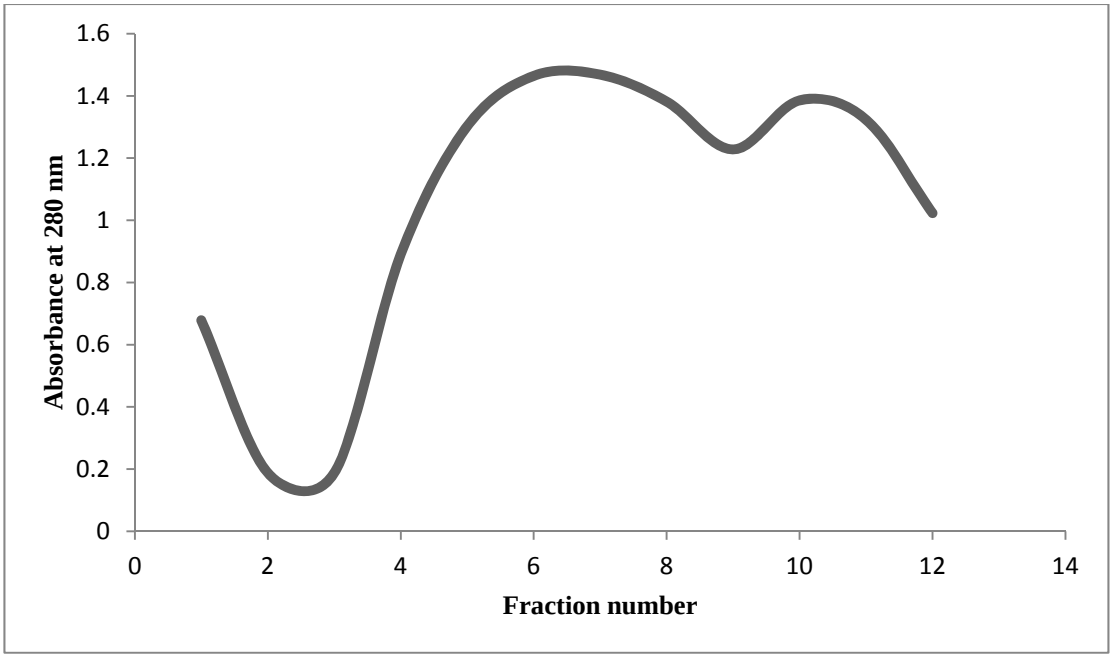


Figure 6. The absorbance at 280 nm of the gel filtration (Sephadex-G75) fractions to measure protein.

An enzyme unit is defined as that amount of protein that produces 1 μmol of product per minute, and the term specific enzyme activity is defined as enzyme unit per μg of protein. Across the gel filtration activity profile, the specific activity of fraction 2 was highest, with that of fraction 5-7 being lowest. A second “peak” of activity was evident in fractions 8-10 (Figure 7). The protein concentrations were measured by using the equation Y=0.597X- 0.012 where Y refer to the absorbance and X refer to the concentration.

3.6 Time frame for the glycosidase reaction in suspension medium

The absorbance reading of the released sugars became constant after one hour of incubation, which means glycosidase is rapidly reacting when brought in close proximity to peroxidase (Figure 7); this will not be the case in normal culture conditions because normally the volume of culture medium is 250 mL which make the interaction between the glycosidase and cationic peanut peroxidase slow.

