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REVIEW

Antiviral activity of hemocyanins

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Abstract

Hemocyanins are giant biological macromolecules acting as oxygen-transporting glycoproteins. Most of them are respiratory proteins of arthropods and mollusks, but besides they also exhibit protecting effects against bacterial, fungal and viral invasions. As discovered by 2-DGE proteomics analyses, several proteins including hemocyanins of hemocytes from virus-infected arthropods increased upon infection, confirming hemocyanin's role as part of the organism's defence system. Based on the structural analyses of molluscan Hcs it is suggested that the carbohydrate chains of the glycoproteins seem to interact with surface-exposed amino acid or carbohydrate residues of the viruses through van der Waals interactions.

Key Words: functional units; hemocyanin; mollusks; arthropods; viruses

Introduction

The persistence of the virus in the organism leads to the development of lymphoproliferative disease, the formation of various carcinomas and to the affection of the peripheral and central nervous system. Antiviral drugs may be divided into acyclic nucleoside analogues (aciclovir, ganciclovir, penciclovir), acyclic nucleotide analogues (cidofovir and adefovir), and substances of natural origin (De Clercq, 2004). Most of the submitted drugs are potent inhibitors of viral reproduction, but not all of them are promising for clinical application, since they are very different in terms of toxicity.

Recently it was found that hemocyanins (Hcs), the oxygen-transporting glycoproteins of many arthropods and mollusks, could be also potential inhibitors of some virus infections (Chongsatja *et al.,* 2007; Flegel *et al.,* 2011). These giant glycoproteins which differ in molecular mass, structure, carbohydrate content, and monosaccharide composition have recently received increasing interest due to their significant immunostimulatory, antitumor, and antiviral properties (Dolashka-Angelova *et al.,* 2009; Dolashka *et al.,* 2010; Mičetić *et al.,* 2010; De Smet *et al.,* 2011; Rehm *et al.,* 2012; Markl *et al.,* 2013). Both species, molluscan and arthropodan Hcs, contain copper-containing active sites in which the Cu(I,I) is oxidized to the Cu(II,II) state, thus accounting for their distinctive deep blue color. The biosynthesis of hemocyanins from e.g. *Concholepas concholepas* (CcH) takes place in the hepatopancreas (Manubens *et al.,* 2010) but *Megathura crenulata's* Hcs originate from rhogocytes (Martin *et al.*, 2011).

Investigations on several molluscan hemocyanins, e.g. from *Haliotis tuberculata* (HtH, Abalon) (Markl *et al.,* 2001); *Helix lucorum* (HlH, garden snail), *Rapana venosa* (RvH, Black sea murex) (Dolashka-Angelova *et al.,* 2003, 2009, 2011; Iliev *et al.*, 2008) or *C. concholepas* (CCH, Loco) from the Pacific Chilean coast (Moltedo *et al.,* 2006, Arancibia *et al.,* 2012) demonstrated their remarkable immunostimulatory properties in experimental animal model and clinical studies. Moreover, hemocyanins have been extensively used as carriers to generate antibodies against diverse hapten molecules and peptides to induce antigen-specific CD8+ and CD4+ T cell responses (Minozzi *et al.,* 2007; Arancibia *et al.,* 2012). Recently, a vaccine potential of *Oncomelania hupensis* Hc against *Schistosoma Japonicum* parasite was also established (Guo *et al.,* 2011).

Probably, due to their high carbohydrate content and specific monosaccharide composition, Hcs were also found to be active against viruses (Dolashka-Angelova *et al.,* 2009, 2010). Proteomic analysis of differentially-expressed proteins in arthropodan *Penaeus vannamei* hemocytes upon

