Final Draft
of the original manuscript:

Helmholz, H.; Naatz, S.; Lassen, S.; Prange, A.:
Isolation of a cytotoxic glycoprotein from the Scyphozoa Cyanea lamarckii by lectin-affinity chromatography and characterization of molecule interactions by surface plasmon resonance
In: Journal of Chromatography B (2008) Elsevier

DOI: 10.1016/j.jchromb.2008.06.040

Isolation of a cytotoxic glycoprotein from the Scyphozoa *Cyanea lamarckii* by lectin affinity chromatography and characterization of molecule interactions by surface plasmon resonance

Heike Helmholz[1], Stefanie Naatz[2], Stephan Lassen[1] and Andreas Prange[1]

1 GKSS Research Centre Geesthacht, Institute for Coastal Research, Department for Marine Bioanalytical Chemistry, Max-Planck-St., D-21502 Geesthacht, Germany

2 University of Applied Sciences Lübeck, FHL Forschungs-GmbH, Mönkhofer Weg 239, 23562 Lübeck, Germany

Corresponding author:

Heike Helmholz

GKSS Research Centre Geesthacht

Institute for Coastal Research

Department for Marine Bioanalytical Chemistry

Max-Planck-St.

D-21502 Geesthacht, Germany

Email: heike.helmholz@gkss.de

Tel: 0049 4152/87-1844

Fax: 0049 4152/87-1875

Key words:

Glycoprotein, SPR, affinity chromatography, lectin, Cyanea

Abstract

A biospecific lectin affinity-based isolation process for a novel glycoprotein (ClGp1) from the venom of the pelagic jellyfish *Cyanea lamarckii*, is described and the isolated glycoprotein is chemically and biologically characterized according to size, molecular interaction and toxicity. The molecular mass of the isolated protein is 25.7 kDa as determined by matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF). The carbohydrate content was calculated after enzymatic deglycosylation as 6.85 kDa. The glycoprotein is cytotoxic and could be isolated from cnidocysts of mesenteric and fishing tentacles. The binding behaviour of the glycoprotein to the lectins Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA) was analyzed by surface plasmon resonance (SPR) and affinity constants in the range of, $K_D=3.0*10^{-7}$ M for ConA and $2.1*10^{-6}$ M (pH 5.0) and $2.6*10^{-6}$ M (pH 7.4) for WGA were obtained.

1. Introduction

Cyanea spec. belong to the most abundant jellyfish of the northern coastal zones. The biochemical composition of their complex venoms and their toxic potency is only poorly understood. *Cyanea* medusea are a major component of the pelagic food web due to their predation on crustacean and fish fry [1]. Their toxins, which are actively utilised for prey capture, are harboured as complex mixtures (venom) in cell organelles (cnidocysts). The cnidocysts are preserved in cnidocytes, specialized cells situated in batteries on fishing and mesenteric tentacles and controlling the extremely fast discharge and envenomation process. In order to identify single venom components for the structural and functional analysis of *Cyanea* toxins preparative purification of the complex venom is performed with special focus on glycosylated proteins.

Glycosylation as a common postranslation modification of proteins is an important function-related structural characteristic in cytotoxic substances [2]. Snake, fish, mollusc and scorpion venoms are known to contain glycoproteins as active compounds [3-6]. Carbohydrate side chains of glycoproteins in snake venoms acting as anticoagulant factors have been characterized due to their affinity to lectins of different specificity [3]. A mannose-containing glycoprotein with mitogenic activity was isolated from a sea urchin [7].

In cnidaria, glycoproteins are known from soft corals to function as structural proteins [8]. Glycoproteins have been obtained from whole body extacts of *Metridium senile* (cnidaria, anthozoa) but the functional significance is unclear [9, 10]. Glycosylated collagens have also been described as structural proteins in the cell wall of cnidocysts and mesoglea tissue from the mesoglea of sea anemones [11-14]. Glycosylated collagens from hydra and also from the scyphozoan jellyfish *Pelagia pacifica* have been detected and compared [15].

An anthozoan glycoprotein with hemolytic activity and affinity to ConA has been isolated from the nematocysts of the sea anemone *Stoichactis helianthus* [16, 17]. Interaction with Concanvalin A was measured by precipitation indicating the occurrence of mannose or

glucose containing carbohydrate side chains. A further cnidarian glycoprotein with identified toxic potency is known from *Physalia physalis* (cnidaria, hydrozoa). The hemolytic activity of this Physalitoxin can be inhibited by ConA [18].