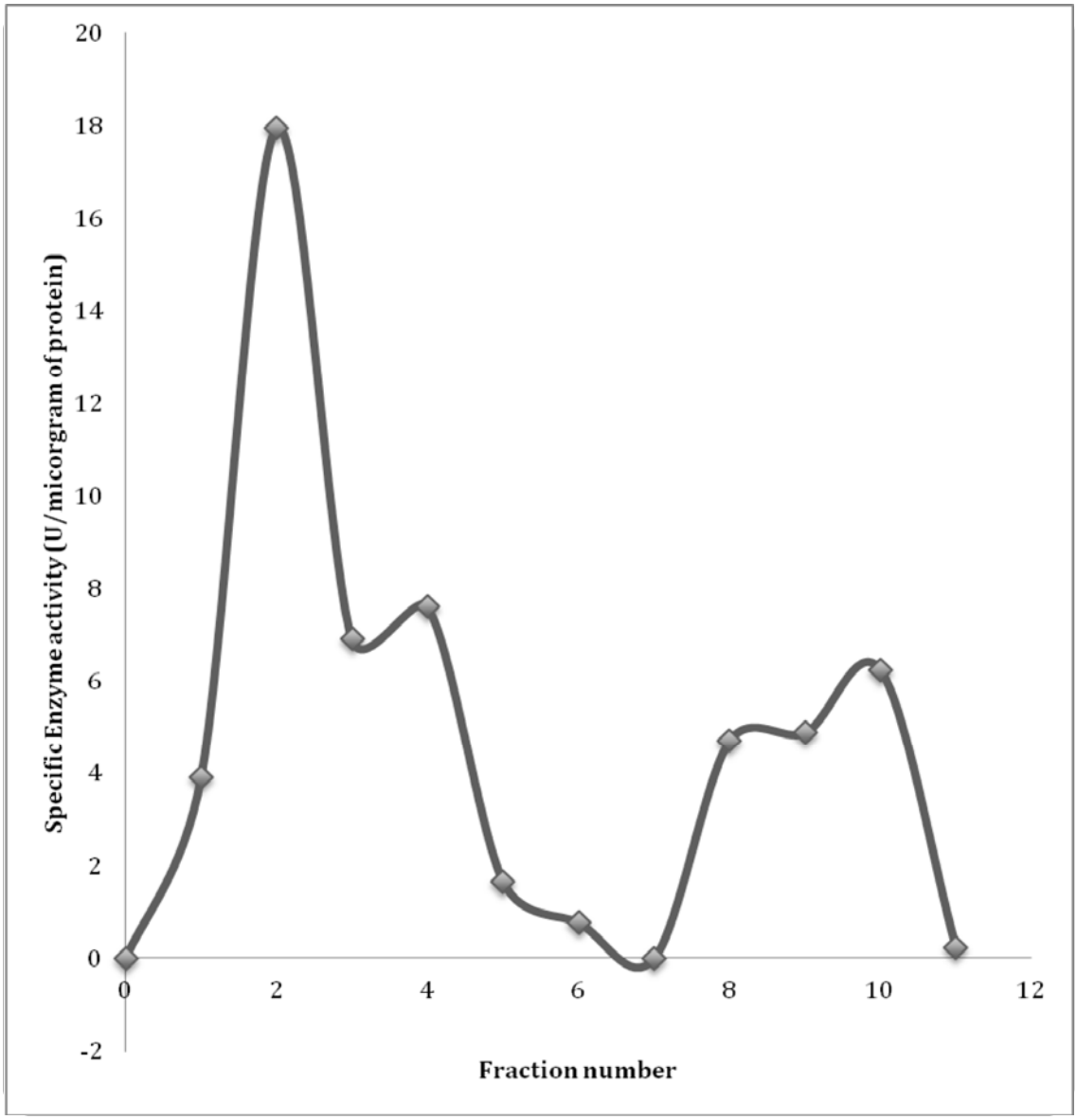


Figure 7. Specific enzyme activity for the gel filtration (Sephadex G-75) fractions showing the possibility of the changing of enzyme activity due to the amount of glycan chains were present.

