Purification of a soluble glycoprotein from the uncalcified ecdysial cuticle of the blue crab *Callinectes sapidus* and its possible role in initial mineralization

Elizabeth P. Tweedie*, Francie E. Coblentz and Thomas H. Shafer†

*Department of Biological Sciences, University of North Carolina at Wilmington, Wilmington, NC, USA

†Author for correspondence (e-mail: shafert@uncw.edu)

Accepted 4 May 2004

Summary

A heavily glycosylated soluble protein was purified using a combination of lectin affinity and size exclusion chromatography from a soluble extract of uncalcified dorsal cuticle of blue crab *Callinectes sapidus* removed at ecdysis. Similarities in apparent molecular mass and carbohydrate composition suggest that this protein is the same species previously shown to disappear from soluble extracts coincidently with the onset of mineral deposition in the newly exposed post-molt cuticle. The amino acid sequence of the N-terminal portion of the core polypeptide was determined and polyclonal antibodies were raised against both the purified glycoprotein and the peptide. Immunoblots of unfraccionated soluble extracts taken at various times post-molt illustrated that the antipeptide antibody recognized several polypeptides with electrophoretic mobilities that differ from the purified glycoprotein. These bands may be deglycosylation products which would not have been purified due to different lectin affinity or size. Immunohistochemical analysis indicated uniform protein distribution in the exocuticle at ecdysis, but decreased antibody binding at the interprismatic septa by 2 h post-molt. The location of the protein is therefore the negative image of the calcification pattern in the exocuticle and provides a spatial pattern to correlate with the previously reported temporal events. This strengthens the hypothesis that the glycoprotein under investigation is an inhibitor of calcite nucleation or of initial amorphous calcium carbonate accumulation.

Key words: blue crab, *Callinectes sapidus*, biomineralization, glycoprotein, post-ecdysial cuticle alteration (PECA).

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Introduction

Biomineralization typically requires heterogeneous nucleation by an organic matrix to lower the energy required for initial crystal formation (Boskey, 1996, 1998). These nucleation sites are often acidic macromolecules such as sulfated or carboxylated glycoproteins (Addadi et al., 1987; Lowenstam and Weiner, 1989; Albeck et al., 1996; Aizenberg et al., 2002). Indeed, many characterized matrix proteins found in the well-studied mineralizing tissues of vertebrates are glycoproteins containing varying amounts of highly heterogeneous carbohydrate moieties. Bone sialoprotein (BSP), for example, is only 50–70% polypeptide by mass and contains both O- and N-linked oligosaccharides (Fisher et al., 1990; Midura and Hascall, 1996; Wuttke et al., 2001). It can act both as a nucleator, promoting crystal formation in agarose or gelatin-based *in vitro* systems, and as a crystal growth inhibitor at greater concentrations or in solution-based assays (Hunter and Goldberg, 1993; Boskey, 1996; Hunter et al., 1996).

The exoskeleton of decapod crustaceans is an excellent model system in which to examine the control of calcification (reviewed in Roer and Dillaman, 1984, 1993). The periodicity of the molt cycle, coupled with the fact that the layers of the cuticle are deposited and calcified at distinctly different times, is advantageous for both biochemical and histological study. Specifically, the two outer layers of the exoskeleton, the epicuticle and the exocuticle, are deposited during pre-molt. This stage begins with apolysis, or the separation of the hypodermis from the existing hard cuticle, and concludes with ecdysis, or the actual emergence of the soft crab from the old cuticle. Since molting is also called exuviation, these layers are referred to as pre-exuvial. They calcify after the molt. By comparison, the endocuticle, which lies proximal to the exocuticle, is not synthesized until after ecdysis and after the pre-exuvial layers have begun to mineralize. Unlike them, it calcifies as it is deposited.