Intergral and membrane-associated proteins extracted from the perirhopalial tissue of *Cyanea capillata* have been positively detected by lectins of different specificities [19]. However toxic glycoproteins have not been described from Scyphozoa of the species *Cyanea lamarckii* which are the object of the present investigation.

In order to purify and prepare glycosylated toxins lectin-affinity adsorbents have been applied. These adsorbents are a powerful tool for the efficient isolation and analysis of glycoproteins from complex mixtures [20-23]. Lectins are proteins or glycoproteins expressing a specific affinity to carbohydrates conjugated to proteins or lipids. These proteins bind to mono- and oligosaccharides reversibly with high specificity, but are devoid of catalytic activity [24, 25]. Lectin-affinity chromatography (LAC) enables the isolation of glycoproteins and the conservation of their function and natural glycosylation pattern by using mild chromatography conditions. A summary of recent development in the preparation and application of lectin adsorbents is given by Monzo et al. [26]. An LAC process starts with the lectin immobilization on a solid support. A mixture containing the glycosylated compound is applied and the unbound material is washed out. Following this, the specifically adsorbed product can be desorbed with an elution buffer containing the corresponding monosaccharide. Lectin affinity chromatography can not only be applied for purification but also for a comprehensive analysis of glycoproteins and glycan profiling which are a fundamental part of the research field of “glycoproteomics” or “glycomics”. For such a comprehensive profiling a wide variety of lectins with different specificities combined with sensitive and high resolution mass spectrometry is needed [27, 28].

If the glycosylation is a function related post translational protein modification lectin interaction can be also used for the functional characterization of glycoproteins by means of a

biospecific interaction analysis based on the physical phenomenon of surface plasmon resonance (SPR) [29]. SPR spectroscopy is a biosensor-based method that observes the interaction of a biomolecule as an analyte in the mobile phase with specific sites immobilized at a functionalized gold-chip surface. The binding of an analyte can be monitored in real time without labelling. Binding characteristics and inhibitor reactions can be measured to determine substance related affinity constants [30]. Carbohydrate-based interactions are an important aspect of the application of biosensor molecular interaction analysis [31]. Oligosaccharide, glycopeptide and glycoprotein binding onto lectins is described in several studies [32-34].

The carbohydrate binding specificity of lectins is utilized for the isolation of a toxic glycoprotein from the venom of *Cyanea lamarckii* named ClGp1 meaning *Cyanea lamarckii* glycoprotein 1. The isolation process by lectin affinity chromatography and the application of biospecific interaction analysis for glycoprotein characterization is described.

2. Experimental

2.1 Materials

The polymer based support Toyopearl AF-Tresyl-650M was obtained as a preactivated bulk material from Tosoh Biosep (Stuttgart, Germany). Sephadex G25, the lectins Wheat Germ Agglutinin (WGA), Concanavalin A (ConA) and Bradford reagent were purchased from Sigma (Taufkirchen, Germany). Horseradish Peroxidase (HRP) (EC 1.11.1.7.), was purchased from Fluka Chemie AG, (Bruchs, Switzerland). All other chemicals were of analytical reagent grade.

2.2 Organisms and venom preparation

Jellyfish of the species *Cyanea lamarckii* (Péron & Léslueur) were collected during a research cruise to the Orkney Islands, Scotland in May/June 2004. Animals collected were larger than

10 cm umbrella diameter. Mesenteric and fishing tentacles were sliced and collected for further processing. Portions of intact oral arms and fishing tentacle tissue were immediately frozen and kept at -80°C.

The deep-frozen intact tissue from oral arms was thawed slowly at 4°C and the cnidocyst containing material was ruptured by sonication in ice cold 10mM acetate buffer pH 5.5 in a cooled sonicator (Branson Sonifier 450, G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany). The suspension was centrifuged at 11000rpm for 5min and 4°C until a almost clear supernatant (extract) could be obtained.

Fresh fishing and mesenteric tentacles were used for the purification of intact cnidocysts. This material was stirred gently in distilled water on ice over night. The resulting organic suspension was filtered through a nylon sieve with a mesh size of 500 µm to remove mesoglea residues. The filtrate was centrifuged at 4°C at 3000 rpm for 5min. After discarding the supernatant, the residues were washed 2-3 times with sterile filtered seawater. The content, purity and integrity of cnidocysts were controlled microscopically and the cnidocyst concentrate was stored at -80°C until extraction. The extraction of cnidocysts was performed by sonication in the same manner as described for tissue samples.