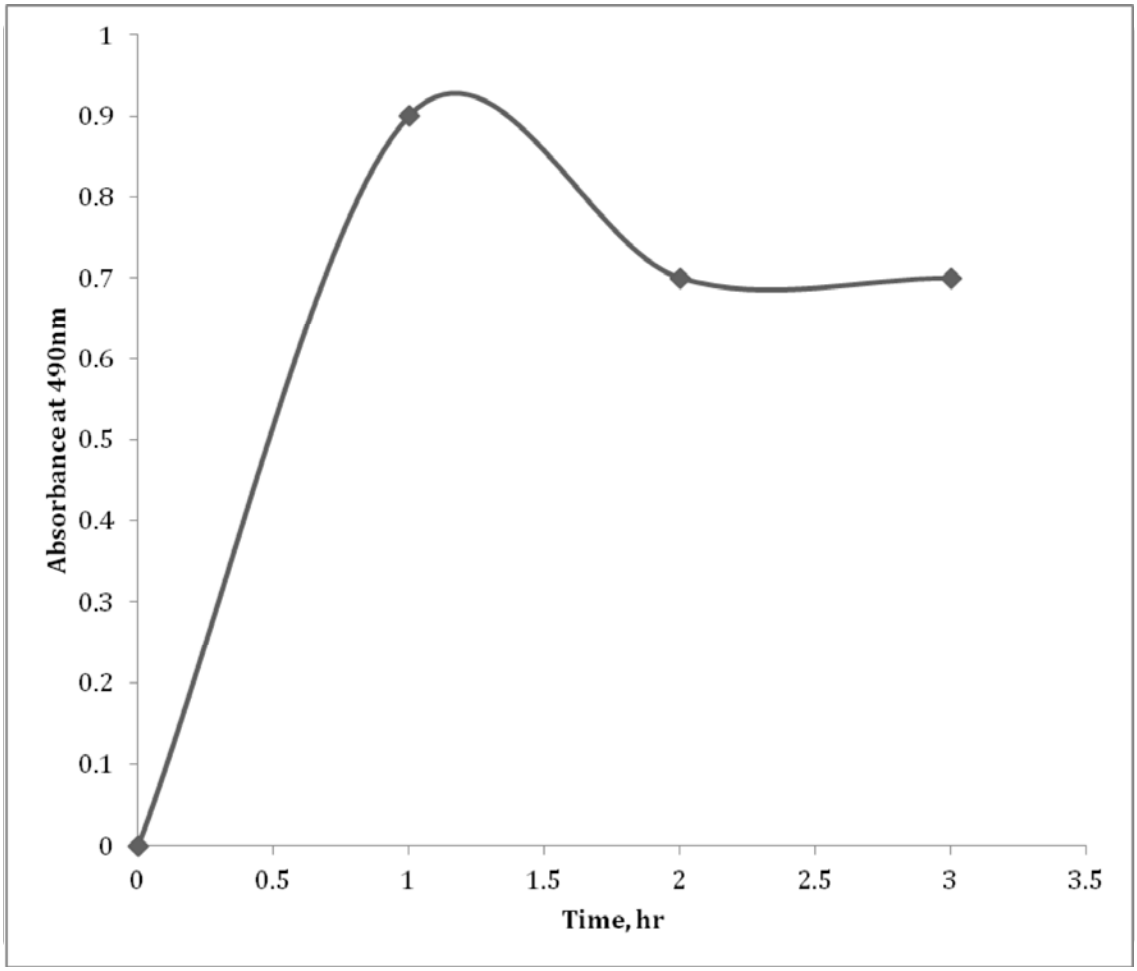


Figure 8. Determination of reaction time for glycosidase; the absorbance reading at 490 nm at varying incubation times for solution which contains 3 mL of Sodium Phosphate Buffer pH5 with the peanut pellet (spent medium). The measurement is for 20 µL of the sample added to 200 µL 5% phenol and 1 mL of concentrated sulphuric acid. The protein (CPRx, glycosidase with the remain 25 peptides in the spent medium) amount is 0.0792 µg/µL.

3.7 Determine the type of exposed sugars in the small fractions by using an Affinity Column (Lectin)

3.7.1 *Concanavalin A* (ConA) affinity chromatography

*Concanavalin A (*Con A) has an affinity to mannose. The presence of terminal galactose in the glycans of CPRx (Figure 3) is supported by Con-A affinity chromatography. The first three gel filtration fractions, pooled together, showed little affinity to the Con-A column (Figure 8). While the amount of mannose in the glycan chains linked to the major cationic peroxidase is higher compared to the other four sugar types (Zhang et al., 2004), it does not all appear to be exposed, as the bulk of the protein did not bind to the column.

3.7.2 *Ricinus communis* (RCA) affinity chromatography

Ricinus communis (RCA) has an affinity to galactose. As with Con A affinity chromatography, the binding of CPRx (fraction 1-3 pooled form gel filtration) to the RCA column suggests that the glycans of CPRx have terminal galactose residues (Figure. 9).