The initial sites of crustacean post-molt mineralization are the epicuticle–exocuticle boundary and within exocuticular regions termed interprismatic septa (IPS; Giraud-Guille, 1984; Dillaman et al., 2001; Hequembourg, 2002). The "prisms" are asymmetrical polygonal columns perpendicular to the lamellae of the cuticle. The IPS, located between the prisms, may correspond to the lateral margins of the hypodermal cells that originally deposited the cuticle. The IPS are rich in carbohydrate, specifically glycosaminoglycans, as determined
Soluble glycoproteins from the organic matrix of the cuticle of brachyurans have been studied at all stages of the molt cycle. Electrophoretic patterns of extracts of lectin-binding proteins from the calcified exoskeleton layers of the Bermuda land crab Gecarcinus lateralis change dramatically during and after apolysis (Kumari and Skinner, 1995). These pre-molt changes in the existing cuticle relate to mineral dissolution and resorption rather than mineral deposition. Various mild aqueous solvents were used to extract glycoproteins from the anec dysial (intermolt) cuticle of the Atlantic shore crab Carcinus maenas, and the complex array of electrophoretic bands described from these extracts contained both O-linked and N-linked glycans (Compère et al., 2002). Fluctuations were observed in both soluble protein and carbohydrate during the molt cycle in the mud crab Scylla serrata, but no electrophoretic fractionation was performed to observe changes in individual glycoproteins (Pratoomchat et al., 2002).

If glycoproteins are to be implicated in controlling calcification in crab cuticle, however, they must be studied during the time and location of initial mineralization, namely in the pre-exuvial layers during and immediately after ecdysis. In a histochemical study of the shore crab Gaetice depressus, Yano (1972) reported that ‘acid mucopolysaccharides’ were found complexed with proteins to a greater degree in the less calcified portion of the exocuticle and proposed that this material could serve as an inhibitor of calcification until its enzymatic modification post-ecdysis. Extracted soluble proteins from the organic matrix of the soft pre-exuvial cuticle of the blue crab Callinectes sapidus inhibited calcium carbonate nucleation in solution but promoted calcite formation when cross-linked by formaldehyde to decalcified carapace tissue (Gunthorpe et al., 1990). The authors speculated that a high concentration of this unbound material existing in pre-molt cuticle could, at ecdysis, become cross-linked onto chitin fibers to promote nucleation in vivo.

A set of modifications of extracellular substances in the dorsal cuticle of C. sapidus termed the post-ecdysial cuticle alteration, or PECA, occurs immediately following ecdysis, in synchrony with a measured increase in the potential for calcite nucleation (Shafer et al., 1994, 1995). Specific changes include the loss of major protein bands at 66 kDa and 79 kDa in the first few hours post-ecdysis. These proteins appear as positive smears on electrobotted membranes stained with periodic acid/Schiff’s reagent (PAS), indicating high levels of glycosylation. As little as 1 h after ecdysis, the glycoprotein bands cease to appear on PAS-stained membranes. Probing electroblots with lectins also demonstrated the PECA, as the two glycoproteins stain intensely at ecdysis with concanavalin A and jacalin, but lose their affinity for those lectins post-ecdysis (Shafer et al., 1995). It is unclear whether proteolysis causes the disappearance of these proteins or if deglycosylation causes the loss of lectin and PAS affinity. Subsequently, Coblenzt et al. (1998) showed that two heavily glycosylated proteins identical in apparent molecular mass to the previously described proteins associate with CaCO₃ crystals in a solution-based mineralization assay, but decrease their affinity for the crystals as time increases post-molt. A model in which these glycoproteins act as pre-molt inhibitors of calcification in the exocuticle was proposed (Coblenz et al., 1998). The proteins are hypothesized to interact with sites of nucleation on the chitin–protein fibers, thereby limiting crystal formation despite supersaturating concentrations of calcium and bicarbonate. At ecdysis, the proteins are presumed to be degraded or deglycosylated, thereby exposing nucleation sites and allowing crystal growth.

This investigation seeks to purify and characterize the key PECA glycoproteins from Callinectes sapidus cuticle at ecdysis, to establish their location in early post-molt cuticle, and to further investigate their temporal changes. Based on the data presented, we speculate that the two previously described PECA bands (Shafer et al., 1995) represent glycosylation variants of the same protein. More importantly, we demonstrate that the polypeptide portion of this glycoprotein begins to disappear from the IPS (but not the prisms themselves) at a time immediately preceding initial mineralization. This spatial correlation supports our hypothesis that we have purified inhibitors of initial calcium carbonate deposition. We suggest that deglycosylation or proteolysis events within the IPS during and after the molt remove this barrier to mineralization.