2.3 Protein purification

2.3.1 Preparation and characterization of affinity adsorbents

The preparation of lectin affinity adsorbents was performed as described previously [23, 35]. Immobilization of ConA onto tresylated polymer supports was carried out within 4 hours using 0.5 M phosphate buffer (pH 8.0) and the remaining tresyl groups were blocked with 0.2 M Tris-HCl buffer (pH 8.0). The coupling yield and surface coverage was calculated by protein determination (section 2.4.1). Three batches of ConA polymer-based adsorbents have been prepared to determine the optimal particle surface coverage for a maximal accessibility

of carbohydrate binding sites and three batches of ConA adsorbents have been prepared for the preparative isolation of ClGp1. The performance of the prepared ConA-adsorbents was controlled by adsorption and specific desorption of HRP as described in section 2.3.2. To determine the accessibility of carbohydrate binding sites the theroretical adsorbents capacity was calculated and HRP was applied excessively.

2.3.2 Lectin affinity chromatography

For ConA affinity chromatography, a low-pressure liquid chromatography system (BioRad, Munich, Germany) was employed. The prepared adsorbents were packed into BioRad glass columns at a bed volume of 1.9 mL (5 mm x. 10 mm width x length). Adsorption was performed in 0.1 M acetate buffer pH 6.0, 1 mM manganese, 1 mM magnesium and 1 mM calcium. Specific desorption was achieved with the same buffer containing 0.1 M methylmannopyranoside. All separations were carried out at a flow rate of 0.6 mL/min. An affinity cycle includes the adsorption, thorough washing of unbound material (washing fraction), and specific desorption (elution fraction), followed by a regeneration step with adsorption buffer. The recovery was determined as protein concentration in the resulting fractions.

2.3.3 Size exclusion chromatography

Further purification of the elution fraction was achieved by size exclusion chromatography on Sephadex G25. SEC (bed volume 27 mL, 1 cm x. 34 cm width x length) was performed on an Äcta prime low-pressure chromatography system (Pharmacia, Uppsala, Sweden). Chromatography parameters were flow 0.6 mL/min, injection volume 0.5 mL, fractionation 1.2 mL in 10 mM acetate buffer pH 5.5. Chromatograms were recorded with an UV detector at 280 nm.

2.4 Protein analysis

2.4.1 Quantitative Protein Assay

To calculate the coupling yield of adsorbent preparation, the ConA concentrations were determined by a dye binding assay (Bradford reagent) adapted to measurements in a microplate reader (Victor 3, 1420 Multilabel Counter, Perkin Elmer, Rodgau - Jügesheim, Germany) with lectin standard. The protein content in crude extract, purified fractions, and purified glycoprotein was measured with bovine serum albumin (BSA) as standard protein [36].

2.4.2 Electrophoresis

Tricine-sodium dodecyl sulfate-gel electrophoresis was performed with self casted polyacrylamide gels. A 10% T, 3% C separating gel was combined with a 4% T, 3% C stacking gel for the separation of proteins in the range of 5-100 kDa [37]. SDS Page protein marker 6.5-200 kDa (Serva electrophoresis GmbH, Heidelberg, Germany) was used for the estimation of molecular weights with the Bio Imaging System Genegenius (Syngene, Cambridge, UK). The one dimensional electrophoresis was performed in the Protein II electrophoresis system (BioRad, Munich, Germany). The gels were coloured by colloidal Comassie Brilliant Blue G250 dye (Roti® Blue, Carl Roth GmbH, Karlsruhe, Germany).

2.4.3 Mass spectrometry

Prior to the molecular weight determination with a matrix assisted laser desorption/ionization – time of flight mass spectrometer MALDI-TOF-MS (Ultraflex II, Bruker Daltonics, Bremen, Germany) the samples were dialyzed against 0.1% trifluoro acetic acid (TFA) and subsequently pre mixed with the matrix sinapinic acid (4-Hydroxy-3,5-dimethoxy-cinnamic

acid) in acetonitrile / 0.1% TFA 1:2 (v/v). 1 µL of the 1:1 (v/v) sample/matrix mixture was spotted on a polished steel target (Bruker). For MS calibration the protein calibration standard II purchased from Bruker was used.

