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A challenging insight on the structural unit 1 of molluscan *Rapana venosa* hemocyanin

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Abstract

Hemocyanins of mollusks are high molecular mass glycoproteins with a complex quaternary structure which still remains to be defined in detail for most of its species as far as number, spatial distribution and interactions of their structural units is concerned. In the present study, we isolated the functional units of the structural subunit RvH1 of *Rapana venosa* hemocyanin, combining enzymatic and non-enzymatic methods. Our results suggest that Hc's carbohydrate moieties play a basic role in the organization of the structural units, resulting from post-translational polymerization of the 50 kDa functional units and involving sugar moieties that link between them.

Keywords: Glycoprotein; Hemocyanin; *Rapana venosa*; Quaternary structure

Hemocyanins (Hcs)[1] are oxygen carrier copper glycoproteins, forming freely dissolved aggregates in the hemolymph of mollusks with extremely high M_w and complex quaternary structure [1,2]. Several aspects of their structural–functional peculiarities make Hcs important materials to address relevant problems of structural biology including molecular recognition among subunits, protein–water interactions or allosteric regulation [3,4].

Molluscan Hcs are oligomers consisting of several 11S basic structural units that have molecular masses ranging between 220 and 400 kDa. The 11S units are composed of five, seven or eight covalently linked functional units (FUs) [5,6], glycoproteins of about 400 amino acid residues (molecular mass ≈ 48 kDa). The FUs are generally thought to be connected by short linker peptides, which are more sensitive to enzymatic cleavage than the globular domains. Individual FUs can thus be obtained by limited proteolysis using trypsin, chymotrypsin, or other specific proteases. Native molluscan Hcs result from the oligomerisation of the 11S basic units, forming hollow cylinders of similar topology, but different dimensions and sedimentation coefficients (105S, 57S, and 49S, depending on the class of the corresponding animal). The quaternary structure of most native Hcs, in particular the spatial distribution and orientation of the 11S building blocks, and their oligosaccharide structures and linkage sites, have so far not been defined, nor have their biomedical relevance [7–9].

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[1] *Abbreviations used:* Hcs, Hemocyanins; FUs, functional units; ESI-MS, electrospray ionization-mass spectrometer.

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In our study, we used the Hc of the Black Sea snail *Rapana venosa* as a paradigm for molluscan Hcs, as the electron micrographs of native *Rapana* Hc show features similar to that of other gastropodan Hcs. Furthermore, it has a carbohydrate content of 8.9% [10–12], but the oligosaccharide structures of only a few of its FUs have been studied so far [13,14].

Materials and methods

Preparation of Rapana venosa hemocyanin and its structural subunits

Rapana venosa hemocyanin was isolated from Black Sea marine snails as already described [11]. The dissociation of native Hc was achieved by dialysing the native protein against a 0.13 M glycine/NaOH buffer, pH 9.6, and the structural subunits RvH1 and RvH2 were purified by means of ion-exchanging chromatography on a Resource Q (Pharmacia) column of 6 ml using an FPLC separation system. Elution was performed as described by Dolashka-Angelova et al. [15]. Two electrophoretically pure structural subunits RvH1 and RvH2 were isolated.

Incubation of RvH1 with urea

Samples of 1 mg each of RvH1 were incubated during 24 h at room temperature with 50 mM Tris/HCl buffer, pH 7.5, containing different concentrations of urea (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 M). The resulting mixtures were separated on a Sephadex G column, eluted with the same buffers.

Enzymatic digestion of structural subunit RvH1

Ten milligrams of structural subunit RvH1 was treated with trypsin in a ratio of 400:1 and eight fractions were isolated from the resulting digest as described previously [10]. Another amount of the subunit was dissolved in 50 mM Tris/HCl buffer, pH 7.0, to which 50 μl PNGase-F (1.5 U in 1 ml) (Roche Diagnostics GmbH, Mannheim, Germany) was added. The samples were applied to 7.5% and 10% gel electrophoresis after one, two and three days of incubation at 37 °C. Both enzymatic digestions were performed in a total volume of 250 μl in 0.7 ml Eppendorf tubes.

Depolymerization of RvH1 with $ZnCl_2$

A sample of structural subunit RvH1 was incubated overnight in 50 mM Gly/NaOH buffer, pH 9.5, in the presence of 2 mM PMSF with three buffer exchanges. The dialysis was extended over 30 days, at room temperature in 50 mM Gly/NaOH buffer, at pH 9.5, in the presence of 0.4 mM $ZnCl_2$. A blank experiment without the presence of $ZnCl_2$ was carried out simultaneously. The samples were checked electrophoretically after 10, 15 and 30 days. At the 15th day of incubation, an aliquot was applied on a Resource Q ion exchange column (Pharmacia), equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and resulting fractions were eluted with a nonlinear gradient (0–0.5 M NaCl in 60 min) at a flow rate of 1 ml min^{-1}. The fractions were further purified by HPLC using a Nucleosil 100 RP-18 column (250 mm × 10 mm; 7 m; Macherey-Nagel, Germany). For elution, a linear gradient from 5% solvent A (0.1% TFA in water) to 100% solvent B (0.085% TFA in acetonitrile) within 70 min, at a flow rate of 1 ml min^{-1} was used. The HPLC fractions, detected at a wavelength of 278 nm, were collected.

Incubation of RvH1 with a yeast culture

These experiments were performed in liquid culture media of strain *Saccharomyces cerevisiae* 90-1 (collection of the Institute of Microbiology, Bulgarian Academy of Sciences). The strain was maintained on malt extract agar and grown in two different media. Medium I contained (expressed in g/L): 10 glucose, 1.0 $(NH_4)_2SO_4$, 0.7 $MgSO_4 \cdot 7H_2O$, 0.5 NaCl, 0.1 $CaCl_2$, 1.0 KH_2PO_4, 0.1 K_2HPO_4 and 0.2 yeast extract (Difco), pH 6.5. Medium II contained in 0.5 M urea and (expressed in g/L): 1.0 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.5 NaCl; 5 $CaCl_2$mg, 2.0 KH_2PO_4, 0.7 K_2HPO_4, pH 6.5. All media were sterilized at 120 °C for 30 min.

