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Protein purification from gastropods

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COORDINATORE DEL DOTTORATO
Prof. Graziano Zocchi

DOTTORANDO
Dott. Ahmed Aufy

TUTOR
Prof. Fabia Rosi

CO-TUTOR
Prof. Erika Staudacher

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- Contents

DEPARTMENT OF ANIMAL SCIENCE
Via Celoria, 2 – 20133 – MILANO (MI)
Ahmed Aufy
Matricola R07937
Settore disciplinare VET/01

Protein purification from gastropods

Gastropods are a class of invertebrates within the mollusks, commonly known as snails and slugs. Recently, scientific interest is directed on snails as pollution indicators, as destroyers of crops, but also as parasites carriers. Furthermore, some species of snails are considered as an important human food source in countries like France and Australia.

In scientific research, snails are used as model animals especially in molecular biology and immunology. Some snails secrete purple matter with anti-cancer property; the snails use it to protect their eggs and scientists hope to create with it a weapon against breast cancer. In the light of this, the aim of the present work was to purify and characterize exoglycosidases, i.e. sugars-hydrolyzing enzymes, extracted from different species of snails and to study the glycosylation pattern of their tissues by using lectins as glycoprotein-specific antibodies.

Purification work started with the screening of 7 different exoglycosidases (α-fucosidase, β-xylosidase, α-mannosidase, β-mannosidase, α-glucosidase, β-glucosidase, β- galactosidase and β-N-acetylglucosaminidase) in 8 different species of snails (*Arion lusitanicus*, *Biomphalaria glabrata*, *Achatina fulica*, *Limax maximus*, *Cepaea hortensis*, *Lymnea stagnalis*, *Arianta arbustorum*, *Planorbarius corneus* and egg from *Achatina fulica*).

In order to create a purification scheme, many trials have been performed on the chosen enzyme, i.e. β-galactosidase from *Arion lusitanicus*. Ten snails were carefully washed after complete removal of the abdomen content to eliminate other sources of the enzyme. Snails were then homogenized and protein was precipitated by 1.2 M ammonium sulfate under cooling centrifugation. Then the precipitate was fractionated by the column hydrophobic interaction chromatography. Fractions exhibiting high enzyme activity were pooled, concentrated, desalted by ultrafiltration and then applied to **affi gel blue**. As the use of affi gel blue resulted in no binding between the enzyme and the gel beads, the unbound fractions were directly applied to **anion exchange chromatography**. As this procedure behaved as the same way of affi gel blue, the unbound fractions were pooled, concentrated to less than 2 ml and applied to **size exclusion chromatography**. Size exclusion chromatography exhibited high resolution purification, because a narrow peak was obtained, after testing the eluate for the enzyme activity. The fractions within this narrow peak were then applied to **cation exchange chromatography**, resulting in

two new very sharp peaks. Fractions of the second peak from cation exchange chromatography were exposed to **SDS-PAGE** that indicated high quality purification, because just two proteins' bands appeared. Finally, **β-galactosidase specific affinity** was the last purification step, where four different buffers were used (50mM sodium citrate pH 4.6, 1M NaCl in50 mM sodium citrate pH 4.6, 50 mM Tris/HCl pH 7.5 and 1M NaCl in 50 mM Tris/HCl pH 7.5) and the enzyme was eluted only with 50 mM Tris/HCl pH 7.5 buffer.
Electrophoresis of this eluate resulted in a single protein band in one fraction, identified by LC-MS as **galactocerebrosidase** with molecular weight about 74 kDa.
β-galactosidase characterization tests indicated that the optimal enzyme activity was at pH range 3.5 to 5.0 regardless to the used salt, i.e. the enzyme needs no special cations to be active, and indicated that the maximum activity of the enzyme is reached after 4 h incubation at 37 °C.
β-galactosidase purification from species other than *Arion lusitanicus* resulted in different purified proteins but not the β-galactosidase.
Moreover, as fucose and sialic acid are frequent and common modifications in snail glycans and they occur in a variety of different linkages and may therefore contribute to a number of recognition and adhesion processes, the glycosylation patterns of snails' tissue were studied by different lectins, i.e. glycoproteins specific antibodies. We analysed eggs and adult land snails and water snails (*Achatina fulica, Arion lusitanicus, Arianta arbustorum, Biomphalaria glabrata, Cepaea hortensis, Limax maximus, Lymnea stagnalis, Planorbarius corneus*) for their N- and O-glycosylation pattern with a focus on their sialylation and fucosylation abilities. Their sialylation potential was investigated by *Sambucus nigra* agglutinin and *Maackia amurensis* agglutinin while their fucosylation potential was investigated by *Aleuria aurantia* lectin, *Lens culinaris* agglutin, *Lotus tetragonolobus* agglutinin and *Ulex europaeus* agglutinin before and after tissues' digestion with glycopeptidase F and β-elimination, respectively.

In conclusion, 1) β-galactosidase purification from *Arion lusitanicus* needs several purification steps and must be conducted with specific β-galactosidase affinity chromatography, otherwise several unwanted proteins will appear as co-purified proteins.
2) The same purification scheme of *Arion lusitanicus*, when adopted in other snails, did not result in purifying β-galactosidase.
3) From the lectin study, it was confirmed that fucose and sialic acid are frequent and common modifications in snail glycans.

Credits evaluation

- Protein engineering
- Doctoral seminar in biochemistry
- Protein chemistry
- Proteomics
- Biochemical and biotechnological methods
- Bioorganic chemistry
- Methods in molecular biology
- Statistical data analysis using SAS
- Practical course in cell biology

Summer school

- Summer School of Theoretical and Practical Proteomics (IV edition). (Dr. Paola Roncada - Prof. Luigi Bonizzi) Istituto Sperimentale Italiano Lazzaro Spallanzani-Sezione di Proteomica, località La Quercia 26027 Rivolta d'Adda (CR). Proteotech s.r.l., località Pixinamanna, 09010 Pula (CA). Pharmaness s.c.a.r.l., località Pixinamanna, 09010 Pula (CA) 30 June to 4 July 2008.

Some of these results have been presented on posters at scientific conferences:

1- **AUFY A.A.**, H. STEPAN, J. RUDOLF and E. STAUDACHER (2009). Glycosylation pattern analysis of snails by lectins and antibodies. European Carbohydrate Symposium 19.

2- STAUDACHER E., H. STEPAN, **A.A. AUFY**, C. BLECKMANN and R. GEYER (2009). Current challenges in glycosylation pattern analysis of snails. GlycoXX-20th International Symposium on Glycoconjugates 29.

3- STEPAN H., **A.A. AUFY**, C. BLECKMANN, R. GEYER and E. STAUDACHER (2010). N- and O-glycosylation pattern analysis of snails. Jahrestagung der ÖGMBT 27-29.9.2010, Vienna, Austria.

4- TAUS C., C. LUCINI, **A.A. AUFY**, R. GRABHERR and E. STAUDACHER (2010). Construction of expression cDNA libraries from snails. Jahrestagung der ÖGMBT 27-29.9.2010, Vienna, Austria.

Oral presentations not related to the thesis work

1- **AUFY A.A**., D. MAGISTRELLI and F. ROSI (2009). Effect of weaning and milk replacer feeding on plasma insulin in goat kids. XVIII Congresso Nazionale ASPA 9–12 June, 2009, Palermo, Italy.

2- MAGISTRELLI D., **A.A. AUFY** and F. ROSI (2010).Effect of weaning and milk replacer on aminotransferase activity in plasma and liver of Saanen goat kids. 3rd EAAP international symposium on energy and protein metabolism and nutrition 6-10 September, 2010. Parma, Italy.

Publications

Protein purification from gastropods.

1- MAGISTRELLI D., **A.A. AUFY** and F. ROSI (2010). Effect of weaning and milk replacer on aminotransferase activity in plasma and liver of Saanen goat kids. In: ENERGY AND PROTEIN METABOLISM AND NUTRITION, (EAAP publication 127), [ed] G. Matteo Crovetto. Wageningen, Wageningen Academic Publishers,20-10-ISBN 9789086861538, pp.189-190.

2- **AUFY A.A**., D. MAGISTRELLI and F. ROSI (2009). Effect of weaning and milk replacer feeding on plasma insulin and related metabolites in Saanen goat kids. ITALIAN JOURNAL OF ANIMAL SCIENCE. ISSN 15944077, vol. 8(sup 1), pp.256-258.

3- MAGISTRELLI D., **A.A. AUFY**, S. MODINA, L. CERRI and F. ROSI (2009). Endocrine Pancreas Development at Weaning in Goat Kids. ITALIAN JOURNAL OF ANIMAL SCIENCE. ISSN 15944077, vol. 8 (sup 1), pp. 310-312.

4- MAGISTRELLI D., **A.A. AUFY** and F. ROSI (2009). Postweaning adaptation of liver activity to solid diet in goat kids. In RUMINANT PHYSIOLOGY : DIGESTION, METABOLISM, AND EFFECTS OF NUTRITION ON REPRODUCTION AND WELFARE. Wageningen, Wageningen Academic Publishers. ISBN/ISSN 978908686-119-4, pp.446-447.

5- ROSI F., **A.A. AUFY** and D. MAGISTRELLI, (2009). Diet influences the content of bioactive peptides in goat milk Journal of Endocrinological Investigation. ISSN 03914097 - ISSN 17208386, 32:6, pp. 486-490.

6- MAGISTRELLI D., **A.A. AUFY** and F. ROSI (2009). Sources of variation in biopeptides and IgG levels in goat milk In 237th ACS NATIONAL MEETING- Salt Lake City, UT: BIOPEPTIDES AND SPECIALITY PROTEINS FOR Health Promotion and Disease Risk Reduction, 22-26 march 2009.

Other scientific activities

1- Teaching activities (2008) with Prof. Fabia Rosi in animal anatomy and physiology course. Dept. of Animal Science, Milan University, Italy.
2- As co-author, Maria Elena Marescotti's thesis (2010) with the title ``PRESENCE OF MAMMARY EPITHELIAL CELLS IN MILK OF HEALTHY COWS IN DIFFERENT STAGES OF LACTATION´´. Dept. of Animal Science, Milan University, Italy.

Tutor
Prof. Fabia Rosi

Protein purification from gastropods.

1- Introduction

1.1 Glycoconjugates

Glycosylation (the term refers to biosynthesis of a glycan) is the enzymatic process that ties different sugars together to constitute the glycans (oligosaccharides are parts of glycoconjugates). The major glycoconjugates are glycoproteins, glycopeptides, peptidoglycans, glycolipids, and lipopolysaccharides.

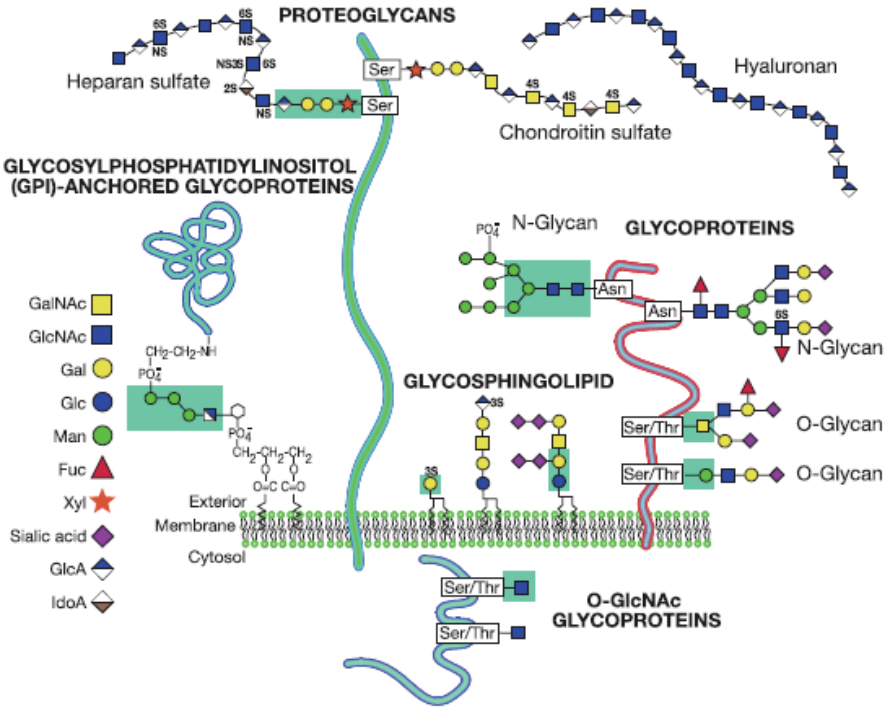


Figure (1). The most abundant glycoconjugates found in animal tissues and as it shown the different linkages between oligosaccharides and different chemical species (Varki et al., 2009).

The glycoconjugates have been clearly described by Varki et al. (2009), they were classified according to the attached biomolecule as follow,

Glycosphigolipid (glycolipids): Oligosaccharide attached via glucose or galactose to the terminal primary hydroxyl group of the lipid moiety.

Glycophospholipid anchor: Glycan Bridge between phosphatidylinositol and a phosphoethanolamine in amide linkage to the carboxyl terminus of a protein.

Proteoglycan: glycoconjugates with covalently attached glycosaminoglycan.

Protein purification from gastropods.

Glycosaminoglycan: long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either of two modified sugars, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc), and a uronic acid such as glucuronate or iduronate.

Glycoprotein: glycoconjugate in which a protein carries one or more glycan chains covalently attached to a polypeptide backbone via *N*-GlcNAc- or *O*-GalNAc-linkages.

1.2 Glycans

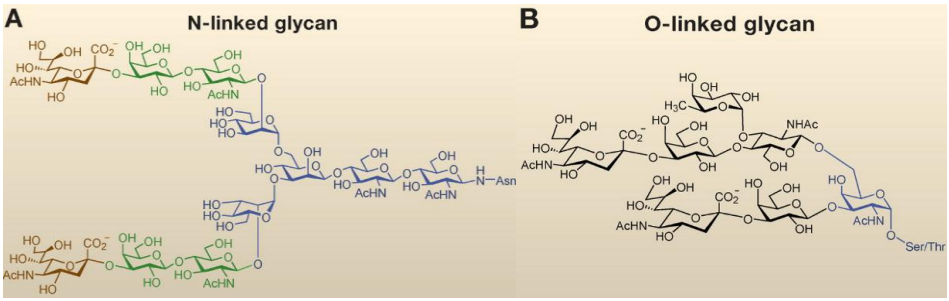
In general glycosylation represents the fast majority of protein posttranslational modifications. Glycans can be divided into two subgroups (Figure 2), O-linked [sugar attached to serine or threonine residue via N-acetylgalactosamine (GalNAc)] and N-linked [quite more complicated sugar structure covalently linked to asparagines residue with the sequence Asn-X-Ser or Asn-X-Thr via N-acetylglucosamine (GlcNAc)].

Asn= asparagines

Ser= serine

Thr=threonine

X= any amino acid except proline



Figue (2) (A) An N-linked glycan. The Man3GlcNAc2 pentasaccharide core (blue) is conserved across all N-glycan structures and can be elaborated with *N*-acetylglucosamine units (green), sialic acid residues (brown), and other epitopes. (B) A mucin-type O-linked glycan. The core GalNAc moiety (blue), attached to a serine/ threonine (Jennifer et al., 2006).

Protein purification from gastropods.

1.3 **N-Glycosylation**

N-Glycans or as they termed asparagines-linked oligosaccharides are quite complex structures which can be categorized into high mannose, hybrid and complex type (Figure 3). The three types have the same core structure, Manαl-3(Manαl-6) Manβ1-4GlcNAcβ1-4 GlcNAc-Asn. The high mannose-type glycans typically have two to six additional mannose residues linked to the pentasaccharide core. The hybrid glycans have features of both high-mannose and complex-type oligosaccharides. Most hybrid glycans contain a "bisecting" N-acetylglucosamine linked β, 4 to the β-1inked mannose residue. The complex-type structure contains two outer branches with the typical Sialyllactosamine sequence and shows two other commonly found substituent, namely a fucose into 1,6 linkage to the N-acetylglucosamine residue, and a "bisecting" N-acetylglucosamine linked β1,4 to the β-1inked mannose residue (Kornfeld and Kornfeld, 1985).

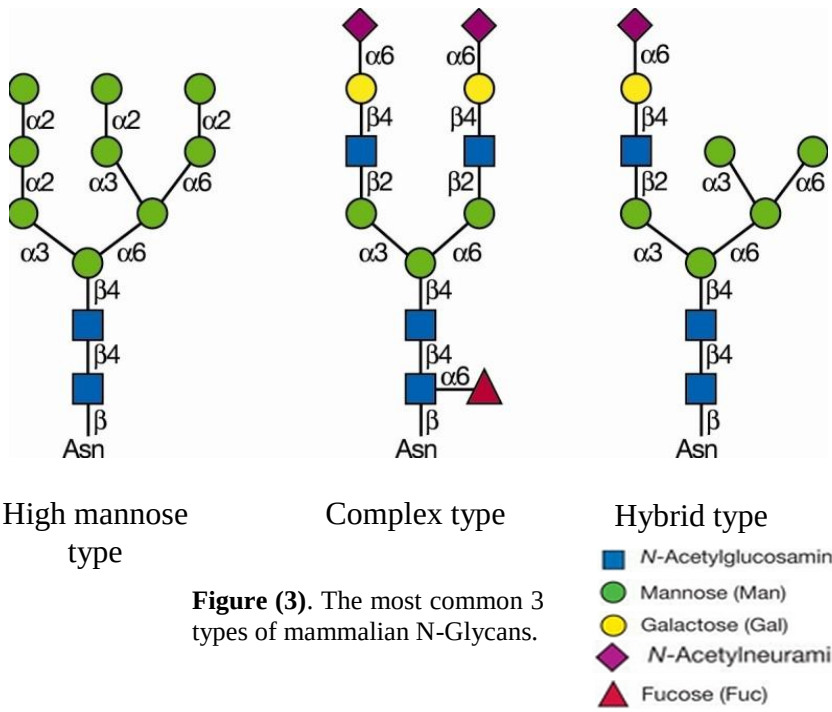


Figure (3). The most common 3 types of mammalian N-Glycans.

Protein purification from gastropods.

Protein N-Glycosylation (Figure 4) biosynthesis starts at the lipid Dolichol phosphate (Dol-P) which is exists in cellular organelles other than endoplasmic reticulum (ER), this lipid structure considered as an oligosaccharide carrier (Hirschberg and Snider, 1987).

Glycosylation mechanism initiates at the cytosolic side of endoplasmic reticulum (ER), where the monosaccharides are added one by one to the Dol-P until it reached specific structure (Man5GlcNAc2-PP-Dol.). Then this structure is translocated or flipped to the luminal side of ER [The Dol-P sugars as well as some of the lipid linked oligosaccharide species have the ability to change the orientation of the membrane (Jensen and Schutzbach,1984)] where it is elongated to Glc3Man9GlcNAc2-PP-Dol. At this point the oligosaccharyl transferase takes place to transfer the oligosaccharide to an asparagines residue with the sequence as mentioned in the glycans section (Gavel and von Heijne 1990).

N-Glycans need Glucosidase I to remove the first glucose and Glucosidase II removes the second and third glucose. Monoglucosylated oligosaccharides are then bind to ER-resident lectins calnexin and carleticulin (Oliver et al., 1997). When the glucosidase II cleaves the last glucose, the protein can be released from calnexin/carleticulin and can leave the ER. The correction process is performed by UDP-Glc: glycoprotein glucosyltransferase which plays very important role in sensing glycoprotein conformation and re-glucosylates glycans with misfolded amino acids (Ritter and Helenius, 2000). Two ER mannosidases can remove one or two mannoses in the ER before the protein is transported to the Golgi apparatus, where further processing proceeds and protein also can be O-glycosylated.

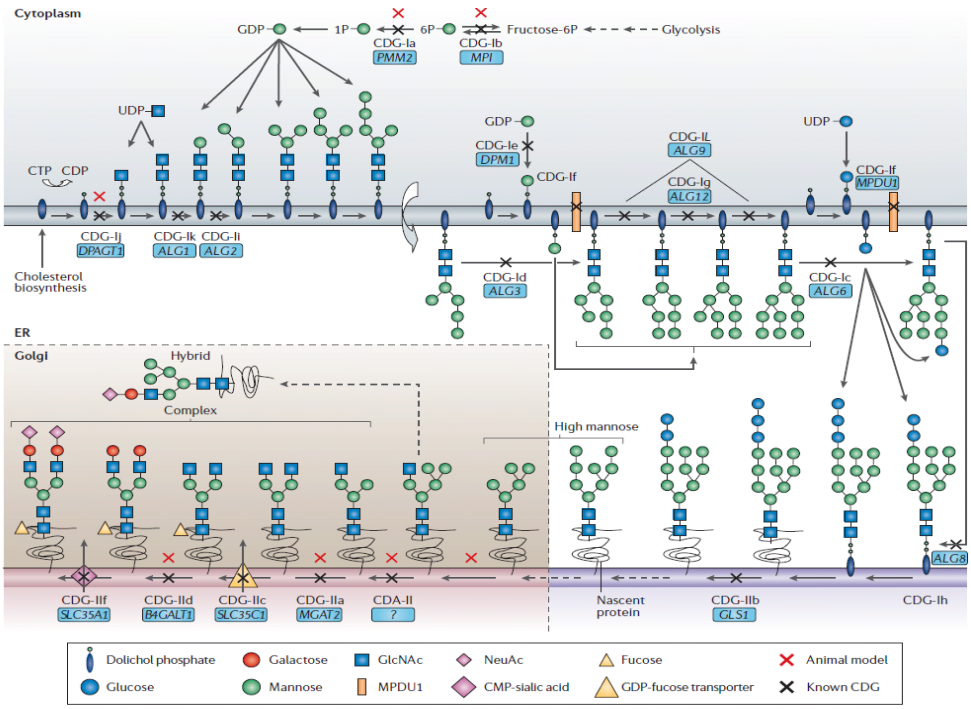


Figure (4) Biosynthesis of N-Glycan (Freeze, 2006)

1.4 Oligosaccharides processing in the Golgi

N-glycans microheterogeneity is driven by series of glycosidases and glycosyltransferases which are located in Golgi apparatus. A mixture of Man_9 and Man_8 oligosaccharides are targeted by the first Golgi's enzyme (α 1,2 mannosidase I) which converts them to Man_5. Then the second enzyme (β 1, 2N-acetyl-glucosaminyltransferase I) takes place to transfer a GlcNAc residue creating the first hybrid oligosaccharide, then the α 1,2 mannosidase II removes one or two Man residues leaving the second branch available for elongation by N-acetyl-glucosaminyltransferase II to give the first biantennary complex oligosaccharide. Now this oligosaccharide can be processed by glucosaminyltransferase IV (GnT IV) or glucosaminyltransferase V (GnTV) that add a GlcNAc residue to the α1,3 mannose or α1,6 Mannose branch respectively creating two different types of complex triantennary oligosaccharides. Another important enzyme

(glucosaminyltransferase III) which modifies any non galactosylated hybrid or complex glycans by transferring β1,4 GlcNAc residue in a linkage to the core Man (Restelli and Butler, 2002).

Marije et al, (2005) indicated that endoglycosidases H (Endo H) is used to monitor the movement of the newly synthesized glycoprotein from ER to Golgi, where glycans on proteins remain sensitive to be digested by Endo H in the ER and the early regions of Golgi. They also added that the enzyme PNGase F has the ability to remove all glycans regardless to glycan's structure; furthermore it converts the glycosylated asparagines to aspartic acid.

PNGase F became widely used enzyme capable of releasing N-glycans; it acts through cleaving the intact glycan and leaves aspartic acid in place of the asparagine at the N-linked site of the protein (Tarentino et al. 1985).

PNGase-F releases most glycans except those that contain fucose α1-3 linked to the reducing-terminal GlcNAc (Altmann et al., 1995) as commonly found in plants. In these situations, PNGase-A is usually effective. This enzyme is also capable of releasing glycans as small as GlcNAc, whereas PNGase-F appears to require at least two GlcNAc residues for effective release. It is important to mention that Endo H is also very useful enzyme in releasing the N-Glycans. It hydrolyses the bond between the two GlcNAc residues of the chitobiose core, leaving the core GlcNAc with any attached fucose attached to the protein.