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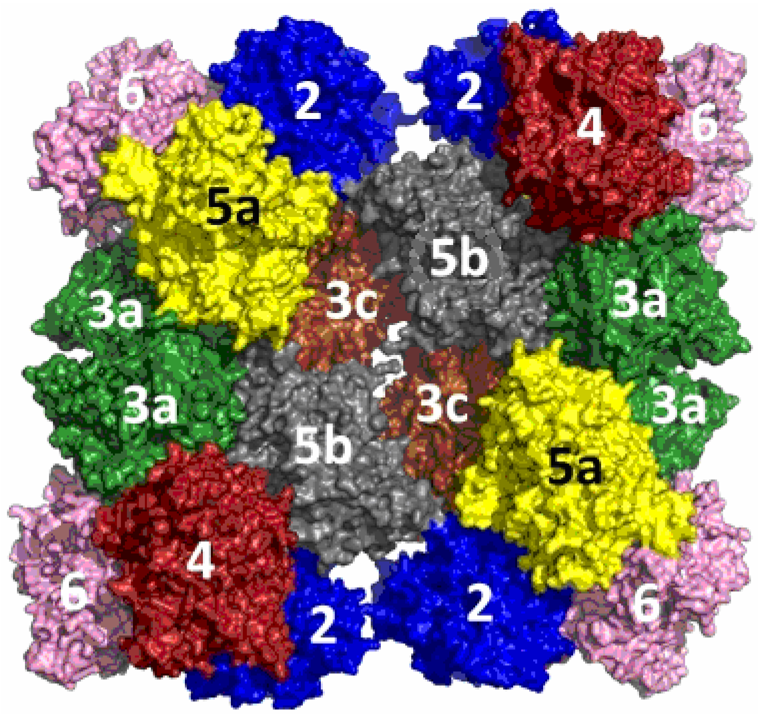

Fig. 1 Pseudoatomic model of emperor scorpion hemocyanin used for molecular replacement (Jaenicke *et al.,* 2012).

Taura syndrome virus (TSV) infection showed increased expression of hemocyanin (Rattanarojpong *et al.,* 2009). The efficacy, against white spot syndrome virus (WSSV) and Singapore grouper iridovirus (SGIV) is also assigned to a protein of the arthropod *Penaeus monodon* (Zhang *et al*., 2004).

Antiviral properties of molluscan Hcs were reported for the first time discribing the inhibition effect of hemocyanins from gastropod *R. venosa* (RvH) against *Respiratory syncytial virus* (RSV) and *Herpes simplex virus* type-1, strain Vic (HSV-1) (Dolashka *et al.,* 2010; Dolashka-Angelova *et al.*, 2011; Nesterova *et al.,* 2011). As this property seems to be associated with the glycosylation of functional units, the oligosaccharide structures of *R. venosa* and related molluscan and arthropodan hemocyanins were determined using different analytical physycochemical thechniques, (Sandra *et al.,* 2007; Dolashka-Angelova *et al*., 2009; Dolashka *et al.,* 2010).

In this short review, current knownledge about structural and biochemical aspects and eventual potential future clinical applications of arthropodan and molluscan hemocyanins as antiviral drugs is presented, and biochemical mechanisms causing the antiviral activity are suggested.

Arthropodan hemocyanins

Structure of arthropodan hemocyanins

Hemocyanins evolved early in the arthropod stem lineage from phenoloxidases, O_2^-consuming enzymes involved in the melanin pathway. They form hexamers or oligo-hexamers of identical or related subunits with a molecular mass of about 75 kDa (Fig. 1) (Dolashka-Angelova *et al.*, 2001; Mičetić *et al.,* 2010; Jaenicke *et al.,* 2012). Each subunit contains three domains and O_2-binding is mediated by two Cu^+ ions which are coordinated by six histidine residues ("type III" copper binding site). Subunit interactions within the multisubunit hemocyanin complex lead to diverse allosteric effects such as the highest cooperativity for oxygen binding found in nature. Based on biochemical, immunochemical and molecular phylogenetic analyses, distinct hemocyanin subunit types have been identified in chelicerata, myriapoda, crustacea and hexapoda (Huang *et al.,* 2008; Rehm *et al.,* 2012). The crystal structure of a native hemocyanin oligomer larger than a hexameric substructure was published for the first time for the 24-meric hemocyanin (MW = 1.8 MDa) from emperor scorpion (*Pandinus imperator*) (Fig. 1) (Jaenicke *et al.,* 2012).

Antiviral activity of arthropodan hemocyanins

Several reports on antiviral effects of arthropodan Hcs appeared in the literature, e.g. Hcs from shrimp *Penaeus japonicus* (PjH) and *P. vannamei* (PvH), against white spot syndrome virus (WSSV), *Taura syndrome* virus (TSV), yellow head virus (YHV) (Lei *et al.,* 2008; Chongsatja *et al.,* 2007; Rattanarojpong *et al.,* 2007; Nesterova *et al*., 2011). Hcs-based antiviral therapies are also reported (Lin, 2005; Balzarini *et al*., 2007).

