# THE IMPLICATION OF THE ISOLATED GLYCOCONJUGATES FROM MASTIC GUM *PISTACIA LENTISCUS* ON THE INHIBITION OF THE NON-OPSONIC EFFECT OF HELICOBACTER PYLORI MEDIATED BY H.P NEUTROPHIL ACTIVATED PROTEIN (HPNAP)

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## Abstract

*The protein glycoconjugates have been extracted from mastic gum var. chia, preliminary identified and antibodies raised against them. In addition, the gene of the strong antigenic membrane protein HPNAP from Helicobacter pylori has been cloned in pET 11a and p-GEX vectors, over expressed heterologous in BL2 E.coli cells and purified in high amount. Its ability to interact with the isolated glycoforms of the mastic gum is under investigation.*

## Introduction

Mastic gum var. *Chia* is a resinous, aromatic substance that comes from the trunk and the thickest branches of the gum mastic tree *Pistacia lentiscus* species. Its bactericidal activity against *Helicobacter pylori* has been reported by Huwez, F.U, et al, (1998), and Marone, P., et al, (2001). Its high content on 69 essential oils and a-pinene (Magiatis, P., et al, 1999) in conjunction with the existence of Zn and high molecular weight glyco-conjugates (Kottakis, Ph., et al, unpublished results) indicates a multifunctional natural product against *Helicobacter pylori*.

There is close association of *H. pylori* gastric inflammation with neutrophil binding (Miller-Podraza, H., et al, 1999) mediated by the neutrophil-activating protein (HPNAP) (Evans, D, et al, 1995), a strong antigen, which upregulates adhesion molecules of the CD11b/CD18 series on human neutrophils, increasing binding of these cells to the endothelium.

On the other hand the sialic acid dependent binding of the bacterium to various glycoconjugates which are abundant in human neutrophils has been suggested to be of great importance for *H. pylori* resistance to phagocytosis but the bacterial cells are not necessarily killed (Chmiela M., et al, (1994), Kist M., et al, (1993) and Rautelin H., et al, (1993)).

The bactericidal activity of the extracts as well as their ability to bind to HPNAP /and or to human neutrophils are presented and discussed.

## Material and Methods

### Extraction of the resin glycoconjugates

The glycoconjugates were extracted after overnight stirring at $4^{o}$ C in 0.1M NaCl, 20mM Tris – HCl, pH 7.5, filtered with 0.45μm type HA filters and dialyzed against water.

### Atomic absorption

The Zn content was estimated by atomic absorption on a Perkin-Elmer atomic absorption spectrometer (Model 403) at 214.5 nm and by using a Zn EDL lamp.

### Production of antibodies

Antiserum to glycoconjugates was produced using the isolated extracts. A 3-month-old rabbit was injected subcutaneously with the extracts from 1gr mastic as described in Harlow and Lane (1988). The resulted rabbit serum collected after the second and third injection and stored in 1ml aliquots at $-20^{0}$C.

### Electrophoresis and Western blotting

SDS_PAGE was performed according to Laemmli U.K. (1970) in 12% polyacrylamide gels and protein transfer onto Immobilon membranes was followed by immunostaining using the produced antiserum in a dilution 1:1000.

### Bactericidal activity of the water extracts against *Helicobacter pylori*

*H. pylori* strains were grown on agar plates containing 10% horse serum in a microaerophilic atmosphere (generated by Campy-Gen, Oxoid,Basingstoke, U. K.) at 37 °C for 48 h. Bacteria where harvested in phosphate-buffered saline (pH 7.4), diluted to a concentration of $2x10^{9}$cells/ ml and 10 μl where plated on 6 cm plates plus 10μl BHI (Brain heart infusion - growth medium) containing agar . The mastic gum water soluble extracts of 1, 2 and 3 gr were dissolved in 50 μl PBS and added to the cultures.

### Cloning and overexpression of HPNAP

The hpnap gene was cloned in pET11a and pGEX vectors. After transformation of BL21 (DE3) cells and induction with 1mM IPTG at $A_{600}$ 0.8 the protein was overproduced as intact and GST-fused, respectively.

### Purification of the HPNAP protein

The recombinant, overproduced HPNAP protein was purified by a one step purification method. After sonication of the *E.coli* cells in 20mM Tris – HCl pH 7.5, 0.5M NaCl, 2M urea, dialysis against 20mM Tris – HCl pH7.5, 0.5M NaCl, 98% saturation with $(NH_4)_2SO_4$ and centrifugation, the protein was collected in the supernatant which was dialysed against 20mM Tris – HCl pH 7.5, 0.1M NaCl.

### Neutrophil isolation and binding experiments

Human neutrophils were prepared from venous blood of healthy donors according to Boyum A. (1974) with some modifications. Neutrophil membranes were prepared from the freshly isolated neutrophils (Kevin L. Moore, et al, 1992). The membranes were solubilised using SDS, the proteins were separated by SDS – PAGE, blotted onto PVDF membranes and used for immunoblot analysis.

## Results and discussion

The extracted – water soluble glycoconjugates – by using saline buffers, as described above in Materials and Methods, have been separated and identified by SDS-PAGE 12%. Figures 1A and 1B show the extracted glycoproteins and the polyclonal antibodies which have been raised against them, respectively.
Their Zn content has been estimated by atomic absorption (see Materials and Methods) which is shown in Figure 2. It has to be noticed that even after extensively dialysis the Zn ion is still detectable due probably to specific or non-specific strong interactions with the glycoconjugates. Further studies concerning the identification of the extracted glycoconjugates are under investigation.