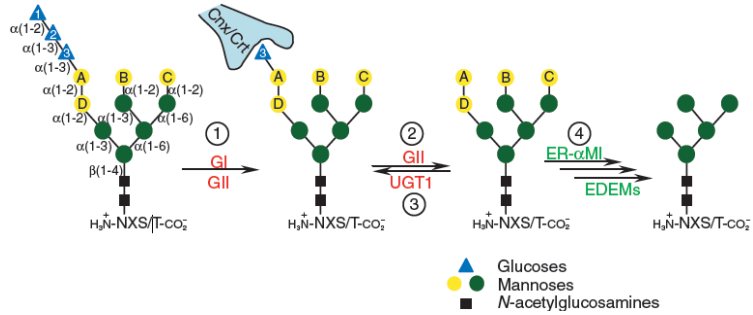


Figure (5) N-glycan processing in ER. Oligosaccharide bound to the side chain of asparagine. Glucosidase I (GI) and glucosidase II (GII) remove glucose 1 and 2, respectively (step 1). The monoglucosylated trimming intermediate associates with calnexin (Cnx) or calreticulin (Crt). After release from the lectins, GII removes glucose 3 (step 2). UGT1 adds back one glucose residue on the terminal mannose A (step 3). α(1-2)-mannose residues are progressively removed by ER-resident α(1-2)-mannosidases (step 4) (Maurizio, 2007).

1.5 Glycosyltransferases and glycosidases

Glycans metabolism is such a complicated process in which several enzymes are incorporated. Those enzymes are classified into two subclasses, glycosyltransferases (EC 2.4) and glycosidases (EC 3.2.1). The fast majority of enzymes involves in glycans biosynthesis are glycosyltransferases and it function through adding the monosaccharides to specific positions on a specific precursor (or by other meaning catalyze the transglycosylation reaction where the monosaccharide component of the nucleotide sugar donor is transferred to the acceptor). Many of the glycosyltransferases are located in ER-Golgi pathway and they have a specific role to export the newly synthesized glycoconjugates.

Glycosyl donor + Acceptor	Glycosyltransferase	Glycosyl-acceptor + Nucleotide or dolichol-P

Glycosyl donors
CMP-Sialic acid
GDP-Fucose
GDP-Mannose
UDP-Galactose
UDP-*N*-acetylgalactosamine
UDP-*N*-acetylglucosamine
UDP-Glucose
UDP-Glucuronic acid
UDP-Xylose
Dolichol-P-Glucose
Dolichol-P-Mannose
Dolichol-P-($Glucose_3$-$Mannose_9$-*N*-acetylglucosamine$_2$)

Acceptors
Oligosaccharides
Monosaccharides
Proteins
Lipids (Ceramides)

Protein purification from gastropods.

Glycosidases are degrading enzymes located in the lysosomes and the degraded sugars are reutilized in the synthesis process (Figure 6). They are also branched into two subclasses endoglycosidases (enzymes that cleave internal linkages in a glycosidic chain, releasing an oligosaccharidic residue) and exoglycosidases (enzymes that cleave a single glycosidic residue at the nonreducing end of an oligosaccharide chain) (Bojarova and Kren, 2009).

The lysosomal O- and N-oligosaccharides contain only six sugars, β-N-acetylglucosamine, α/β-N-acetylgalactosamine, α/β-galactose, α/β-mannose, α-fucose and α-sialic acid. Each one of these sugar require a specific enzyme to release it from the glycan complex.

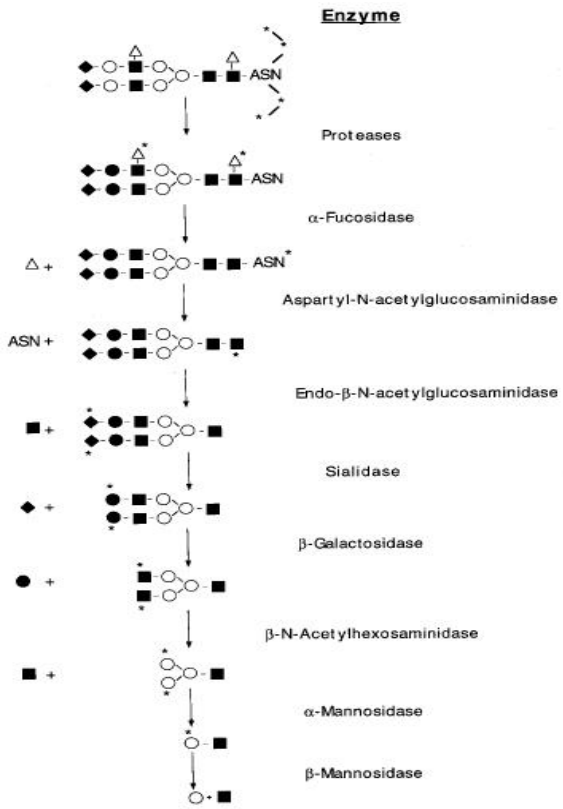


Figure (6). degradation of a complex N-glycan by different lysosomal glycosidases (Jourdian 1996 and Winchester 1996).

1.6 β-galactosidase purification

Glycans are degraded by highly specific Two types of enzymes (endo- and exo-glycosidases). In contrast to endoglycosidases, exoglycosidases cleave sugar chains at the outer (non-reducing) terminal end (Neufeld, 1991; Sandhoff and Kolter, 1996).

In the extracellular matrix, endoglycosidases initiate the degradation of glycoconjugates. Short oligosaccharide fragments, created by action of endoglycosidases, are then transferred to lysosomes where they undergo degradation by exoglycosidases (Ernst *et al*., 1995).

β-Galactosidase (β –D-Galactoside galactohydrolase; EC 3.2.1.23) is one of the most potent exoglycosidases acting through catalyzing the hydrolysis of β-galactoside into monosaccharides. β-Galactosidase has been purified from some organisms like *E.coli* (Steers et al., 1971), *Kluyveromyces lactis* (Dickson *et al*., 1979) and *Penicillium chrysogenum* (Nagy *et al*., 2001) by classical biochemical methods which include different chromatography columns.

Miyata and Suzuki (1975) were able to partially purify β-galactosidase from adult rat brain with the use of actosylceramide, galactosyl-N-acetylgalactosaminyl-galactosyl-glucosylceramide, galactosyl-N-acetylgalactosaminyl- (N-acetylylneuraminyl) galactosyl-glucosylceramide, and 4-methylumbelliferyl β-galactoside as substrates. They were able to achieve proximately 50-fold purification by solubilization, ammonium sulfate fractionation, Sephadex G-200 and DEAE-cellulose column chromatography.

With these previous works, different purification methods have been established (ex: anion-exchange, affinity chromatography and size exclusion chromatography). Establishing the affinity chromatography method in β-galactosidase purification revealed quantitative yields of highly purified enzyme (Steers et al., 1971).

Unlike affinity chromatography, subjecting β-galactosidase to DEAE (diethylaminoethyl) sephadex A-50 ion-exchange chromatography resulted only in additional four fold purification furthermore, this step exhibited two major co-purifying high molecular weight proteins (Nagy *et al*., 2001).

1.7 Lectins

Carbohydrate fine structure is a very challenging task and over the last 3 decades have been intensively studied by those proteins carrying sugars (lectins) which are capable to bind variety of carbohydrate complexes (Barondes,1988). Lectins discovered more than 100 years ago from plant and late from animals and invertebrates. In 1960s and 1970s many lectins were purified from different origins. During these two decades, several animal lectins from slime molds to horseshoe crabs (Marchalonis and Edelman 1968), snails (Hammarstrom and Kabat 1969; Springer and Desai 1971) and vertebrates including mammals (Hudgin et al., 1974; Teichberg et al., 1975) were identified and biochemically characterized. SNA lectin was tested and found to be specific for Neu5Ac_(2,6)Gal, Neu5Ac_(2,6)GalNAc (Shibuya et al., 1987).

Lectins are proteins or glycoproteins that derived from plants, animals, or microorganisms that have specificity for different types of carbohydrate residues. Lectins also defined as proteins that interact non-covalently with.

In the detailed intensive review of Lis and Sharon (1998), they mentioned that each lectin molecule contains two or more carbohydrate-combining sites, so one of these sites will bind to the sugar residue and the other will make cross-linking with another compartment for example cell. They also added that are obtainable in purified form by affinity chromatography and by recombinant DNA techniques, furthermore, lectins represent heterogeneous group of oligomeric proteins that vary widely in size, structure, molecular organization and in the constitution of their combining sites.

On the basis of their specificity, lectins are categorized into five groups, according to the monosaccharide for which they exhibit the highest affinity: mannose, galactose/*N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid.

The affinity of lectins for monosaccharides was known to be weak but with this great jump in molecular biology, we have got broad knowledge indicating very high selectivity of lectins to sugars (Goldstein and Poretz, 1986; Sharon and Lis, 1989).

Recently hundreds of lectins from different sources (plants and animals) were discovered, purified and identified. Animal lectins were classified by Gabius (1997) who stated so many different lectins categorized according to their sources and specificities as follows:

Family	Structural motif	Carbohydrate ligand
C-type	Conserved CRD	variable (mannose, galactose, fucose, heparin tetrasaccharide)
I-type	Immunoglobulin-like CRD	variable (Man,GlcNAc,, HNK-1 epitope, hyaluronic acid, *a2,3/a2,6*- sialy llactose)
Galectins (S-type)	Conserved CRD	β-galactosides
Pentraxins	pentameric subunit arrangement	4,6-cyclic acetal of jl-galactose, galactose, sulfated and hosphorylated Monosaccharides
P-type	similar but not yet strictly defined CRD	mannose-6-phosphate-containing glycoproteins

CRD. Carbohydrate recognition domains.

Protein purification from gastropods.

1.7.1 C-type lectins

Are family of animal proteins (known as Ca^{++} dependent lectins) which control very important biological processes like endocytosis of ligands, cell-to-cell adhesion and serum glycoprotein turnover (Drickamer and Taylor, 1993). Moreover, lectins from this family are also involved in inflammation and tumor infected cells. The carbohydrate recognition domain (CRD) of the C-type lectins is about 115 to 130 amino acid with presence of double folded cysteine residues (Drickamer, 1981; Drickamer and Taylor, 1993).

1.7.2 I-type lectins (this family also called siglecs)

The carbohydrate recognition domain of P-type lectins was detected in two related lectins, the mannose -6-phosphate receptors (Kornfeld 1992; Ludwig *et al.,* 1995*),* they different in molecular weight (one with 300 KDa and the second one is 45 KDa).

1.7.3 P-type lectins

This lectin family contains two members, the first one which is cation dependent (mannose-6-phosphate receptor) and the cation independent one (insulin-like growth factor II/mannose 6-phosphate receptor).

The difference between the cation dependent and cation independent members was clearly shown by Varki (1999) who has reviewed different types of lectins in many papers and also books.

1.7.4 Lectins used in the study

1- *Sambucus nigra* agglutinin (SNA)

The pioneer work of Broekaert et al. (1984) who purified this lectin from Elderberry by affinity chromatography on fetuin-agarose, they found that this molecule is tetrameric composed of two different subunits with molecular weights 34500Da and 37500Da respectively. It is confirmed that SNA binds

specifically to Neu5Acα2-6Gal(NAc)-R (Broekaert *et al.*, 1984; Kaku *et al.*, 1990).

2- *Lens culinaris* agglutinin (LCA)

This lectin is called α-D-mannose or glucose binding lectins and it is so also considered as Man- Gluc- and GlcNAc-recognizing lectins that bind with high affinity to fucosylatedcore regions of N-glycosidically linked glycans.

3- *Aleuria aurantia* agglutinin (AAL)

Debray and Montreuil (1989) have isolated AAL from the mushroom *Aleuria aurantia,* they found that this lectin has specificity toward Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1-4GlcNAc; R_2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R_1.

4- *Ulex europaeus* agglutinin (UEA)

Ulex europaeus agglutinin is binding preferably to α1,2-linked fucose residues (Stephan et al., 1996)

5- *Lotus tetragonolobus* agglutinin (LTA)

Lotus tetragonolobus agglutinin is one of the fucose specific lectins and binds specifically to α1,2-fucosylated H blood group as well as Le^x-structures (Pereira and Kabat, 1974)

1.8 Gastropods

Gastropods is class containing about 35,000 gastropod species (Solem, 1984; van Bruggen, 1995), comprises the largest class of the Mollusca (kingdom, Animalia), it contains for example sea slugs, sea butterflies, slipper shells, slugs and snails. Snails and slugs are gastropod mollusks with and without coiled shells. They can be found in extremely wide environmental range (marine snails, fresh water snails and land snails). Land snails are group of organisms that lives in the upper part of the soil and consist of quite small number of species if compared with fresh water or marine snails.

1.8.1 Growth of Gastropods

Although there is not so much available information about egg-laying, growth and reproduction in snails, in this section we are going to present an overview from the few available literatures in our hands. The egg-laying process has been deeply described by Tompa (1984) who stated that the edible snail families (Helicidae and Achatinidae) started this process by digging a hole in which to incubate the eggs. The egg formation process begins after the hole has been dug, then the oocytes are released from the ovary one by one and joined with the sperms that are earlier has stored from mating. As each egg is laid, it is directly added to the egg pool which located in the hole. In general, snails need about 40 hours to produce 120 eggs (one egg every 20 minute). He also added that the number of egg and egg size are greatly differ within the clutch and also between species, furthermore, the limiting factor in determining the maximum size of the clutch is the amount of material stored in the albumen gland.

The egg-laying velocity is not also the same between species, where the achatinids lay eggs very rapidly and that was in contrast to what was observed in helicids (Hodasi, 1979). The growth rate of young snails in the nature is extremely different to a degree that a snail 12 months old has the same size with other of the same strain only 3 weeks old. There are so many factors affecting growth rate in snails such as

environmental factors, population density and the most important criterion which is the nutrition. The importance of the nutrition comes from the nature of snail's movement because of their very slow movement they rely mainly on available food on their proximity and as it known if the snails found good food, they will eat voraciously while if they found bad food, they will eat just enough to survive and the young snails will be dwarfed.

Snail growth can be limited due to population density probably by two ways, the first is that with too many snails in limited space, the good food will be not available and they will have only the bad food, the second probable reason is that the growth depression by overcrowding is somehow happened by a mechanism involving their mucus (Oosterhoff, 1977; Heller and Ittiel, 1990).

Naturally, snails are active in the dark and after rain, so feeding activity always peaked at the first part of the night. Most of snails have home area in which they spend the night. *Helix pomatia* was found to behave differently due to seasonal migration over sometimes modest distances (Lind, 1989).

1.8.2 Gastropods and ecology

Gastropods were found to respond greatly to the surrounded environment.

Temperature can have an inverse relationship with the time to maturation, lifespan and adult size in some pulmonates (Hommay et al. 2001) or it can have a positive relationship with adult size in other land snails (Wolda 1970; Oosterhoff 1977). Fluctuating temperatures may also result in faster growth compared with predictions from results of snails kept at constant temperatures (South 1982).

Photoperiod may also interact with temperature so that, for example, long-day photoperiods may increase growth rates already elevated by high temperatures or may compensate for the negative effects of low temperatures on reproductive output in some snail species (Gomot de Vaufleury 2001). There is no one-state relationship between snail's growth and photoperiod, where Sokolove and McCrone (1978)

stated that growth of slug like *limax maximus* did not vary with day length and that was in contrast to what observed with *Cantareus asperses* where the growth is strongly regulated by photoperiod (Gomot et al., 1982).

One of the most interesting features of snails that they are really very good indicators to different ecosystem variables, this is may be due to that molluscs are dependent on moisture conditions, food availability and on climate. Well known example is fire, because it removes the surface soil organic layer and has implicated in reducing land y snail numbers (Hawkins et al., 1998), it also decreases overall snail abundance (Berry, 1973), snail diversity (Cain, 1983) and composition (Barker and Mayhill, 1999)

1.8.3 Gastropods as host of pathogens

The well known disease, schistomasis or bilharzias which has severe effect on human health, is known to be transferred by freshwater gastropods which work as pathogen (schistosomes) host. This disease is targeting tropical and sub-tropical regions (Africa, Middle East and South America).

Schistosomiasis is one of the major communicable diseases of public health and Socio-economic importance in the developing world. Despite control efforts in a number of countries, still an estimated 200 million people are infected, of which 120 million are symptomatic and 20 million have severe debilitating disease. An estimated 85% of all cases, and most of the severely affected, are now concentrated in Africa. Water resources development projects in Africa have been often linked with an increase in schistosomiasis transmission. Sustainable agricultural development in Africa may be hampered if schistosomiasis is not brought under control (WHO 1998).

The strategy to control schistomasis was achieved by several ways which included controlling snail populations by environmental management and molluscicides (Lardans and Dissous, 1998).

Protein purification from gastropods.

The main snail carrying schistosomes is *Biomphalaria glabrata* or precisly we van say the most infected snail. Its infected with 2 types of schistosomes, *Schistosoma mansoni* and *Echinostoma paraensei* (Loker et al, 2010).

2. Materials and Methods

2.1 Preparation of β-galactosidase (methodology)

2.1.1 Homogenization

Ten frozen snails (*Arion lusitanicus*) were washed carefully, abdominal cavity was opened and the whole digestion system including food was discarded. The snail tissues (about 23g) were suspended in 100 ml of 50 mM Tris/HCl pH 7.5 and homogenized with an IKA Ultra-Turrax T25 (IKA-Labortechnik, Janke and Kunkel GmbH, Staufen, Germany) at 15 000 r.p.m. for 30s.

2.1.2 Ammonium sulfate precipitation

Protein precipitation by ammonium sulfate is one of these classical efficient methods, it is also named salting out and works through disrupting water molecules surrounding proteins and filling it with ions that lead to a decrease of the solubility of non-polar molecules. This method is also important to maintain the native protein structure therefore increasing protein stability.

The homogenized mix was adjusted to 20% (w/v) of ammonium sulfate and stirred for 30 minutes at room temperature. Then the mixture was centrifuged at 5000 r.p.m. for 20min at 4°C. The precipitate was discarded and the supernatant adjusted to 80% (w/v) of ammonium sulfate, stirred for 2 hours at room temperature and centrifuged at 11000 r.p.m. for 35min (Sorvall / Rotor SLA-1500) at 4°C.

2.1.3 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography is a method created from the fact that most of proteins contain one or more hydrophobic groups. The hydrophobic interaction between protein and a hydrophobic ligand can be created by increasing the ionic strength. Salt concentration was set to a high level to control this interaction. The hydrophobic proteins are released or eluted by just decreasing the ionic strength later.

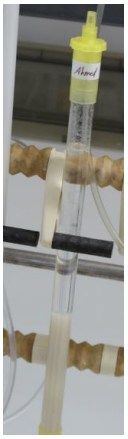


Figure (7). HIC column

- Material:

Gel:	Octyl-sepharose (Amersham Pharmacia, Biotech AB, Sweden)
Starting buffer:	1.2 M ammonium sulfate in 10mM phosphate buffer pH 7.0, 100ml
Elution buffer:	10 mM phosphate buffer pH 7.0, 100ml
Column:	25 ml
Fraction size:	1.7 ml

High salt concentration is a perquisite for HIC. Therefore after ammonium sulfate precipitation the sample was in a suitable form for HIC. After centrifugation, the supernatant was discarded and the precipitate was dissolved in an appropriate amount of starting buffer. The gel was packed into a glass column (1.5 x 14 cm), and then the column was washed with starting buffer. The sample was applied to the column and washed into the column by 50ml of the starting buffer, then the gradient mixer containing both starting and elution buffers (each 100ml) was conducted to the column. Fractions of 90 drops were collected and checked for protein content and enzyme activity.

2.1.4 Ultra-Filtration

Fractions which exhibited high enzyme activity were pooled and concentrated by ultrafiltration [(Amicon, ultra-filtration cell) using an ultra-filtration membrane (Millipore, 50mm regenerated cellulose with exclusion molecular weight of 10,000 Da). For buffer exchange, the sample was washed 3 times by the starting buffer of the next step.

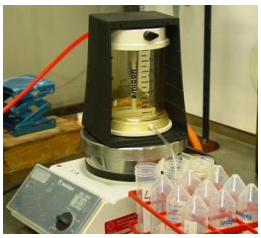


Figure (8). Ultra filtration cell for 50mm filter paper.

2.1.5 Affinity chromatography using Affi Gel Blue

Affi Gel Blue is a cross linked Agarose gel with covalently attached Cibacron Blue F3GA dye. It has been used as a bifunctional affinity/ion exchange chromatography (specifically cation exchange chromatography). This column is frequently used for enzyme purification.

Gel:	Affi Gel Blue [sepharose 4B (Amersham Pharmacia, Biotech AB, Sweden)]
Starting buffer:	50 mM Tris/HCl pH 7.5
Elution buffer:	1M NaCl in 50 mM Tris/HCl pH 7.5
Column:	5.5 ml
Fraction size:	1.2 ml

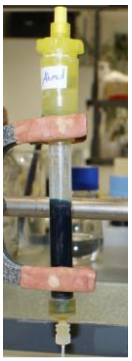


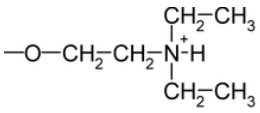
Figure (9). Affi gel blue column.

After desalting the target fractions produced by HIC by ultrafiltration; the sample was applied to the affi Gel blue column and washed with 1 column volume of the starting buffer. The flow through and the washing eluate were collected in one sample each, whereas the following gradient (30 ml starting buffer and 30 ml elution buffer) was fractionated. All fractions were checked for protein content and enzyme activity.

2.1.6 Anion exchange chromatography

Figure (10).
DEAE column.

Gel: DEAE (diethylaminoethyl)



Starting buffer: 50 mM Tris/HCl pH 7.5

Elution buffer: 1M NaCl in 50 mM tris/HCl pH 7.5

Column: 21 ml

Fraction size: 1.2

Anion exchange chromatography is a very potent purification method because it relies on charge to charge interaction. In this case the interaction will be between the negative charged ions (protein) and the positive charged resin (DEAE). This gel is functioning very well at neutral pH, where the sample is applied to the column in a salt free form to give the resin a chance to bind later the bound proteins are eluted by increasing the ionic strength through increasing salt concentration (1M NaCl).

Those fractions exhibiting high enzyme activities, produced by affi Gel blue column were pooled and applied to the anion exchange chromatography column. The flow through and washing eluate were collected in one sample each whereas the following gradient (30ml starting buffer and 30ml elution buffer) was fractionated. All fractions were checked for protein content and enzyme activity.

2.1.7 Size Exclusion Chromatography (Gel Filtration)

Size exclusion chromatography or also called gel filtration is a technique to separate the molecules according to their sizes. Using Sephacryl gel at neutral pH allows

protein fractionation according to the molecular weight where the high molecular weight proteins elute early and the lower ones elute late.

Figure (11). Gel filtration column.

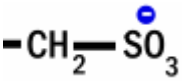
Gel:	Sephacryl S-200
Elution buffer:	50 mM Tris/HCl pH 7.5
Column:	212 ml, 1.5 x 120 cm
Fraction size:	1.3 ml

The enzymatically active fractions after anion-exchange chromatography were pooled, concentrated down less than 2ml and applied to the gel filtration column. Fractions of 60 drops were collected and checked for protein content and enzyme activity.

2.1.8 Cation Exchange Chromatography (AG50)

Figure (12). Cation exchange chromatography column.

Gel:	AG 50 resin (composed of sulfonic acid functional groups attached to a styrene divinylbenzene copolymer lattice) BIORAD



Starting buffer:	Acetic acid 2% (20ml)
Elution buffer:	0.4 M ammonium acetate (NH_4Ac) pH 6.0 (20 ml)
Column:	21 ml
Fraction size:	1.0 ml

Alike to the anion exchange chromatography, the positive charged proteins will bind to the negative charged gel.