3.7.3 *Triticum vulgare* (WGA) affinity chromatography

N-acetylglucosamine is present in many places in the glycan chains of CPRx as reported earlier (Figure 3) (Sun et al., 1997). Gel filtration fraction number 5 showed an affinity to the WGA (Figure 10). However, it was small compared to the galactose indicating that little of the N-acetylglucosamine is exposed.

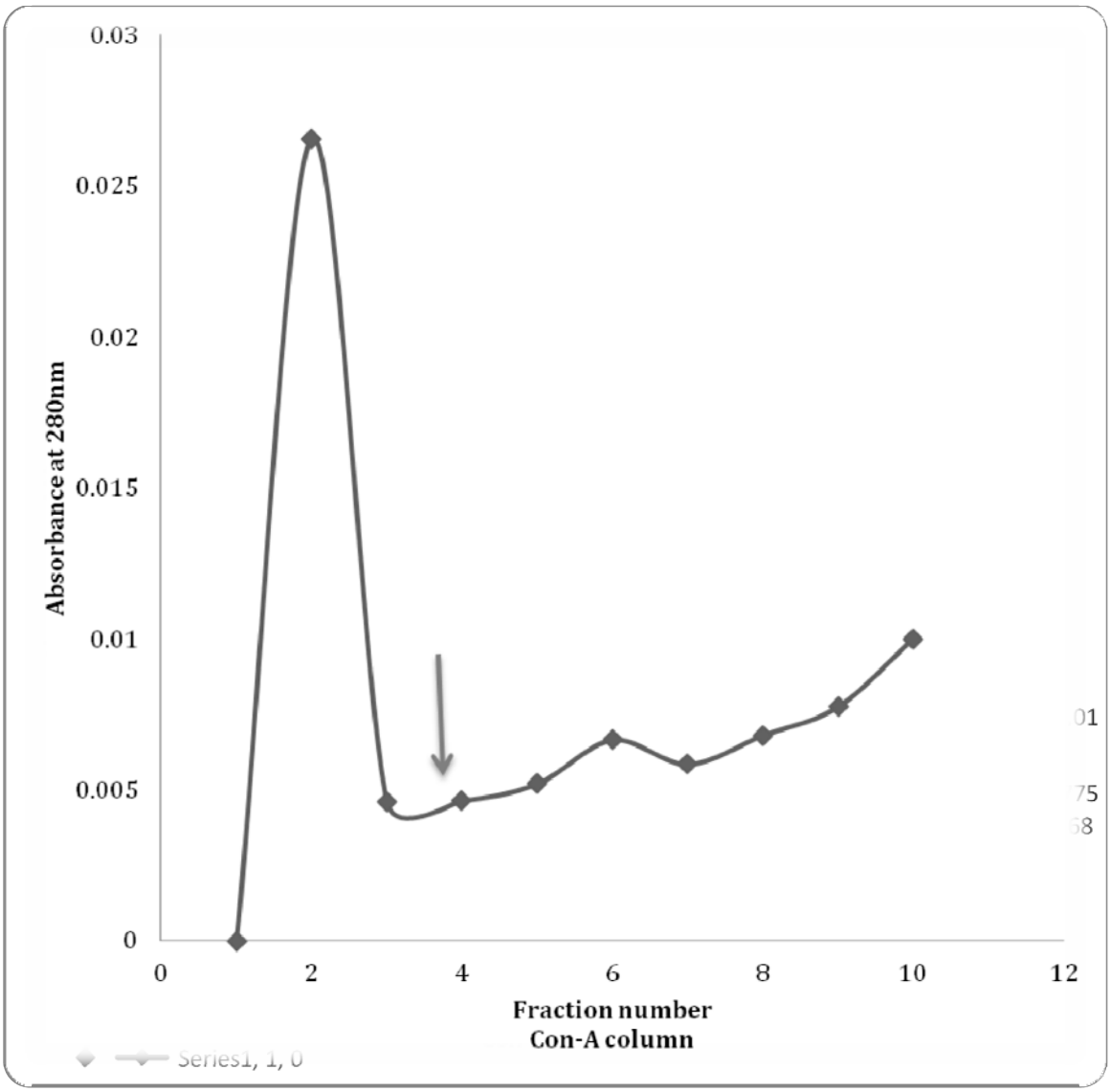


Figure 9. The absorbance at 280 nm for a protein quantification of the Con-A lectin column fractions, the first peak was washed with TCM-saline buffer pH 7.5, at the arrow 5 mL of 0.2 M Mannose was added.

