PARTICIPATION OF CARBOHYDRATE STRUCTURES IN THE FORMATION OF COMPLEX STRUCTURE OF HEMOCYANINS FROM HALIOTIS TUBERCULATA

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Received on November 20, 2021
Presented by Ch. Tsvetanov, Member of BAS, on January 27, 2022

Abstract

Glycosylation plays an important role in many important processes in organisms. Their participation in the construction of the tertiary structure and immunotherapeutic activity of the hemocyanin Rapana venosa (RvH) from the Black Sea has been presented. Based on these results, the importance of glycans in organization of the structure of hemocyanin from the abdominal mollusk Haliotis tuberculata (HtH) was also studied.

After incubation of both isoforms HtH1 and HtH2 with yeast Saccharomyces cerevisiae and enzyme Zymolyase, fractions with lower molecular weight (MW) between 45 and 65 kDa, which corresponds to MW of functional units (FUs) of hemocyanins, was identified. A comparative analysis of 10% SDS PAGE assays confirmed the depolymerization of the subunits HtH1 and HtH2 due to the specific glycosidase in the yeast S. cerevisiae and the enzyme Zymolyase. The presented results confirm our hypothesis that glycans are involved in the formation of the structural subunits of HtH.

Key words: Saccharomyces cerevisiae yeast, SDS PAGE, hemocyanins, functional units, Haliotis tuberculata
Introduction. In comparison to vertebrate organisms which count on the iron of the hemoglobin in the red blood cells, most mollusk and arthropods breathe through the respiratory protein hemocyanins. With their immense size, the respiratory proteins belong to the group of the “multimeric glycoproteins containing a type-3 copper centre that occur freely dissolved in the hemolymph”, and vary deeply in structure and carbohydrate content. The structure of the molluscan hemocyanins is comprised by decamers with molecular masses (MW) \( \sim 4 \text{ MDa} \) or multidecamers with MW > 8 MDa \[^1,2\].

A complementary feature of these respiratory macromolecules is the abundant carbohydrate content which composes 3–4% (w/w) of the MW of hemocyanins. N-glycosylation motifs of the hemocyanins are found near the active sites and between the binding sites of their subunits. Moreover, the N-glycans are responsible for the assembly of the quaternary structure of molluscan hemocyanins \[^3\] which contributes to their structural stability and immunomodulatory properties in mammals \[^4,5\]. This assumption is confirmed by Dolashka-Angelova et al. \[^6\].

The involvement of an oligosaccharide structure in the formation of the organization of structural subunit RvH1 of \( R. \) *venosa* has been suggested \[^6\]. Several hemocyanin fragments, functional units (FUs), were eluted by ion exchange chromatography (Resource Q column) after fourth day of incubation of structural subunit RvH1 in culture media of yeast strain \( S. \) *cerevisiae* 90-1. Formation of FUs was also observed after the depolymerization of RvH1 in the presence of \( \text{Zn}^{2+} \) ions, urea, as well as the yeast \( S. \) *cerevisiae*. This process does not involve breaking the covalent peptide bonds of the subunit RvH1. Only the bonds of the carbohydrate moiety are broken down \[^1\].

This article presents information on the tertiary structure of the HtH1 subunit of hemocyanin from the gastropod mollusk \( H. \) *tuberculata* (HtH). The relationship between the individual FUs and the formation of the HtH subunits is of great interest. In this study, we shed light on the idea that FUs in the HtH1 subunit are associated with oligosaccharide structures between them.

Materials and methods. Preparation of \( H. \) *tuberculata* hemocyanin and its structural subunits. Extracts from the hemolymph of the Abalones were provided by Prof. Bernard Lieb of the Johannes Gutenberg University in Mainz (JGU). Both subunits, HtH1 and HtH2, were purified by gel filtration on Sephadex G100 as was described by Dolashka-Angelova et al. \[^6\].