Materials and methods
Preparation of cuticle
Callinectes sapidus Rathbun specimens were obtained from a commercial ‘shedding’ operation in Kill Devil Hills, NC, USA. Animals were killed at ecdysis (0 h) or at 1 h, 2 h or 3 h post-ecdysis. For biochemical analysis, the dorsobranchial carapace was removed, stripped of underlying hypodermal tissue, rinsed in distilled water, frozen in liquid nitrogen, and stored at −70°C for protein extraction. For histochemical analysis, carapace pieces were removed, rinsed in distilled water, and immediately fixed in either 2.5% glacialaldehyde in phosphate-buffered saline (PBS); 0.1 mol l⁻¹ sodium phosphate, 0.9% NaCl, pH 7.4) for general protein staining or Rossman’s fixative for immunohistochemistry (Presnell and Schreibman, 1997).

Extraction of cuticular protein
Frozen cuticle samples were lyophilized, manually macerated and weighed. Pieces from several 0 h crabs (10–30 at ~30 mg each) were pooled to obtain soluble material for protein purification, but cuticle from individual crabs was used for electrophoresis and blotting of unfractionated extracts. In either case, the lyophilized soft cuticle was extracted in Tris-EDTA buffer (0.05 mol l⁻¹ Tris-Cl, 0.1 mol l⁻¹ EDTA, pH 7.4), 0.5 ml per piece, for 4 h at 4°C with constant shaking.
Protease inhibitors were not included since no protease activity has been detected in extracts prepared in this manner (Shafer et al., 1994). Insoluble material was removed by centrifugation for 5 min at room temperature, and the supernatant dialyzed (MWCO 2000) against 50 mmol l⁻¹ ammonium bicarbonate for 48 h at 4°C. Sodium azide was added to the dialysate to a final concentration of 0.02%. Protein concentration was measured using the dye-binding Bio-Rad (Hercules, CA, USA) microtiter plate protein assay (based on Bradford, 1976), with bovine serum albumin as a standard. Protein samples were lyophilized and stored at −70°C.

**Purification of glycoprotein**

Pooled 0 h protein extract was fractionated on a jacalin lectin affinity column according to the method of Hortin and Trimpe (1990) with modifications. Jacalin was chosen because of its affinity for O-linked oligosaccharides. Briefly, a 10 ml Bio-Rad disposable column (approximately 8 mm diameter) was packed with 2 ml of agarose-bound jacalin slurry (4 mg jacalin ml⁻¹ gel; Vector Labs, Burlingame, CA, USA) diluted 1:1 in TBS buffer (0.5 mol l⁻¹ NaCl, 100 mmol l⁻¹ Tris-Cl, 0.02% sodium azide, pH 7.5). The column was rinsed with a minimum of 20 ml TBS before application of sample. Lyophilized protein was dissolved in distilled water (2.0 mg ml⁻¹) and applied to the column 1 ml at a time. Absorbance was monitored at 280 nm using a Bio-Rad BioLogic LP chromatography system. Unbound material was eluted using TBS, and bound material was subsequently eluted using 10 mmol l⁻¹ α-methylgalactose in TBS. Bound fractions (Jac⁺) were dialyzed against 50 mmol l⁻¹ ammonium bicarbonate at 4°C and lyophilized.

Jacalin-positive material was further purified by fractionation on a Sephacryl S-200 (Amersham Biosciences, Piscataway, NJ, USA) gel-filtration column (47.5 cm x 1.4 cm) using the BioLogic LP chromatography system. The column was eluted with buffer consisting of 0.25 mol l⁻¹ NaCl, 50 mmol l⁻¹ Tris-Cl and 0.02% sodium azide (pH 7.4) at a flow rate of 0.3 ml min⁻¹. Due to the low solubility of the glycoprotein samples, approximately 1.0 mg lyophilized protein dissolved in 1.3 ml distilled water was applied to the column.