After collecting the target fractions resulted from gel filtration, the sample was applied to the cation exchange column and washed with 1 column volume of the starting buffer. The flow through and the washing elute were collected in one sample each, whereas the following gradient (20 ml starting buffer and 20 ml elution buffer) was fractionated. Fractions of 35 drops were collected and checked for protein content and enzyme activity.

2.1.9 β-Galactosidase Specific Affinity Chromatography

Figure (13). β-galactosidase specific affinity columns.

Gel1:	*p*- Aminophenyl β-D-thiogalactopyranoside- Agarose, Sigma.
Gel2:	*p*-Aminobenzyl 1-thio-β-D-Galactopyranoside-Agarose, Sigma.
First buffer:	50mM sodium citrate pH 4.6 (6ml)
Second buffer:	1M NaCl in50 mM sodium citrat pH 4.6 (6ml)
Third buffer:	50 mM Tris/HCl pH 7.5 (6ml)
Fourth buffer:	1M NaCl in 50 mM Tris/HCl pH 7.5 (6ml)
Column:	2 ml
Fraction size:	2ml

Note: these 2 gels are suspended in 0.5 M NaCl and 0.02% thimerosal, so before starting, the gels have to be rinsed first with the first buffer.

After gel filtration the enzyme is in 50 mM Tris/HCl pH 7.5, so first the target fractions were pooled and washed 3 times with the first buffer (50mM sodium citrat pH 4.6). Then the sample was applied to the column and eluted with the first buffer.

Then the buffers 2-4 were applied sequential. All of the five 2 ml-fractions were collected. All fractions were tested for protein content and enzyme activity.

2.2 Analytical methods

2.2.1 Enzyme activity determination

Enzyme activity assay was performed using 0.05 M 4-Nitrophenyl-sugars from Sigma, USA. The substrate was dissolved in 0.1M sodium citrate + 0.04% sodium azide pH 4.6. enzyme activity was determined by the following reaction.

Substrate: 25µl

Sample: 25µl

The mixture was incubated at 37 °C for 2h. Then the reaction was blocked by 250µl of Glycin/NaOH pH 10.4 and the hydrolysis was determined spectrophotometrically at 405nm.

The following substrates were used

a- For β-galactosidase: 4-nitrophenyl-β-D-galactoside.

b- For β-hexosaminidase: 4-nitrophenyl-N-acetyl-β-D-glucosaminide.

c- For α-mannosidase: 4-nitrophenyl-α-D-mannopyranoside.

d- For β-mannosidase: 4-nitrophenyl-β-D-mannopyranoside.

e- For α-glucosidase: p-nitrophenyl-α-D-glucoside.

f- For β-glucosidase: p-nitrophenyl-β-D-glucoside.

2.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins.

Samples containing about 10 µg of protein were dried in the speed vac, dissolved in 300 µl of ddH_2O and precipitated by methanol.

Methanol precipitation was done by freezing samples containing 4 folds methanol at 80°C for 2h and then centrifuged for 30min at 14000 r.p.m.

The supernatant was discarded, the pellet was dried at room temperature. The dried protein after methanol precipitation is dissolved in SDS-sample buffer and denatured at 95°C for 7min.

Components	Separating gel	Stacking gel
1% (w/v) Bisacrylamide [BioRad]	780 µl	390 µl
1.5M Tris/HCl pH 8.8	1500 µl	-
0.5M Tris/HCl pH 8.8	-	750 µl
H2O	1140µl	1280 µl
SDS 10%	60 µl	30 µl
Ammonium persulfate 10%	36 µl	24 µl
TEMED	3.6 µl	2.4 µl

- Sample buffer (2x)

Dithierithritol :	31 mg
SDS :	200 mg
0.5 M tris/HCl pH 6.8:	5 ml
Glycerin (87%):	2.8 ml
H_2O:	2.7 ml

The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad laboratories, Watfad, UK). 12% polyacrlamide gel was prepared (2 Gels 80 x 70 x 0.75mm) as follows;

The separating gel was poured firstly and overlaid by isopropanol, after 1h the isopropanol was removed, the stacking gel is poured and the comb is settled (it needs 30min for polymerization).

- Electrophoresis buffer (5x)

Tris 18 g/l
Glycin 72 g/l
SDS 5 g/l

The electrophoresis was performed at 200V in the electrophoresis buffer at RT until the tracking dye reaches the bottom of the glass plates.

a) Staining

Gels were fixed in fixation buffer (50% methanol, 7% HAc and 43% H_2O) for 10 min, stained by Coomassie brilliant blue R-250 [0.1% (w/v) Coomassie brilliant blue R-250 in 40% (w/v) methanol and 10% (w/v) acetic acid for 20min]. The gel is then destained with 5% acetic acid until the protein bands are clearly visible.

b) Silver staining

- Reagents (stock)

50% methanol + 12%TCA + 2%$CuCl_2$
10% methanol + 5% acetic acid
0.01% $KMnO_4$
10% EtOH

- Reagents (freshly prepared)

  0.1% $AgNO_3$ (50ml per gel)

  1% K_2CO_3 + 0.01% formaldehyde (50 per gel)

The gel washed by the previously mentioned reagents as follow:

1- 50% methanol + 12%TCA + 2%$CuCl_2$ (5min)

2- 10% methanol + 5% acetic acid (5min)

3- 0.01% $KMnO_4$ (5min)

4- 10% methanol + 5% acetic acid (1min)

5- 10% EtOH (5min)

6- ddH_2O (5min)

7- 0.1% $AgNO_3$ (5min)

8- ddH_2O (30sec)

9- 1% K_2CO_3 + 0.01% formaldehyde (50 per gel) in dark room until the bands are clearly seen.

10- The staining was stopped by 5% HAc

2.2.3 Enzyme characterization

- pH optimum determination

Reaction:-

Substrate	25 µl
Buffer	20 µl
Enzyme	5 µl

For the pH optimum determination, the enzyme was incubated with its substrate in the presence of the following buffers

0.4 M acetate with pH between 3.5 and 5.5

0.4 M citrate with pH between 3.5 and 6.5

0.4 M morpholinoethanesulfonic acid (MES) with pH between 5.5 and 7.0

0.4 M phosphate/NaOH with pH between 6.0 and 7.5

0.4 M tris/HCl with pH between 3.5 and 5.5

The mixture was incubated at 37°C for 2h, then stopped by 250µl Glycin/NaOH pH 10.4 and measured spectrophotometrically at 405nm.

- Metal dependency

Reaction

Substrate:	25 µl
Enzyme:	10 µl
Cation:	10 µl
NaCl 0.9%:	5 µl

In order to test the effect of metal ions on the enzyme activity, the enzyme was incubated with its substrate in the presence of different metals ($MnCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, $NiCl_2$, $CoCl_2$, $SnCl_2$ and $BaCl_2$), furthermore, EDTA was used to see activity without any metal. The mixture was incubated at 37°C for 2h, then stopped by 250µl Glycin/NaOH pH 10.4 and measured spectrophotometrically at 405nm.

- Time course

Reaction

Substrate:	25 µl
Enzyme:	5 µl
NaCl 0.9%:	20 µl

This mixture was incubated at 37°C for different incubation times (0, 10min, 30min, 1h, 2h, and 4h). The mixture was stopped by 250µl Glycin/NaOH pH 10.4 and measured spectrophotometrically at 405nm.

- (Thermal) Stability

For the determination of thermal stability assay, the enzyme was incubated at different temperatures (-20°C, 4°C, RT, 37°C, 50°C and 80°C) for 12h. Aliquots of these tubes were taken and the standard activity assay was performed.

2.2.4 Quantification of enzyme activity by p-4-nitrophenol standard curve

Eight points p-4-nitrophenol standard curve (0nmol, 5nmol, 10nmol, 15nmol, 20nmol, 25nmol, 30nmol, 35nmol and 40nmol) were prepared by dissolving nitrophenol in 0.1M sodium citrate and 0.04% sodium azide, pH 4.6. The different nitrophenol concentrations, prepared in microtiter plate were stopped immediately by 250µl 0.4 M glycine/NaOH, pH 10.4 and measured spectrophotometrically at 405nm (figure 14).

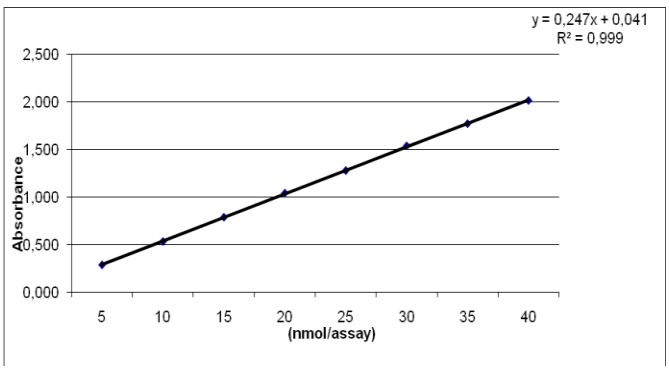


Figure (14) p-4-nitrophenol standard curve.

2.2.5 Protein quantification (BCA method)

BCA protein quantification was done according to the manufacturer's protocol (PIERCE, USA). The test was performed in a microtiter plate with BSA calibration curve which ranged between 5 to 50µg/100µl (10 standard points). 100µl of the BSA or unknown sample was mixed with 100µl working reagent. The reaction components in the microtiter plate were incubated at 37°C for 2h and the absorbance was measured spectrophotometrically at 570nm. The protein concentration was quantified by using the BSA calibration curve (figure 15).

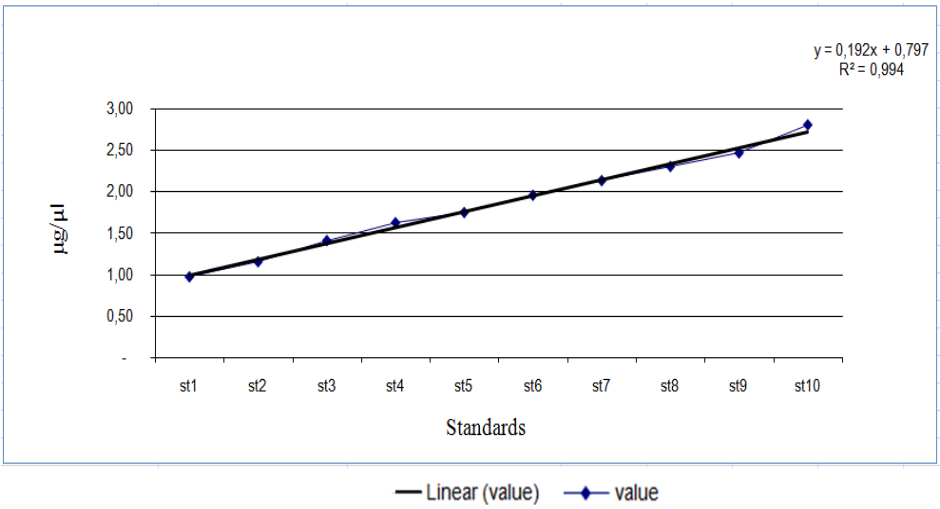


Figure (15) Bovine serum albumin (BSA) standard curve.

2.2.6 Sample preparation for peptide analysis

Target bands were cut from the SDS-gel and fragmented in very small pieces. Gel pieces containing target protein were rinsed with ddH_2O. Fragmented bands were destained by washing it twice by 50µl of 50% AcCN (acetonitrile), (each washing lasted

for 15min and the liquid was discarded). Then the slices were washed by 100% AcCN but only for few seconds and again the liquid was discarded. Gel was re-

swelled for 5min in 30µl of 0.1M NH_4HCO_3, washed again for 15min by 30µl of 100% AcCN and dried in the speed vac without heating lamp.

After destaining, the carbamidomethylation took place by firstly re-swelling the gel for 5min in 50µl 10mM DTT (Dithiothreitol), then the mixture was incubated for 45min at 56°C and the liquid was discarded. 50µl of 55mM iodoacetamide was added to the gel and incubated for 30min at RT in the dark.

Again samples were washed twice by 50µl of 50% AcCN for 15min and the liquid was discarded, 100% AcCN was added for few seconds and the liquid was discarded. The gels were re-swelled by 30µl 0.1M NH_4HCO_3 for 5min, then washed again by 30µl 100% AcCN for 15min and dried in speed vac without heating lamp.

The tryptic digestion was done by 10µl trypsin solution [1part from the trypsin stock (50ng/µl sequencing grade trypsin) mixed with 2 parts 25mM NH_4CO_3] which pipetted directly on the dried gel pieces, left for re-swelling for 10min and incubated

Addition of 30µl of 25mM NH4HCO3:	15min shaking
Adding 30µl of 100% AcCN:	15min shaking and the supernatant to be collected
30 µl of 5% Formic acid	15min shaking and the supernatant is collected and added to the first supernatant
30 µl of 5% Formic acid	15min shaking and the supernatant is collected and added to the first supernatant

over night at 37°C after addition of 10-20µl 25mM NH_4HCO_3.

- Peptides were extracted by the following steps;

2.2.7 MALDI (matrix assisted laser desorption) –MS- analysis of peptides.

Matrix solution: 1% α-cyano-4-hydroxy-cynnamic acid in 70% acetonitrile in water. The dried peptides were taken in 20µl 5% formic acid and 0.5µl was spotted on MALDI target which dried firstly before analysis by MALDI-TOF-MS.

(This analysis was carried out by Dr. Johannes Stadlmann)

2.3 Western blot analysis of snail proteins

2.4 Sample preparation

About 60 to 80mg of snail's tissue (skin or viscera or egg) were soaked in RIPA buffer (100µl for each 10mg). This mixture was incubated on ice for 15min, then the homogenization was performed by Ultra Turrax at 15 000 r.p.m. for 30s. The homogenized mixture was sonicated by Ultra Sonic for 10min and incubated at RT for 10min. The samples were then centrifuged at 14 000 r.p.m for 20min at 4°C. 5µl was taken from the supernatant to be loaded to the electrophoretic gel.

RIPA buffer

150 mM NaCl

0.1% SDS

0.5% Cholate

10% Glycerol

0.4 M EDTA

2.5 Transfer to nitrocellulose membranes

Figure (16). Semi-dry blotting apparatus.

The electrophoresis was carried out as described in 1.2.2.

After the electrophoresis, the protein was transferred to nitrocellulose membranes using a mini Protean II kit (Bio-RAD). The nitrocellulose membrane was first presoaked in blotting buffer (25mM Tris and 192mM glycine). The gel, membrane and filter papers were wetted in the blotting buffer, all

the components were placed in semi-dry blotting apparatus (Semi-transfer blot, Bio-RAD) the transfer was done at 15V for 20min (for 1 gel). After the transfer, the membranes were blocked in the blocking buffer [0.5% BSA (bovine serum albumin) in TTBS (20mM Tris/HCl pH 7.6, 8g/l NaCl and 0.05% Tween)].

2.6 Blotting

Blotting buffer:	25 mM Tris, 192 mM glycine
Blocking buffer:	0.5% BSA in TTBS
TTBS:	M Tris/HCl pH7.6, 8 g/l NaCl, 0.05 Tween

The membranes were then incubated with the primary antibody in dilution of 1: 1000 TTBS for 1h after that washed 3 times by TTBS 5min each. The membranes were then incubated with the secondary antibody in dilution of 1: 500 TTBS. Membranes were stained (Sigma fast BCIP/NBT diluted in 10ml ddH_2O. The color was developed in dark room and the reaction was then stopped by 5% HAc.

- **Lectins (*SIGMA*) are used in the study and their specificities**

LCA	*Lens culinaris*	mannosyl residues with an enhanced binding if there is an α1,6-fucose linked to the N-acetylglucosamine
MAA	*Maackia amurensis*	α 2,3 linkages in sialylated glycans
SNA	*Sambucus nigra*	α2,6-linked sialic acid linked to galactose or N-acetylagalactosamine residues
AAL	*Aleuria aurantia*	core α1,6- fucosylated N-glycans

Protein purification from gastropods.

LTA	*Lotus tetragonolobus*	α1,2-fucosylated H blood group as well as Le^x-structures
UEA	*Ulex europeus*	α1,2-fucosylated H blood group glycans

The primary antibodies were used in dilution 1:1000 while alkaline phosphatase was used as secondary antibody in dilution 1:500.

2.7 PNGase F digestion

N-glycans were removed to analyze their influence on the size of the proteins. About 100 µg of each tissue was dried completely in speed vac (Thermo Savant) at 45°C. 40µl of the digestion buffer (50mM Tris and 50mM EDTA, pH 8.4) were added and incubated at 95°C for 30min. The mixture was left to cool down for 10min at RT and 1.5µl of the enzyme (N-Glycosidase F, 100 units, Roche Diagnostics GmbH) were added. The sample was incubated at 37°C overnight and after that the protein was precipitated by methanol and loaded on the gel.

2.8 Digestion by β-elimination

O-glycans were removed to analyze their influence on the size of the proteins.

β-elimination reagent mixture (*SIGMA*)

β-elimination reagent mixture (total volume)	β-elimination reagent	Sodium hydroxide solution
40 µl	37.6 µl	2.4 µl
100 µl	94 µl	6 µl

10 µg of each tissue were dried and then re-dissolved in 15µl ddH_2O and cooked for 30min at 95°C, then the mixture was left to cool down at RT for 10min. 2.7µl of the β-elimination mixture was added and the vials were kept overnight at 4°C, then the protein was precipitated by methanol and the sample was loaded on the gel.

2.9 Native Gel Electrophoresis

Reagents

- Acrylamide solution: 30% acrylamide + 0.8% bis-acrylamide
- Separating gel buffer: 1.5M Tris/HCl, pH 8.8
- Stacking gel buffer: 0.5M Tris/HCl, pH 6.8

After pouring the separating gel, it was covered by a layer of isopropanol. The gel was kept for 30min to polymerize and after the polymerization, the isopropanol was removed in order to pour the stacking gel.

Stacking gel

Acrylamide solution:	2499 µl
Separating gel buffer:	2499 µl
H_2O:	4950 µl
Ammonium persulfate (10%):	50 µl
TEMED:	3 µl

Separating gel

Acrylamide solution:	500 µl
Stacking gel buffer:	1000 µl
H_2O	2466 µl
Ammonium persulfate (10%):	33.3 µl
TEMED	5 µl

- Sample buffer (5x)

Tris/HCl pH 6.8:	15.5 ml
1% Bromophenol blue:	2.5 ml
H_2O:	7 ml
Glycerol:	25 ml

Note: Sample was mixed with the sample buffer in dilution of 1: 4 (sample buffer to sample).

- Electrophoresis buffer

Tris:	3g
Glycine:	14.4g
H_2O:	Up to 1L, pH 8.3

The electrophoresis was performed at 100V (until the tracking dye reaches the bottom of the glass plates which takes about 2h: 30min).

2.10 Protein elution from the native gel

The same sample was applied in 2 or 3 lanes, the first lane was subjected to staining with coomassie to see the bands pattern. After that and according to the stained lane we cut the different pieces from the unstained gel (in very small pieces) which were soaked in about 500µl of the electrophoresis buffer and left to rotate overnight at 4°C (passive elution). Buffer containing gel was centrifuged at 5000 r.p.m for 5min, then the supernatant was taken and the enzyme assay was performed as previously mentioned. All resulted supernatants were precipitated by methanol and protein exposed to SDS-poly acrylamide gel electrophoresis.

3 Results

3.1 Preliminary study

Before starting the direct work on β-galactosidase purification, some of the available exoglycosidases [α-mannosidase (α Man), β-mannosidase (β Man), α-glucosidase (α Glc), β-glucosidase (β Glc), β-galactosidase (β Gal) and β-N-acetylglucosaminidase (β GlcNAc)] were examined in different snails (*Arion lusitanicus*, *Biomphalaria glabrata*, *Achatina fulica*, *Limax maximus*, *Cepaea hortensis*, *Arianta arbustorum*, *Planorbarius corneus* and *Lymnea stagnalis*).

After homogenization and protein precipitation by ammonium sulfate, the precipitate was dialyzed and fractionated by anion exchange chromatography which was performed by batch purification (by modifying the pH of the dialyzed precipitate to 7.5 by 500mM Tris/HCl pH 8.2, loading into the ion exchange column and receiving the flow through and the bound fraction which was eluted by 1 M NaCl in 50 mM Tris/HCl, pH 7.5). All resulted fractions were tested for previously mentioned Exoglycosidases (Figures 17, 18, 19, 20, 21, 22, 23 and 24)

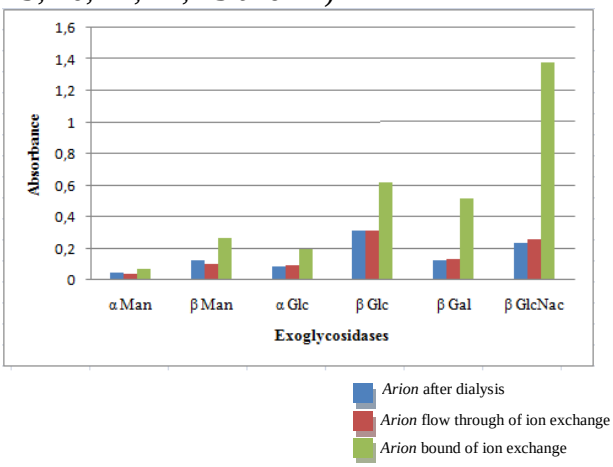


Figure (17)
Exoglcosidases screening in *Arion lusitanicus*

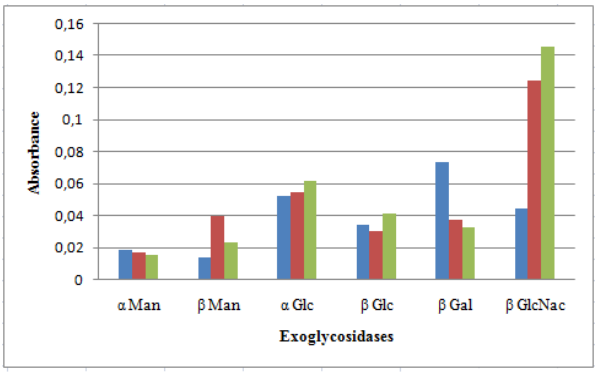


Figure (18) Exoglcosidases screening in *Biomphalaria glabrata.*

Protein purification from gastropods.

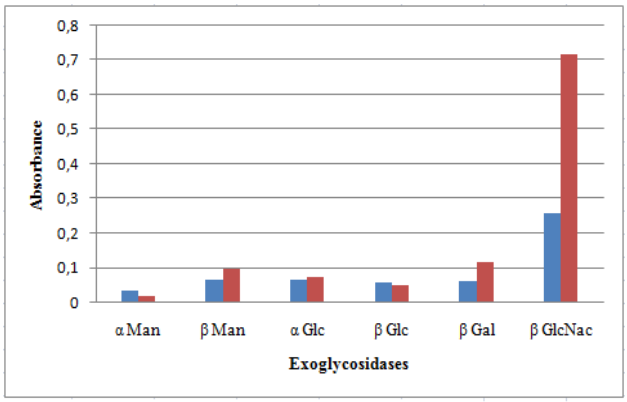


Figure (19) Exoglcosidases screening in *Achatina fulica*

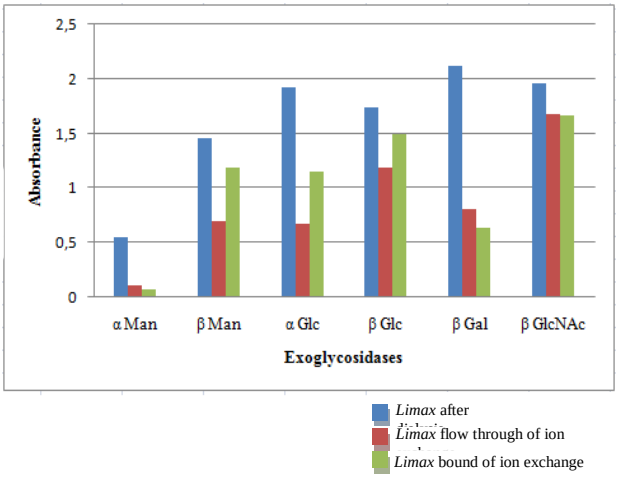


Figure (20) Exoglcosidases screening in *Limax maximus*

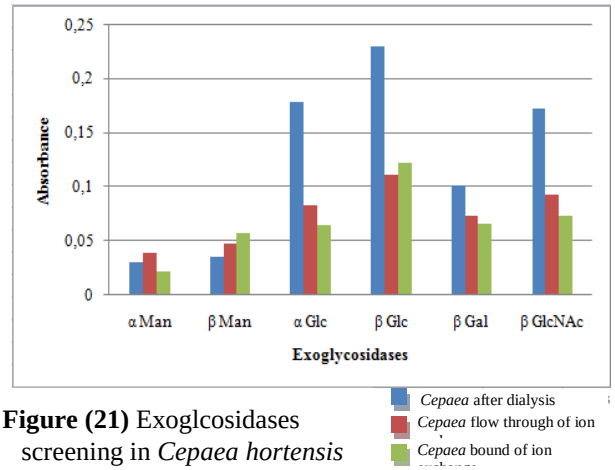


Figure (21) Exoglcosidases screening in *Cepaea hortensis*

Protein purification from gastropods.