Incubation of HtH1 and HtH2 with \( S. \) *cerevisiae* studies with the subunits were performed in liquid culture medium from strain \( S. \) *cerevisiae* 90-1, as described by Dolashka-Angelova et al. \[^6\]. The growth medium of the strain contains \((\text{NH}_4)_2\text{SO}_4, \text{MgSO}_4.7\text{H}_2\text{O}, \text{NaCl, CaCl}_2, \text{KH}_2\text{PO}_4, \text{K}_2\text{HPO}_4, 0.5 \text{ M H}_2\text{NCONH}_2 \) at pH 6.5. Malt agar cells were grown in 200 mL flasks containing 50 mL of medium at 28 °C for 20 h. The fermentation medium is inoculated with 10% (v/v) inoculum. Cultivation was performed at 28 °C for 24 h in tubes.
containing 5 mL of growth medium on a rotary shaker (220 min \(^{-1}\)). The biomass was removed after centrifugation at 4000 \(\times\) g for 20 min and washed three times with sterilized water. Cultivation was continued after HtH (1 mg.mL\(^{-1}\)) and the two subunits (HtH1 and HtH2) were added to the cells in a 2:1 ratio. The biodegradability of HtH was observed in the supernatants obtained after 2 and 3 days of cultivation.

After the 2nd and the 3rd day, the cells were removed by centrifugation and the supernatant was analyzed by SDS–PAGE to detect the presence of hemocyanin fragments.

**Incubation of HtH1 and HtH2 with Zymolyase.** Protein concentration of the \(H.\) tuberculata hemocyanin and structural subunits HtH1 and HtH2 was adjusted to 0.07 mg/mL by 50 mM Tris/HCl (pH 7.0) and then treated with Zymolyase-20T (MP Biomedicals, Aurora, Ohio). Fifty microliters hemocyanins sample was mixed with 1 mg Zymolyase and was incubated at 37\(^\circ\)C for 1 h, 3 h, 6 h and 24 h. The size of the obtained hemocyanins fragments was determined by SDS-PAGE according to the work of LAEMMLI \(^7\).

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished through the procedure, described by Laemmli \(^7\), with slight changes, using 10% gels at pH 8.6. To stain the protein bands, Coomassie Blue R-250 was used. A quantity of 5 \(\mu\)L from the protein ladder Blue Plus\(^\circledR\) II was applied in the wells of the mini gel as a molecular standard. Quality assessment of the SDS-PAGE was performed by the image software (Image Quant TL, v. 8.1) after scanning the gels on a transmission scanner (ImageScanner III, GE Healthcare).

**Results and discussion.** The hemocyanins of arthropods and mollusks attract scientific interest with their structure and potential applications in pharmacy and medicine \(^8\). The structure of molluscan hemocyanins is represented by subunits that covalently bind 7 or 8 FUs by linkage peptides \(^9,10\). There is also a hypothesis about the role of carbohydrate structures in the formation of the tertiary structure of Hcs from mollusk, presented by Dolashka-Angelova et al. \(^6\). To test this hypothesis, we have applied partial experiments from the strategy, following the fragmentation of the subunits of the gastropod mollusk \(H.\) tuberculata.

Only two approaches have been applied: cultivation of \(Saccharomyces\) cerevisiae in the presence of both subunits of the HtH and use of Zymolyase as an enzyme to cleave oligosaccharide residues from the hemocyanin.

An advantage of the studies is that the primary structures of the two subunits, HtH1 and HtH2, are known, as well the 14 carbohydrate structures of the HtH1, which are composed mainly of mannose-rich N-glycans as well as N-mixed carbohydrates with fucose, galactose, GlcNAc and clones for glycosylation \(^11\).

**Analyses of subunits HtH1 and HtH2 before and after incubation with \(S.\) cerevisiae strain.** Various approaches to deglycosylation of hemocyanin...
subunits are known in the literature, mainly with PNGase F or trypsin. It is known that glucans are the main components of the yeast cell wall and during budding and wall growth or ascus formation, they undergo limited hydrolysis of the wall directed to the site by the medium of β-glucanases [12–15]. Two classes of 1,3 β-glucanases (exoglucanases and endoglucanases) are found in yeast, where exo-1,3-β-glucanases have greater activity and hydrolyze β-O-glycosidic bonds [15]. Therefore, our suggestion was that this characteristic of S. cerevisiae may be applicable to the deglycosylation of hemocyanin subunits.

