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Involvement of glycan chains in the antigenicity of *Rapana thomasiana* hemocyanin

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Abstract

Functional unit (FU) *Rt*H2-*e* from *Rapana thomasiana* hemocyanin (Hc) was degraded into small fragments with chymotrypsin. The glycopeptides were separated from the non-glycosylated peptides by chromatography on Concanavalin-A–Sepharose and characterized by mass spectrometry. The glycan part of the glycopeptides (all with common peptide stretch of 14 amino acids) consists of the classical trimannosyl-*N,N*-diacetylchitobiose core for *N*-glycosylation, predominantly extended with a unique tetrasaccharide that is branched on fucose. In inhibition ELISA experiments, the glycopeptides interfered in the complex formation between FU *Rt*H2-*e* and rabbit antibodies against *Rapana* Hc (about 30% of inhibition). The inhibition also was retained after treatment of the glycopeptides with pronase in order to completely destroy the peptide part. The inhibitory effect of the non-glycosylated peptides, on the other hand, was very low. This study thus demonstrates that the glycans attached to FU *Rt*H2-*e* contribute to the antigenicity of *Rapana* Hc.

Keywords: Antigenicity; Electrospray ionization-mass spectrometry; ELISA; Gastropod; Glycan; *Rapana thomasiana*; Hemocyanin; Mollusc

The hemocyanins (Hcs) are extracellular type-3 copper proteins occurring in high concentration in the hemolymph of molluscs and arthropods. Molluscan Hcs exist as cylindrical structures that are built up of 10 (cephalopods) or 20 (gastropods) 350–450 kDa subunits. These subunits are composed of seven or eight globular functional units (FUs) denoted by the letters *a* to *g* (or *h*) read from the N-terminus on. Each FU has an average molecular mass of about 50 kDa and carries a pair of copper atoms enabling the reversible binding of molecular oxygen [1,2]. In addition to their function as oxygen transporting proteins, Hcs play a role in the innate immune system of the animals as they possess some phenoloxidase activity, which can be enhanced *e.g.* by limited proteolysis [3,4].

Hcs are able to stimulate the immune system in many organisms including human. In particular, this was studied for the Hc of the marine gastropod *Megathura crenulata*, commonly known as keyhole limpet hemocyanin (KLH), which has frequently been used as an immunotherapeutic agent in the treatment of certain kinds of cancer (mainly bladder carcinoma) and as a carrier for vaccines [5]. Hc from another marine gastropod, *Haliotis tuberculata* is considered to be a possible substitute for KLH as immunostimulant [6]. Recently, also the Hc from a Chilean gastropod, *Concholepas concholepas* [7], was shown to have significant antitumor activity against mouse bladder carcinoma cells [8].

Studies, basically made on KLH, showed that the glycan structures present on the protein play a significant role in the antigenicity [5]. These glycans are more abundant on molluscan Hcs (2–6% carbohydrate, w/w) than

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on arthropodan Hcs [9]. Within the molluscan phylum, gastropodan Hcs are richer in sugar than cephalopodan ones [9].

The successful use of KLH as antitumor agent and vaccine carrier and the growing evidence for the antigenic potential of its sugar motifs have made it interesting to explore the carbohydrate based antigenicity of other related Hcs, especially from gastropods. In this context, our present study deals with the antigenicity of the Hc of *Rapana thomasiana* (*Rt*), a marine gastropod living along the west coast of the Black Sea. This Hc exists in the hemolymph as two distinct isoforms, *Rt*H1 and *Rt*H2, both characterized by the typical quaternary structure of gastropodan Hcs [10] and a carbohydrate content of 2.6% (w/w) [11]. For FU *Rt*H2-*e* (*i.e.* FU *e* from the second isoform), the primary structure [12] and crystal structure (PDB code 1LNL) [13] are known. Recently, our group characterized the *N*-linked glycans of this FU [14]. A novel motif was discovered, consisting of a central fucose moiety that is substituted with 3-*O*-methylgalactose and *N*-acetylgalactosamine and linked to *N*-acetylglucosamine at the reducing end and that is further connected to one of the mannose arms of the core structure. The purpose of this work was to study the involvement of this peculiar glycan structure in the antigenicity of *Rapana* Hc.

Materials and methods

Chymotrypsinolysis of functional unit RtH2-e and separation of fractions. FU *Rt*H2-*e* was isolated from *R. thomasiana* Hc [15] and next denatured, reduced and *S*-pyridylethylated as described [14]. After dialysis against 0.1 M NH_4HCO_3, pH 8.2, the solution (~7 mg in 2 mL) was treated with α-chymotrypsin (Sigma, St. Louis, MI, USA) at an enzyme/substrate ratio of 1/50 (w/w) for 18 h at room temperature. The digest was applied on a column (9.6 × 0.9 cm) of Concanavalin-A–Sepharose 4B (Sigma), equilibrated with binding buffer (20 mM Tris/HCl, pH 7.4, containing 0.5 M NaCl, 1 mM $MnCl_2$ 1 mM $CaCl_2$, and 1 mM $MgCl_2$). The column was eluted with binding buffer for recovery of the non-glycosylated peptides, and subsequently with the same buffer containing 0.5 M methyl-α-D-glucopyranoside for desorption of the glycopeptides.