**Protein and carbohydrate detection**

The purity and apparent size of column fractions and glycoprotein samples were confirmed by reducing SDS–PAGE. Samples containing approximately 10 μg protein were denatured, reduced, and applied to pre-cast 8 cm x 8 cm (1.0 mm thick) polyacrylamide gels using the NuPAGE system (Invitrogen, Carlsbad, CA, USA). The gels contained a 4–12% acrylamide gradient in Bis–Tris-Cl buffer, and were run for approximately 50 min at 200 V in MOPS denaturing running buffer. Bands were visualized in gels using the Colloidal (Coomassie) Blue staining system (Invitrogen). Proteins with associated carbohydrate moieties were detected by first electroblotting (1 h at 30 V) from an SDS-PAGE gel onto polyvinyl difluoride (PVDF) membranes, and then staining with periodic acid/Schiff’s (PAS) reagent (Strömqvist and Gruffman, 1992).

**Carbohydrate quantification**

The amount of O-linked carbohydrate in the purified glycoprotein was determined following the methodology of Crowther and Wetmore (1987). Briefly, oligosaccharides were removed and derivatized via β-elimination with 2-cyanoaceticamide. Fluorescence at 383 nm was measured in the presence of borate ions at an excitation wavelength of 336 nm. Bovine submaxillary mucin, with an O-linked carbohydrate content of ~65% of its total mass (Jiang et al., 1998), was used to create a standard curve.

**Enzymatic treatments**

The presence of N-linked glycans was determined by treating <5.0 μg purified glycoprotein with peptide-N-glycosidase-F (PNGase-F; Prozyme) at 37°C for 3 h according to manufacturer’s specifications. Similar broad-range O-glycosidases are unavailable; however, since O-linked glycans can be classified as either proteoglycan-type or mucin-type, a battery of proteoglycan-specific glycosidases was used to test for the presence of proteoglycans. Samples containing <5.0 μg glycoprotein were treated at 37°C for 3 h with 0.02 units of either chondroitinase-ABC, keratanase or heparinase II (Sigma; Midura et al., 1989; Takeuchi et al., 1992). After treatment, samples were subjected to reducing SDS-PAGE, blotted and stained with PAS to evaluate any carbohydrate loss or electrophoretic mobility changes (Strömqvist and Gruffman, 1992).

**Antibody production and western blotting**

Automated Edman degradation was performed on the purified glycoprotein material by the Protein Chemistry Core Facility, Biotechnology Program, University of Florida, and a 15-amino-acid N-terminal sequence was determined. Two polyclonal antibodies were produced in hens by Aves Laboratories, Inc. (Tigard, OR, USA): one against the purified glycoprotein (hereafter called ‘anti-glycoprotein’) and the other against the 15-amino-acid sequence synthesized and affixed to bovine serum albumin as a carrier (‘anti-peptide’).

Unfractionated protein extracts or purified glycoprotein preparations were blotted to nitrocellulose using the NuPAGE western blotting system (Invitrogen). Blots were probed with one or the other primary antibody diluted to either 1:500 or 1:1000 (v/v) in blocking solution. The secondary antibody was alkaline phosphatase-labeled rabbit anti-chicken IgG diluted to either 1:1000 or 1:5000 (v/v) in blocking solution. Blots were incubated and developed at room temperature using CDP-Star ready-to-use chemiluminescent substrate with Nitro-Block II enhancer (Tropix/PE Biosystems, Bedford, MA, USA) according to the manufacturer’s specifications. The alkaline phosphatase signals were imaged by exposure to Kodak X-OMAT film. Developed and fixed films were digitally scanned at 600 d.p.i. resolution and the contrast in the resultant images was enhanced using Adobe Photoshop software.
**Immunohistochemistry and histology**

Carapace pieces fixed in Rossman’s fixative were decalcified (10% EDTA, 0.1 mol·l⁻¹ Tris-Cl, pH 7.4) for 7 days, dehydrated and embedded in paraffin. Thin sections (10 μm) were deparaffinized with toluene, rehydrated to distilled water through a descending acetone series, and rinsed in PBS. Sections were then incubated in primary antibody (1:100 dilution in PBS with 0.1% Tween-20) for 1 h, rinsed for 30 min in PBS, and labeled with a fluorescent secondary antibody (10 μg·ml⁻¹ goat anti-chicken IgG labeled with ALEXA-FLUOR 488; Molecular Probes, Eugene, OR, USA) for 1 h. Slides were examined with an Olympus BH-2 (GMI Inc., Albertville, MN, USA) epifluorescence microscope using blue excitation, and digital images were captured with a Diagnostics (Sterling Heights, MI, USA) SPOT-RT camera. In order to allow accurate comparisons in signal distribution and intensity among various treatments, all exposures were identical times and the images were not digitally enhanced unless so indicated.