The change in MW of the native molecule HtH and subunits, HtH1 and HtH2, before and after incubation with a S. cerevisiae strain was monitored by 10% SDS-PAGE (Fig. 1). The comparative analysis showed no changes in MW of non-incubated HtH and two subunits (∼392 kDa) (Fig. 1, Lane 1 (L1), Lane 4 (L4) and Lane 7 (L7), respectively). Therefore, their tertiary structure is preserved. After incubation of S. cerevisiae strain in the presence of HtH, HtH1 and HtH2, changes in their MW were observed after the 2nd day (Fig. 1, Lane 2 (L2), Lane 5 (L5) and Lane 8 (L8), respectively) and the 3rd day (Fig. 1, Lane 3 (L3), Lane 6 (L6) and Lane 9 (L9), respectively) from the incubation. The highest yield of depolymerized native HtH and HtH1 and HtH2 subunits was obtained after the
Table 1
Comparative analyses of calculated MW of the obtained fractions after incubation with *S. cerevisiae*, using software Image Quant TL

<table>
<thead>
<tr>
<th>Band No</th>
<th>Lane 1 (MW kDa)</th>
<th>Lane 2 (After incubation of HtH, MW kDa)</th>
<th>Lane 3 (After incubation of HtH1 MW kDa)</th>
<th>Lane 4 (After incubation of HtH2 MW kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>390.2</td>
<td>238.8</td>
<td>72.9</td>
<td>69.9</td>
</tr>
<tr>
<td>2</td>
<td>234.6</td>
<td>121.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>214.7</td>
<td>111.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>75.6</td>
<td>74.1</td>
<td>70.1</td>
<td>69.8</td>
</tr>
<tr>
<td>5</td>
<td>58.7</td>
<td>52.9</td>
<td>53.3</td>
<td>53.1</td>
</tr>
<tr>
<td>6</td>
<td>45.1</td>
<td>44.7</td>
<td>47.7</td>
<td>43.1</td>
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<tr>
<td>7</td>
<td>40.9</td>
<td>39.6</td>
<td>41.9</td>
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<tr>
<td>8</td>
<td>39.6</td>
<td>41.9</td>
<td>40.6</td>
<td>41.1</td>
</tr>
</tbody>
</table>

3rd day, with several fractions having a MW ∼ 50 kDa (Fig. 1, L3, L6 and L9, respectively).

A more accurate analysis of the change in molecular weights of the fractions was presented using Image Quant TL software, v. 8.1 in Table 1.

New fractions with an MW of 40 to 100 kDa were observed after incubation of the strain in the presence of HtH, HtH1 and HtH2 subunits (L5; L6 and L8; L9, respectively). The change in HtH1 after 48 h is demonstrated in the expression of mainly five proteins with MW 70.1 kDa, 59.8 kDa, 53.3 kDa, 43.1 kDa and 40.6 kDa (Fig. 1, L5), but after 72 h of incubation, only four proteins with MW 69.8 kDa, 59.4 kDa and 53.1 kDa and 41.1 kDa were reported (Fig. 1, L6).

The behaviour of the HtH2 is similar, where the change after 48 h is expressed in five proteins with MW 72.9 kDa, 59.1 kDa, 53.7 kDa, 43.2 kDa and 41.1 kDa (Fig. 1, L8), which after 72 h decreased to three proteins with MW 59.1 kDa, 53.7 kDa, 41.1 kDa (Fig. 1, L9).

From the presented results we can conclude that the enzymatic activity of yeast is better represented in HtH2, where after the 3rd day of enzymatic hydrolysis fractions with MW are observed, which correspond to the data on the molecular weight of functional units of HtH. Cultivation of the subunits also produced fragments with a higher MW of about 70 kDa, as well as lower MW of 40 kDa (Fig. 1, L6 and L9), which may be due to incomplete enzymatic hydrolysis caused by the enzyme in the yeast chitinase Cts1 (1,4-beta-poly-N-acetylglucosaminidase), which is responsible for the mother/daughter division, acting mainly on the glycosidic bonds in the chitin of the *S. cerevisiae* cell wall [16].