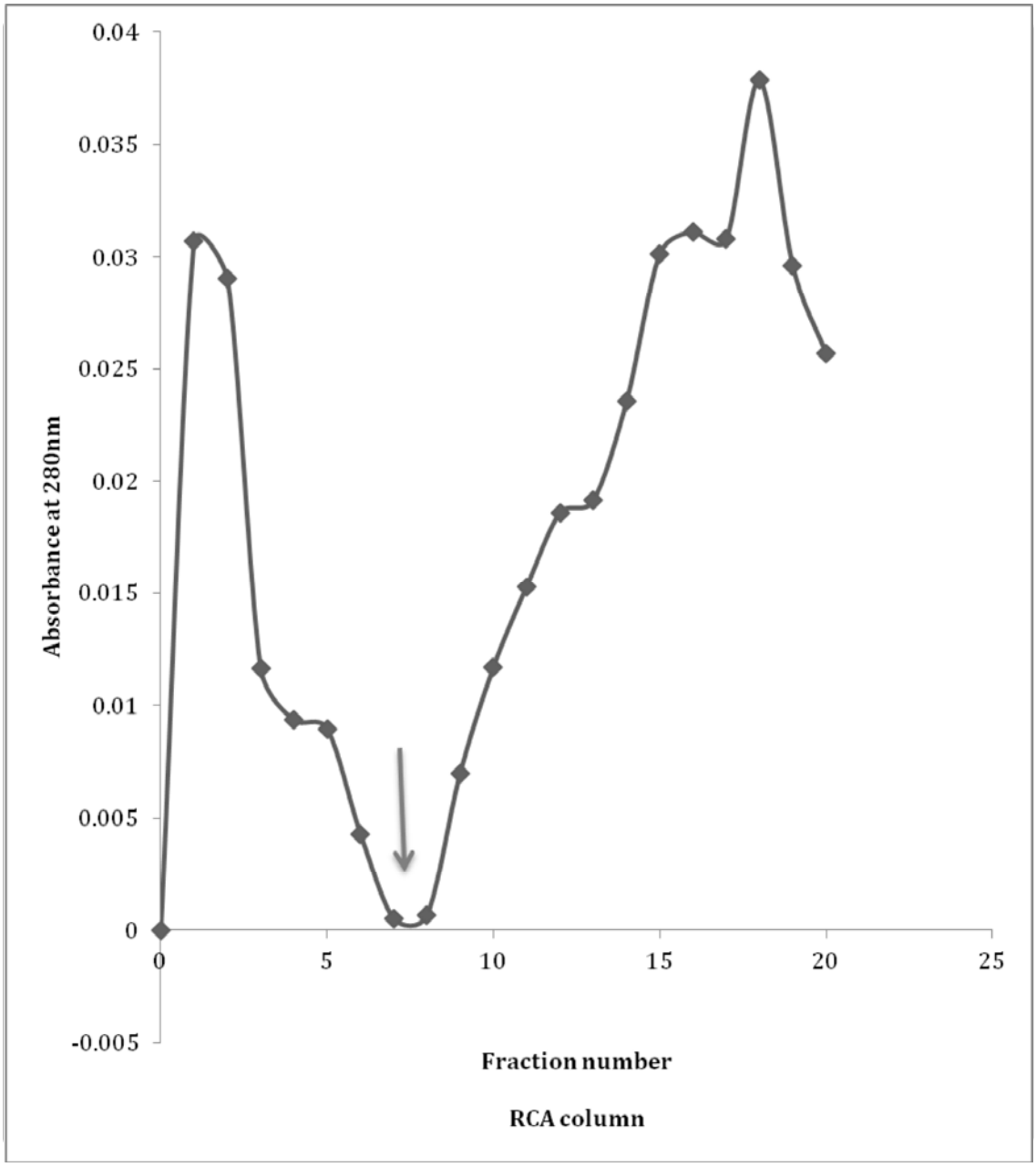


Figure 10. The absorbance at 280 nm for a protein quantification of the RCA lectin column fractions, the first peak was washed with TCM-saline buffer pH 7.5, at the arrow 5 mL of 0.2 M Galactose was added.