One of these viruses, the white spot syndrome virus (WSSV) caused billions of dollars of losses for

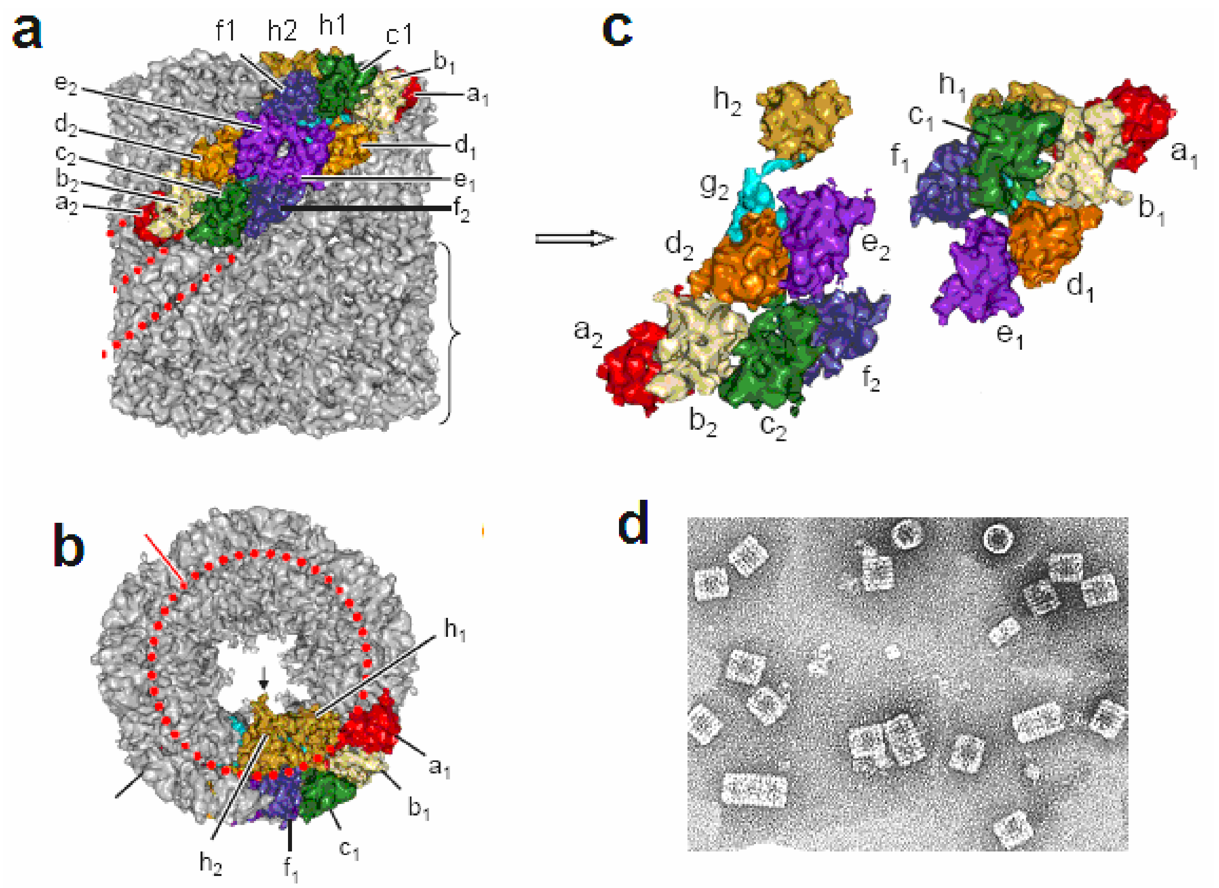

Fig. 2 Quaternary structure of molluscan hemocyanins: a) side view of didecamer; b) top view of decamer; c) structural subunits and functional units; d) electron microscopic picture of the native molecule of molluscan hemocyanin (Lieb *et al*. 2008).

shrimp farmers. To reduce shrimp production losses, stimulated since 2000 a plethora of research programmes to test shrimp's responses against viral pathogens at the molecular level. Humoral responses, binding studies between shrimp and viral structural proteins including intracellular responses, viral persistence co-interactions as well as viral sequence determination of the shrimp genome were subject of these investigations. Based on these results, a novel practical method for improved disease control was developed (Flegel *et al.,* 2011).