Cuticle pieces fixed in glutaraldehyde were similarly decalcified, embedded, sectioned and deparaffinized for general protein staining. The sections were rehydrated through a descending ethanol series and stained with Toluidine Blue in 2% sodium borate (pH 9.2).

**Results**

A two-step glycoprotein purification employing jacalin lectin affinity and gel filtration (Fig. 1) was used to purify certain heavily glycosylated proteins from an extract of blue crab soft cuticle taken at ecdysis (0 h). A single major Coomassie Blue-positive band with an apparent molecular mass of approximately 66 kDa was detected (Fig. 2A,C). When the purified material was electrobotted and stained with PAS, sometimes one carbohydrate-containing band was seen at the 66 kDa position (Fig. 2B), but more often two broad carbohydrate bands were present with apparent molecular mass ranging from >100 kDa down to about 66 kDa (Fig. 2D). This inconsistency in the results was observed despite using identical protocols for all protein purifications. The varying amounts of carbohydrate in the purified samples correlated with the amount of carbohydrate and number of carbohydrate-containing bands in the original unfractionated protein extract as determined by PAS staining prior to purification (Fig. 2B,D, lane 1). Thus, whenever the two broad glycoprotein bands (PAS⁺) were present, they copurified in this two-step system. O-linked oligosaccharides were chemically removed from known amounts of the purified glycoprotein and assayed to determine their contribution to the total mass of the material. When compared to a bovine submaxillary mucin standard, the glycoprotein was calculated to contain 53% O-linked carbohydrate by mass. These O-linkages are predominantly mucin-type, as indicated by the unchanged electrophoretic pattern of the PAS⁺ bands after treatment with enzymes specific for proteoglycan-type linkages (Fig. 3A). Also, no change in electrophoretic pattern was detected after treatment with PNGase-F, which degrades N-linkages (Fig. 3B). Positive controls, i.e. known substrates, were cleaved by each enzyme and resulted in shifts in the detected electrophoretic patterns (data not shown).

Automated Edman degradation of the purified glycoprotein produced the following 15-amino-acid N-terminal sequence: DKYERLYEPYDQRV. One polyclonal antibody was raised against the purified glycoprotein itself (‘anti-glycoprotein’), and another polyclonal antibody was raised against a synthetic peptide corresponding to the known 15-amino-acid sequence (‘anti-peptide’).

Immunoblots of a 0 h extract similar to the material from which the glycoprotein was originally purified were probed with both antibodies and compared with an SDS-PAGE gel of the same extract stained for total protein (Fig. 4). Overall, the anti-glycoprotein antibody bound to the extract with much lower affinity than the anti-peptide antibody since the chemiluminescent detection product had to be exposed to film four times as long to give approximately equal band intensity. The anti-glycoprotein antibody also detected a qualitatively different pattern of proteins than the anti-peptide antibody in immunoblots of the same extract (Fig. 4, lanes 2 and 3). The anti-glycoprotein antibody did recognize a broad band of material with apparent molecular mass between 90–115 kDa (Fig. 4, lane 2), similar to the purified material often seen on PAS blots (compare with Fig. 2D). However, the anti-

![Fig. 1. Chromatography of 0 h protein extract on a jacalin lectin affinity column (A) and subsequent chromatography of the jacalin-bound fraction on a Sephacryl S-200 gel filtration column (B). Arrows indicate peaks containing the glycoprotein of interest, as confirmed by reducing SDS-PAGE.](image-url)
Glycoprotein and mineralization in blue crab

The glycoprotein antibody did not recognize a 66 kDa band in the 0 h extract, even though the purified antigen contained a protein at this position. Instead it recognized a protein with apparent molecular mass of 55 kDa, not seen in the purified antigen.