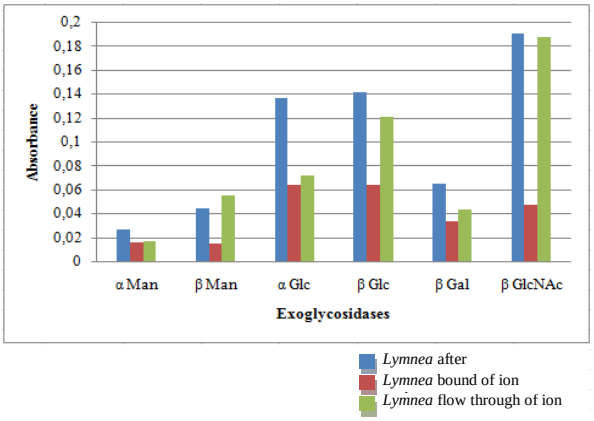
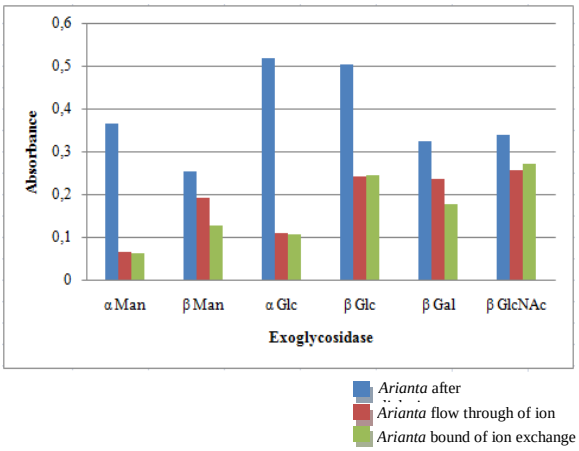


Figure (22) Exoglcosidases screening in *Lymnea stagnalis*

Figure (23) Exoglcosidases screening in *Arianta arbustorum*



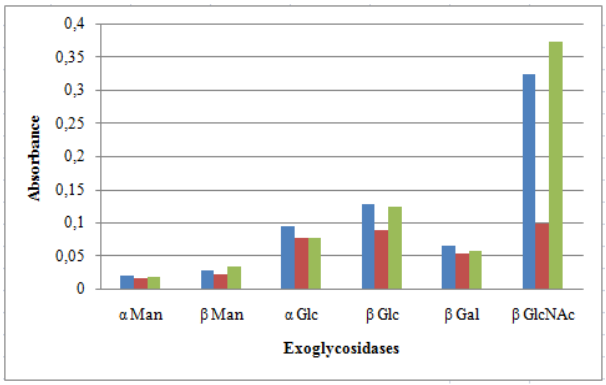


Figure (24) Exoglcosidases screening in *Planorbarius corneus*

Eggs also were checked for exoglycosidases activity. *Achatina fulica* eggs, were homogenized, precipitated and chromatographed by anion exchange chromatography (by batch). The flow through and the bound fractions were collected and exoglycosidases activity were determined (figure 25).

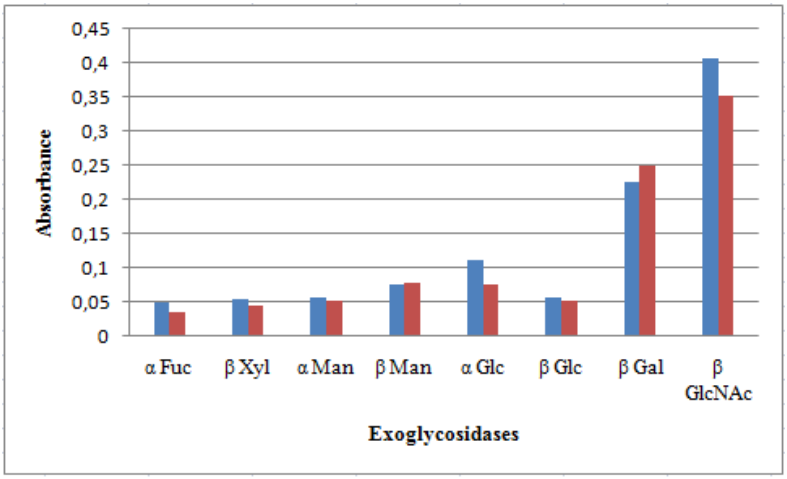


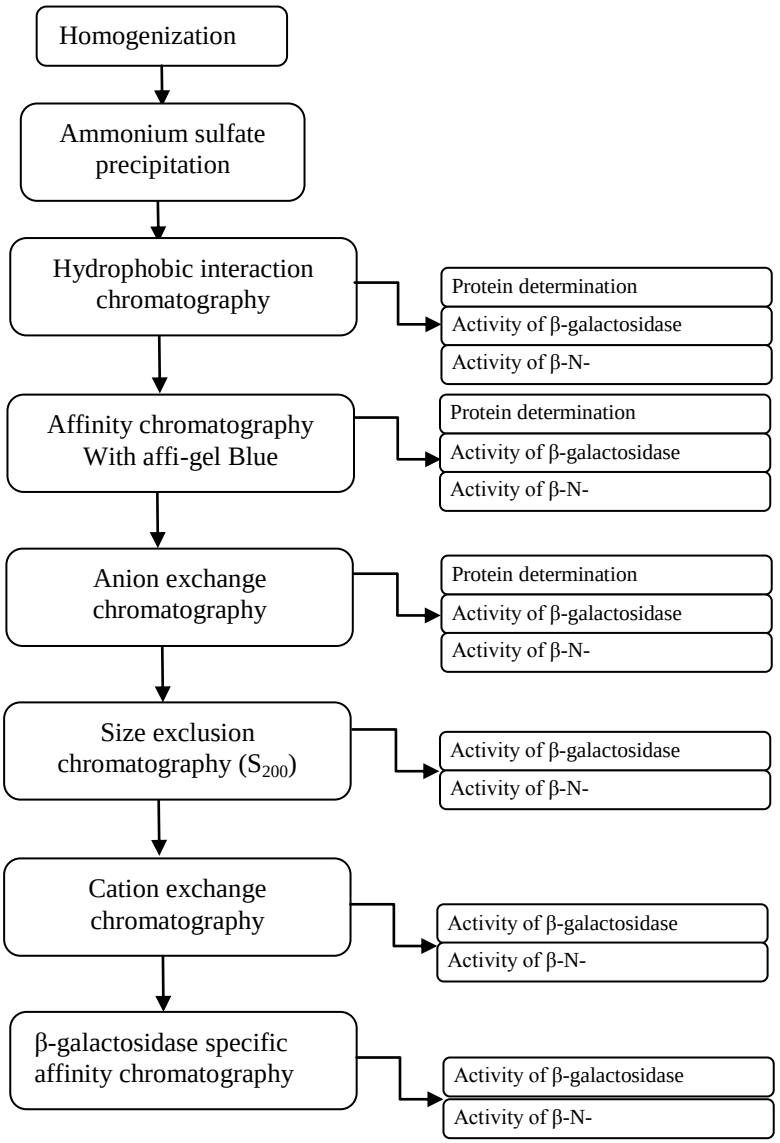
Figure (25) Exoglcosidases screening of the egg of *Achatina fulica*

Data obtained after exoglycosidases screening indicated that not all tested enzymes have similar activities and also they showed different activities patterns among different snails. Some of these enzymes were sometimes undetectable (α-Man and β-Man) and other higher in most of the cases (β-GlcNAc). On the other hand the fractionation by anion exchange chromatography has not much effect as the flow through and the bound fractions contain quite similar amount of most of the enzymes. Testing these enzymes after dialysis gave fluctuating values. this showed higher enzyme activity in *Arion lusitanicus, Limax maximus, Lymnea stagnalis, Cepaea hortensis and Arianta arbustorum* and lower enzyme activity in *Achatina fulica* and *Biomphalaria glabrata*. The screened exoglycosidases followed quite a similar pattern of activity in *Achatina fulica* eggs with an important observation that among these exoglycosidases, β-Gal and β-GlcNAc were the highest in the activity.

In the light of all screened exoglycosidases, it was valuable and possible to work on β-galactosidase purification and characterization because as it was observed that this enzyme is a highly abundant enzyme due to its presence in all snail´s tissues.

3.2 Purification of the enzyme from *Arion lusitanicus*

Snails may be a potential source of enzymes and glycan structures with novel specificities and unusual determinants. Therefore this work presents one of the most frequent enzymes in these tissues a β-galactosidase which was purified according to the following scheme:



Protein purification from gastropods.

The purification of β-galactosidase started after homogenization with 100 ml of crude extract (from 10 animals of *Arion lusitanicus*). The first step was an ammonium sulfate precipitation where the 80% precipitate was taken for further analysis (In the next step).

Hydrophobic interaction chromatography: The crude extract was applied to the column, the elution of flow through as one fraction, then 50ml of starting buffer was added and eluted also in one fraction. Finally the gradient was started and fractions of 2 ml were collected and analyzed for protein content and exoglycosidase activities. (Figure 26)

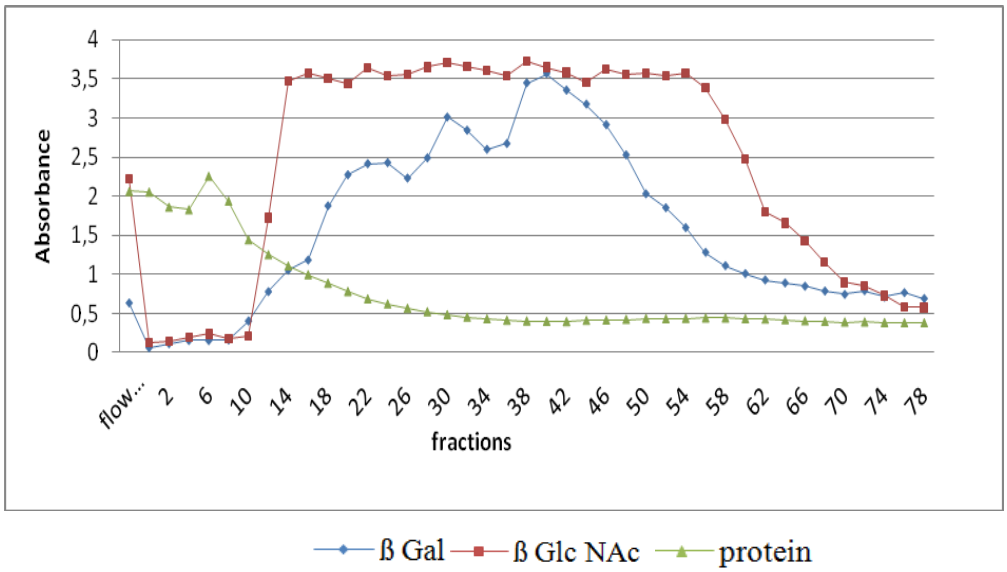


Figure (26). Enzyme activity (405mAbs) and protein (280 mAbs) determination assay fraction after HIC

The chromatography by HIC shows that the enzyme activities elute after the main protein amount which means a significant purification in this first chromatography.

Those fractions which showed high enzyme activity (18-58) were pooled (90ml), concentrated, washed by 50mM Tris/HCl pH 7.5 (to remove salts) and concentrated to less than 25 ml by ultrafiltration.

The third purification step was performed by an affi-gel Blue column with a gradient elution. The sample was applied and the flow through was collected in one fraction, then one column volume of the starting buffer was added to wash the column (collected also in one fraction) and then the gradient was applied to the column. As it is observed from the chromatography by affi-gel blue (Figure 27), there is no affinity or binding capacity between the gel and tested enzymes, where the fast majority of the enzymes were in the flow through and the washing Tris fractions and the rest of the fractions were seen to contain no activity.

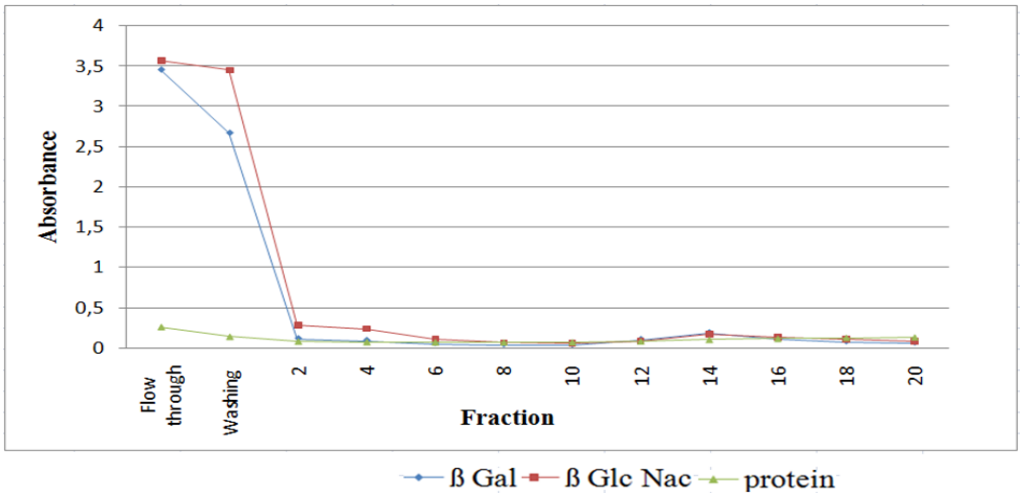


Figure (27). Enzyme activity (405mAbs) and protein (280 mAbs) determination assay of the resulted fraction after Affi-gell-blue.

Flow through and Tris eluted fraction were pooled (35ml) together and directly applied to anion-exchange column (Figure 28) which showed quite similar results to the affi-gel blue column. However β-N-Acetylglucosaminidase activity was also observed at the end of the gradient elution.

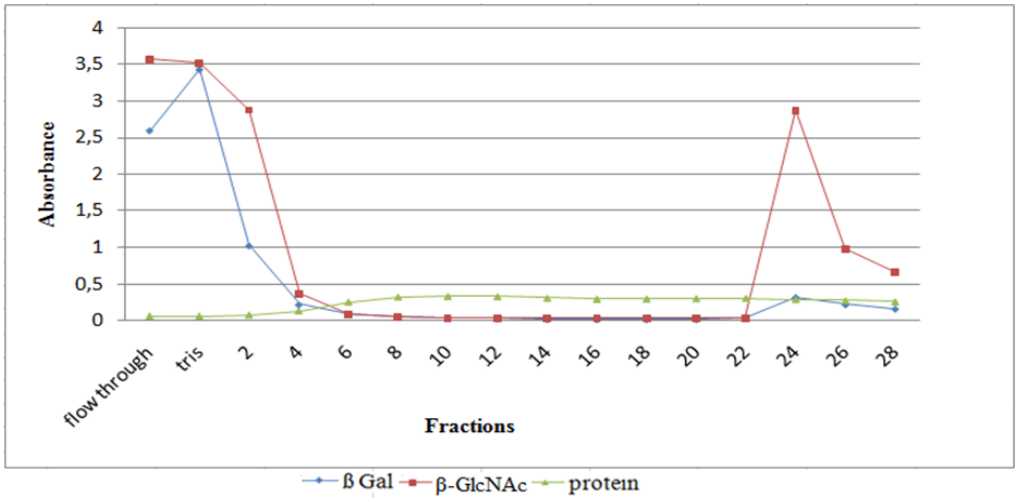


Figure (28). Enzyme activity assay of the resulted fraction after anion-exchange chromatography.

All of the early obtained peaks were pooled, concentrated to down less than 2ml and applied on Sephacryl column (S_{200}) (Figure 29**)**.

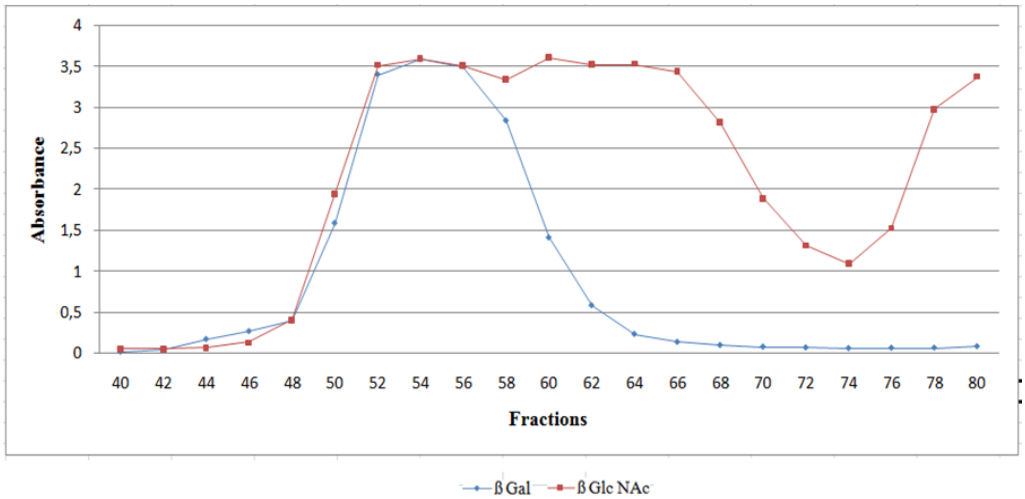


Figure (29). Enzyme activity assay of the resulted fraction after size exclusion chromatography.

Fractions 50 to 60 (22ml) from size exclusion chromatography were pooled, concentrated and, divided into 2 parts, the first part was exposed to the cation exchange chromatography and the resulted fractions were checked on SDS-PAGE. After electrophoresis only 2 bands appeared at molecular weights about 55 and 75 kDa.

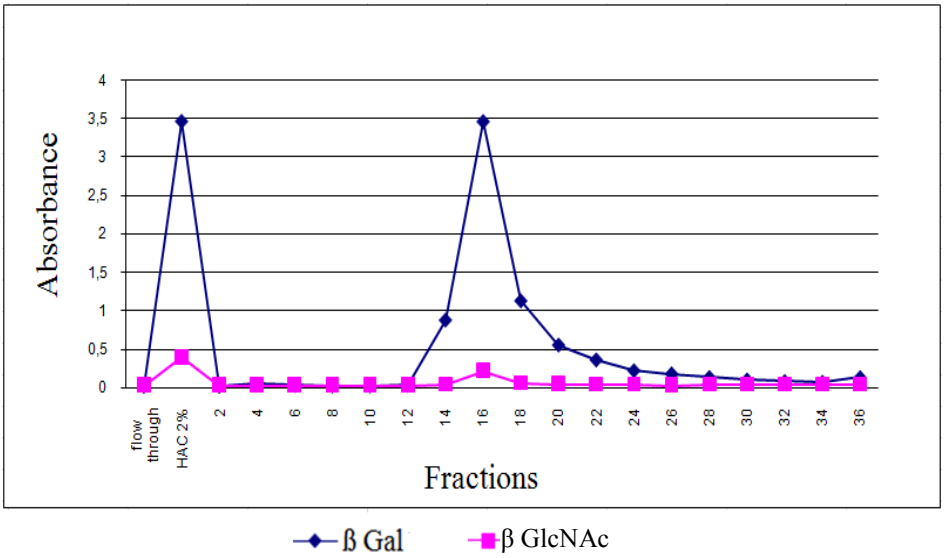


Figure (30). Enzyme activity assay of the resulted fraction after cation-exchange chromatography.

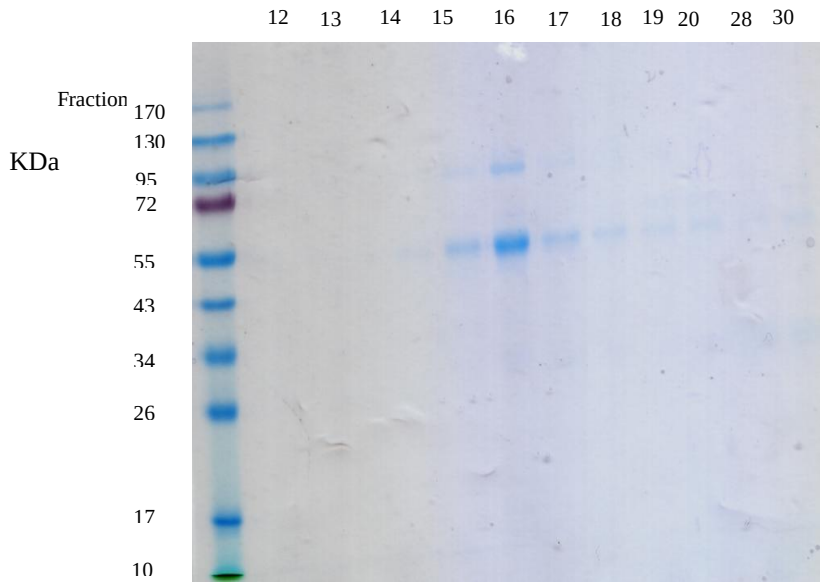


Figure (31) SDS-PAGE cation exchange fractions.

The second part was transferred into 50 mM sodium citrate buffer pH 4.6 and concentrated again to 4.5ml to be applied to specific β-Galactosidase affinity column. After applying the sample to the column, a series of different buffers differing in salt concentrations and pH's were applied to the column and the resulting fractions were analyzed for β-galactosidase activity (figure 32).

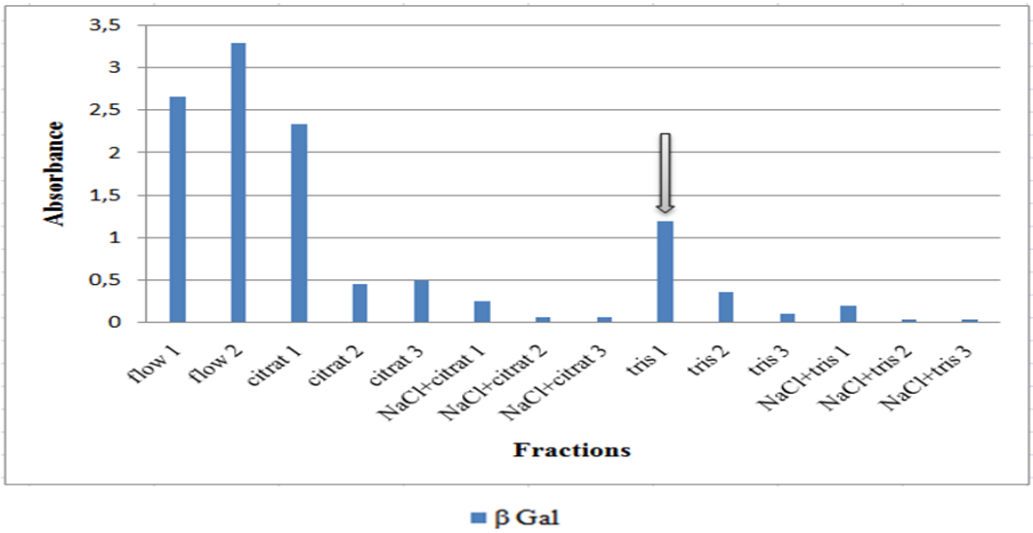


Figure (32). β-galactosidase activity assay of the resulted fractions after specific affinity chromatography.