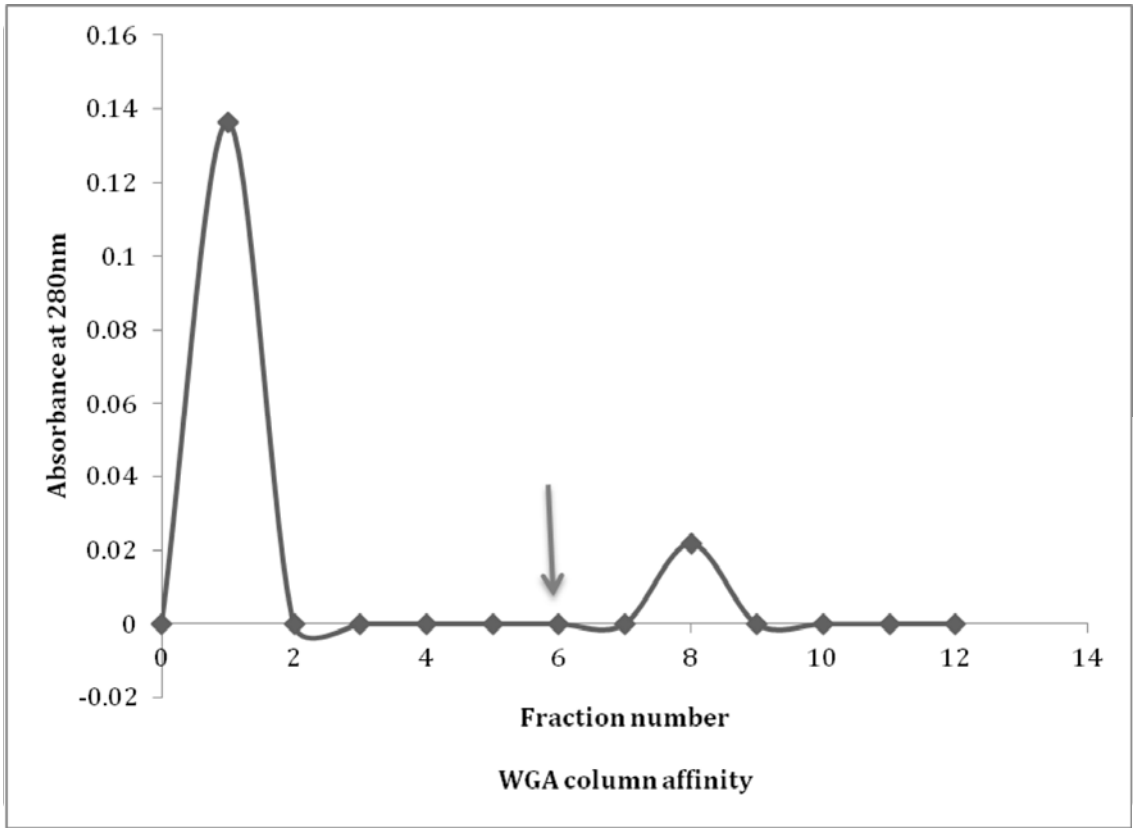


Figure 11. The absorbance at 280 nm for a protein quantification of the WGA lectin column fractions, the first peak was washed with TCM-saline buffer pH 7.5, at the arrow 5 mL of 0.1 M N-acetylglucoseamine was added.

3.7.4 *Ulex europaeus* (UEA-I) affinity chromatography

Fucose is present in much lower amount in plant protein glycan chains. Gel filtration fraction 5 showed slight affinity to the UAE-I (Figure 11).