2.4.4 Deglycosylation

The N-glycosylation carbohydrate content of ClGp1 was determined by enzymatic deglycosylation with PNGase F (Glycoprofile TM II Sigma, Munich, Germany). According to the manual the lyophilized sample was solubilized in reaction buffer and denaturated by heating in 2% octyl-β-D-glucopyranosid, 100 mM mercaptoethanol for 10 min at 100°C. Enzymatic carbohydrate cleavage was performed at 37°C for 24 h. Prior to the analysis an 800µm AnchorChipTM target (Bruker) was prepared with “super DHB” (sDHB). 2,5-Dihydroxy benzoic acid (10 mg/mL in 20% acetonitrile) and 5-methoxy salicylic acid (10 mg/mL 50% acetonitrile) were first mixed at a ratio of 9:1 (v/v). 1 µL of the resulting matrix were deposited on the anchors and allowed to crystallize at room temperature. 0.5 µL of the reaction mixture were then applied onto the matrix surface and after drying the spots were analysed.

2.4.5 Biospecific interaction analysis

The characterization of the binding behaviour of the isolated glycoprotein was performed with a Biacore 3000 system (GE Healthcare Europe, Freiburg, Germany).

Immobilization of ligands

The immobilization of ligands was performed according to the Biacore immobilization protocol V1.0 for amino coupling using a CM5 chip. Each of the four flow cells was activated, functionalized and deactivated in a single procedure. In detail the sensor chip surface was activated by 35 µL of a 1:1 (v/v) mixture of 200 mM N-ethyl-

N(dimethylaminopropyl)carbodiimide (EDC) and 400 mM N-hydroxysuccinimide (NHS) followed by manual injection of ligands and injection of 20µL 1 M ethanolamine pH 8.5 for deactivation. The functionalized chip surface was equilibrated for 30min with 50 mM 3-morpholinopropane sulfonic acid (MOPS), pH 7.5 150 mM sodium chloride, 3 mM magnesium, 10 µM calcium, 10 µM manganese. Prior to binding assays, the chip was washed with adsorption buffer (0.1 M sodium acetate; 0.5 M sodium chloride; 1 mM manganese, calcium, magnesium pH 5) for several hours, for baseline stabilization.

Binding assays and data analysis

The evaluation of affinity constants was performed for HRP as model substance and for the isolated glycoprotein in steady-state at a continuous flow rate of 10 µL/min. As reference flow cells, a blank (activation and deactivation without ligand) and a bovine serum albumin (BSA) as ligand were used.

Different concentrations of analytes (HRP, ClGp1 and lectins) were applied. Unspecific adsorption was controlled by injection of BSA under the same conditions. Adsorption was performed in a running buffer of 0.1 M sodium acetate pH 5.0 with 0.5 M sodium chloride and 1 mM magnesium, manganese and calcium. Regeneration conditions were 0.5 M and 1.0 M methylmannopyranoside with multiple injections.

Binding analysis with WGA was conducted in 0.1 M acetate buffer (pH 5.0) and 0.1 M phosphate buffered saline PBS (pH 7.4) with 0.2 M N-acetylglucosamin for regeneration.

The resulting sensogramms were analyzed with the BIAevaluation software version 4.1 The curves for the different concentrations were synchronized, signals of reference cell and blank buffer injection were subtracted. Req was plotted against the concentration and evaluated with the Biacore steady state affinity model.

2.5 Cell toxicity assay

A dye uptake test system was used to detect the acute toxicity of the extracts as described in [38]. Cells of a continuous HepG2 culture were sowed into a microtiter plate in a density of 10^5 cells per well in 150 µL RPMI medium, allowed to equilibrate for 24h and different venom and ClGp1 concentrations were applied. After an incubation of 48h, the cells were incubated, for 3 h with a 0.005% Neutral Red solution in RPMI medium and cell disruption was performed with lysis solution containing 50% (v/v) ethanol 1% (v/v) acetic acid and rigorous shaking. The released Neutral Red was measured in a microtiter plate reader at 550 nm (Victor 3, 1420 Multilabel Counter, Perkin Elmer, Rodgau - Jügesheim, Germany). Each extract concentration was measured in six replicates. The percentage of viable cells was estimated by setting the absorbance of the buffer controls minus the values of the negative control to 100%.

3. Results

3.1 Isolation of the glycoprotein

Extracts of different samples of *C. lamarckii* (umbrella diameter >10 cm) were utilized for glycoprotein isolation. The preparative affinity and separation procedures have been developed with frozen intact tissue material derived from mesenteric tentacles. The purification process was also applied for material from purified cnidocyst suspensions of mesenteric and fishing tentacles for comparison.