The inoculum was prepared by growing cells from malt agar in 200 ml Erlenmeyer flasks in a rotary shaker containing 50 ml of medium I at 28 °C for 20 h. The medium was inoculated with 10% (v/v) of inoculum. Cultivation was carried out in tubes on a rotary shaker (220 min^{-1}) at 28 °C containing 5 ml of medium I for 24 h. The biomass was separated by centrifugation at 4000g for 20 min and washed three times with sterilized water. Medium II and hemocyanin (1 mg ml^{-1}), at a ratio of 1:2, were added to the cells and cultivation was continued for 4 days. After 1, 2, 3 and 4 days, respectively, the supernatants were analyzed by SDS–PAGE to detect the presence hemocyanin fragments.

The fragments were separated on a Sephadex G-100 column, eluted with 50 mM Tris/HCl buffer, pH 8.2. At the 4th day of incubation, an aliquot was applied on a Resource Q ion exchange column (Pharmacia), equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and eluted with a nonlinear gradient (0–0.5 M NaCl in 60 min) at a flow rate of 1 ml min^{-1}. Fractions were further purified by HPLC using a Nucleosil 100 RP-18 column (250 mm × 10 mm; 7 μm; Macherey-Nagel, Germany). For elution, a linear gradient from 5% solvent A (0.1% TFA in water) to 100% solvent B (0.085% TFA in acetonitrile) within 70 min, at a flow rate of 1 ml min^{-1}, was used. Also here, the HPLC fractions were detected at a wavelength of 278 nm and collected.

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli [16] using either 7.5% or 10% gels at pH 8.6. Coomassie Blue R-250 was used to stain the proteins bands.

MALDI-TOF mass spectrometry

A 4700 TOF/TOF Analyser was used in this study (Applied Biosystems, Framingham, MA) with a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. About 5 pmol protein material in the HPLC fractions containing the polypeptide fragments of the enzymatic or non-enzymatic cleavages were dissolved in 0.1% (v/v) TFA and applied to the target. Analysis was carried out using α-cyano-4-hydroxycinnamic acid as a matrix. A total of 4500 shots were acquired in the MS mode at a laser energy of 5180. Human albumin (66347.7 Da) and rabbit actin (43 kDa) were used to calibrate the mass scale. The mass values assigned to the amino acid residues are average masses.

Electrospray ionization mass spectrometry

Mass spectra were also performed on an electrospray ionization mass spectrometer (ESI-MS) Q-TOF, equipped with a nanospray source. Protein samples were prepared by diluting the protein stock solution in 10 mM ammonium acetate buffer. The solutions were kept at room temperature prior to analysis. ESI source settings were kept constant throughout all measurements to avoid changes in the processes of ion desorption and transmission. The spectra were recorded at a rate of 5 s. To ensure a high signal-to-noise ratio, typically 180–280 scans were averaged to generate each spectrum.

Amino acid sequence determination

Isolated HPLC fractions were dried and after dissolving them in 40% methanol/1% formic acid, they were subjected to automated Edman N-terminal sequencing on a Pulsed Liquid Protein Sequencer (Applied Biosystems GmbH, Foster City, CA).

Glycoprotein staining

Lyophilized fractions, obtained after enzymatic digestion with trypsin, depolymerization with Zn^{2+} ions, or incubation with *Saccharomyces* were dissolved in water. Some 2 μL of these solutions were applied to a silicagel plate (spot size restricted to 2–3 mm in diameter) which were dried, sprayed with orcinol/H_2SO_4, and heated for 20 min at 100 °C to detect the presence of glycopeptides [17].

Results

To test our hypothesis on the role of the carbohydrate moieties in the quaternary structure of molluscan Hcs, we followed the strategy outlined in Fig. 1.

Isolation of FUs after enzymatic digestion

Native Hc was purified from the hemolymph of *Rapana venosa* Hc using ion-exchange chromatography as described previously [11]. Electrophoretic analysis confirmed a good separation of the two structural subunits RvH1 (420 kDa) and RvH2 (400 kDa), which are glycoproteins as confirmed by the orcinol/H_2SO_4 test (not shown). Fig. 2 shows that the isolated RvH1 ($M_w \approx 420$ kDa) appeared as a single band. To remove the carbohydrate chains from RvH1, 0.25 mg protein was incubated at 37 °C with 75 mU PNGase F (peptide *N*4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F) for 48 and 72 h, respectively, and the resulting digest mixtures were applied onto 7.5% and 10% SDS–PAGE gels. Fig. 2 shows that a series of bands with lower molecular masses appeared, all of them (after 48 h), and most of them (after 72 h), having a molecular masses lower than 420 kDa but higher that 40 kDa.

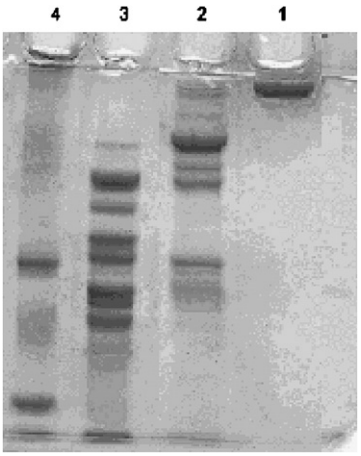

Fig. 2. SDS–polyacrylamide gel electrophoresis (10% gel) of structural subunit RvH1 before and after treatment with glycosidase PNGase F. (Lane 1) RvH1 ($M_w \approx 420$ kDa) before treatment with the enzyme. (Lanes 2 and 3) RvH1 after 2, respectively, 3 days of treatment with the enzyme. (Lane 4, a standard) Bovine serum albumin (66 kDa) and hen white lysozyme (14.4 kDa).