Studies on shrimp *P. japonicus* (PjH) showed that its hemocyanin could delay the infection of white spot syndrome virus (WSSV) *in vivo* and its subunits have different behavior in anti-WSSV defense (Table 1). During WSSV infection a strong induced one also cloned subunits, PjHcL was observed in contrast to subunit Pj-HcY. These findings suggest a possible discrepancy between the two subunits in shrimp innate immunity (Lei *et al.,* 2008).

To understand the molecular responses of crustacean hemocytes to virus infection, a two-dimensional electrophoresis (2-DE) proteomics approach was applied to determine altered proteins in hemocytes of *P. vannamei* during *Taura syndrome* virus (TSV) and yellow head virus (YHV) infections (Rattanarojpong *et al.,* 2007; Chongsatja *et al.,* 2007).

Proteomic analysis of *P. vannamei* hemocytes (PvH) upon YHV and TSV infection show the differentially-expressed proteins from the hemocytes. In the proteomic analysis of gills from yellow head virus-infected *P. vannamei*, 13 spots with up-regulated protein expression levels and five spots with down-regulated levels were identified. LC-nano-ESI-MS/MS indicated that the up-regulated proteins included enzymes in the glycolytic pathway, the tricarboxylic acid cycle and amino acid metabolism. The other upregulated proteins were arginine kinase, imaginal disk growth factor (IDGF) and a Ras-like GTP protein. These results provide preliminary data, however, it was not able to assign specific YHV-response status to any of the up-regulated or down-regulated genes identified after YHV challenge (Rattanarojpong *et al.,* 2007).

Proteomic analysis was also applied to explain the antiviral effect of *P. vannamei* hemocyanin (PvH) against *Taura syndrome* virus (TSV) (Chongsatja *et al.,* 2007). At 24 h post infection of PvH with *Taura syndrome* virus (TSV), quantitative

Table 1 Antiviral activity of several molluscan and arthropodan hemocyanins against different viruses

Hemocyanins	Viruses	Efficacy	References
	Arthropodan Hcs		
P. japonicus (PjH),	White spot syndrome virus (WSSV)	-	Lei *et al*., 2008
PjHcY, PjHcL	WSSV	+	Lei *et al*., 2008
P. vannamei (PvH),	*Taura syndrome* virus (TSV)	+	Chongsatja *et al*., 2007
PvH	Yellow head virus (YHV)	+	Rattanarojpong *et al*., 2007
P. monodon	Singapore grouper iridovirus (SGIV)		Zhang *et al*. 2004
	Molluscan Hcs		
Rapana venosa			
RvH, RvH1 and RvH2	Polio virus type 1(LSc-2ab)	-	Dolashka-Angelova 2009
RvH, RvH1 and RvH2	Respiratory syncytial virus (RSV)	-	Dolashka-Angelova 2009
RvH, RvH1 and RvH2	Coxsackie virus B1 (CV-B1)		Dolashka-Angelova 2009
Gglycosylated RvH-b	RSV	-	Dolashka-Angelova 2009
Nonglycosylated RvH-b	RSV	-	Dolashka-Angelova 2009
Gglycosylated RvH-c	RSV	+	Dolashka-Angelova 2009
Nonglycosylated RvH-c	RSV	-	Dolashka-Angelova 2009
RvH, RvH1 and RvH2	Epstein Barr virus	-	Velkova *et al.,* 2009
Helix vulgaris (HvH)	Epstein Barr virus	-	Zagorodnya *et al.,* 2011
RvH, RvH1 and RvH2	HSV-1	-	Nesterova *et al*., 2010
Helix vulgaris (HvH)	HSV-1	-	Nesterova *et al*., 2010
RvH2-e	HSV-1	+	Nesterova *et al*., 2010

intensity analysis and nano-LC-ESI-MS/MS revealed 8 proteins that were significantly up-regulated, whereas 5 proteins were significantly down-regulated in the infected shrimps. All of these proteins (hemocyanin, catalase, carboxylesterase, transglutaminase, and glutathione transferase) play important roles in host defense, signal transduction, carbohydrate metabolism, cellular structure and ER-stress response. However, hemocyanin, is one of these up-regulated proteins indicating a relation between this protein and the organism's defence system.