Specific adsorbents for lectin affinity chromatography were self-prepared by functionalization of activated polymeric porous particles. The amount of ConA immobilization varied for 3 different experiments from 53 to 74%, resulting in a ligand density of 32 -52% particle surface coverage as calculated by a ConA area of 0.2745 m^2/mg (pdb file 5CNA) and an active surface of 42 m^2/g adsorbent. The obtained particle surface coverage allowed an favorable utilization and accessibility of the carbohydrate binding sites of the ligand ConA as

documented in figure 1, where an accessibility optimum of binding sites is obtained at a particle surface coverage about 50%.

The prepared adsorbents could be used for the purification of crude extracts for up to 30 affinity cycles without capacity loss. The percentage of ConA recognizing glycoproteins in the venom varies between 2.4 – 6.7% when determined as protein concentration in the elution fraction setting the amount of protein content of the applied extract volume as 100%. Extract protein concentration and affinity chromatography protein yields are summarized in table 1. Due to the increased protein content in the tissue extract compared to the extracts obtained from the purified cnidocysts suspension the amount of glycoprotein in the elution fraction is higher whereas the percentage ratio is comparable.

SEC was performed in order to remove the methylmannopyranoside in the affinity chromatography elution fraction and to achieve a separation of contaminants like other glycoprotein with affinity to ConA or proteins with weak unspecific interaction (figure 2). *Cyanea lamarckii* glycoprotein 1 (ClGp1) appeared in the first peak at 18 min and the smaller carbohydrate molecules indicated by the increase in conductivity can be eliminated.

The isolation process has led to a bioactive, cytotoxic glycoprotein. The toxicity was measured in a cell based assay and a total loss of cell growth could be determined at a concentration of 5µg protein equivalents per 10^5 cells per microtiter well corresponding to a protein concentration of 17 µg/mL. Compared to the crude extract a 5 fold increase of activity was achieved.

3.2 Glycoprotein identification

Molecular weight and purity of the isolated glycoprotein were determined by SDS PAGE and MALDI-TOF (fig 3). According to the molecular weight marker the MW was determined as 27 kDa by SDS Page and 25.7 kDa by MALDI-TOF. Analysis of the glycoprotein isolated from extracts of cnidocyst concentrates from mesenteric tentacles and also of cnidocyst

extracts from fishing tentacles showed the same molecular weight compared to the ClGp1 from whole tissue extracts of oral arms (data not shown).

Enzymatic deglycosylation with PNGase F has led to a molecular weight loss of 6.85 kDa indicating a carbohydrate content in ClGp1 of 26.8%. The MALDI-TOF mass spectrum of the deglycosylated sample is given in figure 4. The molecule ion signals of the deglycosylated protein at 9.5 $[M+2H]^{2+}$ and 18.7 $[M+H]^{+}$ could be detected. The remaining residue of the glycosylated ClGp1 can also be observed in very low intensities.

3.3. Biospecific interaction analysis

Binding constants were determined as a substance specific parameter for the characterization of molecule interactions between lectins and the isolated glycoprotein. The conserved binding activity of ClGp1 onto ConA indicates that the isolation procedure is mild enough to preserve the protein structure.

According to the preparative lectin affinity chromatography Horseradish peroxidise has been selected as standard glycoprotein for the verification of the biomolecular interaction analysis with the unknown glycoprotein. Binding constants of HRP onto immobilized Concanavalin A were measured in the range of 0.3 µM to 600 µM, comprising 4 potencies as recommended for the determination of affinity constants in a steady state. The concentration dependant binding of HRP onto ConA is documented in figure 5. Summarizing three replicates, a binding constant of $K_D = 7.0*10^{-6}$ M ± $2.4*10^{-6}$ M was obtained. There was negligible non-specific adsorption of BSA onto the ConA and the reference flow cell.

For the affinity analysis of ClGp1 onto ConA the ClGp1 was immobilized as ligand and ConA was applied at increasing concentrations as analyte. An approximately steady-state could be achieved resulting, in a $K_D = 3.0*10^{-7}$ M. The resulting affinity constants are summarized in table 2. Comparing the two instrumental designs a change in association and

dissociation kinetics can be observed. This could be due to an increased accessibility of carbohydrate recognition domains when ConA is used as analyte in a soluble form.
In order to identify other carbohydrate side chains, the affinity of ClGp1 to Wheat Germ Agglutinin with specificity to N-acetylglucosamin and sialic acid residues was tested in two different buffer systems. ClGp1 showed a distinct, reversible and concentration dependent affinity to WGA in acetate buffer pH 5.0 and phosphate buffer pH 7.4 (figure 7). Affinity constants were determined as $K_D=2.1*10^{-6}$ M in acetate buffer and $K_D=2.6*10^{-6}$ M in phosphate buffer, respectively (table 2).