When compared to the immunoblot results for the anti-glycoprotein antibody, the proteins in the unfractionated 0 h extract that are immunopositive for the anti-peptide antibody are more numerous (Fig. 4, lane 3). They include a number of closely spaced but distinct bands between 70–75 kDa and a smaller protein with apparent molecular mass of about 33 kDa. These bands correspond with some of the abundant proteins detected by Coomassie Blue staining of the same 0 h extract (Fig. 4, lane 1). These distinct bands appear superimposed on a fainter pattern that is similar to the PAS image of the purified glycoprotein, i.e. a broad smear of material with highest

Fig. 2. Two glycoprotein purifications (A,B and C,D) from 0 h cuticle extracts performed identically yet yielded different results. The purified material retains one major Coomassie Blue-stained band but either one (A,B) or two (C,D) broad carbohydrate bands detectable by PAS staining. A and C are SDS-PAGE gels stained with Coomassie Blue for protein. B and D are PVDF membrane blots of similar gels stained with PAS for carbohydrate. In all cases, the first lane contains molecular mass standards (MWS), lane 1 is unfractionated 0 h cuticle protein extract, lane 2 is the Jacalin-positive fraction (Jac⁺) following lectin affinity chromatography, and lane 3 is the final purified product after gel filtration chromatography of the Jac⁺ fraction.

Fig. 3. Enzyme-treated purified glycoprotein subjected to SDS-PAGE and visualized by PAS staining of electroblotted membranes. (A) Lane 1, untreated glycoprotein; lane 2, chondroitinase-ABC treatment; lane 3, keratanase treatment; lane 4, heparinase II treatment. (B) Lane 1, untreated glycoprotein, lane 2, PNGase-F treatment. See Materials and methods for details.
apparent molecular mass greater than 100 kDa and a narrower band at about 66 kDa. When the anti-peptide antibody was used to probe a blot of the purified glycoprotein from which its antigen sequence was derived, it recognized only regions of the gel similar to the PAS image itself (Fig. 4, lane 4). In other words, the sharp bands between 70–75 kDa and at 33 kDa were removed from the extract by purification, either because they were not jacalin-positive or because in their native state they had smaller hydrodynamic volumes than the material in the peak collected from the gel filtration column.

The anti-peptide antibody was used to probe western blots of unfractionated proteins extracted from cuticle material harvested at various times in the interval between ecdysis (0 h) and 3 h post-molt. These immunoblots were compared to the patterns of total protein in the same extracts stained with colloidal Coomassie. Variability among individual crabs was distinguished from consistent temporal changes in protein content by repeating the experiment with several sets of animals. A representative experiment is shown in Fig. 5. Invariably, immunopositive proteins at apparent molecular mass of 33 kDa and 70–75 kDa were detected at all time points. However, the individual bands within the 70–75 kDa range were not consistent between experimental groups. One subtle difference among time periods was the loss of rather diffuse immunopositive material migrating with apparent molecular mass greater than 100 kDa in ‘older’ individuals (2–3 h post-molt, Fig. 5, lanes 3 and 4). The distinct band at 55 kDa in the 1 h extract of Fig. 5 (lane 2) seemed similar to a band recognized by the anti-glycoprotein.

**Fig. 4.** An immunoblot experiment on unfractionated proteins from 0-h cuticle. Coomassie Blue staining of molecular mass standards (MWS) and cuticle extract (lane 1) are compared with immunoblots of the same extract probed with either anti-glycoprotein (lane 2) or anti-peptide (lane 3) antibodies. Lane 4 is an immunoblot of the purified glycoprotein probed with the anti-peptide antibody. Detection of the chemiluminescent product required 2 min exposure to film for the anti-glycoprotein blot (lane 2) and 30 s exposure for the anti-peptide (lanes 3 and 4).

**Fig. 5.** Coomassie Blue-stained gel (A) and corresponding immunoblot (B) of soluble proteins extracted at ecdysis and at various times immediately thereafter. The immunoblot was probed with antibodies raised against the Edman degradation-derived N-terminal amino acid sequence (anti-peptide). In both A and B, lane 1 contains 0 h extract, lane 2 contains 1 h extract, lane 3 contains 2 h extract, and lane 4 contains 3 h extract. MWS, molecular mass standards (kDa).
antibodies (compare with Fig. 4, lane 2) but was not consistent, appearing only once in a 1 h and once in a 3 h extract in a total of four such experiments.