The proteolytic enzyme trypsin was used to cleave structural unit RvH1 in a ratio 1/400, for 1 h at room temperature [10]. The tryptic hydrolysate was separated on a 6 ml Resource Q column using 50 mM Tris/HCl buffer, pH 8.2. The resulting fractions were eluted within 60 min with a linear gradient (0–0.5 M NaCl) at a flow rate of 1 ml min^{-1}. Several fractions were isolated, each one, as determined by SDS–PAGE (Fig. 3) having a molecular mass of at least 45–50 kDa, which is the molecular mass of FUs, isolated from molluscan Hcs in general [1,2]. However, most fractions also contained polypeptides with molecular masses

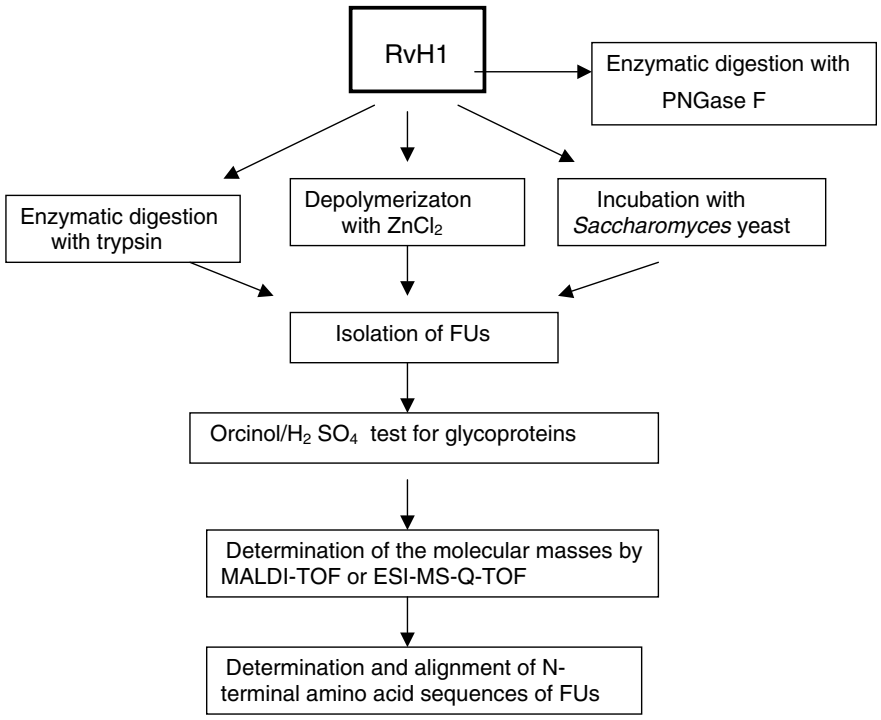

Fig. 1. Strategies used for the isolation and characterization of functional units from structural subunit RvH1 of *Rapana venosa* hemocyanin.

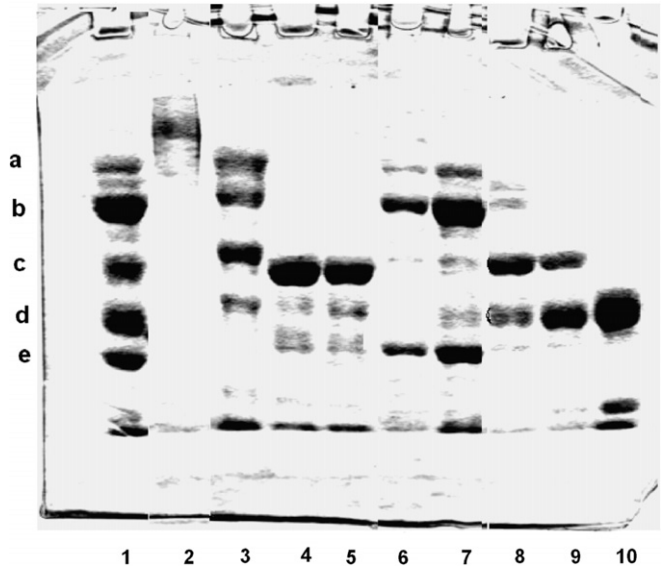

Fig. 3. SDS–polyacrylamide gel electrophoresis (10% gel) fractions resulting from a treatment of structural subunit RvH1 with trypsin. Lane 1, a standard: (a) β-galactosidase (116.3 kDa), (b) phosphorylase b (97.4 kDa), (c) ovotransferrin (78.0 kDa), (d) glutamate dehydrogenase (56.0 kDa), (e) ovalbumin (42.7 kDa). (Lane 2) RvH1 ($M_w \approx 420$ kDa) before treatment with the enzyme. (Lanes 3–10) Eight fractions isolated by ion-exchange chromatography.

lower than 40 kDa (Fig. 3, lanes 3–10). After additional purification on a Nucleosil 100 RP-18 column the masses of polypeptides in each fraction were determined with more precision by MALDI-TOF (Fig. 4; Table 1, fractions: 2, 5, 6). For example, the molecular mass of fraction 5 was found to be 31,652 Da, but ESI-MS-Q-TOF revealed that this fraction contained other species which differed from 31,652 Da by 162 Da or multiples thereof (corresponding to the mass of one hexose residue or multiples thereof (Fig. 5). Fig. 6 (row 1) shows that all the fractions isolated by trypsin treatment of RvH1 were glycosylated, as shown by the orcinol/H_2SO_4 assay.

Isolation of fractions after depolymerization with $ZnCl_2$

In order to find the best conditions to obtain FUs in highest yield, using this method, various parameters were studied. RvH1 was incubated in alkaline buffer, pH 9.5, and 0.4 mM Zn^{2+} was added to the medium to initiate the reaction. The treatment was extended over 15 days at room temperature, with 3 changes of the dialysis solution. The generated fractions were isolated on a Resource Q column. Five of them had molecular masses of about 50 kDa, as shown in Fig. 7. They were still fully functional after the 15th day of incubation, showing ratios of absorbance at 340–280 nm of about 0.26 ± 0.02, corresponding to a solution containing 100% oxygenated Hc (Table 1). Some depolymerization products also had molecular masses of about 100 and 150 kDa, as shown by the electrophoresis of fractions 1 and 6 (Fig. 7). In Fig. 8, we present the MALDI spectrum of fraction 3 after additional purification on a Nucleosil 100 RP-18 column, showing a molecular mass of 47,560 Da, with a doubly charged ion at *m/z* 23,790. The molecular masses of the other fractions of 48–51 kDa are higher as given in Table 1. These higher molecular masses of about 90 and 140 kDa indicate that several FUs were associated into dimers or trimers. According to the orcinol/H_2SO_4 assay, all isolated FUs (Fig. 6, row 2, positions 1–9) were glycosylated. It is remarkable that 2 fractions showed N-terminal sequences that are the same as the FUs RvH1-a and RvH1-f generated by trypsin (Table 2).