4. Discussion

A structure specific separation process was developed for the isolation of a toxic glycoprotein from the cnidocysts of the jellyfish, *Cyanea lamarckii*. Information on the biochemical composition of *Cyanea* spec. venoms is limited and a glycoprotein with cytotoxic potency from this species has not been described before. As structural information for protein identification, the molecular weight of 25.7 kDa was determined and, according to the biospecific interaction analysis, the occurrence of glucose/mannose and N-acetylglucosamin/sialic acid in carbohydrate side chains of the cytotoxin can be supposed. Glycosylation is known as a function-related, posttranslational protein modification. However the application of biospecific lectin affinity chromatography combined with a polishing step, such as SEC, for the isolation of such toxins is infrequent. This process has the advantages of high-resolution, fast processing, and mild chromatography conditions which favour the maintenance of bioactivity, as documented by the cell-based toxicity assay. Hemolytic activity, as published for anthozoan glycoproteins [17, 39], could not be detected (data not shown), although the crude venom of mesenteric and fishing tentacles from *Cyanea lamarckii* expressed hemolytic activity [38].

GlGp1 was detected in cnidocyst extracts of frozen whole tissue samples as well as in extracts of purified cnidocyst suspensions from both mesenteric and fishing tentacles. The toxin ClGp1 was isolated from the soluble fraction after cnidocysts discharge without any supplementation of detergents. These results confirmed the hypothesis that this isolated glycoprotein can be considered as a component of *C. lamarckii* venom acting as a toxic protein and not a structural membrane protein.

The glycosylation pattern of membrane proteins of the related species *C. capillata*, which were analyzed by means of their function in sensory and nerve tissues, show a limited pattern of glycosylation. In tissue staining experiments both ConA and WGA gave a positive response indicating the distribution of mannose/glucose and N-acetylglucosamin/sialic acid in *Cyanea* glycoproteins [19].

N-glycosylated carbohydrate side chains were determined by enzymatic deglycosylation and mass spectrometric analysis of the resulting protein back bone. The measured carbohydrate moiety was 26.8% of the total protein mass which is relatively high compared to 3% in stone fish venom and 3.7% in the anthozoan glycoprotein [4, 17]. However, the carbohydrate content in Physalitoxin from *P. physalis* (10.6%), in Cx3 from cobra venom (≅ 29%), as well as in Pachymatismin from the marine sponge *P. johnstonii* (30%), indicate that a high degree of glycosylation in marine and terrestrial toxins may occur [2, 18]. SDS Page analysis with carbohydrate staining showed the majority of glycoproteins in the venom of the sea anemone *Aiptasia pallida* and *Physalia* venom supporting the necessity for the application of glycoprotein specific procedures for venom purification [18, 39].

The function and stability of toxins can be affected by the glycosylation pattern. Therefore the carbohydrate-based molecular interaction with two glycoconjugate binding lectins of different specificities has been analyzed. Characterization of binding strength to ConA could also be used to optimize the preparative affinity separation procedures and to prove the structure-

preserving quality of the separation process. Interaction analysis with Wheat Germ Agglutinin was performed to obtain additional structural information on carbohydrate side chains.

The biosensor based lectin interaction analysis with surface plasmon resonance was used for the acquisition of binding constants for substance characterization and identification. Prior to the analysis of the unknown substance, the ConA – immobilization and binding behaviour must be verified with a standard glycoprotein. Horseradish Peroxidase was selected. ConA is a plant lectin from Jack Bean (*Canavalia ensiformis*) requiring manganese, calcium and magnesium for the recognition of terminal mannose and glucose in complex carbohydrate side chains. Only a few biosensor studies have been conducted with immobilized ConA [31, 40, 41]. The lectin immobilization procedure has been adapted from Rebois et al. (2002) [42]. Due to the dissociation of ConA tetramers into dimers with two carbohydrate binding domains at pH 5 it is recommended to run the immobilization and a thorough equilibration at lower pH to ensure a stable ligand conformation [42]. The binding analysis was performed under these stabilized conditions and pH or buffer shifts were avoided. Covalent amino coupling of ConA and surface regeneration with several injections of 0.5 M methylmannopyranoside enabled a surface functionalization feasible for the application of repeating affinity cycles. Affinity constants between HRP and ConA are only available from batch studies with particle-based ConA-affinity adsorbents and not from SPR experiments. Miranda et al (2003) found $K_D = 4.4*10^{-5}$ M which is comparable to the present result of $7.0*10^{-6}$ M, taking into account that it was a particle-based approach with different instrumental and analytical conditions [43].