In addition to the fact that both subunits are glycosylated and bind complex carbohydrate structures [11], an orcinol/H$_2$SO$_4$ analysis of the resulting depolymerized fractions after each day of cultivation is presented.
screening was performed to assess the presence of traces of carbohydrates in the resulting fractions after incubation with *S. cerevisiae*.

The analyses in Fig. 2 show a positive test before incubation of HtH1 and HtH2 (position 4, position 5), but the test for subunits HtH1 and HtH2 after the 2nd (position 1 and position 2, respectively) and the 3rd day (position 6 and position 7, respectively) becomes negative. The fractions showed no sugars at the end of the experiment (day 3). Traces of glycosylation were observed for the native molecule HtH after the 2nd and 3rd day (position 3 and position 9, respectively).

The lack of carbohydrates in the hemocyanin isoforms after culturing in medium of *S. cerevisiae* is evidence of the enzymatic hydrolysis that is caused by the specific glycosidases present in the medium.

**Analyses of subunits HtH1 and HtH2 before and after incubation with Zymolyase.** After the obtained fragments of depolymerization of HtH1 and HtH2 subunits under the influence of specific enzymes produced by the *S. cerevisiae* strain 90-1, the experiment continues with application of Zymolyase upon native HtH and its subunits HtH1 and HtH2. Characteristic feature of the enzyme complex Zymolyase is its main activity β-1,3 glucanlaminaripentaohydrolase and auxiliary activities: β-1,3-glucanase, protease, mannanase (Fig. 3, L1) [17].

There are four representative bands expressed on the gel electrophoresis (Fig. 3, L1) with MW 67.8 kDa, 53.9 kDa, 35.4 kDa and 32.3 kDa, which coincide with MW of enzyme complex Zymolyase [18]. It is known that the action of this enzyme is directed at wall components other than chitin [17]. The results presented in Fig. 3 confirm the obtained results after incubation of HtH and isoforms with *S. cerevisiae* strain 90-1. Fractions with MW between 45–50 kDa (Fig. 3, L2; L4 and L6) were not observed, because they are the native molecules.

After treatment of hemocyanins with Zymolyase, fractions with a molecular weight lower than 70 kDa were obtained for HtH, HtH1 and HtH2 (Fig. 3,
L3, L5 and L7, respectively). Of particular importance is the fraction with MW of 48.6 kDa, which can correspond to FU HtH1-b (48.07 kDa) or HtH1-e (48.36 kDa) [19].

An interesting result is also the fraction with MW 58.7 kDa, which is undoubtedly relevant for HtH1-h (L5-MW 59.2 kDa). This FU is of great importance as it consists of three domains [20].

L7 shows also fractions with MW lower than 50 kDa, obtained after depolymerization of the HtH2 subunit with the complex enzyme Zymolyase. The fraction with MW of 50.1 kDa can be addressed to HtH2-a, b, c (≈ 50 kDa) FU, respectively, fraction of MW 54.4 kDa to HtH2-e (55 kDa) and fraction 59.7 kDa to HtH2-h (60 kDa). All comparative data were determined on the basis of cDNA of the two subunits by LIEB et al. [1-19], which partly explains the lower molecular weights of the hemocyanin fraction determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The reason why fractions with MW below 40 kDa (L5 and L7) are still observed may be due to the resulting fragments with MW above 60 kDa from partial enzymatic hydrolysis.
Conclusion. The present work is focused on the participation of N-glycosylation in the construction of the tertiary structure of hemocyanin *H. tuberculosis*. It has been shown that after incubation of the native molecule and both subunits with the *S. cerevisiae* strain, fractions with lower MW are obtained, which correspond to the MW characteristic of FUs. This is probably due to the hydrolysis of the carbohydrate structures by the specific endoglucanases present in the strain. This process was confirmed after the enzymatic hydrolysis of HtH and both structural subunits HtH1 and HtH2 with the specific enzyme Zymolyase.

Electrophoretic analyses and screening with orcinol/H$_2$SO$_4$ confirm our assumption that the individual FUs are linked by glycans and form the structural subunits of HtH. Additional mass spectrometric analyses of the fragments obtained after hydrolysis of HtH are envisaged to confirm the stated assumption.

REFERENCES