Quite a lot of β-galactosidase activity can be found in the flow through and the first washing fractions, but also a significant amount binds to the column and is finally eluted by changing the pH to pH 7.5.

In order to determine the molecular weight of the enzyme, SDS-PAGE electrophoresis was performed.

Lane (1). Flow1 + flow2 + Citrate1

Lane (2). Citrat2 + Citrat3 + NaCl Citrate1

Lane (3). NaCl Citrate2 + NaCl Citrate3

Lane (4). Tris1 + Tris2

Lane (5). Tris + NaCl Tris1 + NaCl Tris 2 + NaCl Tris 3.

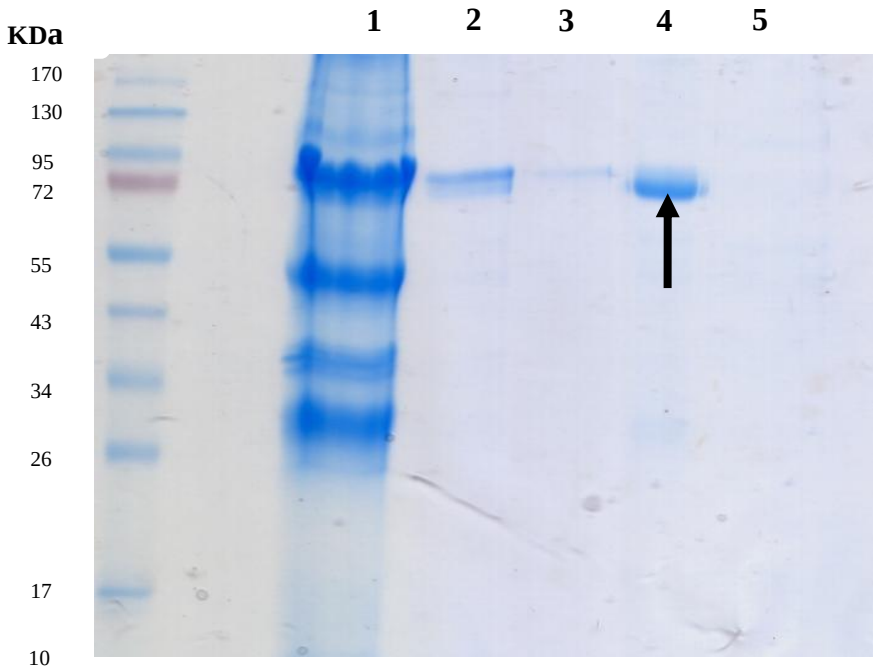
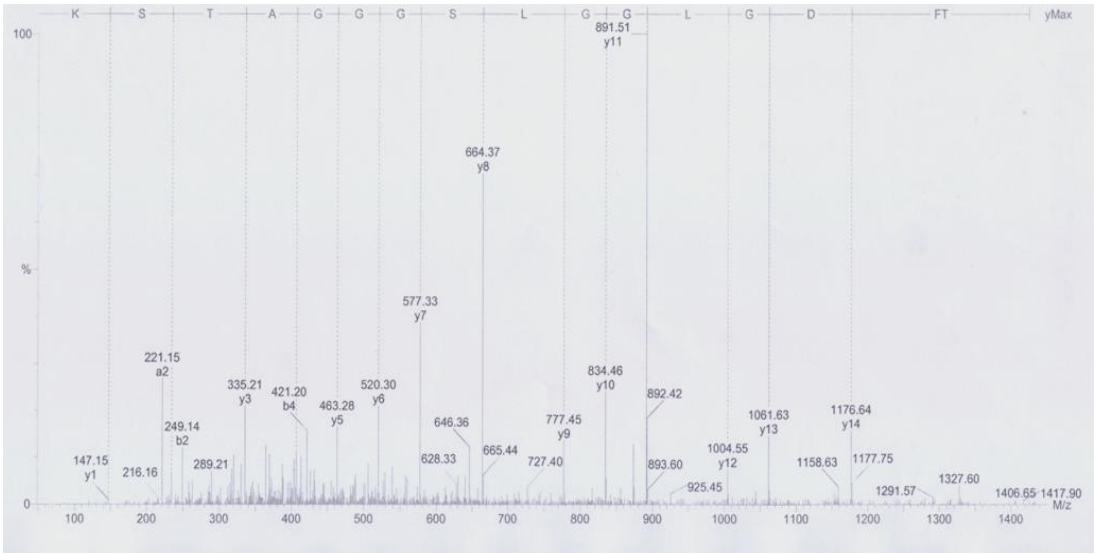


Figure (33) SDS-PAGE of specific affinity fractions.

Electrophoresis displayed only one single band in lane number four with a molecular weight of approximately 74 KDa. This one was cut out, the protein extracted and identified by LC-MS. After analysis data obtained from mass spectrometry and from homology search for two analyzed peptides of this protein it was verified protein is a homologue to a galactocerebrosidase.



The obtained peptide sequence from this mass is [FTDGLGGLSGGGATSK]. The second obtained peptide with sequence [LLVNYPK] which was quite a short peptide, and from homology search, it was found to be homologue to also a galactocerebrosidase.

After each purification step an aliquot was taken and stored to determine protein content and enzyme activity throughout the whole experimental steps (Table 1). From data shown in table 1, it was obvious that the specific activity and the purification (expressed by fold) reached their maximal values after gel filtration step. Unfortunately, it was not possible to calculate the specific activity and also folds of purification at the last step (specific affinity) because the protein value for the last resulted fraction was under the minimal detection limit. It is also important to mention that the protein concentration (mg) and total enzyme activity were reduced with the purification step after step to reach the lowest values after gel filtration for protein content and after the specific affinity for total enzyme activity.

Table 1 Purification of β-galactosidase from 23g of *Arion lusitanicus*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Homogenization	549,30	26000	47,33	1,00
20% precipitation	389,08	44030	113,16	2,39
80% precipitation	208,45	51480	246,96	5,22
HIC	190,14	43920	230,99	4,88
Affi gell blue	120,42	24930	207,02	4,37
Ion exchange	98,59	24880	252,35	5,33
Gel filtration	54,23	15400	284,00	6,00
Specific affinity	-	2100	-	-

3.2.1 Determination of the pH optimum

The β-galactosidase was incubated for 2h at 37 °C at various pH values. The optimal activity of the enzyme was at low pH (3.5-5.0). At pH above 8.0 there was a complete loss of the enzyme activity (Figure 34). The kind of salt responsible for the certain pH seems to have no significant influence on the activity.

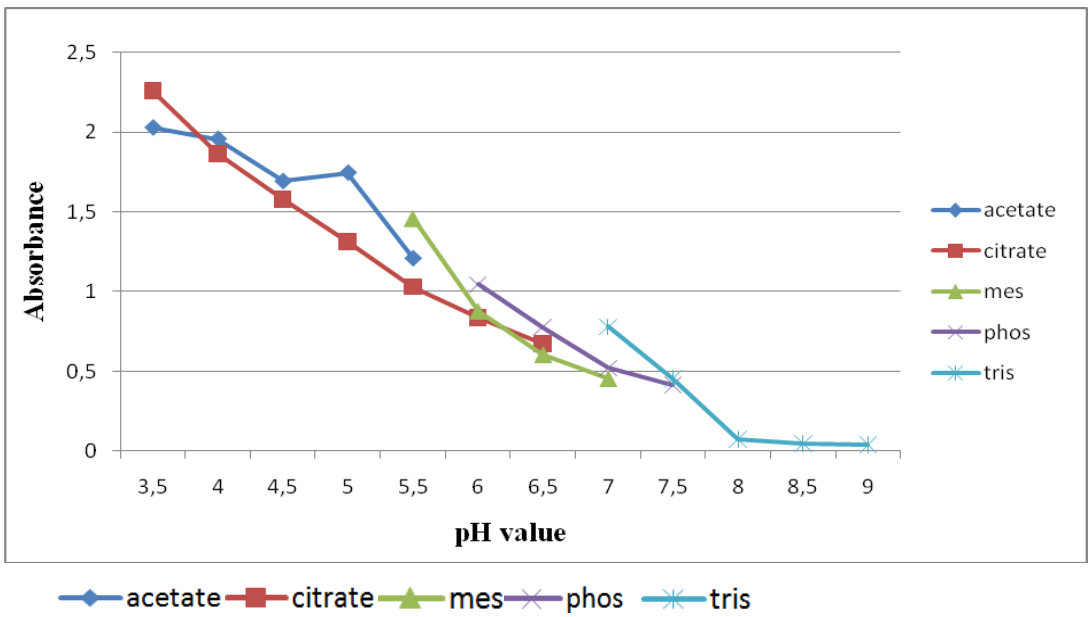


Figure (34). pH optima for β-galactosidase.

3.2.2 β-Galactosidase Stability

In this test the enzyme was exposed to different temperatures, solvents and **(**Figure 35**).** After incubating the enzyme in different environments for 20h, results showed that the enzyme activity sharply decreased after incubation at 50°C. At 80°C there was no activity while the highest activity was shown at 37°C. β-galactosidase incubation with different solvents (methanol, glycerin and acetonitrile) showed no obvious effect on the enzyme activity.

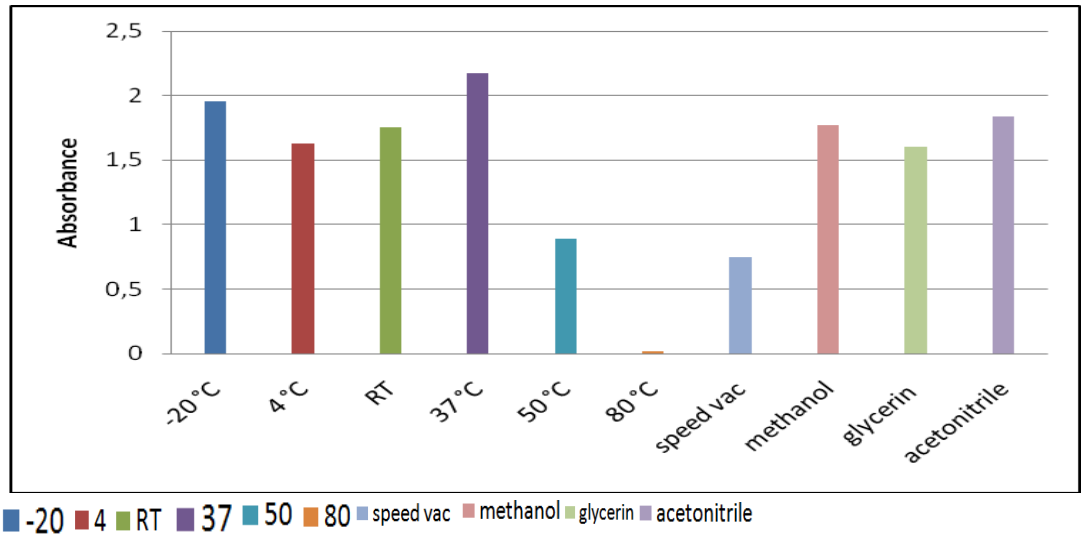


Figure (35). β-galactosidase stability.

3.2.3 Metal Dependency

The metal cation requirements of β-galactosidase from *Arion lusitanicus* were examined **(**Figure 36**)** No effect for the different cations on the enzyme activity except for zinc which resulted in a pronounced decrease in β-galactosidase activity. Complexing any metals by EDTA also does not slow any effect which makes clear, that the enzyme does not depend on metal ions.

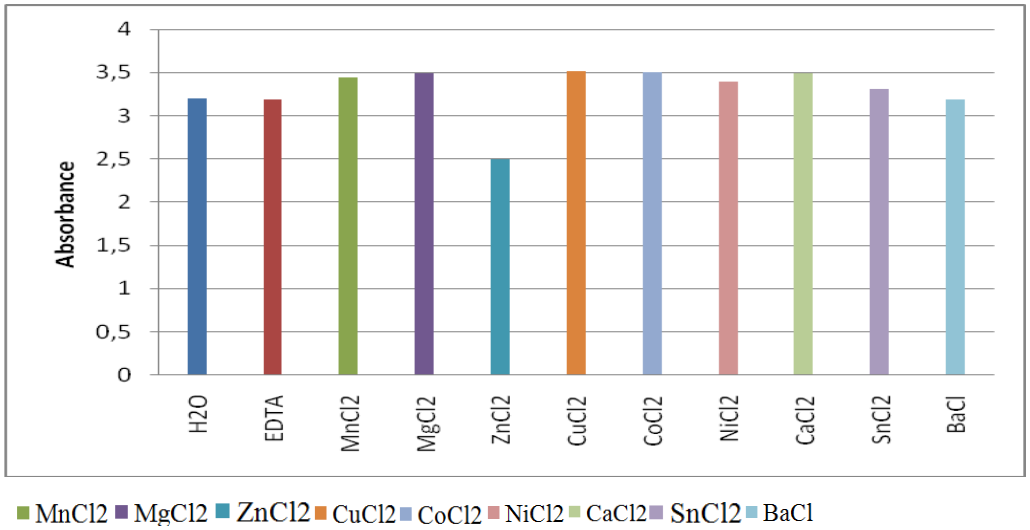


Figure (36). β-galactosidase activity in the presence of divalent cations and EDTA.

3.2.4 Time course

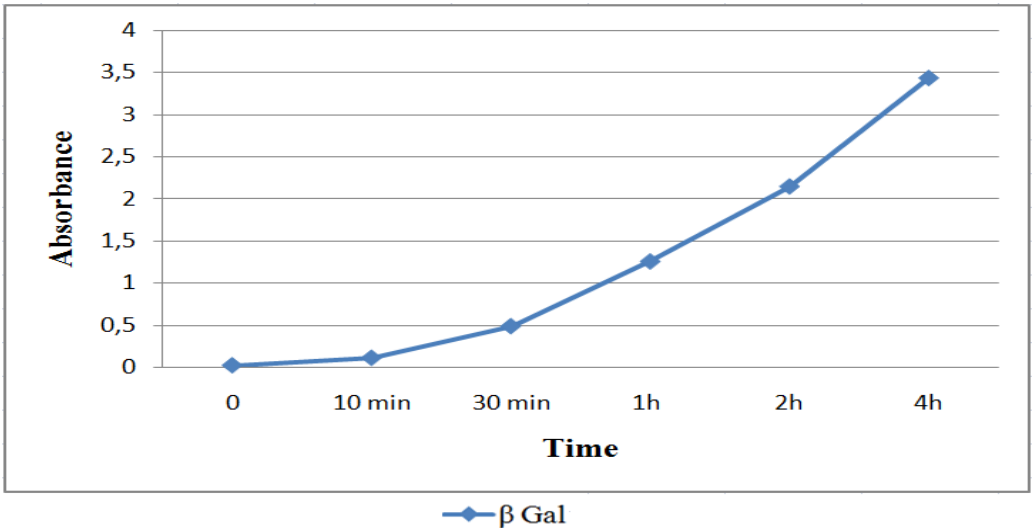


Figure (37). β-galactosidase activity after incubation at 37°C for several times.

Enzyme incubation at 37°C indicated that β-galactosidase reached its maximum activity after 4 hours.

3.3 Purification of a β-galactosidase from *Planorbarius corneus*

Thirty eight snails were taken from the shells; abdominal cavity was removed and the rest was weighed (40g). Sample was prepared and protein extracted as described previously in *Arion lusitanicus* section.

3.3.1 Hydrophobic interaction chromatography

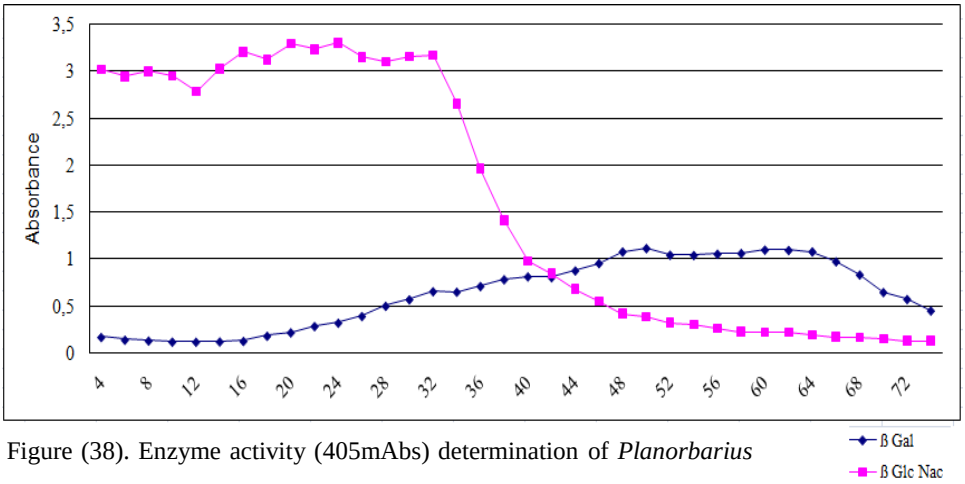


Figure (38). Enzyme activity (405mAbs) determination of *Planorbarius* after HIC

Fractions 40 to 70 (70ml) were pooled, concentrated and desalted by ultrafiltration, the buffer was changed to 50 mM Tris/Hcl pH 7.5 and applied to the next step.

3.3.2 Affi-gel blue

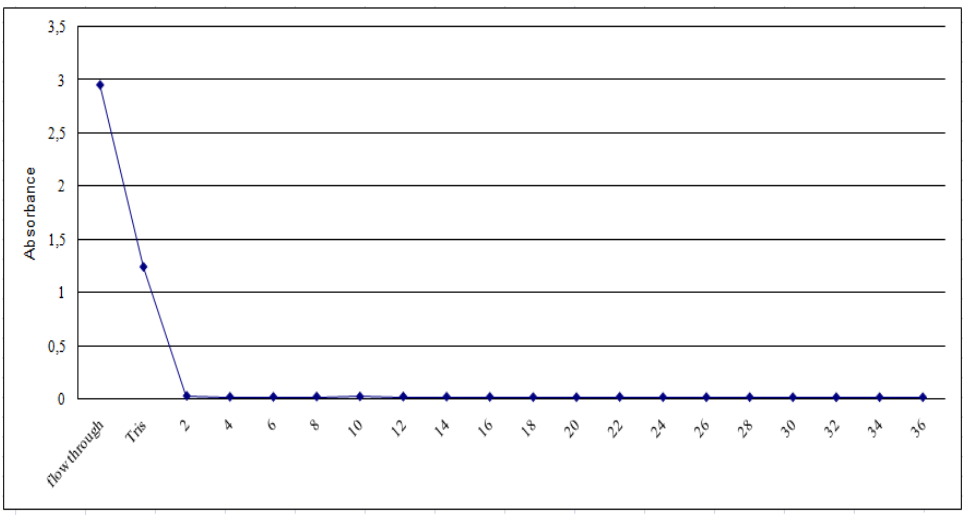


Figure (39). Enzyme activity (405mAbs) determination of *Planorbarius* after affi-gel blue.

Protein purification from gastropods.

The first 2 fractions were pooled and applied directly to the next step.

3.3.3 Anion exchange chromatography

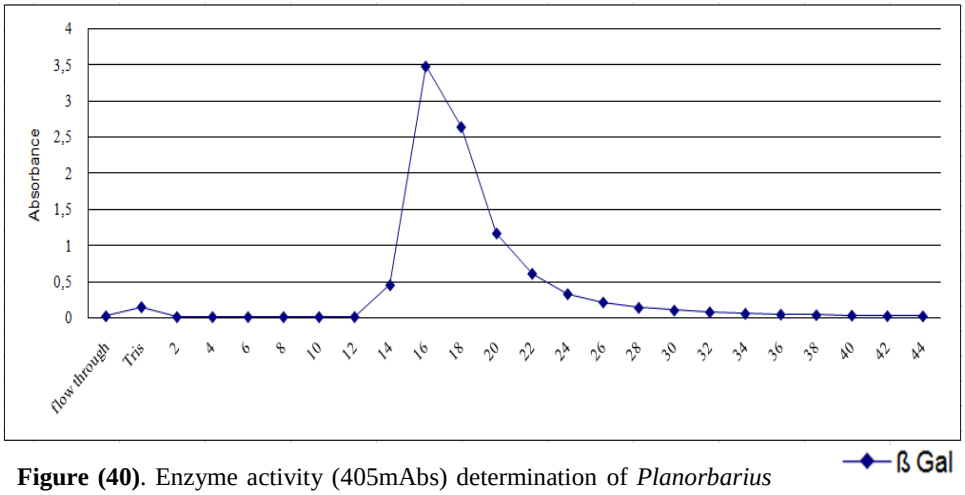


Figure (40). Enzyme activity (405mAbs) determination of *Planorbarius* after anion exchange chromatography.

Fractions 14 to 22 (11 ml) were pooled, concentrated to about 1.8 ml and applied directly to the last purification step.

3.3.4 Size exclusion chromatography

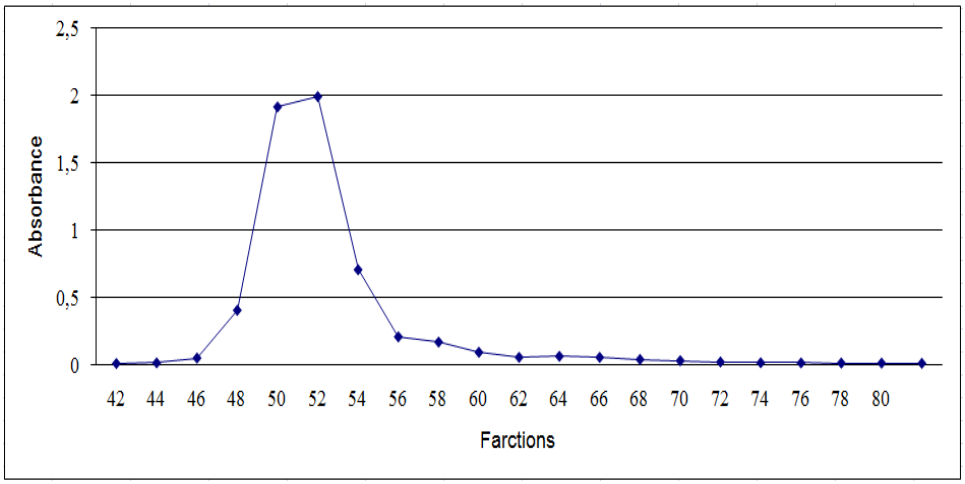


Figure (41). Enzyme activity (405mAbs) determination of *Planorbarius* after size exclusion chromatography.

Those fractions showed high enzyme activity were exposed to 1D SDS-PAGE.

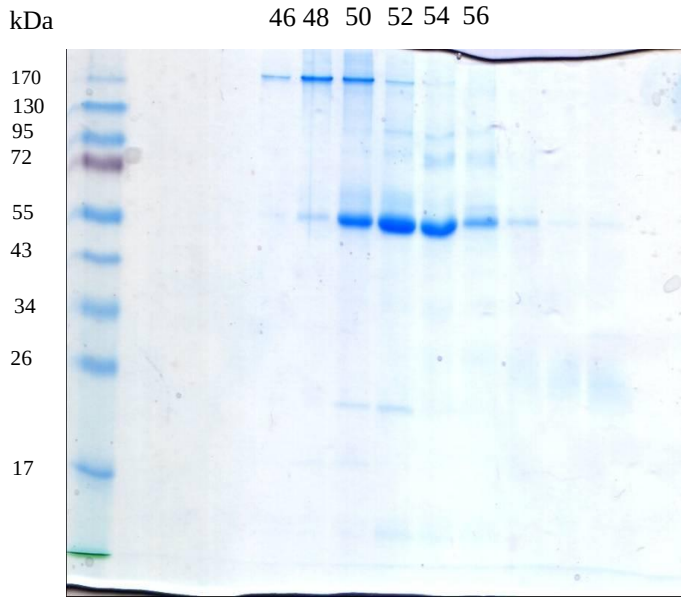


Figure (42) SDS-PAGE of Planorbarius fractions from S_{200}

The same SDS-PAGE was exposed to silver staining in order to visualize the very small protein bands.