The isolated GlGp1 expressed a slightly stronger affinity to ConA when compared to HRP, indicating a high degree of mannosyl/glucosylation. GlGp1 also showed a binding activity to WGA. WGA has been used in several SPR studies for carbohydrate-protein interaction analysis [44, 45]. Different buffer systems, at pH 5.0 and 7.4 respectively, were used to compare the binding behaviour between ConA and WGA and to apply the optimized

conditions routinely used for preparative WGA affinity separations [46]. No significant impact of a pH (range of 5.0 and 7.4) on the binding strength of ClGp1 onto WGA could be detected. The affinity constants show a decreased binding strength compared to ConA. According to the affinity constants in the micromolecular range the application of a weak affinity separation could also be possible, as it has been shown for particulate WGA adsorbents [47]. The affinity of ClGp1 to WGA indicated the occurrence of N-acetylglucosamine or sialic acid in carbohydrate side chains of the isolated cytotoxin. Considering the high degree of glycosylation, different glycosylation sites in the toxin molecule are likely. Hence ClGp1 has to be viewed as a heterogenous mixture of closely related glycosylation variants, so-called glycoisoforms, as indicated by the relative broad molecule ion mass signal (figure 3) which would imply different glycoisoforms.
Further bioanalytical investigation will be carried out for the structure elucidation of the isolated ClGp1 and to clarify the observed cytotoxic effects on a molecular level.

5. Conclusion

Species of the genus *Cyanea* belong to the most abundant scyphozoa in the northern coastal territories. The stinging capacity of *Cyanea* spec. is described as low but in vitro studies to characterize the true toxic potency and information on the biochemical composition of the venom are not available for *C. lamarckii*. Due to the importance of glycosylation as a function-related protein modification a biospecific separation process based on lectin-carbohydrate interactions have been applied and a novel glycoprotein with cytotoxic potency could be isolated. The functionality of a carbohydrate-specific isolation procedure as a tool for the preparation of a bioactive compound has been demonstrated. Biosensor based SPR studies have been used to describe the binding behaviour of the isolated glycoprotein to different lectins as a substance-related characteristic. This method is often used for the characterization of well-defined model-glycoproteins or synthetic oligosaccharide structures.

With the application of this micro-affinity analytical method indications for the structure of carbohydrate side chains and binding conditions for the optimization of the preparative process could be obtained for an unknown, natural glycoprotein