Isolation of FUs after incubation of RvH1 with Saccharomyces

The conditions for depolymerisation of RvH1 by *S. cerevisiae* were optimized testing different media. The best medium, medium II, containing 0.5 M urea at pH 6.5 was incubated with the hemocyanin solution at a ratio of 2:1 (v/v) for a period of 4 days, after which the cells were

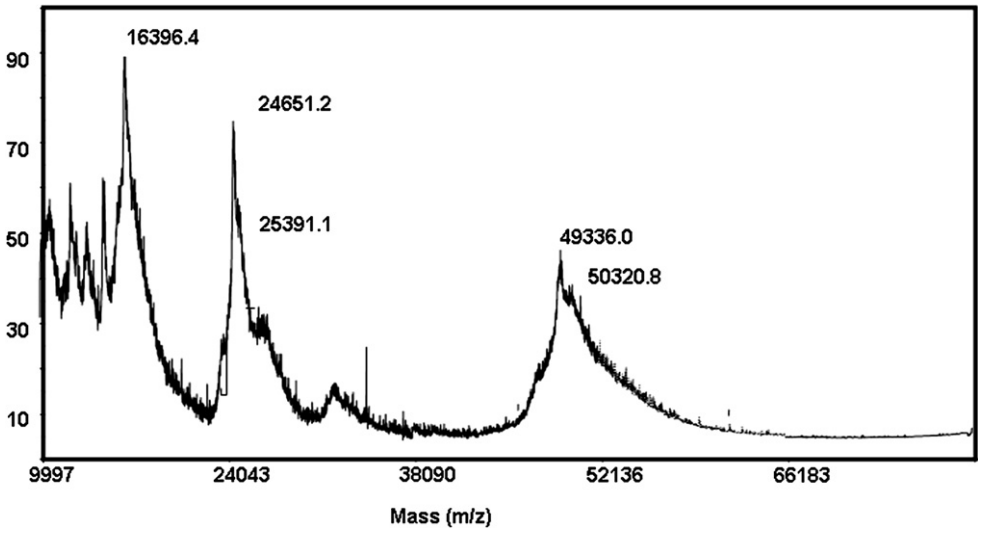

Fig. 4. MALDI-TOF spectrum of fraction 6 isolated after treatment of RvH1 with trypsin. Aside of the molecular mass at *m/z* 49,336 Da, also the doubly charged and the triple-charged ions are detected at *m/z* 24,651 and 16,396 Da, respectively.

Table 1
Molecular masses determined by MALDI-TOF/MS of fractions isolated after treatment with trypsin, $ZnCl_2$ or *Saccharomyces* on a Resource Q column and additionally purified on a Nucleosil 100 RP-18 column

No.	Fractions	Molecular masses (Da ± 100)				A_{345}/A_{280} [d]
1	[a] Fr. 1—trypsin		47,581 ± 50			0.0203
2	[a] Fr. 2—trypsin	35,415 ± 50	50,014 ± 50	99,710 ± 100	141,476 ± 150	0.0210
3	[a] Fr. 3—trypsin		47,592 ± 50	95,078 ± 100		0.0198
4	[a] Fr. 4—trypsin		50,398 ± 50	100,053 ± 100		0.0205
5	[a] Fr. 5—trypsin	31, 652 ± 50		95,053 ± 100	126,512 ± 150	0.0208
6	[a] Fr. 6—trypsin	16,396 ± 50	49,336 ± 50			0.0201
7	[a] Fr. 7—trypsin		48,142 ± 50	95,393 ± 100		0.0198
8	[a] Fr. 8—trypsin		46,739 ± 50			0.0204
9	[b] Fr. 1—$ZnCl_2$-f		48,631 ± 50		143,211 ± 150	0.0210
10	[b] Fr. 2—$ZnCl_2$ –e		51,418 ± 50			0.0202
11	[b] Fr. 3—$ZnCl_2$ -b		47,560 ± 50	95,043 ± 100	140,930 ± 150	0.0205
12	[b] Fr. 4—$ZnCl_2$ –a		50,491 ± 50			0.0201
13	[b] Fr. 5—$ZnCl_2$ –c		49,736 ± 50	97,245 ± 100	144,670 ± 150	0.0202
14	[c] Fr. 1—yeast –a		47,277 ± 50	98,461 ± 100	146,193 ± 150	0.0199
15	[c] Fr. 2—yeast-f		47,680 ± 50			0.0203
16	[c] Fr. 3—yeast-d		48,078 ± 50			0.0207
17	[c] Fr. 4—yeast-b		50,491 ± 50			0.0211
18	[c] Fr. 5—yeast-g		45,659 ± 50	110,083 ± 100		0.0202
19	[c] Fr. 6—yeast-c		51,418 ± 50			0.0193
20	[c] Fr. 7—yeast-e		49,726 ± 50	97,847 ± 100	147,548 ± 150	0.0203

[a] Fractions isolated by HPLC after treated of RvH1 with trypsin.
[b] Fractions isolated by HPLC after incubation of RvH1 with $ZnCl_2$.
[c] Fraction isolated by HPLC after incubation of RvH1 with *Saccharomyces* yeast.
[d] $A_{345}/A_{280} = 0.21$ giving information about the oxygenated state of FUs.