The relatively small amounts of exposed N-acetylglucoseamine and fucose indicate that for one or both degradation is slow and that relatively little is found at the surface in comparison to galactose. Furthermore, the relatively high amount of galactose compared with other sugars means that the action of glycosidase in a large volume of liquid is low. At day 14 there are many more cells than earlier in the growth cycle; presumably thesecontinue to secrete CPRx with intact glycans. Thus it is expected that late in the culture cycle, CPRx with complete glycans would be prevalent.

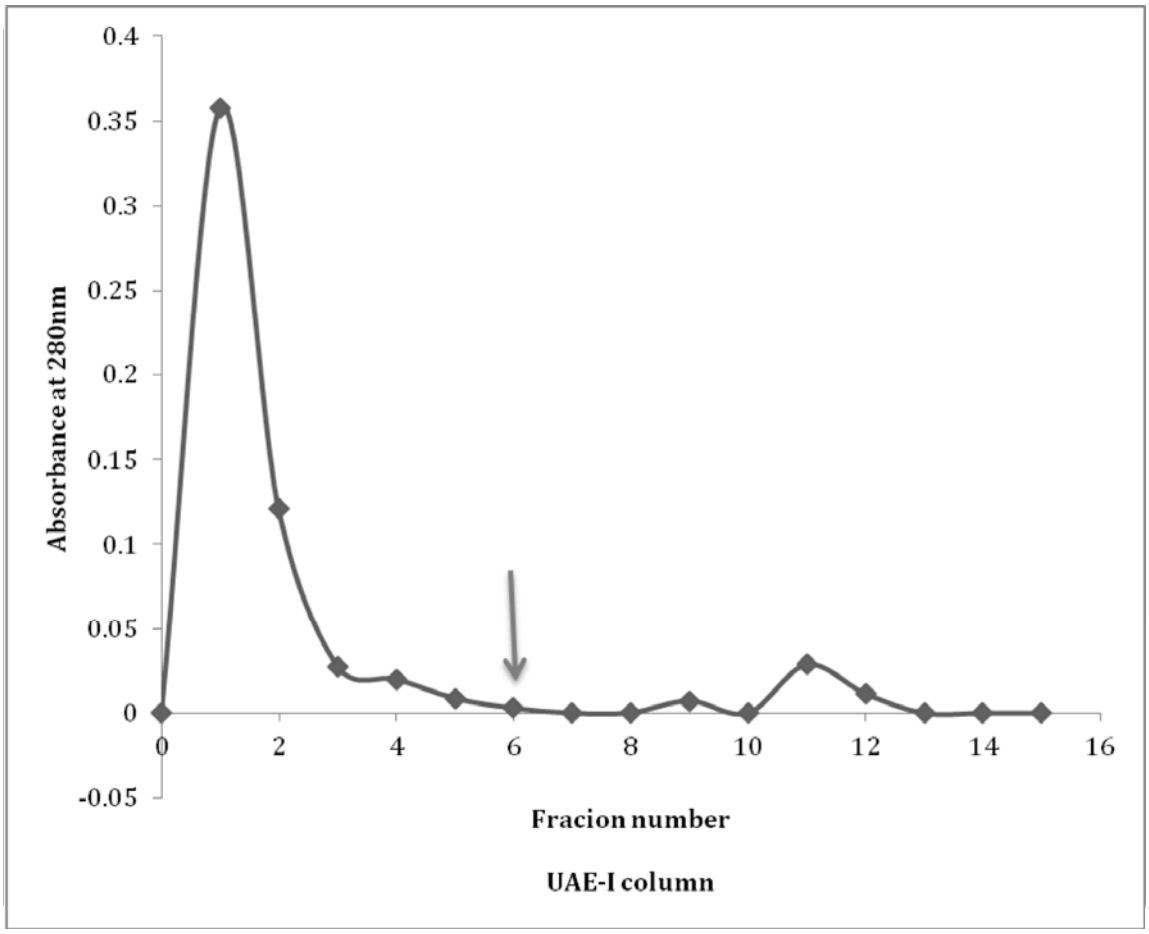


Figure 12. The absorbance at 280 nm for a protein quantification of the UAE-I lectin column fractions, the first peak was washed with TCM-saline buffer pH 7.5, at the arrow 5 mL of 0.05 M Fucose was added.