Immunohistochemistry results illustrated homogenous binding across the entire exocuticle for both antibodies when used to probe sections of cuticle taken from crabs killed at ecdysis (0 h) and at 1 h post-molt (Fig. 6A–F). Beginning at 2 h, and becoming more prominent at 3 h post-molt, unstained lines apparently representing the IPS could be seen (Fig. 6G–L). This loss of antigen reactivity in the IPS during first few hours was more pronounced with the anti-peptide antibody than with the less specific anti-glycoprotein antibody. In addition to the specific loss of staining in the IPS, the overall intensity of binding decreased somewhat at 3 h post-molt with
the anti-peptide antibody (Fig. 6K). Control sections show that autofluorescence or non-specific secondary antibody binding occurred in the hypodermis tissue and in the epicuticle, but was never a concern in the comparisons involving the exocuticle layer. The decreased binding of the anti-peptide antibody in the IPS regions at 3 h post-molt became more obvious when the contrast in the immunohistochemistry images were digitally enhanced (Fig. 7A). This differential loss of antigen was not simply due to a general loss of protein from the IPS during early post-molt because the IPS of 3 h post-molt exocuticle stained more intensely with Toluidine Blue than did the ‘prisms’ they surround (Fig. 7B). Furthermore, the IPS could not be distinguished morphologically by phase contrast microscopy in unstained sections taken at either 0 h or 3 h post-molt (data not shown).

Discussion

The *C. sapidus* glycoprotein material previously studied (Shafer et al., 1994, 1995; Coblentz et al., 1998) was purified from total soluble protein extracts of cuticle from crabs killed at ecdysis. A jacalin lectin affinity column was used to separate glycoproteins with O-linked carbohydrates, which were then separated on the basis of size using a gel filtration column (Fig. 1). Purity was determined by reducing SDS-PAGE, in which protein and carbohydrate visualization detected one or two broad bands (Fig. 2). Since the two bands have the same hydrodynamic volume (Fig. 1B), it is assumed that the difference in their electrophoretic mobility is due to differing charge densities on sugar moieties not complexed with SDS (Thornton et al., 1995). However, this phenomenon could also be the result of a deglycosylation event occurring either as an artifact of the extraction process, or as an endogenous event beginning at ecdysis and occurring at different rates in different crabs. The purified glycoprotein was calculated to contain 53% O-linked carbohydrate by mass. It is resistant to enzymes specific for N-linked glycans and O-linked glycans typical of proteoglycan-type proteins (Fig. 3). Based on these results, mucin-type glycans are probably the predominant structural feature of this molecule. However, since the glycoproteins that lose PAS reactivity during the first few hours post-molt do bind Concanavalin A as well as jacalin on lectin blots (Shafer et al., 1995), they presumably contain N-linked as well as O-linked oligosaccharides. The resistance to PNGase-F may be due to the presence of N-linked glycans α1,3-fucosylated on the asparagine-linked N-acetylglucosamine, a configuration found in insect glycoproteins and known to be unaffected by PNGase-F (Altmann et al., 1995, 1999).

Immunoblots of unfractionated soluble extract of cuticle taken at ecdysis were probed with an antibody raised against the purified glycoprotein material. Though the binding was weak, the antibody recognized the larger of the two broad bands that purify from the unfractionated material by the scheme employed (Fig. 4). The anti-glycoprotein antibody did not bind to the smaller purified band (66 kDa) but did have affinity for a 55 kDa band that is never seen in the purified material. Perhaps a major epitope for this polyclonal antibody is a jacalin-negative glycan. Then the 55 kDa band would be a polypeptide containing this moiety but no O-linked carbohydrates. Furthermore, the difference between the larger (>100–70 kDa) and the smaller (66 kDa) purified bands could simply be the presence or absence of the jacalin-negative glycan. The fact that the larger band is missing in some purified preparations stained with PAS could be explained by assuming that the presence of this jacalin-negative glycan is inconsistent from sample to sample.