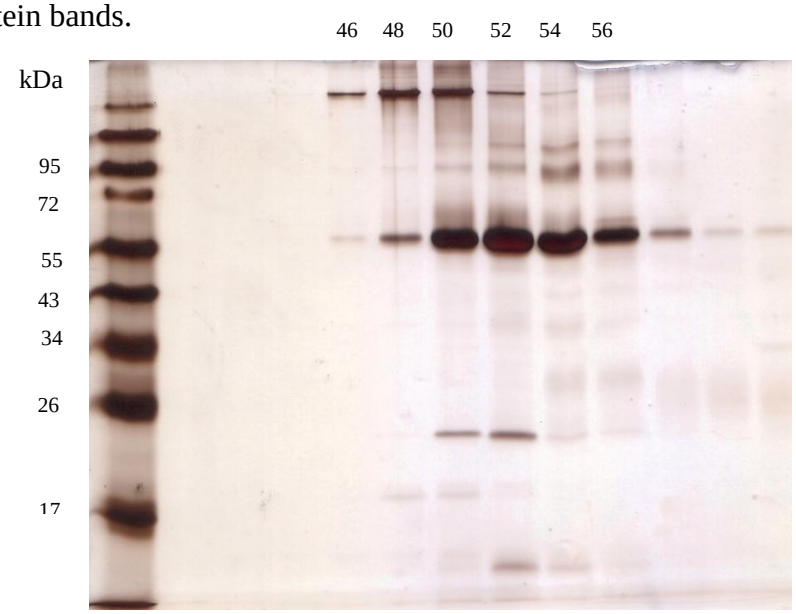


Figure (43) Silver staining of the SDS-PAGE of Planorbarius fractions from S_{200}

Protein purification from gastropods.

There was no any probability to find our target enzyme according to this gel especially when it compared with the activity curve (figure 41).

3.4 Purification of the enzyme from *Achatina fulica*

Snails were used in this experiment; they were removed from the shell, the digestive tract was removed and protein was prepared as previously described.

3.4.1 Hydrophobic interaction chromatography

The hydrophobic interaction chromatography was performed by batch purification.

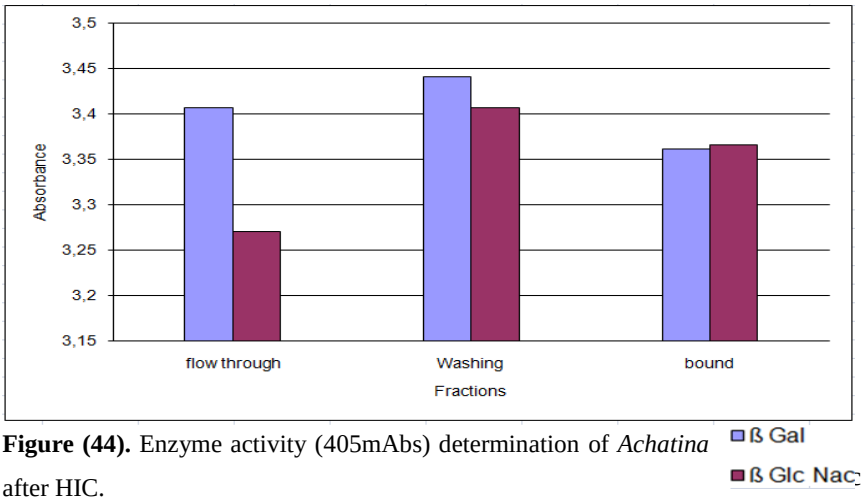


Figure (44). Enzyme activity (405mAbs) determination of *Achatina* after HIC.

The bound fraction was desalted by ultrafiltration and applied to the next purification step.

3.4.2 Affi-gel blue

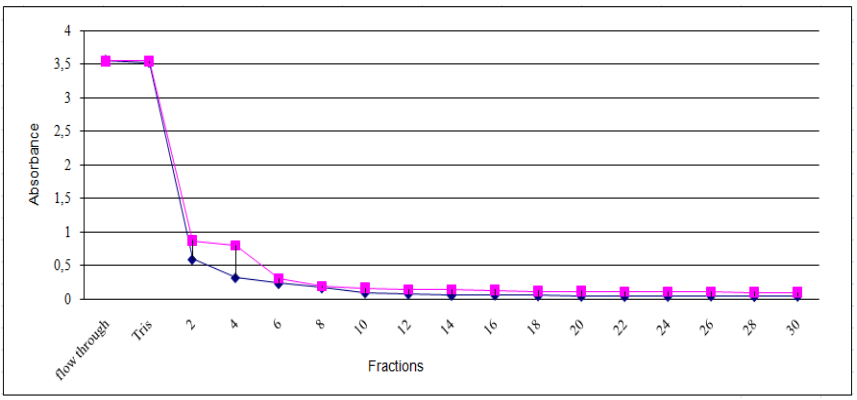


Figure (45). Enzyme activity (405mAbs) determination of *Achatina* after affi-gel blue.

The first two fractions were applied directly to the next step.

3.4.3 Anion exchange chromatography

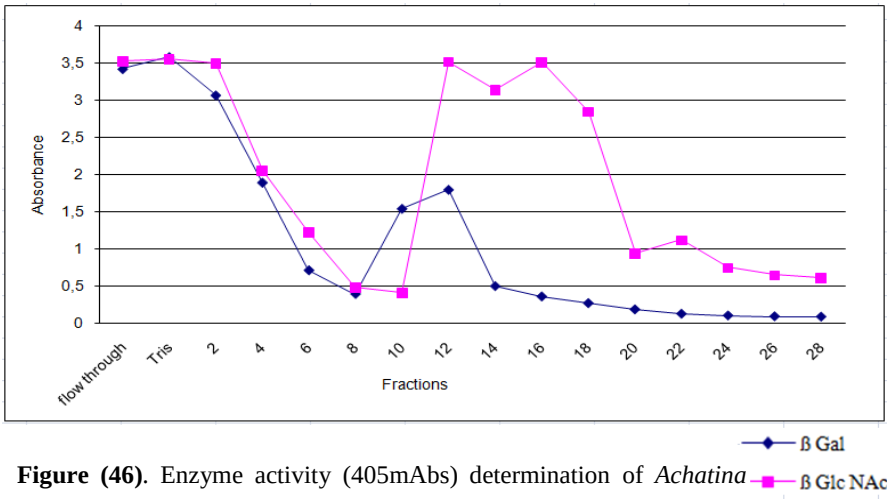


Figure (46). Enzyme activity (405mAbs) determination of *Achatina* after anion exchange.

The first 6 fractions were pooled (56 ml), concentrated to about 2 ml and applied to the next step.

3.4.4 Size exclusion chromatography

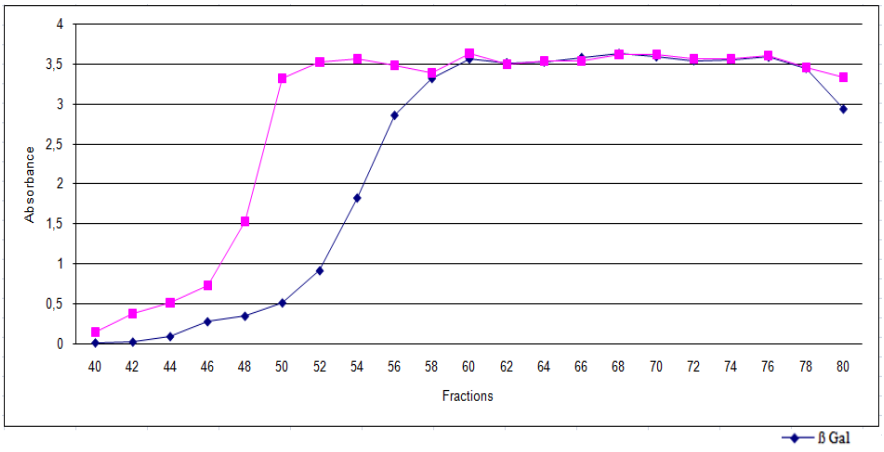


Figure (47). Enzyme activity (405mAbs) determination of *Achatina* after gel filtration.

Fractions 55 to 70 were equilibrated by NaCitrate pH 4.6, concentrated to 4 ml and applied to β-galactosidase affinity column.

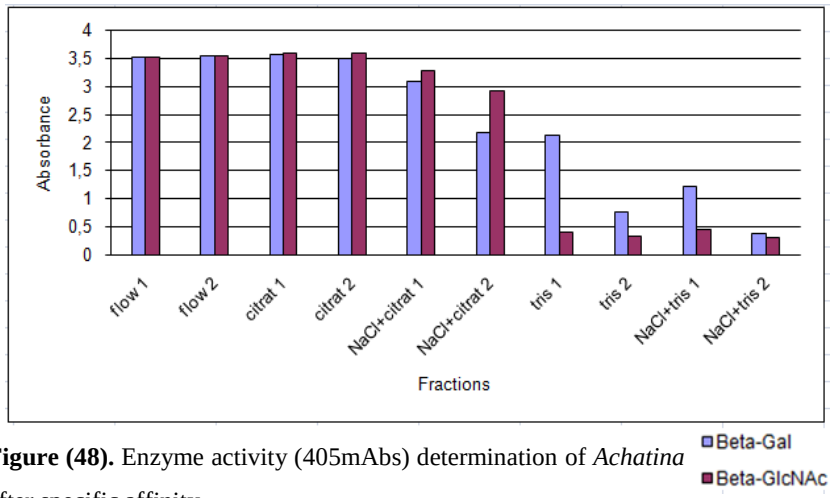
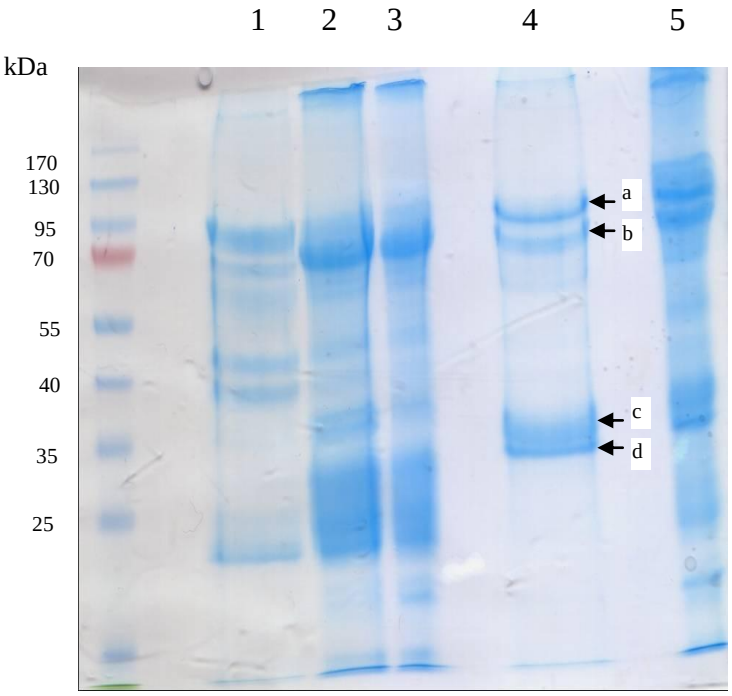


Figure (48). Enzyme activity (405mAbs) determination of *Achatina* after specific affinity.



1- NaCl Citrate fraction
2- Tris fraction
3- Tris fraction
4- NaCl Tris fraction
5- NaCl Tris fraction

Figure (49) SDS-PAGE of *Achatina fulica.*

Different proteins were identified by LC-MS (pointed by black arrows). Proteins from top to down are (a) tropomyosin, (b) paramyosin, (c) tropomyosin and (d) paramyosin.

3.5 Purification of the enzyme from *Biomphalaria glabrata*

28 snails were used in this study and the experiment was performed as previously done in other experiments.

3.5.1 Hydrophobic interaction chromatography

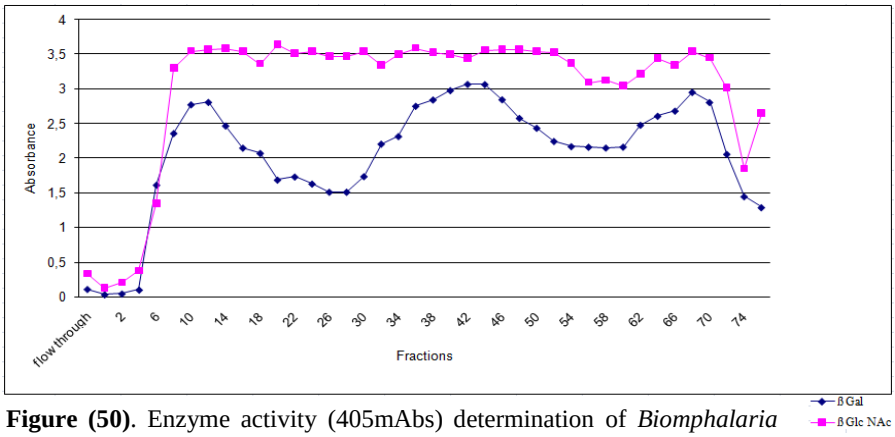


Figure (50). Enzyme activity (405mAbs) determination of *Biomphalaria*

Fractions 7 to 27 were pooled, desalted by ultrafiltration using 50 mM Tris/HCl, pH 7,5 and applied to affi-gel blue.

3.5.2 Affi-gel blue

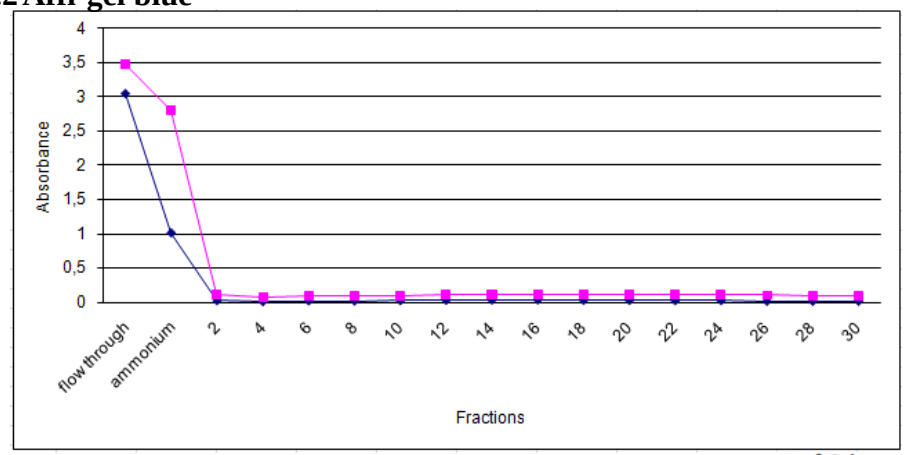
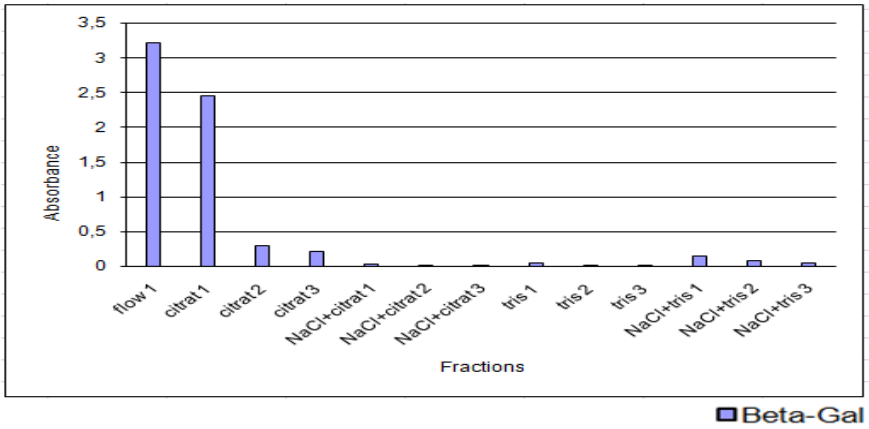


Figure (51). Enzyme activity (405mAbs) determination of *Biomphalaria* after affi-gel blue.

The first 2 fractions were applied to anion exchange chromatography column.

3.5.3 Anion exchange chromatography



The first 2 fractions (45 ml) were concentrated to about 2 ml and applied to gel filtration column.

3.5.4 Size exclusion chromatography

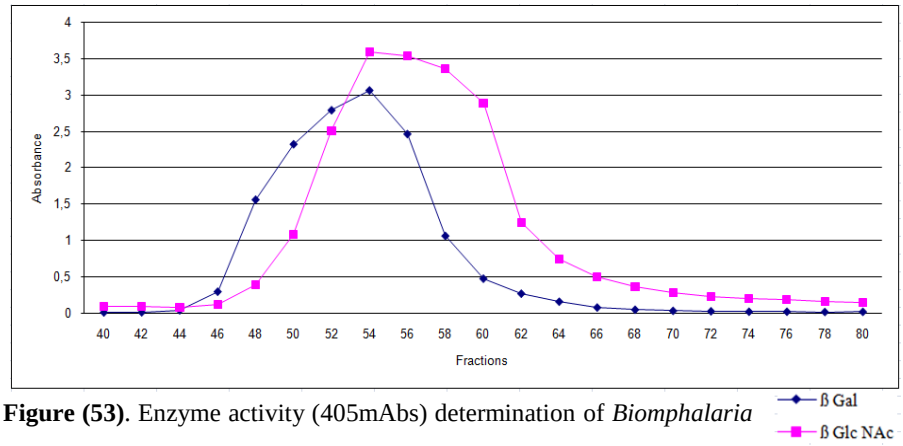


Figure (53). Enzyme activity (405mAbs) determination of *Biomphalaria* after size exclusion chromatography.

Fraction 47 to 60 were pooled, equilibrated by 50 mM NaCitrate pH 4.6 and applied to β-galactosidase affinity column (Aminophenyl gel).

3.5.5 β-galactosidase affinity chromatography

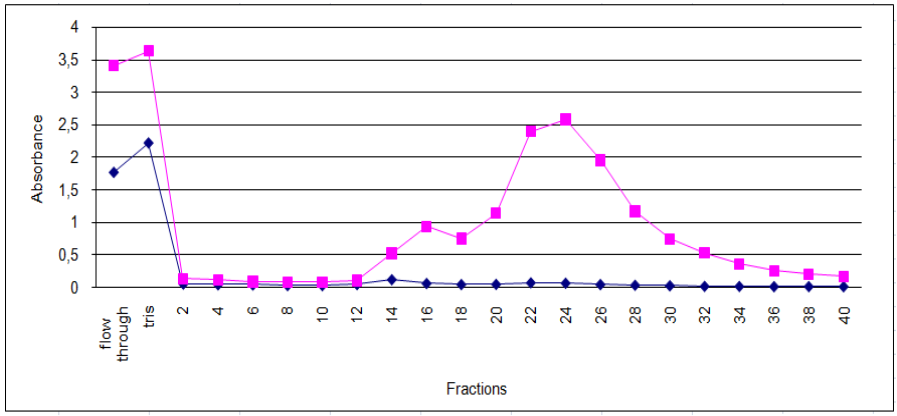
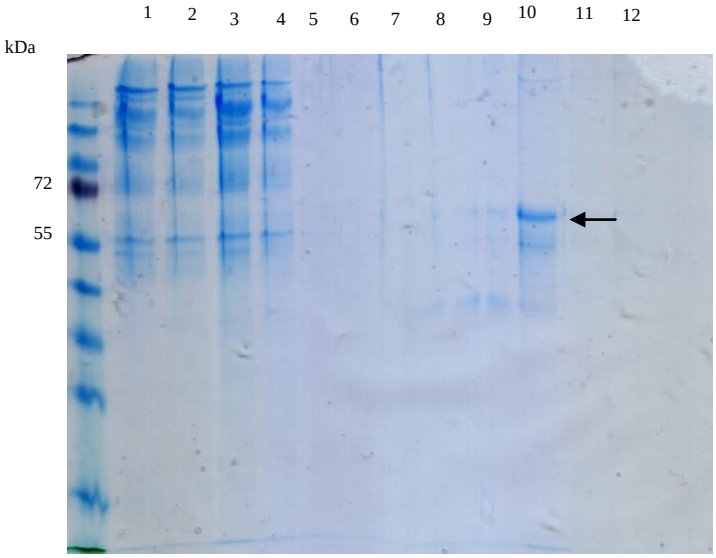


Figure (52). Enzyme activity (405mAbs) determination of *Biomphalaria* after anion exchange chromatography.



Figre (55) SDS-PAGE of *Biomphalaria glabrata*

NaCl Tris fraction (10). The band pointed by the black arrow was analyzed by LC-MS and identified as keratin.

4. Native gel to catch β-galactosidase

Some of the residual S200 fractions of Arion lusitanicus were pooled (10 ml), concentrated to 0.5 ml by ultrafiltration and applied to native gel electrophoresis. Only the first lane from the gel stained by coomassie to use it as a marker for the non-stained lanes (figure 56).

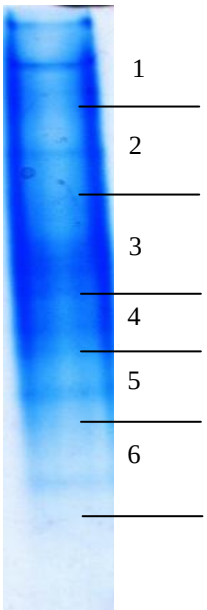


Figure (56). The coomassie stained lane from native gel electrophoresis.

The non-stained lanes were cut horizontally in 6 different pieces according to the most visible bands in the coomassie stained lane. Protein from these pieces was separately eluted by passive elution (overnight at 4°C) and tested for the activities of β-galactosidase and β-N-acetylglucosaminidase (figure 57).

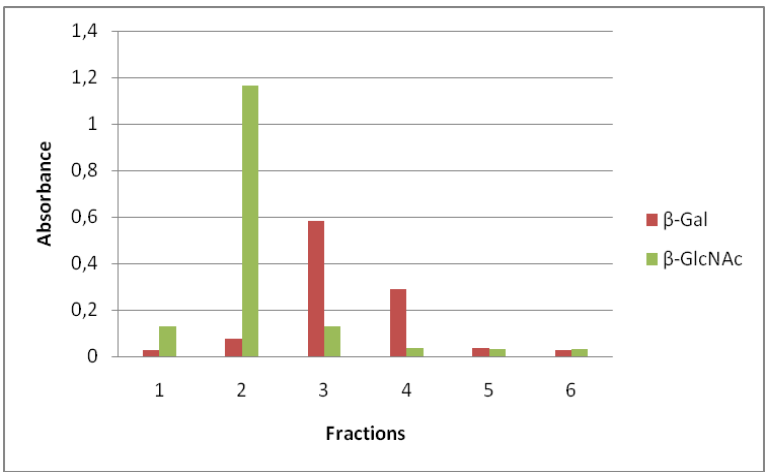


Figure (57). Activity of passively eluted β-gal and β-GlcNAc from the native gel electrophoresis.

As shown in figure that the highest activity of β-galactosidase is existed in fraction 3. The resulted fractions were exposed to normal SDS-PAGE (figure 58) to visualize the enzyme.

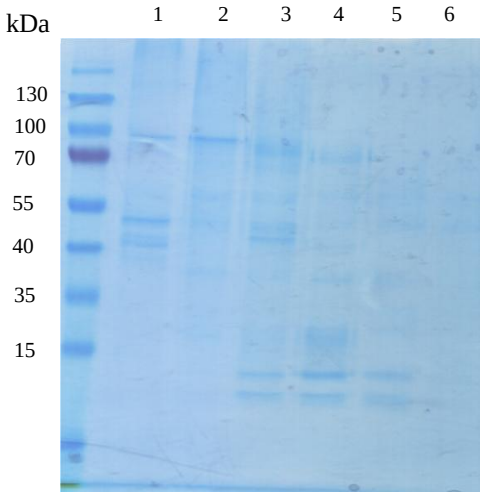


Figure (58) SDS-PAGE of the passively eluted proteins after native gel electrophoresis.

From the previous gel it was clear that there are still remaining many co-purified proteins which made it difficult to recognize the target enzyme.