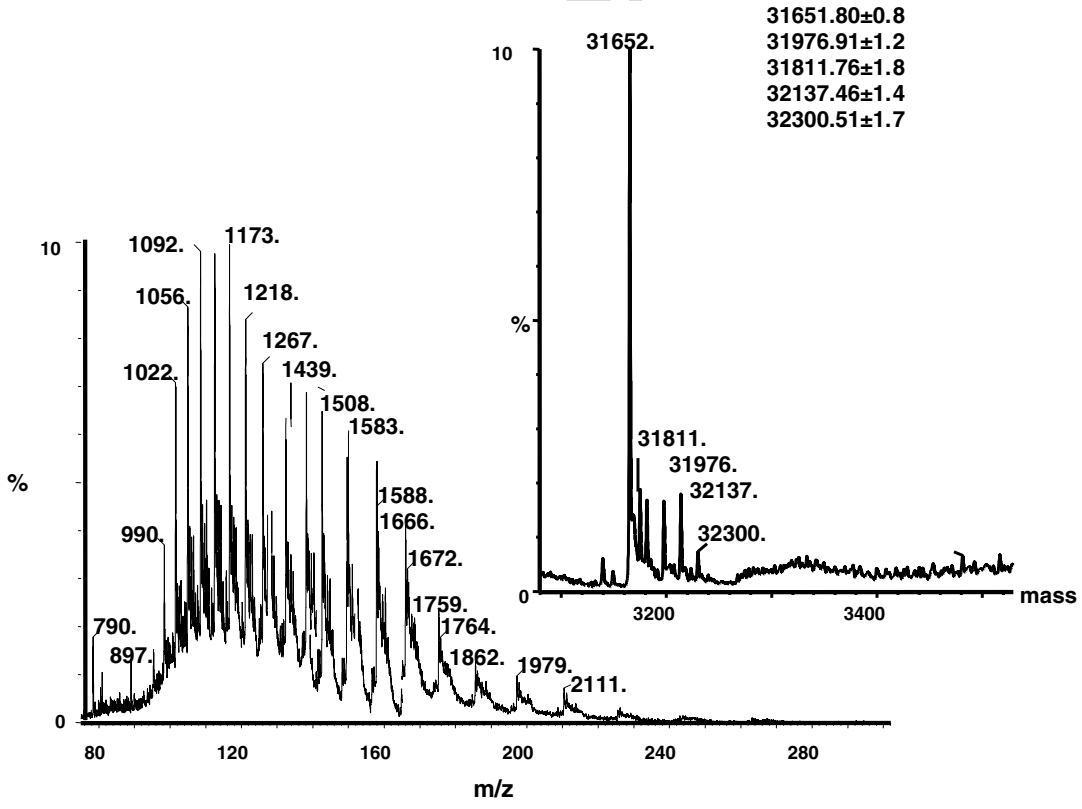

Fig. 5. ESI-Q-TOF mass spectrum, converted using MaxEnt software (inset), of fraction 5 isolated after treatment of RvH1 with trypsin.

removed by centrifugation. From the supernatant culture it was possible to isolate single FUs after 2–3 days of incubation. The highest yield of depolymerised Hc was obtained after the third day, with several fractions having molecular masses of around 50 kDa, as observed with 10% SDS–PAGE (results not shown). No molecular spe-

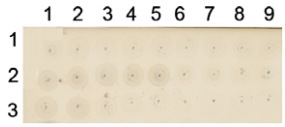

Fig. 6. Test of several fractions on a silica gel plate using the orcinol/H_2SO_4 test: Row 1, positions 1–9: FUs isolated after treatment of RvH1 with trypsin. Row 2, positions 1–9: fractions isolated after depolymerization of RvH1 with $ZnCl_2$. Row 3, positions 1–4: fractions isolated after the first and the second day of incubation with *Saccharomyces* yeast. Row 3, positions 5–9 fractions isolated after the third day of incubation of RvH1 with *Saccharomyces* yeast growth medium.

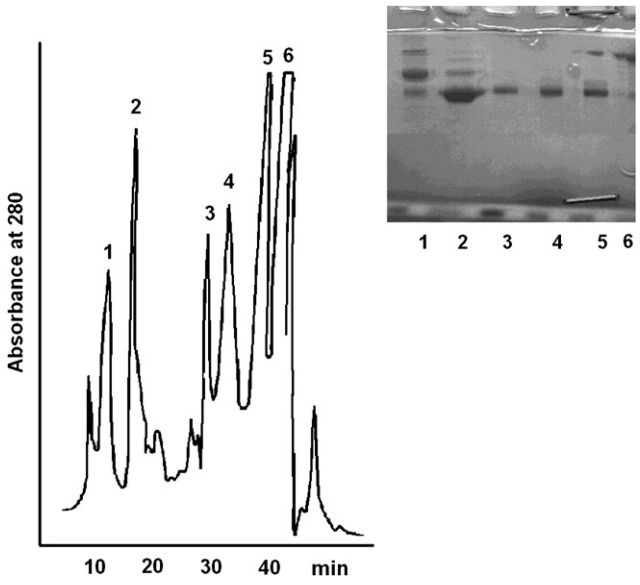

Fig. 7. Isolation of different fractions on a Resource Q column (Life Science, Germany) after depolymerization of RvH1with $ZnCl_2$. The ion-exchange column, equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and the FUs were eluted with a nonlinear gradient (0–0.5 M NaCl in 60 min) at a flow rate of 1 ml min^{-1}. (Inset) SDS–polyacrylamide gel electrophoresis (10% gel) of structural subunit RvH1 incubation with $ZnCl_2$. (Lanes 1–6) Several fractions isolated on a Resource Q column after depolymerization of RvH1 with $ZnCl_2$.

cies of this mass were observed in a blank experiment in which no yeast cells had been added. After each day of cultivation, the medium was assayed with orcinol/H_2SO_4 to detect the presence of carbohydrates. The test was positive after the first and second day (Fig. 6, row 3, positions 1–4), but from the third day the test became negative (Fig. 6, row 3, positions 5–9). After the fourth day, the cells were removed by centrifugation and the supernatant was analyzed on a Sephadex G-100 column. As shown in Fig. 9a, FUs with a molecular mass of around 50 kDa were effectively observed. By further performing ion-exchange chromatography, seven different fractions were isolated (see e.g., Fig. 9b) which were negative for orcinol/H_2SO_4. After additional purification on a Nucleosil C18 reversed phase column, the molecular masses (Fig. 10) as well as the N-terminal sequences were determined of each fractions (Tables 1 and 2).