When immunoblots of soluble proteins extracted from the cuticle at ecdysis or during the time period of the PECA (Shafer et al., 1995) were probed with an antibody against the N-terminal peptide of the purified glycoprotein (anti-peptide), a relatively large number of bands were detected (Figs 4, 5). This does not appear to be due to non-specific binding because not all bands in Coomassie Blue-stained gels from the same extracts appeared and because more stringent washing conditions or lower primary antibody concentrations could not change the pattern (data not shown). It is possible that these multiple bands, specifically the group of reactive species between 70–75 kDa and the strongly positive band at 33 kDa, represent different proteins all with antigenic amino acid sequences similar to the known N-terminal portion of the core.
polypeptide of the purified glycoprotein. However, partial-to-complete deglycosylation products of the purified material (glycoforms) seems more reasonable. If the latter hypothesis is true, the data indicate that these varied glycosylation states of the same core peptide are present in the extracts throughout the time period studied. We speculate that they do not purify in the two-step scheme employed because the deglycosylation events that produce them render them either jacalin-negative or smaller than the two heavily glycosylated bands remaining in the purified material.

In contrast to these sharp immunopositive bands, the heavily glycosylated material greater than 100 KDa is also detected by the anti-peptide antibody, but tends to appear in the extracts collected up to 1 h after ecdisis and not in later samples (Fig. 5). We speculate, therefore, that a series of deglycosylation events has already begun before the molt and is only completed at the PECA. An N-hexosaminidase from the extracellular cuticle that could play a role in these hypothetical deglycosylation events has been described (Roer et al., 2001). This enzyme is not present in intermolt crabs, and its activity rises in pre-molt and peaks in early post-molt.

Perhaps the most significant finding reported is that anti-peptide antibody (as well as the less specific anti-glycoprotein antibody) detected a uniform distribution of antigen in the exocuticle only at ecdisis and at 1 h post-molt. In the immunohistochemistry preparations from 2 h and 3 h post-molt, the IPS reacted less intensely (Figs 6, 7). This observation is in contrast to Toluidine Blue-stained cuticle sections, which showed positive staining of the IPS. Though total protein did not decrease in the septa relative to the prisms, proteins containing the amino acid sequence deduced from the N terminus of the purified material did, in fact, decrease. Since the IPS are the first regions to calcify in the exocuticle (Giraud-Guille, 1984; Dillaman et al., 2001; Hequembourg, 2002), the 3 h post-molt antigen localization represents the negative image of the calcification pattern that will appear somewhat later. This finding adds a spatial correlation to the temporal correlation we have described between changes in these glycoprotein bands and mineralization (Shafer et al., 1995), and strengthens our hypothesis that the unaltered glycoproteins represent inhibitors of crystal nucleation (Coblentz et al., 1995), and supports the idea that the altered glycoproteins are more readily available for reaction with the calcium carbonate precursor. The early post-molt alteration in the IPS, but not in the prisms, could control the pattern of mineralization in the exocuticle.

Evidence has appeared suggesting that the post-molt mineralization in the C. sapidus exocuticle might be initiated by an amorphous form of calcium carbonate that rapidly transforms to more stable calcite (Dillaman et al., 2001). This evidence includes the existence of a highly soluble initial phase, especially in the IPS, which is consistent with reports of similar transient amorphous forms in sea urchin spicules (Beniash et al., 1997) and larval mollusc shells (Weiss et al., 2002), and more stable forms of amorphous calcium carbonate in ascidian spicules (Aizenberg et al., 2002) and the calcium storing organ of an isopod (Raz et al., 2003). Therefore, the glycoprotein(s) described in this research and predicted to inhibit calcite nucleation here and in the past (Shafer et al., 1995; Coblentz et al., 1998) may actually inhibit the initial deposition of amorphous calcium carbonate.

Support for this research was provided by the National Science Foundation (Grants IBN-9807804 and IBN-0114597). We are grateful to R. M. Dillaman and R. D. Roer for many helpful discussions and critiques of this work. We also wish to thank Endurance Seafood, Kill Devil Hills, NC for permission to collect material; the Protein Chemistry Core Facility, University of Florida, Gainesville, FL, USA for Edman sequencing; Aves Laboratories, Inc., Tigard, OR, USA for antibody production; and M. Gay, K. Powell, Y. Kahn, N. Marschhauser and M. Torre for technical assistance.

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