5. Other identified snails' proteins

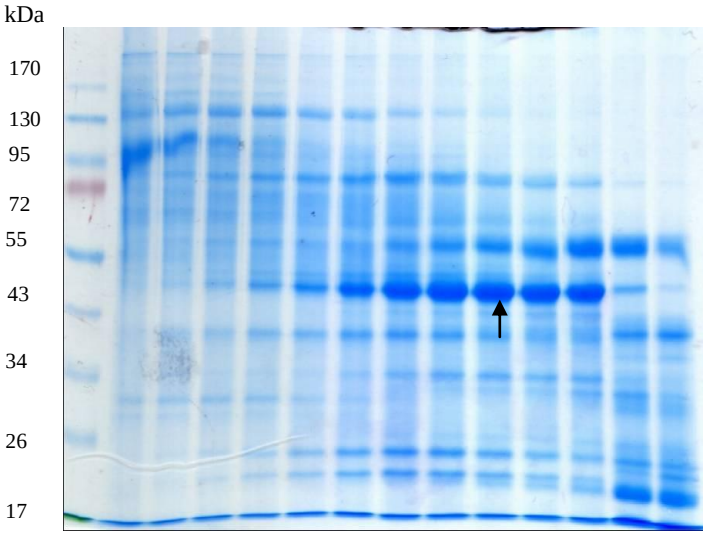


Figure (59) SDS-PAGE with identified Enolase of *Arion lusitanicus.*

Protein purification from gastropods.

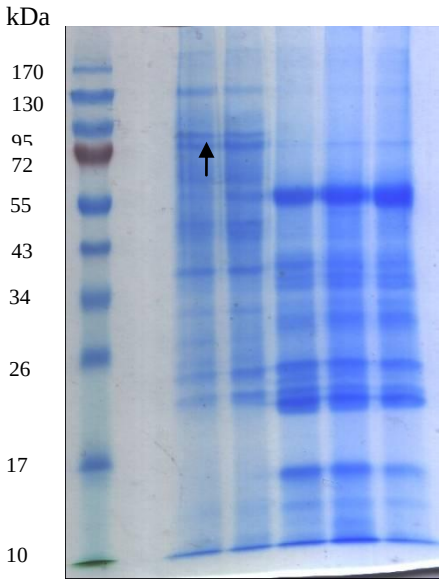


Figure (60) SDS-PAGE with identified α-N-acetylglucosaminidase of *Arion lusitanicus*

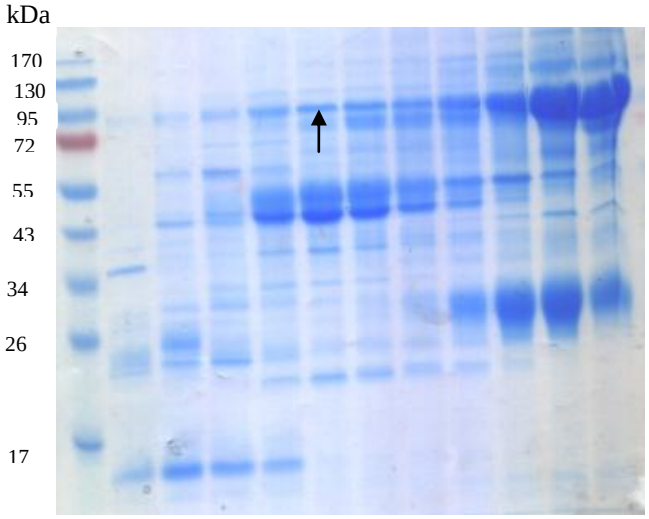


Figure (61) SDS-PAGE with identified Glycosyl transferase of *Achatina fulica.*

6. Lectins

Snail´s tissues (skin and viscera) and eggs from *Arion* egg, *Achatina* egg, *Biomphalaria* egg, *Planorbarius* skin, *Planorbarius* viscera and *Planorbarius* egg were analyzed for their glycosylation patterns and in order to enhance our knowledge about their glycosylation capacity, two different digestions were performed. The first one was PNGase digestion (to release the N-glycans) and second one was a chemical digestion by β-elimination (to remove the O-glycans).

In order to study the fucosylation capacity of snail´s tissue, four different fucose recognizing lectins were used, *Lotus tetragonolobus* (LTA, recognizes α1,2-fucosylated H blood group as well as Le^x-structures), *Aleuria aurantia* (AAL, recognizes core α1,6- fucosylated N-glycans), *Ulex europaeus* (UEA, specific for α1,2-fucosylated H blood group glycans) and *Lens Culinaris* (LCA, recognizes α-mannosyl residues with an enhanced binding if there is an α1,6-fucose linked to the N-acetylglucosamine). Furthermore *Sambucus nigra agglutinin* (SNA) was also used to investigate the existence of α2,6-linked sialic acid linked to galactose or N-acetylgalactosamine residues.

For each tissue the protein pattern and the digests are shown on SDS-PAGE (Figures 62 and 63), four blots with fucose specific lectins (Figures 64, 66, 67 and 68) and one blot with the sialic acid specific lectin (Figure 65).

Blots (A) and (B) showed that except for AAL, *Achatina* egg expressed many positive signals, where it was clear that many proteins are fucosylated. The patterns of binding of the fucose specific lectins were quite weak in *Arion* egg, *Biomphalaria* egg and *Planorbarius* egg. Unlike all fucose recognizing lectins used, AAL gave no signal or positive bands with different tissues as well as eggs.

Digestion by PNGase F and β-elimination in gel (A) showed no major differences in the pattern of binding while it was observed that the digested protein gave quite lower signal intensity. In the blots of gel (B), the digestion was more pronounced

where the undigested *Planorbarius* skin had completely different fucosylation and sialylation capacity in the time the digested ones lost the fast majority of the signal. Surprisingly, although that SNA, UEA and LTA have different specificities they expressed quite similar patterns in blots of gel (B) and with a lesser extent in the blots of gel (A).

- **Protein digestion and electrophoresis**

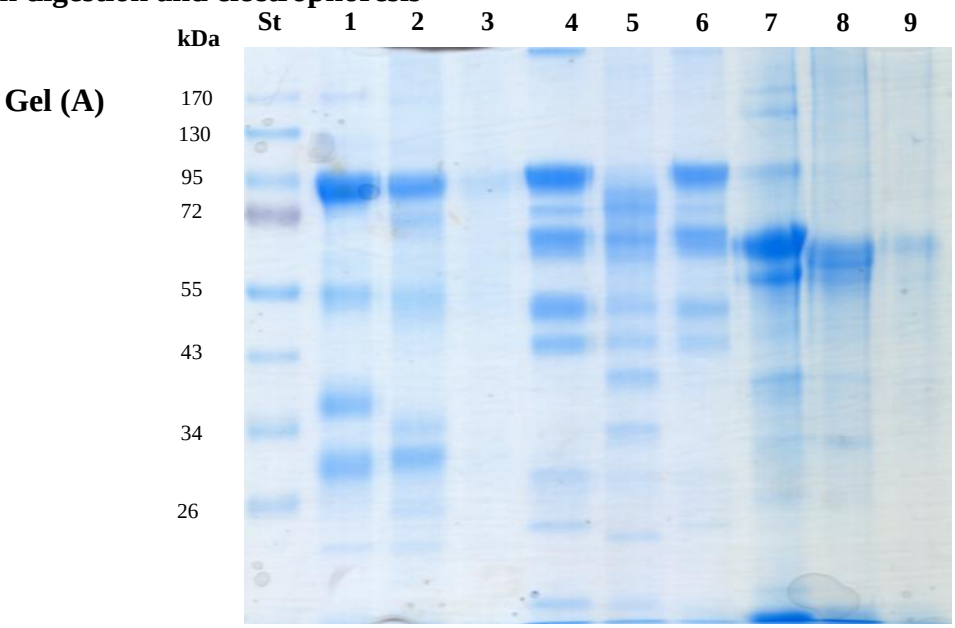


Figure (62) SDS-PAGE of eggs from *Arion*, *Achatina* and *Biomphalaria* (undigested and digested with PNGaseF and β-elimination).

St- Protein ladder
1- *Arion* egg (undigested)
2- *Arion* egg (PNGase F)
3- *Arion* egg (β-elimination)
4- *Biomphalaria* egg (PNGase F)
5- *Biomphalaria* egg (β-elimination)
6- *Biomphalaria* egg (undigested)
7- *Achatina* egg (undigested)
8- *Achatina* egg (PNGase F)
9- *Achatina* egg (β-elimination)

Gel (B)

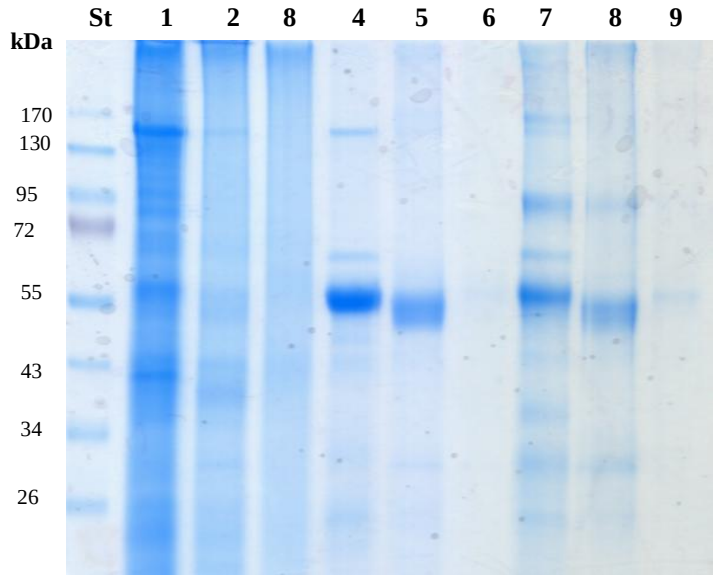


Figure (63) SDS-PAGE of *Planorbarius* skin, viscera and egg (undigested and digested with PNGaseF and β-elimination)

St- Protein ladder
1- Planorbarius skin (undigested)
2- Planorbarius skin (PNGase F)
3- Planorbarius skin (β-elimination)
4- Planorbarius viscera (undigested)
5- Planorbarius viscera (PNGase F)
6- Planorbarius viscera (β-elimination)
7- Planorbarius egg (undigested)
8- Planorbarius egg (PNGase F)
9- Planorbarius egg (β-elimination)

Blots of ge (A)

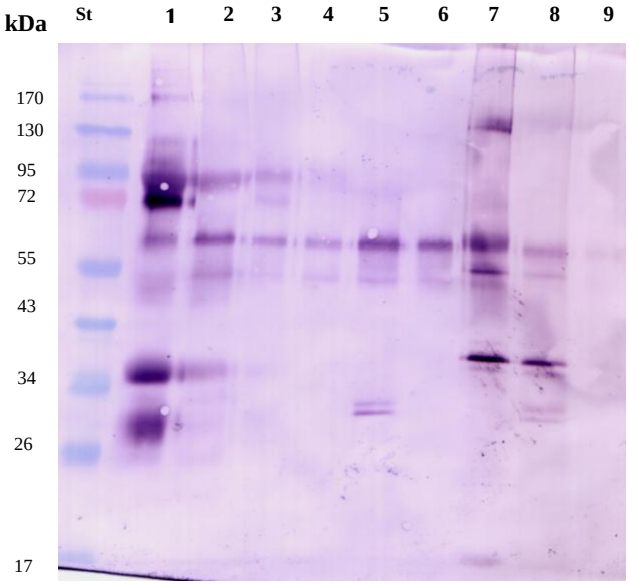


Figure (64) Lectin blot with *Lens culinaris* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

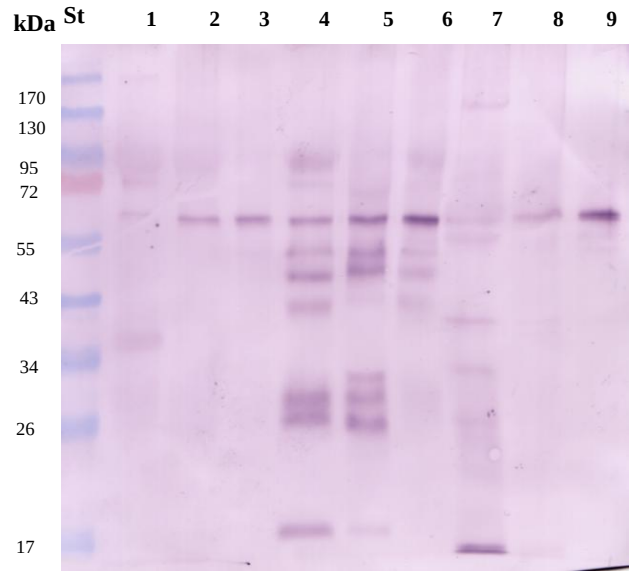


Figure (65) Lectin blot with *Sambucus nigra* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

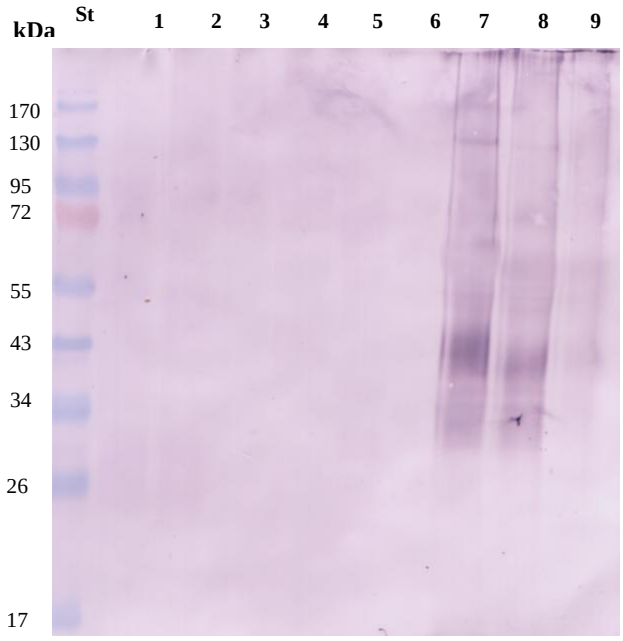


Figure (66) Lectin blot with *Aleuria aurantia* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

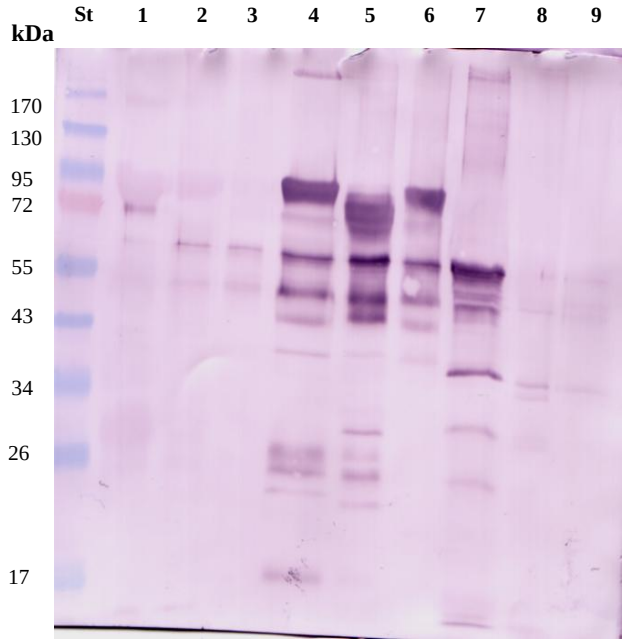


Figure (67) Lectin blot with *Ulex europaeus* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3.6.9 after mild ß-elimination.

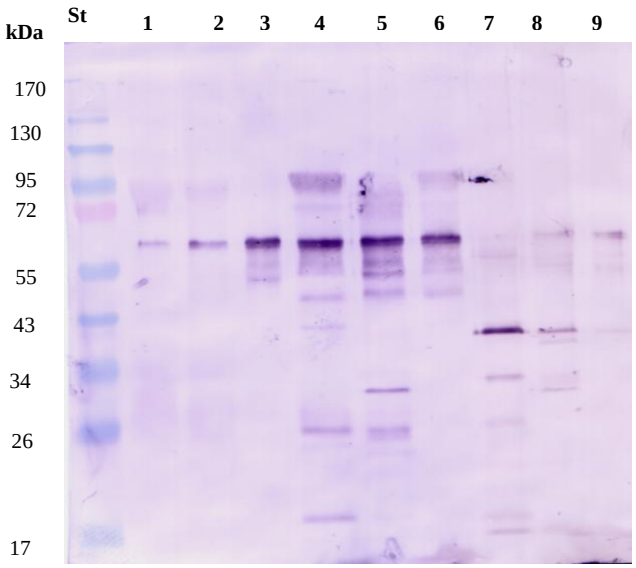


Figure (68) Lectin blot with *lotus tetragonolobus* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3.6.9 after mild ß-elimination.

St- Protein ladder

5- *Arion* egg (undigested)

6- *Arion* egg (PNGase F)

7- *Arion* egg (β-elimination)

8- *Achatina* egg (undigested)

9- *Achatina* egg (PNGase F)

10- *Achatina* egg (β-elimination)

11- *Biomphalaria* egg (undigested)

12- *Biomphalaria* egg (PNGase F)

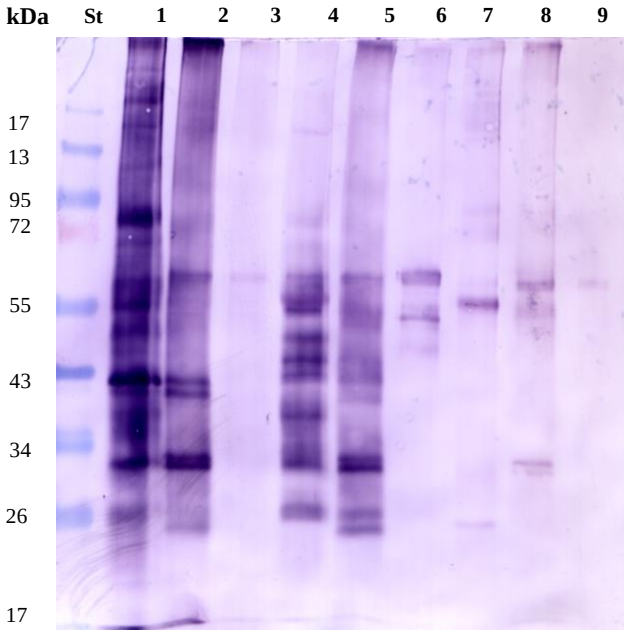


Figure (69) Lectin blot with *Lens culinaris* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

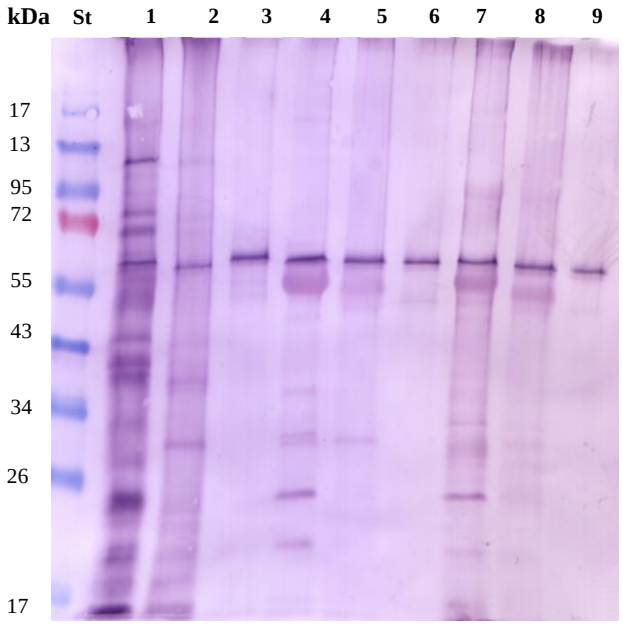


Figure (70) Lectin blot with *Sambucus nigra* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

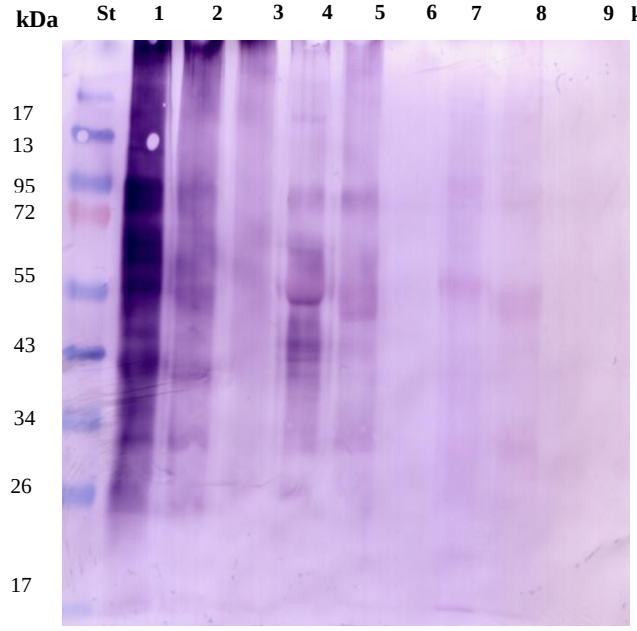


Figure (71) Lectin blot with *Aleuria aurantia* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

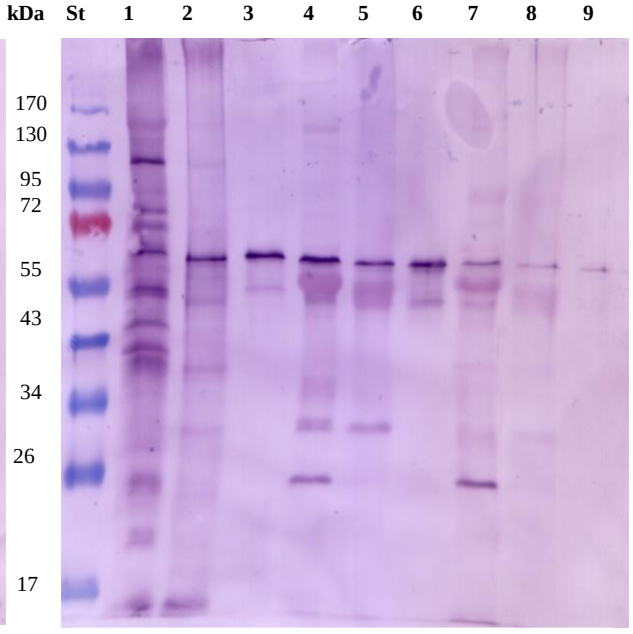
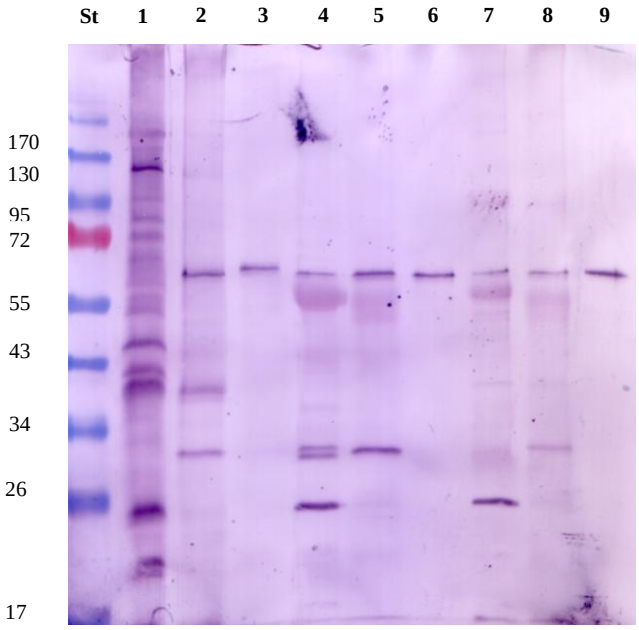


Figure (72) Lectin blot with *Ulex europaeus* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.