Isolation of FUs after incubation of RvH1 with urea

Upon incubation of the structural unit with increasing concentrations of up to 5.0 M urea, and separating the resulting reaction mixture on a Sephadex G-100 column, we found that a peak at an elution time corresponding to molecular masses of around 50 kDa raised in intensity (Fig. 11), suggesting the generation of the functional units.

Discussion

According to available genomic data, the different functional units of the several structural units of molluscan Hc, each of 40–45 kDa molecular mass, are sequentially linked to each other by short linker peptides, resulting in the molecular masses of 350 kDa to 400 kDa for a structural unit [18]. This interpretation does not explain why the structural unit of all molluscan Hcs has a sedimentation coefficient of 11S, which accounts for a lower molecular mass of about 250 kDa. Evidence for this structural organisation is provided by the fact that trypsin treatment of the structural units produces fragments, the so called

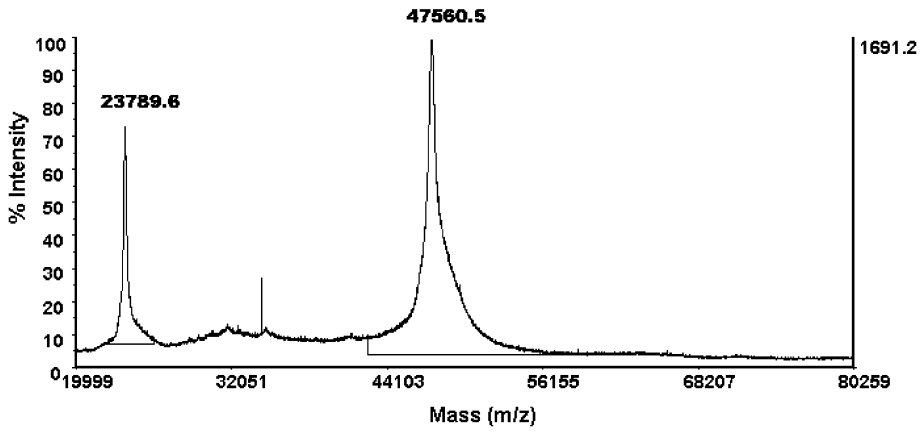

Fig. 8. MALDI spectrum of reversed phase purified fraction 3 ($ZnCl_2$-b) isolated after non-enzymatic treatment of RvH1 with $ZnCl_2$ and additional purification on a Nucleosil 100 RP-18 column, showing a molecular mass at *m/z* 47,560 Da of the doubly charged ion at *m/z* 23,789.

Table 2
N-terminal sequences of FUs isolated after treatment with trypsin [10,18], $ZnCl_2$ or *Saccharomyces* on a Resource Q column and additionally purified on a Nucleosil 100 RP-18 column

RvH1-**a** [a]	S	L	L	R	K	N	V	D	T	L	T	E	Q	E	I	L								
RvH1-**a** [b]	S	L	L	R	K	N	V	D	T	L	T	E	Q	E	I	L								
RvH1[c]	S	L	L	R	K	N	V	D	T	L	T	E	Q	E	I	L								
RvH1 [d]	S	L	L	R	K	N	V	D	T	L	T	E	Q	E	I	L								
RvH1-**b** [a]										A	L	T	R	K	N	V	D	T	L	N	E			
RvH1[c]	I	H	V	A	G	G	A	M	K	G	F	V	R	K	D	I	P	R	I	H				
RvH1 [d]	I	H	V	A	G	G	A	M	K	G	F	V	R	K	D	I	P	K						
RtH1-**c** [a]					S	L	T	R	K	N	V	E	F	L										
RvH1[c]	S	I	A	G	V	G	V	R	K	N	I	N	S	L	T	D	A	E	M					
RvH1 [d]	S	I	A	G	V	G	V	R	K	N	I	N	S	L	T	D	A	E	M					
RtH1-**d** [a]	A	P	V		R		V	R	K	N	L	N	V	L	T	D								
RvH1 [b]	V	R	H	S	S	H	V	R	R	N	L	I	S	L	R	X	E							
RvH1 [d]		S	V	H	R	H	S	R	X	D	L	T	T	G	G									
RtH1-**e** [a]					A	E	V	R	H	N	V	D	R	L	T	D	Q	E	I	L	D			
RvH1 [b]	G	X	P	I	S	D	L	V	X	K	S	V	X	T	L	S	P							
RvH1 [c]	G	X	P	I	S	D	L	V	X	K	S	V	X	T	L	S	P							
RvH1 [d]	G	X	P	I	S	D	L	V	X	K	S	V	X	T	L	S	P							
RvH1-**f** [a]	G	V	G	V	R	K	D	L	N	T	L	T	A	A	E									
RvH1 [b]	G	V	G	V	R	K	D	L	N	T	L	T	A	A	E									
RvH1 [c]	G	V	G	V	R	K	D	L	N	T	L	T	A	A	E									
RvH1 [d]	G	V	G	V	R	K	D	L	N	T	L	T	A	A	E									
RvH1-**g**[a]					V	T	A	L	E	E	E	H	R	K	E	V	D	D	L	N	D	R	D	I
RvH1 [d]		X	F	D	V	T		E	T	M	E	H	M	T	Y									

The letters in bold refer to the different FUs isolated and labeled in previous work [10,20]. *Rapana thomasiana* (RtH) was renamed as *Rapana venosa* (RvH).

[a] FUs isolated after treatment of RvH1 with trypsin (Ref. [10]).
[b] FUs isolated after treatment of RvH1 with trypsin (Ref. [20]).
[c] FUs isolated after treatment of RvH1 with $ZnCl_2$ (this work).
[d] Fractions isolated after incubation of RvH1 with *Saccharomyces* yeast (this work).