To get more insight into the molecular mechanism of the immune response of *P. vannamei* during Taura syndrome virus (TSV) infection, expression and functional proteomics studies were performed on its hemocyanin, which is a major abundant protein in shrimp hemocytes. Two-dimensional electrophoresis revealed up-regulation of several C-terminal fragments of hemocyanin, whereas the N-terminal fragments were down-regulated during TSV infection. 2-D Western blot analysis showed that the C-terminal hemocyanin fragments had more acidic isoelectric points (pI), whereas the N-terminal fragments had less acidic pIs. By motif scanning an important motif was discovered in the C-terminal hemocyanin, the ERK D-domain, of required for activation of ERK1/2 effector kinase, as a kinase-binding site at Val527 in the hemocyanins C-terminus, whereas no functional domain was found in the N-terminus. Co-immunoprecipitation confirmed the interaction between the C-terminal hemocyanin and ERK1/2 which was also up-regulated during TSV infection. These findings demonstrate for the first time that the ERK1/2 signaling pathway may play an important role in molecular immune response of *P. vannamei* upon TSV infection through its interaction with the C-terminal hemocyanin (Havanapan *et al.,* 2009).

Molluscan hemocyanins

Structure of molluscan hemocyanins

Molluscan hemocyanins (Hcs) are giant biological macromolecules acting as oxygen-transporting glycoproteins. They have recently received particular interest due to their immunostimulatory and antitumoral properties. Most of them are glycoproteins with molecular mass around 9000 kDa organized as decamers or didecamers (Fig. 2) (Lieb *et al.* 2008; Markl, 2013). The native hemocyanins contain one or two structural subunits with molecular masses around 350 - 450 kDa. Seven or eighth functional units with molecular masses of 50 kDa organize one structural subunits (Fig. 2) (Dolashka-Angelova *et al*., 2003a; Lieb *et al.* 2008; De Smet *et al*., 2011).

Molluscan hemocyanins possess certain inhibitory properties against tumor cells and viruses (Iliev *et al*., 2008; Toshkova *et al*., 2009; Dolashka *et al*., 2011), as also found for the molluscan hemocyanins *R. venosa* (RvH) and *H. lucorum* (HlH) against different viruses (Dolashka-Angelova *et al.,* 2009, 2010; Nesterova *et al.,* 2010 2011; Zagorodnya *et al.,* 2011; Velkova *et al.,* 2011).

Antiviral activity of molluscan hemocyanins

The antiviral effect of RvH and its isoforms was studied against polio virus type 1 (LSc-2ab), coxsackie virus B1 (CV-B1), respiratory syncytial virus (RSV) and Epstein Barr virus (EBV). After treatment of the viruses with the native RvH, the non-glycosylated and glycosylated functional units RvH-b and RvH-c, the quantitative virucidal suspension test did not show any effect against polio virus type 1 (LSc-2ab), coxsackie virus B1 (CV-B1). Also no antiviral effects of native RvH against RSV was observed. However, the results presented in Table 1 show that only the glycosylated