**Figure 1B**
Immunoblot of the glycoconjugates shown in figure 1A.

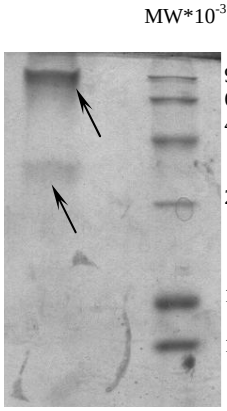


**Figure 1A**
SDS-PAGE 12% of the glycoconjugates which are shown by arrows.

As it has been mentioned above, the bactericidal activity of the mastic gum has been proved in the total resin (Huwez, F.U., et al, 1998 and Marone, P., et al, 2001) which -except the essential oils- contains a- and b- pinene, known for its bactericidal activity due to their terpene classification.
The bactericidal activity of the water soluble extracts from mastic gum has been proved as described above in Materials and Methods. The extracts contained in 1gr mastic inhibited slightly the growth of the bacteria. In contrast the extracts contained in 2gr and 3gr,respectively, inhibited the growth completely .
Our findings, concerning the existence of the water soluble glycoconjugates in the resin, which is universal in the resins from other tested plants (data not shown) as well as their antibacterial activity against *Helicobacter pylori* (see Materials and Methods) lead to the suggestion that the bacterial growth inhibition should be attributed to a number of substances contained in this resin. Namely, the Zn ion probably is transferred inside the cell and substitutes the two Ni ions which are essential for urease activity (Harry L.T. Mobley, et al, 1995 and Fulkerson JF Jr, et al, 1998).
In addition, a binding of the mastic glycoconjugates with bacterial membrane proteins would also be possible, taking into account our experimental data concerning the MIC of the extracts for the *Helicobacter pylori* (see Materials and Methods). This suggestion is corroborated by the evidence that the HPNAP binds in a sialic acid

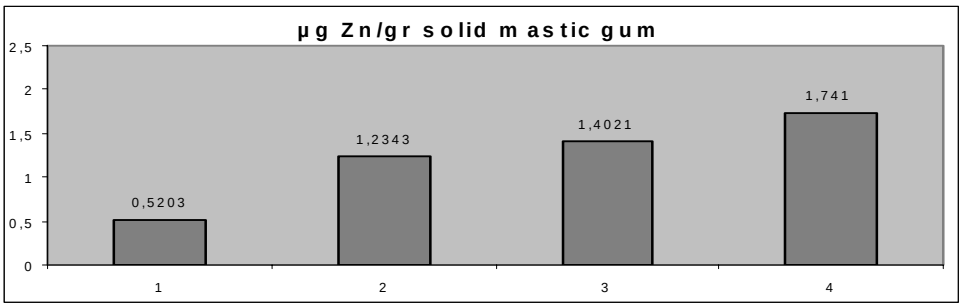


**Figure 2**

**1: extraction with 0.1M NaCl, 20mM Tris – HCl, pH 7.5**
**2: extraction with 0.1N HCl**
**3: extraction with 0.1N $HNO_3$**
**4: extraction with 6M Guanidine hydrochloride**

dependent manner on the neutrophils (Miller-Podraza, H., et al, 1999) (Evans, D, et al, 1995).

In order to study possible implication of the HPNAP binding with the glycoconjugates or its neutrophil binding mediated by the glycoconjugates the hpnap gene has been cloned in the pGEX and pET11a vectors and overexpressed as GST-fused or intact protein, respectively.

The overproduced HPNAP protein in BL21 *E.coli* host cells (Figure 3) in quite high level enabled its one step purification by using ammonium sulfate precipitation. The purified protein (99% degree of purification) will be used for structural studies and antiserum production.

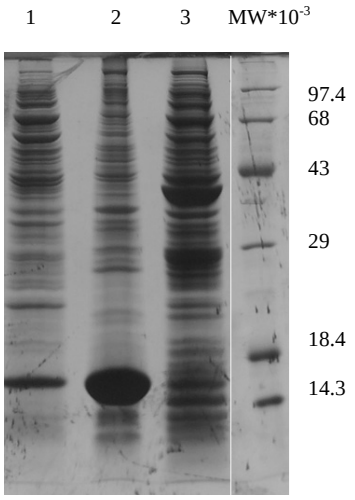


**Figure 3**
1. non induced protein
2. IPTG induced
3. pET11a proteins before IPTG induction

Concerning the binding experiments of the mastic glycoconjugates with the fused GST-HPNAP a non-specific binding even with the glutathione -sepharose beads has been observed. It could be attributed to a kind of affinity to carbohydrates. On the other hand this first "negative" observation would indicate a possible affinity to the glycoconjugates of human neutrophils which has to be further studied.

In Figure 4 is shown the recognition of some neutrophil proteins from the antibodies produced against the glycoconjugates (see Materials and Methods). These preliminary experiments show a putative binding of the glycoconjugates to the membrane proteins of neutrophils and not a direct binding to the HPNAP protein. The observed inhibitory effect of the glycoconjugates *in vitro*, mentioned above, indicates probably the transfer of Zn ions into the cells or a direct binding of the glycoconjugates with other membrane proteins from *Helicobacter pylori* which have to be further elucidated. In addition, the implication of the glycoconjugates on the non-opsonic effect could be another explanation taking into account the sialic acid dependent binding of HPNAP with the neutrophils, which is also under investigation.

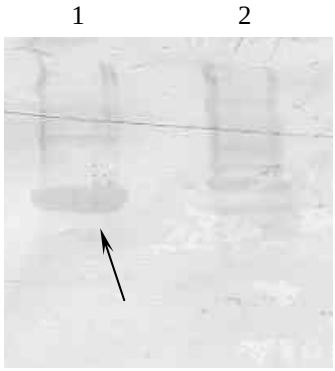


**Figure 4**
1. Arrow shows the GST-HPNAP immunostained by the glycoconjugates' anti-serum.
2. Proteins from neutrophils immunostained an in 1.

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