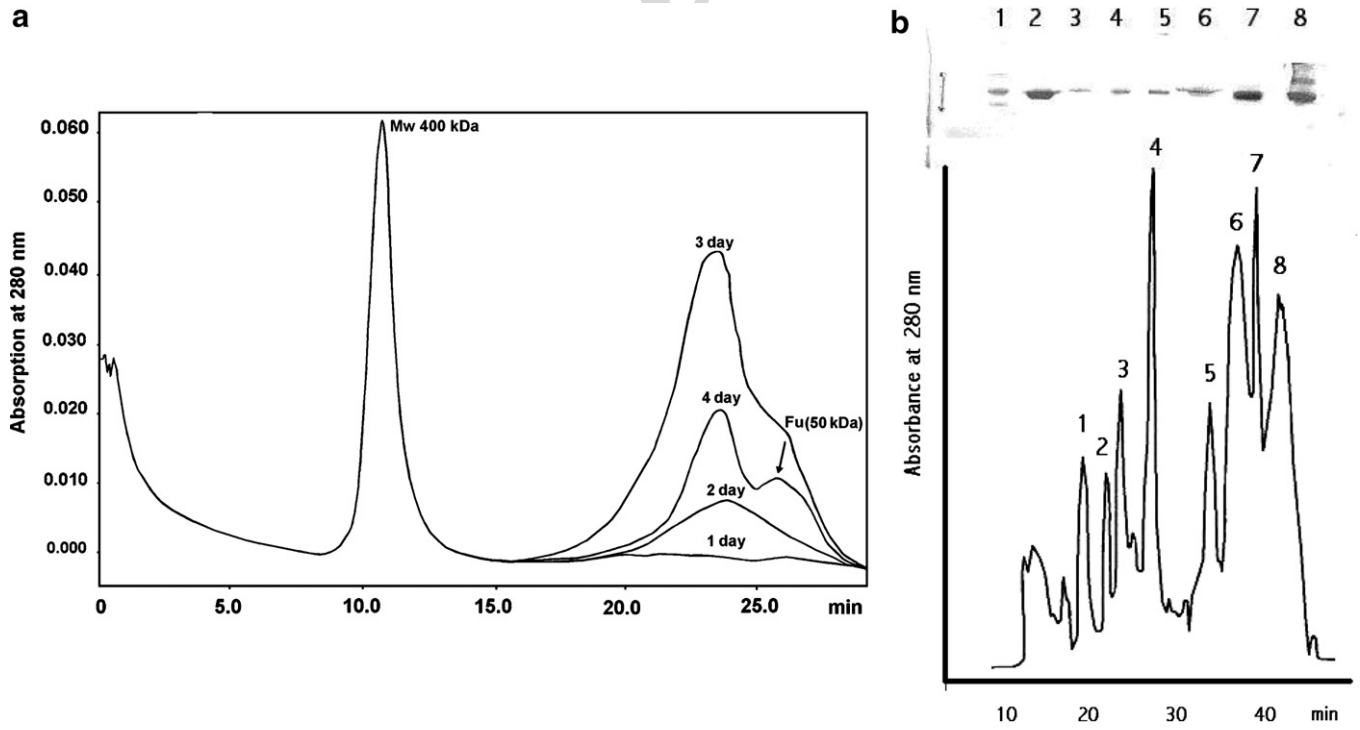

Fig. 9. (a) Isolation of different fractions on a Sephadex G-100 column after incubation of RvH1with *Saccharomyces*. The column, equilibrated with 50 mM Tris/HCl buffer, pH 8.2, was eluted with the same buffer at a flow rate of 1 ml min^{-1}. The peak coming after 26 min are combined fractions of 50 kDa. (b) Isolation of different fractions on Resource column (Life Science, Germany) after incubation of RvH1with *Saccharomyces*. The ion-exchange column was equilibrated with 50 mM Tris/HCl buffer, pH 8.2, and the FUs were eluted with a nonlinear gradient (0–0.5 M NaCl in 60 min) at a flow rate of 1 ml min^{-1}. (Inset) SDS–polyacrylamide gel electrophoresis (10% gel) of the different fractions.

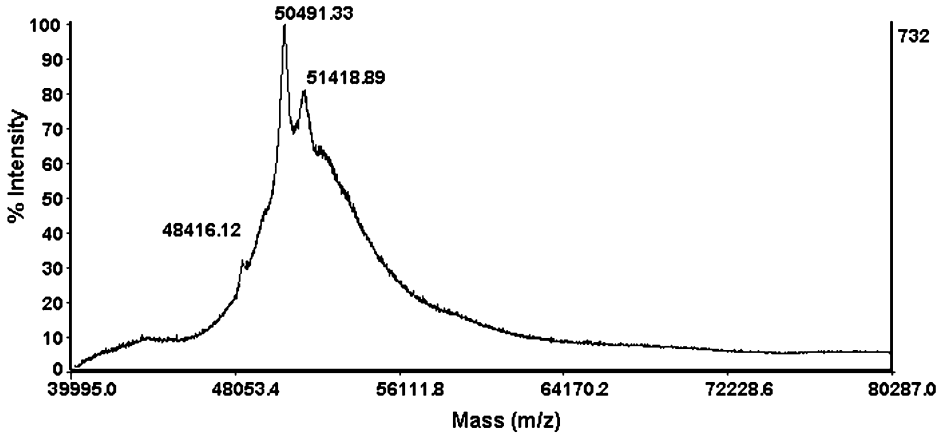

Fig. 10. MALDI spectrum of several fractions isolated on a Resource Q 6 ml column after incubation of RvH1 with *Saccharomyces* yeast. After additional purification on Nucleosil column RP C18, the sample was measured by MALDI MS using conditions as in Fig. 8.