CHAPTER FOUR

DISCUSSION

Oligosaccharide-peptide Linkages

One of the most distinctive features of glycoproteins is the primary linkage between the oligosaccharide and peptide. These include the O-linkage and N-linkage. The N-linkage, which is commonly referred to as N-glycosylation, is the glycan attachment that connects through the anomeric hydroxyl group of a GLcNAc moietyto the amide-N of Asn. Recent studies have gone further to discover linkages between other sugars like D-Glc, L-Rha and D-GalNAc and Asn in bacterial glycoproteins (Neuhoff, 1999).

Glycan Structural Features

Glycans that are N-linked have a common mannotriose and N-acetylchitobiose structural pattern, which is linked to the amide-N of an Asn amino acid. Most glycoproteins have a residue of Xyl that is attached via 3-1 to 2 linkages extending towards the core of the β-linked Man. The link in some cases extends to GLcNAc proximal via an α-1, 3 bond involving the glycoside. Glycans that are N-linked, with reference to the antenna structures they have, can be put into three categories (Neuhoff, 1999):

1. The oligomannose kind, where the antennae substitute can only be achieved through adding Man residues
2. Complex type where the antennae is made up of Fuc, GlcNac together with Man residues

3. Hybrid type; this is a representation of the two above types. In the past, the above was believed to only take place in animals yeast and in some cases fungal glycoproteins.

Measuring the total sugars by using sulphuric acid - phenol assays shows that glycosylated proteins eluted within the first four fractions of the gel filtration column (Figure 8). These proteins, which have a Mr in the 32-40 kDa range likely have longer glycan chains than subsequent CPRx proteins.

Figure 7 shows significant specific enzyme activity in the first three fractions, but that this activity then decreases through fractions 5-7. A later eluting, and therefore smaller CPRx is evident in fractions 8-10. These changes in enzyme specific activity agree with earlier studies suggesting that glycans provide structural protection to the enzyme (Hu and van Huystee, 1989: Lige et al 1999). Therefore, the initial hypothesis that decreasing glycan chains affects CPRx activity is supported. This view of the CPRx activity is not new considering that cleavage of the glycan chains by PNGase treatment resulted in the loss of nearly all enzyme activity (Hu and van Huystee, 1989). However, a reduction of the glycan chain length does not have the same effcet as removing a single chain (Lige et al, 2001).

Moreover, determining glycosidase reaction time (Figure. 8) showed that the amount of free sugars increases during the first hour of incubation, and remained constant for the remaining 2 to 3 hours of incubation. This suggests that the glycosidases react more rapidly in a small volume of sample than in the larger, more dilute culture volume. The small volume causes greater release of sugar, which means also that there may be a wide variety of glycosidases active against the hybrid chain. Future work could involve 1H-NMR to see the alterations caused by the loss of sugars,

as has been done for CPRx when calcium was removed (Barber et al, 1995). A total loss of enzyme activity was noted with the loss of calcium.

Figure 9 shows an initial high peak of protein eluted from the Con-A lectin chromatography. Then, a relatively small amount of protein with exposed mannose was eluted by 5 ml 0.02 M mannose. This data agree well with the previous study (Wan et al 1994). Without the treatment of galactosidase no affinity for Con-A was noted. That indicates that there is little mannose exposed at the ends of the glycan chains. Figure 10 shows also that there was the high affinity for galactose on the RCA column, confirming that this is the hexose found at the end of the glycan chains (Zhang et al, 2004). There was little affinity for either fucose or N-acetyl glucosamine (Figure. 11 and 12). These have been found in the peripheral side of the glycan chains (Zhang et al, 2004) But there linkages to their lectins is minimal compared to the galactose to the RCA column., suggesting that they are not readily accessible.

In future, it will be useful to examine the CPRx with the shortest glycans by 1H-NMR as has been done for the loss of calcium, wherein it was clearly shown how the enzyme site was affected (Barber et al.,1995). Ideally a crystal of the shortest CPRx from the gel flitration should have been X-ray difracted and the structure compared with the fully glycosylated one (Schuller et al, 1996).

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