Determination of (glyco)peptide concentration. The concentration of the peptide and glycopeptide fractions (expressed in μg peptide/μL) was estimated by amino acid analysis on a 4151 Alpha Plus amino acid analyzer (LKB, Uppsala, Sweden), equipped with an Alpha Plus PEEK column. For the glycopeptide fraction the mass of the bound carbohydrates is thus not taken into account. The hydrolyses were performed in the presence of norleucine (5 nmol) as internal standard with azeotropic HCl, 0.1% phenol, in evacuated sealed tubes at 110 °C for 24 h.

Liquid chromatography/electrospray ionization-mass spectrometry. The LC system was equipped with an Agilent 1100 binary pump, degasser, and autosampler. A C-18 Alltech Prevail 5 μm LC column (150 × 2.1 mm) was used. Ten microliters of sample (glycopeptide fraction from Concanavalin-A chromatography; peptide concentration ~0.02 μg/μL) was injected in the LC apparatus and a linear gradient of acetonitrile (from 10% to 80% in 30 min) in 0.1% aqueous HCOOH was applied. The flow rate was maintained at 0.2 mL/min. The system was directly coupled with a LCQ Advantage ion trap mass spectrometer of Thermo Finnigan (San Jose, CA, USA) equipped with an ESI (electrospray ionization) source operating in the positive-ion mode to generate molecular ions $(M+xH)^{x+}$ of the glycopeptides. Besides full MS scans (upper mass limit *m/z* 2000), also MS/MS spectra were recorded. Spray voltage was maintained at approximately 4.5 kV, and N_2 was used as a nebulizing gas. The collision energy was set as 25%.

Preparation of antiserum. The antiserum against total *Rapana* Hc was prepared according to the protocol described in [16]. For the ELISA experiments the antiserum (anti-*Rapana* Hc) was diluted in phosphate-buffered saline (PBS).

Competitive enzyme linked immunosorbent assay. For each competitive ELISA experiment two microtiter (96 wells) plates were used. A first plate was coated with the antigen by application of a solution (100 μL per well) of FU *Rt*H2-*e* at a concentration of 50 nM in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$), pH 9.7, at 4 °C overnight. The plate was rinsed three times with wash buffer (0.05% Tween 20 in PBS), treated for 1 h at room temperature with a 1% casein solution in PBS (200 μL per well) to block the unoccupied places and rinsed three times with wash buffer. A second plate was coated with 1% casein solution (200 μL per well) for 1 h at room temperature. This plate was used for pre-incubation of the antibodies with the potential competitors (peptide and glycopeptide fractions) in the reaction with FU *Rt*H2-*e*.

In the classical competitive ELISA experiments, 158 μL of antiserum, 500× diluted in PBS, was added in the first well of the row and a semi-logarithmic dilution series was made by transferring 50 μL to the successive wells filled with 108 μL PBS. Then to each well of a dilution series 0.2 μg peptide, *i.e.* 10 μL of (glyco)peptide fraction brought to a concentration of 0.02 μg peptide/μL with PBS, was added. In a second approach [17], increasing amounts (0.02–0.4 μg peptide; in a final volume of 20 μL) were added to 108 μL of 5000× diluted antiserum (corresponding to the third point in the dilution series in the first approach). In parallel series FU *Rt*H2-*e* was added instead of the (glyco)peptide fraction to obtain conditions of 100% inhibition. After 3 h at room temperature the mixtures were transferred to the wells of the antigen-coated plate (first plate). This plate was then incubated for 1 h at room temperature (for reaction of the free antibodies with the coated *Rt*H2-*e*) and then rinsed with wash buffer. Next, 100 μL of secondary antibodies, namely goat anti-rabbit antibodies conjugated with alkaline phosphatase (GaR-AP) (ICN, Aurora, OH, USA), diluted to 1:10,000, v/v, in PBS with 0.1% casein, was added to each well of the plate. After 1 h at room temperature the plate was washed and 100 μL of *para*-nitrophenylphosphate dissolved at 0.5 mg/mL in 0.1 M glycine–NaOH buffer, pH 10.4, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, was added per well as substrate. Finally, the plate was measured at 405 nm by a multichannel-spectrophotometer (ELX800, Bio-Tek Instruments, Winooski, VT, USA). The absorbance values were plotted against $-\log_{10}$(dilution).

Pronase treatment. The (glyco)peptide fractions (pH 7.4) were treated with pronase (Calbiochem, La Jolla, CA, USA) in an enzyme to substrate ratio of 1/1 (w/w) for 48 h at 37 °C.

Results

Separation and MS analysis of chymotryptic glycopeptides

In our earlier work, making use of trypsin, the glycans attached to FU *Rt*H2-*e* were determined [14]. Four main glycopeptides, Gp Ia, Gp Ib, Gp IIa, and Gp IIb, all with the same peptide part (52 amino acids long; peptide stretch 92–143 in the sequence of *Rt*H2-*e*) were found. The glycan structures, deduced by a thorough MS and MS/MS analysis, are schematised in Fig. 1A. As the peptide part of these glycopeptides could interfere in the study of the antigenic properties of the glycans, we now used chymotrypsin instead of trypsin to degrade FU *Rt*H2-*e* so as to obtain glycopeptides with shorter peptide stretch.

The glycopeptides (ConA+ fraction) were separated from the non-glycosylated peptides (ConA− fraction) as described in Materials and methods. On LC/ESI-MS of the ConA+ fraction four main elution peaks were observed