6. References

[1] C. Lynam, S. Hay, A. Brierley, Limnology and Oceanography 49 (2004) 637.
[2] A. Osipov, M. Astrapova, V. Tsetlin, Y. Utkin, Europaen Journal of Biochemistry 271 (2004) 2018.
[3] G.W. Birrell, S. Earl, P.P. Masci, J. de Jersey, T.P. Wallis, J.J. Gorman, M.F. Lavin, Mol Cell Proteomics 5 (2006) 379.
[4] P. Garnier, F. Goudey-Perriere, P. Breton, C. Dewulf, F. Petek, C. Perriere, Toxicon 33 (1995) 143.
[5] B. Olivera, L. Cruz, Toxicon 39 (2001) 7.
[6] N. Valdez-Cruz, C.V.F. Batista, L.D. Possani, European Journal of Biochemistry 271 (2004) 1453.
[7] H. Nakagawa, T. Tanigawa, K. Tomita, Y. Tomihara, Y. Araki, E. Tachikawa, Toxin Reviews 22 (2003) 633.
[8] P. Meikle, G. Richards, D. Yellowlees, The Journal of Biological Chemistry 262 (1987) 16941.
[9] R. Hilderbrand, T. Henderson, T. Glonek, T. Myers, Biochemistry 12 (1973) 4756.
[10] D.S. Kirkpatrick, S.H. Bishop, Biochemistry 12 (1973) 2829.
[11] D.D. Brand, R.S. Blanquet, M.A. Phelan, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 106 (1993) 115.
[12] R.L. Katzman, A.H. Kang, J. Biol. Chem. 247 (1972) 5486.
[13] H. Nowack, A. Nordwig, European Journal of Biochemistry 45 (1974) 333.
[14] A. Nordwig, U. Hayduk, Journal of Molecular Biology 44 (1969) 161.
[15] B. Barzansky, H.M. Lenhoff, H. Bode, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 50 (1975) 419.
[16] J. Devlin, Journal of Pharmaceutical Sciences 63 (1974) 1478.
[17] A.W. Bernheimer, L. Avigad, Proceedings of the National Academy of Science USA 73 (1976) 467.
[18] M.M. Tamkun, D.A. Hessinger, Biochimica et Biophysica Acta (BBA) - Protein Structure 667 (1981) 87.
[19] J. Schetz, P. Anderson, Cell and Tissue Research 279 (1995) 315.
[20] M. Madera, Y. Mechref, I. Klouckova, M.V. Novotny, Journal of Chromatography B 845 (2007) 121.
[21] R. Qiu, X. Zhang, F.E. Regnier, Journal of Chromatography B 845 (2007) 143.
[22] F. Okanda, Z. El Rassi, ELECTROPHORESIS 27 (2006) 1020.
[23] H. Helmholz, S. Cartellieri, L. He, P. Thiesen, B. Niemeyer, Journal of Chromatography A 1006 (2003) 127.
[24] A. Naeem, M. Saleemuddin, R. Khan, Current Protein and Peptide Science 8 (2007) 261.
[25] H. Lis, N. Sharon, Chem. Rev. 98 (1998) 637.
[26] A. Monzo, G.K. Bonn, A. Guttman, TrAC Trends in Analytical Chemistry 26 (2007) 423.
[27] J. Hirabayashi, Glycoconjugate Journal 21 (2004) 35.

[28] R.R. Drake, E.E. Schwegler, G. Malik, J. Diaz, T. Block, A. Mehta, O.J. Semmes, Mol Cell Proteomics 5 (2006) 1957.
[29] R. Karlsson, Journal of Molecular Recognition 17 (2004) 151.
[30] L.G. Fagerstam, A. Frostell-Karlsson, R. Karlsson, B. Persson, I. Ronnberg, Journal of Chromatography A 597 (1992) 397.
[31] E. Duverger, N. Frison, A.C. Roche, M. Monsigny, Biochimie 85 (2003) 167.
[32] Y. Shinohara, F. Kim, M. Goto, M. Tosu, Y. Hasegawa, European Journal of Biochemistry 223 (1994) 189.
[33] A. Hutchinson, Analytical Biochemistry 220 (1994) 303.
[34] I. Blikstad, L. Fägerstam, R. Bhikhabhai, H. Lindblom, Analytical Biochemistry 233 (1996) 42.
[35] S. Cartellieri, H. Helmholz, B. Niemeyer, Analytical Biochemistry 295 (2001) 66.
[36] M. Bradford, Analytical Biochemistry 72 (1976) 248.
[37] H. Schagger, G. von Jagow, Analytical Biochemistry 166 (1987) 368.
[38] H. Helmholz, C. Ruhnau, C. Schutt, A. Prange, Toxicon 50 (2007) 53.
[39] G.R. Grotendorst, D.A. Hessinger, Toxicon 37 (1999) 1779.
[40] D. Mislovicova, J. Masarova, J. Svitel, P. Gemeiner, International Journal of Biological Macromolecules 30 (2002) 251.
[41] R. Gutierrez Gallego, S.R. Haseley, V.F.L. van Miegem, J.F.G. Vliegenthart, J.P. Kamerling, Glycobiology 14 (2004) 373.
[42] R. Rebois, P. Schuck, J. Northup, Methods in Enzymology 344 (2002) 15.
[43] M. Miranda, M. Magri, R. Cabrera, H. Fernandez - Lahore, O. Cascone, Latin American Applied Research 33 (2003) 67.
[44] J.L.J. Blanco, S.R. Haseley, J.P. Kamerling, J.F.G. Vliegenthart, Biochimie 83 (2001) 653.
[45] S. Haseley, P. talaga, J. Kamerling, J. Vliegenthart, Analytical Biochemistry 274 (1999) 203.
[46] S. Cartellieri, O. Hamer, H. Helmholz, B. Niemeyer, Biotechnology and Applied Biochemistry 35 (2002) 83.
[47] L. Leickt, B. M, D. Zopf, S. Ohlson, Analytical Biochemistry 253 (1997) 135.