St- Protein ladder

1- *Planorbarius* skin (undigested)
2- *Planorbarius* skin (PNGase F)
3- *Planorbarius* skin (β-elimination)
4- *Planorbarius* viscera (undigested)
5- *Planorbarius* viscera (PNGase F)
6- *Planorbarius* viscera (β-elimination)
7- *Planorbarius* egg (undigested)
8- *Planorbarius* egg (PNGase F)
9- *Planorbarius* egg (β-eliminatio)

Figure (73) Lectin blot with *lotus tetragonolobus* agglutinin. 1,4,7 are undigested; 2,5,8 after digestion with PNGaseF; 3,6,9 after mild β-elimination.

7. Discussion

β-galactosidase is one of the major players in the exo-glycosidases group which has been shown to function on the secreted glycoprotein and lead to a significant change of the heterogeneity of the glycoforms. With the evolution in glycobiology, it became very necessary to characterize those e enzymes which are involved in the glycosylation process and to tie them with other cellular functions.

Among different references concerning β-galactosidase purification and characterization it was found that the purification of this enzyme needs several steps even with the simple or lower organisms (for example different *E.coli* strains) and it was quite a challenging task to purify the enzyme from a complete and complex (with wide proteome map) organism like *Arion lusitanicus*. Several preliminary experiments were performed in order to optimize all purification steps until the purification scheme is established. The nature of *Arion´s* tissue was also a big problem especially with the column chromatography because it was always probable that the protein mixture stuck to the column or may be other unknown materials interfere with different beads and lead to bad resolution or not effective purification.

Our preliminary work on exoglycosidases screening indicated that β-N-acetylglucosaminidase is a co-purified enzyme and always eluted with β-galactosidase which may be due to the facts that they share some characteristics therefore we have always determined this enzyme at the same time with our target enzyme in order to eliminate as much as possible from this co-purifying enzyme.

The first purification step was done by ammonium sulfate fractionation (precipitation), so the resulting sample is already in very high salt concentration and is ready to be loaded onto a HIC column. Theoretically the amino acids which contain hydrophobic groups will interact with the stationary column and this interaction is very strong with high salt concentration media and neutral pH, therefore decreasing salt concentrations result in separation and elution of those proteins containing these amino acids (El Rassi et al., 1990). Not surprisingly,

decreasing ammonium sulfate concentration in the performed HIC resulted in elution of the enzyme which may be due to the fact that β-galactosidase contains quite a lot of hydrophobic groups which need a decrease in salt concentration to be released from gel beads.

The majority of protein was eluted in the flow through which resulted in a efficient purification effect for the target enzyme.

There was only weak binding between β-galactosidase and gel beads of affi-gell blue. This was inconsistent to the finding of Steers et al. 1971 who indicated that the affinity chromatography yielded high quantitively purified enzyme. The exact reason of this weak binding may be probably due to the used pH, as it is known that the affinity chromatography is functioning well at pH higher than 10. But this represents a critical risk to the β-galactosidase activity which displays relatively low pH optima. There seems to be no competition with the other proteins for binding sites in the column.

The 4^{th} purification step was ion-exchange chromatography or specifically anion-exchange chromatography which revealed two different peaks; the flow through, tris and salt gradient eluted enzyme which indicate quite complicated purification step. It seems that the eluted β-galactosidase from salt gradient expressed slight reduction in specific activity; this reduction was due to unknown reasons and probably due to enzyme denaturation during the chromatography process, on the other hand and according to our knowledge denaturation can happen as a consequence of inadequate buffering or extreme pH.

Size exclusion chromatography is considered as final purification step and particularly suited when the purified extract has low impurities and the target protein has been partially purified and concentrated by earlier chromatography steps. In order to check the efficiency of the S_{200} column, the enzymatically active fractions were exposed to electrophoresis which unfortunately exhibited different bands with wide range of molecular weights in the fractions. So at this step our enzyme was not

100% pure but we were able to detect our enzyme on the gel by comparing bands existence and strength with the enzyme activity curve. At this point we have found ourselves in front of one explanation based on S_{200} function; it is well known that S200 column separate biological macromolecules by hydrodynamic size and we strongly believe from the electrophoresis of earlier purification steps that our purified proteins may occur also in a polymeric. This could be an explanation for the electrophoretic obtained results.

Electrophoresis after gel filtration showed us how complex the enzyme purification from snail´s tissues is which led us to use the last card in the purification system which is affinity chromatography using *p*- Aminophenyl β-D-thiogalactopyranoside-Agarose and *p*-Aminobenzyl 1-thio-β-D-Galactopyranoside-Agarose. Among different purifications studies concerning β-galactosidase purification and characterization for example from *E.Coli* (Gazorla et al., 2001) and *Penicillium chrysogenum* (Nagy et al., 2001), it seems that it will be easy to use such columns but in the real purification life not. We have tried to purify the same enzyme from snails like *Achatina fulica* and *Biomphalaria glabrata*, unfortunately we couldn´t and this is may be due to the nature of the enzyme existed in such organisms. Fortunately, we were able to purify the enzyme using these columns only from *Arion lusitanicus* which was quite tricky because there is no stated purification procedure or known buffers to be used in order to elute the bound enzyme. In some publications, they used high pH buffers (10 or more) and also some detergents like mercaptoethanol, on the other hand using such detergent and high pH is very dangerous for enzyme activity.

Although that very big amount of β-galactosidase was eluted in the very early fractions; we have missed the activity in the middle fractions while the late obtained peaks indicate that the enzyme bound specifically to the gel beads. The early eluted enzyme may be due to existence of other forms of the β-galactosidase.

The advantageous pH optimum for β-galactosidase from *Arion lusitanicus* is between 3.5 and 4.5. however the optimal pH for activity is not the optimal pH for long term storage, the enzyme half life may be become shorter. Through many published studies, we have found so many contradictions where it was so clear that our enzyme behave uniquely with the surrounded pH in contrast to others who observed that their β-Galactosidase is active in very wide pH range (Crueger and Crueger 1984) and also for those who found that the activity of β-galactosidase is falling on higher pH values (Miyazaki, 1988; Fischer *et al*., 1995). According to that we can suggest that the activity of β-galactosidase activity is completely depending on the organism which has been extracted from.

Exposing β-galactosidase to different temperatures clearly indicated that the enzyme is considered as weak enzyme or by other description is not strong a thermo-tolerant enzyme. By going back to the few literature concerning this issue, it was observed that the studied thermo-stable β-galactosidase was extracted from thermophilic organisms regardless to localization of the enzyme (intra or extracellular. This is supported by the finding of Lind et al. (1989) who found that β-galactosidase from *thermoanaerobacter* sp. has half life of 2.58h at 75°C. This previous observation indicates also that our enzyme is sensitive to high temperatures.

β-galactosidase from *Arion lusitanicus* needs no cations in order to be activated. This is supported by the fact that the chelating agent, EDTA also has no influence on the activity. Metals also do not play any role in β-galactosidase activation. This finding is concomitant with the finding of Fischer et al. (1995).

Fucose specific lectins

Blots obtained from fucose specific lectins (LCA, LTA, AAL and UEA) indicated a wide pattern of fucosylated proteins with an exception for AAL which showed no binding capacity with the snail´s protein which was quite unexpected because it binds preferentially to core α1,6- fucosylated N-glycans which may indicate a lack of this core fucose in snail´s N-glycans (Figure 70).

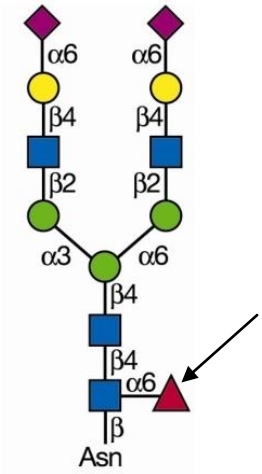


Figure (74) fucosylated N-glycan.

But although that AAL and LCA share some common features in terms of specificities but they exhibit completely different pattern of binding (Matsumura et al, 2007).

Fucose residues have been found in three different types of linkage (α1,2, α1,3 or α1,4) in terminal position of N-glycans as well as α1,3- or/and α1,6 linked to the inner N-acetylglucosamine, therefore it is one of the most variable sugar compounds in glycans.

The LCA blot was in all cases the most intense one in terms of number of recognised bands. *Lotus tetagonolobus* agglutinin (LTA) is specific for the α1,2-fucosylated H blood group glycan as well as Le^x-structures (Iskratsch et al. 2009). Also with this lectin all snail tissues gave some stained bands. *Aleuria aurantia* lectin (AAL) shows high affinity to core α1,6-fucosylated N-glycans which is enhanced by an additional α1,3-linked fucose residue in terminal position (Debray and Montreuil, 1989).

It was also clear that *Ulex europaeus* agglutinin (UEA-I) interacted positively with many proteins from different tissues but mainly those from egg. This observation indicates that α1,2-fucosylated glycans are quite abundant in snail´s tissues. Fortunately and as its observed from blots, *Lotus tetragonolobus* (LTA) and UEA have similar specificities (α1,2-fucosylated H blood group glycans), this was

obvious because they have a similar pattern but with quite different bands intensity. This is concomitant with other published observation concerning both lectins (Lis and Sharon, 1998).

Bark lectin from eldberry or *Sambucus nigra agglutinin* (SNA) was known to bind specifically to Neu5Acα2,6 Gal/GalNAc structure and is intensively used to study the sialoglycoconjugates.

It was quite confusing because that all studied eggs showed similar pattern in SNA, LTA and UEA although they have different specificities. It was also observed that blotting eggs with SNA showed similar pattern with UEA and the same observation was also between SNA, UEA and LTA in *Planorbarius corneus*.

8. Reference

Altmann, F., Schweiszer, S., and Weber, C. (1995) Kinetic comparison of peptide: N-glycosidases F and A reveals several differences in substrate specificity." Glycoconjugate Journal 12: 84-93.

Barker, G. M. and Mayhill, P. C., (1999). Patterns and habitat relationships in terrestrial mollusk communities of the pukeamaru Ecological District, northeastern New Zealand. *J. Biography.* 26: 215-238.

Barondes, S.H. (1988) Bifunctional properties of lectins: Lectins redefined. *Trends Biochem. Sci*. 3: 480–482.

Berry, F. G., (1973). Patterns of snail distribution at Ham Street Woods National Nature Reserve, East Kent. *J. Choncology*. 28: 23-35.

Bojarova´, P and Kr˘en, V. (2009). Glycosidases: a key to tailored carbohydrates. Trends in Biotechnology. 27:199-209

Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B. and W. J. Peumans (1984*) A lectin from elder* (Sambucus nigra L.) Bark. Biochem. J. 221: 163-169

Cain, A. J., (1983). Ecology and ecogenetics of terrestrial Molluscan populations. In: Russel-Hunter, Ed. *The Mollusca. Vol. 6, Ecology*. Academic press, New York, 597-647.

Cazorla D., Feliu J. X., Villaverde A. (2001). Variable specific activity of Escherichia coli beta-galactosidase in bacterial cells. Biotechnology and bioengineering 72(3):255-60.

Crueger, A., and Crueger, W. **(**1984). Carbohydrates, p. 421–457. *In* K. Kieslich (ed.), Biotechnology, vol. 6a. Verlag Chemie, Weinheim, Germany.

Debray, H. and Montreuil, J. (1989) Aleuria aurantia agglutinin. A new isolation procedure and further study of its specificity towards various glycopeptides and oligosaccharides. Carbohydr Res. 185(1):15–26.

Dickson, R. C., Dickson, L R. and Marklin J. S. (1979). Purification and properties of an inducible, B-galactosidase isolated from the yeast Kluyveromyces lactis. *J. Bacteriol*. 137:51-61.

Drickamer. K. (1981). Complete amino acid sequence of a membrane receptor for glycoproteins. Sequence of the chicken hepatic lectin *J. Biol. Chem.* 256: 5827-5839.

Drickamer, K. and Taylor, M. E. (1993). Biology of animal lectins. Annu Rev Cell Biol. 9: 237-264.

El Rassi, Z., De Ocampo, L. F. and Bacolod, M. D. (1990). Binary and ternary salt gradients in hydrophobic interaction chromatography of proteins. *J. Chromatog*. 499: 141-152.

Ernst S., Langer R., Cooney CL. and Sasisekhran R (1995) Enzymatic degradation of glycosaminoglycans. *Crit. Rev. Biochem. Mol. Biol.* 30:387–444.

Fischer, L., Scheckermann, C. and Wagner, F. (1995). Purification and characterization of a thermotolerant β-Galactosidase from *thermomyces lanuginosus.* Appl. Environ. Microbiol. 61: 1497-1501.

Freeze H. H. (2006) Genetic defects in the human glycome. *Nature Reviews Genetics.* 7: 537–551.

Gabius H.J. (1997). Animal lectins. Eur J Biochem; 243**:**543-576.

Gavel, Y. and von Heijne, G. (1990). Sequence differences between glycosylated and non-glycosylated Asn- X-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng 3: 433–442.

Goldstein, I. J., Poretz, R. D. (1986). In The Lectins: Properties, Functions and Applications in Biology and Medicine; Liener, I. E., Sharon, N., Goldstein, I. J., Eds.; Academic Press, Inc.: Orlando; p 35.

Gomot, L., Enée, J. and Laurent, J. (1982). Influence de la photopériode sur la croissance pondéralede l'escargot *Helix aspersa* Müller en milieu contrôlé. *Comptes Redus de l'Académie de sciences, Paris* 294: 749-752.

Gomot de Vaufleury, A. (2001). Regulation of growth and reproduction. Pp. 331–355 in Barker, G.M. 2001: The Biology of Terrestrial Molluscs. CABI Publishing, Wallingford, U.K.

Hammarstrom, S. and Kabat, E.A. (1969). Purification and characterization of a blood-group A reactive hemagglutinin from the snail *Helix pomatia* and a study of its combining site. *Biochemistry* 8: 2696–2705.

Hawkins, J. W., Lankester, M. W. and Nelson, R. R. A. (1998). Sampling terrestrial gastropods using cardboard sheets. *Malacologia,* 39: 1-9.

Heller, J. and Ittiel, H. (1990). Natural history and population dynamics of the land snail *helix texta* in Israel. J. Molluscan studies 56: 189-204.

Hirschberg, C.B. and Snider, M.D. (1987). Topography of glycosylation in the rough endoplasmic reticulu and Golgi apparatus *Annu. Rev. Biochem.* 56: 63-87.

Hodasi, J.K.M. (1979). Life history studies of Achatina achatina (Linè). J. Molluscan studies. 45: 328-339.

Hommay, G., Kienlen, J.C., Gertz, C. and Hill, A. (2001). Growth and reproduction of the slug *Limax valentianus* Férussac in experimental conditions. *Journal of Molluscan Studies* 67: 191–207.

Hudgin, R.L., Pricer, W.E. Jr., Ashwell, G., Stockert, R.J., and Morell, A.G. (1974). The isolation and properties of a rabbit liver binding protein specifi c for asialoglycoproteins. *J. Biol. Chem*. 249: 5536–5543.

Iskratsch T., Braun A, Paschinger K, Wilson IB. (2009). Specificity analysis of lectins and antibodies using remodeled glycoproteins. Anal Biochem. 15:133-146.

Jennifer A., Prescher and Bertozzi R. (2006) Chemical Technologies for Probing Glycans. *Cell* 126: 851-854.

Jensen, J. W. and Schutzbach, J. S. (1984) *Activation of mannosyltransferase II by nonbilayer* phospholipids. *Biochemistry* 23: 1115–1119.

Jourdian, G.W. (1996). Normal and pathological catabolism of glycoproteins *New Compr. Biochem.* 30: 3-54.

Kaku, H., Peumans, W. J, and Goldstein, I. J. (1990). Isolation and characterization of a second lectin (SNA-II) present in elderberry (*Sambucus nigra* L.) bark, *Archives of Biochemistry and Biophysics,* 277: 255–62.

Kornfeld, R., Kornfeld, S. (1985). Assembly of asparagines-linked oligosaccharides. *Ann. Rev. Biochem*. 54: 631-664.

Kornfeld S. (1992*).* Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem*. ;*61*:*307*–330.

Lardans V., Dissous C. (1998). Snail control strategies for reduction of schistomasis transmission. *Parasitology today.* 4: 413-417.

Lind H. (1989). Homing to hibernating sites in Helix pomatia involving detailed long term memory. Ethology 81: 221-234.

Lis H. and Sharon, N. (1998). Lectins: carbohydrate-specific proteins that mediate cellular recogni-tion, *Chem*. *Rev*., 98: 637-674.

Loker, E. S., Hanington, P. C., Lun, C. M. and Adema, C. M. (2010). Time series analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the course of intramolluscan development of *Scistosoma mansoni* and *Echinostoma paraensei.* Int. J. Parasitology. 10: 1-13.

Lowe, J. B. and Varki, A. (1999). Glycosyltransferases, in Essentials of Glycobiology. 17: 279-292.

Ludwig T, Le Borgne R, and Hoflack B. *(*1995). Roles for mannose-6-phosphate receptors in lysosomal enzyme sorting, IGF-II binding and clathrin-coat assembly. *Trends Cell Biol*; 5:202–206.

Marchalonis, J.J. and Edelman, G.M. (1968) Isolation and characterization of a hemagglutinin from *Limulus polyphemus. J. Mol. Biol*. 32: 453–465.

Marije L. Bertrand K.and Ineke B. (2005). Studying protein folding *in-vivo*. In: Protein Folding Handbook. 1: 73-104.

Matsumura K, Higashida K, Ishida H, Hata Y, Yamamoto K, Shigeta M, Mizuno-Horikawa Y, Wang X, Miyoshi E, Gu J, and Taniguchi N. (2007). Carbohydrate binding specificity of a fucose-specific lectin from Aspergillus oryzae: a novel probe for core fucose. *J. Biol. Chem.* 282: 15700-15708.

Maurizio M. (2007). N-glycan structure dictates extension of protein folding or onset of disposal. *Nature Chemical Biology* 3: 313–320.

Miyata T. and Suzuki K. (1975). Partial purification and characterization of β-galactosidase from rat brain hydrolyzing glycosphingolipids. *J. Biol. Chem*. 250: 585-592.

Miyazaki, Y. (1988). Purification, crystallization and properties of β-galactosidase from *Bacillus macerans*. Agric. Biol. Chem. 52:625–631.

Nagy, Z., T. Kiss, A. Szentirmai and S. Biro (2001). β-Galactosidase of *Penicillium chrysogenum:* Production, purification and characterization of the enzyme. *J. Protein expression and purification.* 21: 24-29.

Neufeld, E.F. (1991). Lysosomal storage diseases *Annu. Rev. Biochem.* 60: 257-280.

Oliver, J. D., van der Wal, F. J., Bulleid, N. J. and High, S. (1997). Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science* 275: 86–88.

Oosterhoff L.M. (1977). Variation in growth rate in as an ecological factor in the land snail *Cepeae nemoralis* (L). Netherland J. Zoology 27: 1-132.

Pereira M. E., and Kabat E. A. (1974). Specificity of purified hemagglutinin (lectin) from Lotus tetragonolobus Biochemistry. (15):3184–3192.

Prescher, J. A. and Bertozzi, C. R. (2006).Chemical Technologies for Probing Glycans, *Cell*. 126: 851-854.

Restelli, V. and Butler, M. (2002). The effect of cell culture parameters *on* protein glycosylation. In: Cell Engineering, Glycosylation. 3: 61-92.

Ritter, C. and Helenius, A. (2000). Recognition of local glycoprotein misfolding by the ER folding sensor UDP-glucose:glycoprotein glucosyltransferase. Nat Struct Biol 7: 278–280.

Sandhoff, K. and Kolter, T. (1996). Topology of glycosphingolipid degradation *Trends Cell Biol.* 6: 98-103.

Sharon, N. and Lis, H. (1989) Lectins. Chapman & Hall, London; (2003)*Lectin's*, 2nd Ed., Kluwer Academic Publishers, Dordrecht, The Netherlands

Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsima-Lubaki, M., Peeters B. and W.J. Peumans, (1987). Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry (*Sambucus nigra L.*) bark lectin. *J. Biol. Chem*., 262: 1596–1601.

Sokolove, P. G. and McCrone, E. J. (1978). Reproductive maturation in the slug *Limax maximus* and effect of artificial photoperiod. *J. Comparative Phsiology* 125A: 317-325.

Solem, A. (1984). A world model of land snail diversity and abundance. In: Solem, A. and van Bruggen, A.C. (eds) World Wide Snails: Biogeographical studies on non-marine Mollusca. Brill, Leiden, 6-22.

South, A. (1982). A comparison of the life cycles of *Dendroceras reticulatus* (Müller) and *Arion intermedius* (Normand) (Pulmonata: Stylommatophora) at different temperatures under laboratory conditions. *Journal of Molluscan Studies* 48: 233–244.

Springer, G.F. and Desai, P.R. (1971). Monosaccharides as specific precipitinogens of eel anti-human blood group H (O) antibody. *Biochemistry* 10: 3749–3760.

Steers, E. J., Cuatrecasas, P., and Pollard, H. B. (1971). Purification of b-galactosidase from *E. coli* by affinity chromatography. *J. Biol. Chem.* 246: 196–200.

Stephan, E. B., Juergen, T. Young-Ok, Franz-George, P. Jacques, H. B. and Robert, F. (1996). Characterization of the binding specificity of Anguilla anguilla

agglutinin (AAA) in comparison to Ulex europaeus agglutinin I (UEA-I) *Glycoconj. J.* 13: 585-590.

Tarentino, A. L., Gomez, C. M. and Plummer, T. H. (1985). Deglycosylation of asparaginve-linked glycans by peptide:N-glycosidase F. Biochem 24: 4665-4671.

Teichberg, V.I., Silman, I., Beitsch, D.D., and Resheff, G. (1975). A β-Dgalactoside binding protein from electric organ tissue of *Electrophorus electricus. Proc. Natl Acad. Sci. USA* 72: 1383–1387.

Tompa A. S. (1984). Land snails (stylommatophora). The Mollusca. Vol. 7. Reproduction. P: 48-127, Academic Press Inc.

Van Bruggen, A.C. (1995). Biodiversity of the Mollusca: time for a new approach. In: van Bruggen, A.C., Wells, S.M. and Kemperman, Th.C.M. (eds) *Biodiversity and conservation of the Mollusca*. 1-19.

Varki, A. (1999). I-Type Lectins, in Essentials of Glycobiology. 23: 395-412.

Varki, A., Freeze, H., and Manzi, A. (2009a). Overview of glycoconjugate analysis. Curr Protoc Protein Sci Chapter 12, Unit 12.11 12.11.11-18.

WHO (1998). Report of the WHO informal consultation on schistomasis control WHO/CDS/CPC/SIP/99.2

Winchester, B.G. (1996). Lysosomal metabolism of glycoconjugates *Subcell. Biochem.* 27: 191- 238.

Wolda, H. (1970). Variation in growth rate in the landsnail *Cepaea nemoralis*. *Researches on Population Ecology* 12: 185–204.

Conclusion

Although gastropods comprise over 35,000 living species of marine, terrestrial, and fresh water snails and slugs, there is a big lack of research concerning their importance in science. From this point, there was a need to lighting a dark point in these organisms and we have decided to purify and characterize all available exoglycosidases, furthermore, studying the glycosylation pattern of snails' tissues and eggs. Our work on β-galactosidase purification indicated that purifying such enzyme from an organism is very complex and full of tricks since it needs many purification steps which started with salt precipitation by ammonium sulfate (80% saturation). The second step was hydrophobic interaction chromatography which is a very powerful tool to eliminate many unwanted proteins due to the separation according to hydrophobicity. In our purification scheme, we also used anion and cation exchange chromatography to get use of the existed charges on protein molecules and their interactions with the charged beads. Size exclusion chromatography was also useful to separate the molecules according to their sizes and by this method, the enzyme was purified with very few numbers of other co-purified proteins but still our target protein is unknown. We have purified β-galactosidase by one more purification step which was β-galactosidase specific affinity chromatography, these columns contain substrate (β-galactopyranoside) to binds to the enzyme and then we eluted it by different buffers.

The purified β-galactosidase has molecular weight about 74kDa.

It was not the same to purify β-galactosidase from snails other than *Arion lusitanicus* where, the enzyme from each snail behaves in a very different way and this is why we could not purify this enzyme from other snails.

Studying the glycosylation patterns in snail tissues and eggs showed wide range of fucosylated and sialylated structures.

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