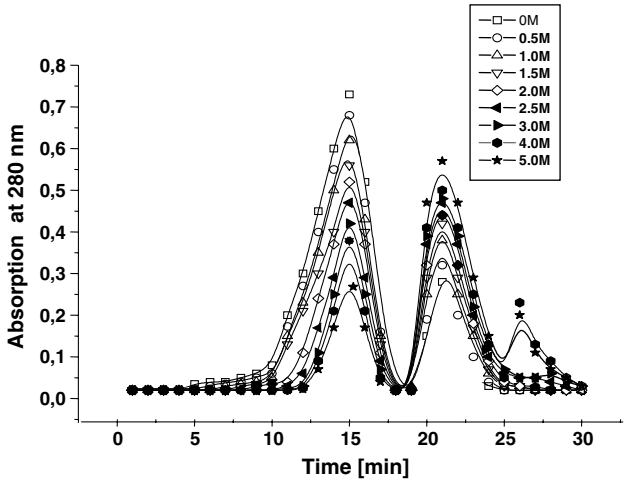

Fig. 11. Isolation of different fractions on a Sephadex column after 24 h incubation of RvH1with different concentrations of urea (0–5 M). The column, equilibrated with 50 mM Tris/HCl buffer, pH 8.2, was eluted with the same buffer at a flow rate of 1 ml min^{-1}. The peak after 22 min are fractions of 50 kDa.

functional units, of masses around 40 kDa. The N-terminal sequences of the FUs of *Rapana venosa* Hc have been determined [10], and are mentioned again in our Table 2. In the present work on the same structural unit, we aim to challenge this viewpoint and provide evidence for the alternative interpretation that the functional units exist as separate polypeptides of 40–45 kDa which are linked amongst each other by N-linked oligosaccharide trees.

The experiment at the start of this hypothesis is the fact that, upon treatment of RvH1 with up to 5 M increasing amounts of urea, fragments are produced having molecular masses of around 50 kDa. The fragments coelute upon separation of the reaction mixture on a Sephadex G-100 column (Fig. 11). It is extremely unlikely that the so-called linker peptides in the one-gene scenario for RvH1 would be sensitive to a cleavage reaction under influence of urea.

The second argument is in favour of our hypothesis comes from the observation that Zn^{2+}-ions are able to cleave RvH1 into fragments that also have molecular masses of 45–50 kDa (Table 1) and moreover, for some of them, have the same N-terminal sequences as the FUs generated by trypsin. Such is the case, for example, for the fractions that resemble the tryptic FUs indicated in Table 2 as RvH1-a and RvH1-f. It is extremely unlikely that $ZnCl_2$ would cleave the intersubunit connecting peptides in the one-gene scenario, but it is quite reasonable to assume that the Zn^{2+} ions cause the basic hydrolysis of sugar–protein bonds, since these are energetically less stable than peptide bonds. The depolymerisation of RvH1 may be due to the fact that Zn^{2+} ions are good Lewis acids which may locally increase OH^- concentrations, causing bond cleavage. Since RvH1 is about 8% glycosylated [19], the higher than 45–50 kDa molecular masses we detected by MALDI-TOF MS (Table 1) can be explained by an incomplete cleavage of all of the polypeptide–sugar bonds.

The third line of evidence to support our hypothesis is provided by the effect that Hc supplemented *Saccharomyces cerevisiae* growth medium generates fragments that also have molecular masses of around 50 kDa (Table 1). This type of experiment, not yet been carried out so far, is based on the assumption that the yeast produces externally secreted glycosidases which cleave the oligosaccharides linking the different functional units. It is remarkable, moreover, that N-terminal sequences of the different fractions generated by this type of digestion are identical to those of the fragments generated by the treatment with $ZnCl_2$, certainly for the fractions that are aligned in Table 2 with the typical FUs called RvH1-a, RvH1-b and RvH1-f. We interpret the fact that the orcinol test is negative after the third day of incubation, but that higher molecular masses were nevertheless detected upon MALDI-TOFMS for fractions 5 and 7, is due to the limited sensitivity of the test.

Yet another feature that corroborates our challenging view is the fact that glycosidase PNGase F, being given RvH1 as a substrate, generates molecular species with molecular masses lower than 400 kDa but higher than 50 kDa after 2 days of incubation (Fig. 2). In our model,

this can be explained by the incomplete cleavage of each one of the oligosaccharides in the several FUs, such that several of them remain linked to each other. Also the latter apparently get further cleaved off, as we see the higher molecular mass species gradually decreasing in amount after 3 days of incubation (Fig. 2).

Upon repeating the digest of RvH1 with trypsin, as carried out before [10], we found an additional argument for our hypothesis in the results of the SDS–polyacrylamide gel electrophoresis of the resulting FUs (Fig. 3), combined with the fact that these fragments are glycosylated (Fig. 6). Apparently, trypsin can cleave the structural unit RvH1 at several different sites, giving rise to several different fragments depending on the conditions of the digest (see e.g., the difference in N-terminal sequence results for RvH1-d and RvH1-e obtained by us [10] and others [20]). The higher molecular masses of around 100 kDa for the species found in the tryptic fragments 2, 3, 4, 5 and 7 may thus arise from FUs that remain linked to oligosaccharide trees.

A final argument, being of less weight than the previous ones, in favour of our hypothesis may be found in a comparison of the molecular masses of 50,491 Da for the fraction 4 obtained by treatment with $ZnCl_2$ and 47,777 Da for the fraction 1 obtained by depolymerisation with yeast (Table 1). The difference of 3214 Da closely corresponds to the mass of the oligosaccharide tree, published before [13].

In conclusion, we presented 5 types of experimental data, resulting from digests with different agents (urea, $ZnCl_2$, yeast, trypsin, PNGase F) that the structural unit RvH1 of the mollusc *Rapana venosa* Hc is not composed of a single polypeptide chain in which the different functional units are linked by protease sensitive peptide linkers. We rather propose the model that the individual polypeptides of the different FUs are linked to each other in a network generated by oligosaccharides. The structure of one N-linkedglycopeptide of a subunit has already been determined by MALDI-TOF/TOF-MS [21] to be SVNGTLLGSQILGKPY. The authors also sequenced a peptide of 14 residues containing 3 histidine residues, occurring at the positions 6, 7 and 13, respectively. We propose that some of these histidine residues are involved in the linkage of the oligosaccharide trees which generate the RvH1 network of FUs. The linkage of the imidazole side chain of histidine with OH groups in general is well documented [22] and should, from the chemical point of view, be quite susceptible to cleavage with Zn^{2+}-ions, forming metal–chelate complexes. Whether this is effectively the case for the RvH1 remains to be proven by further work.

Acknowledgments

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