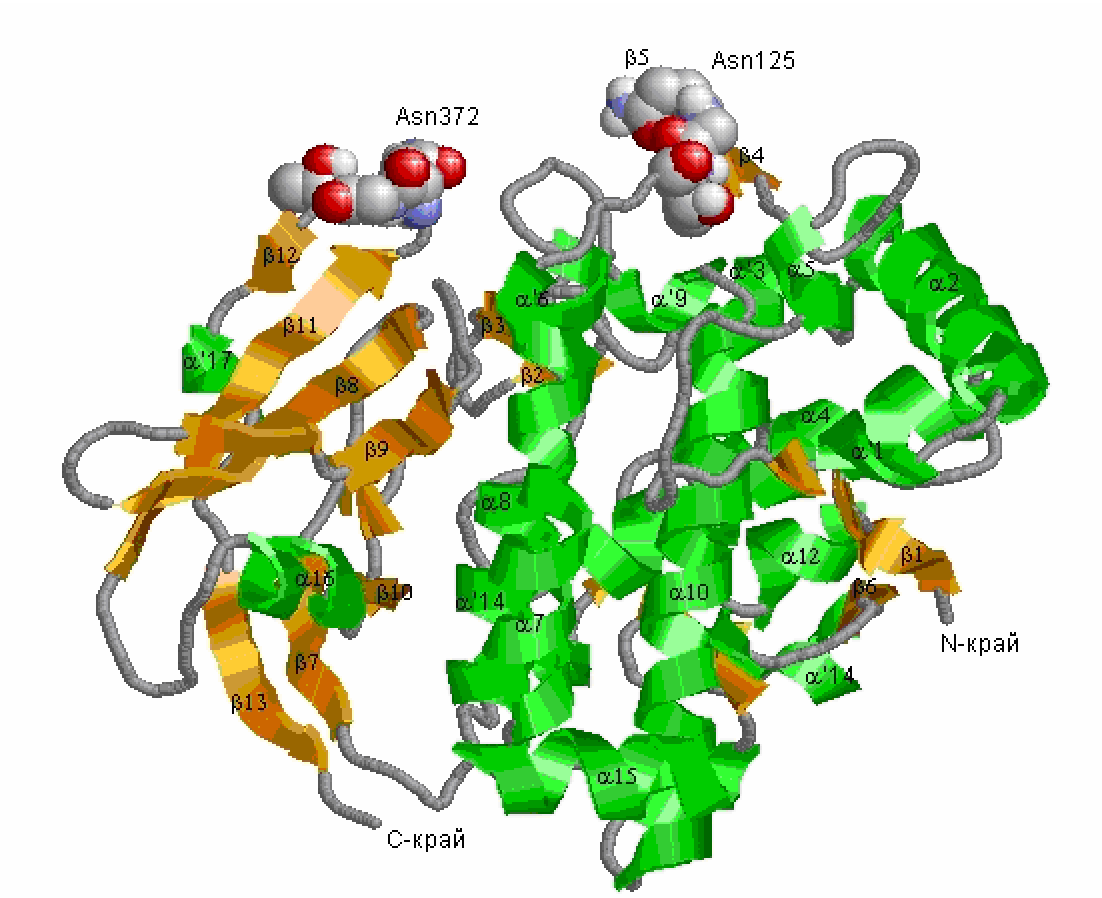

Fig. 3 3D model of βcHlH-g created by using the Swiss PDB viewer and the model of functional unit "g" from *Octopus dofleini* (OdH-g) hemocyanin. Glycans and the putative glycosilated sites N125 and N245 are represent as balls (Kostadinova *et al*., 2013).

FU, RvH-c, possesses some antiviral effect against the replication of RSV, at both viral doses tested (Dolashka-Angelova *et al.,* 2009). In contrast, non-glycosylated FU RvH-b does not reveal any antiviral activity against the replication of RSV, poliovirus type 1 (LSc-2ab) and CV-B1. This is the first report of the fact that molluscan hemocyanin functional units with specific glycosylation possess antiviral activity (Dolashka-Angelova *et al.,* 2009).

The properties of native hemocyanins from *R. venosa* and *Helix vulgaris* (HvH) snails and their structural subunits RvH1, RvH2, HvH1, and HvH2 have been studied *in vitro* as substances with feasible antiEBV activity (Velkova *et al.,* 2009). Epstein Barr virus (family Herpesveridae) is one of the etiologic agents that cause Burkitt's lymphoma and nasopharyngeal carcinoma, but drugs for antiEBV treatment are limited. There are few approaches for the treatment of this viral infection, namely, the use of effective antiviral chemotherapy, serotherapy or seroprevention which supposes the use of specific human immunoglobulins and vaccines (Pagano and Gershburg, 2005). The influence of *R. venosa* hemocyanin on viability and proliferative activity of lymphoblastoid cells was characterized by cytomorphological and colorimetric methods and an antiviral effect was observed after treatment with FUs RvH1-a and RvH2-e (Nesterova *et al.,* 2010).

Antiviral effects on *R. venosa* and *H. vulgaris* (HvH) hemocyanins, their structural subunits, the glycosylated functional unit RvH2-e and the non-glycosylated unit RvH2-c were also investigated against HSV virus type 1: only the glycosylated FU RvH2-e exhibited an antiviral activity, which probably is due to its high carbohydrate content and specific monosaccharide composition (Zagorodnya *et al.,* 2011; Velkova *et al.,* 2011).

Based on the obtained results, we suggested for the first time that the complete native RvH molecule lacks any antiviral activity because the carbohydrate chains are buried in between the structural subunits of the global protein and therefore, are unable to interact with the viral surface glycoproteins. To support this hypothesis, we found that two putative glycosylated sites in FUs βcHlH-g are exposed on the surface of the molecule (Fig. 3) (Kostadinova *et al*., 2013) and that FU RvH1-a, exhibits antiviral activity and bears two N-glycosidic resirues attached to Asn262 and Asn401.

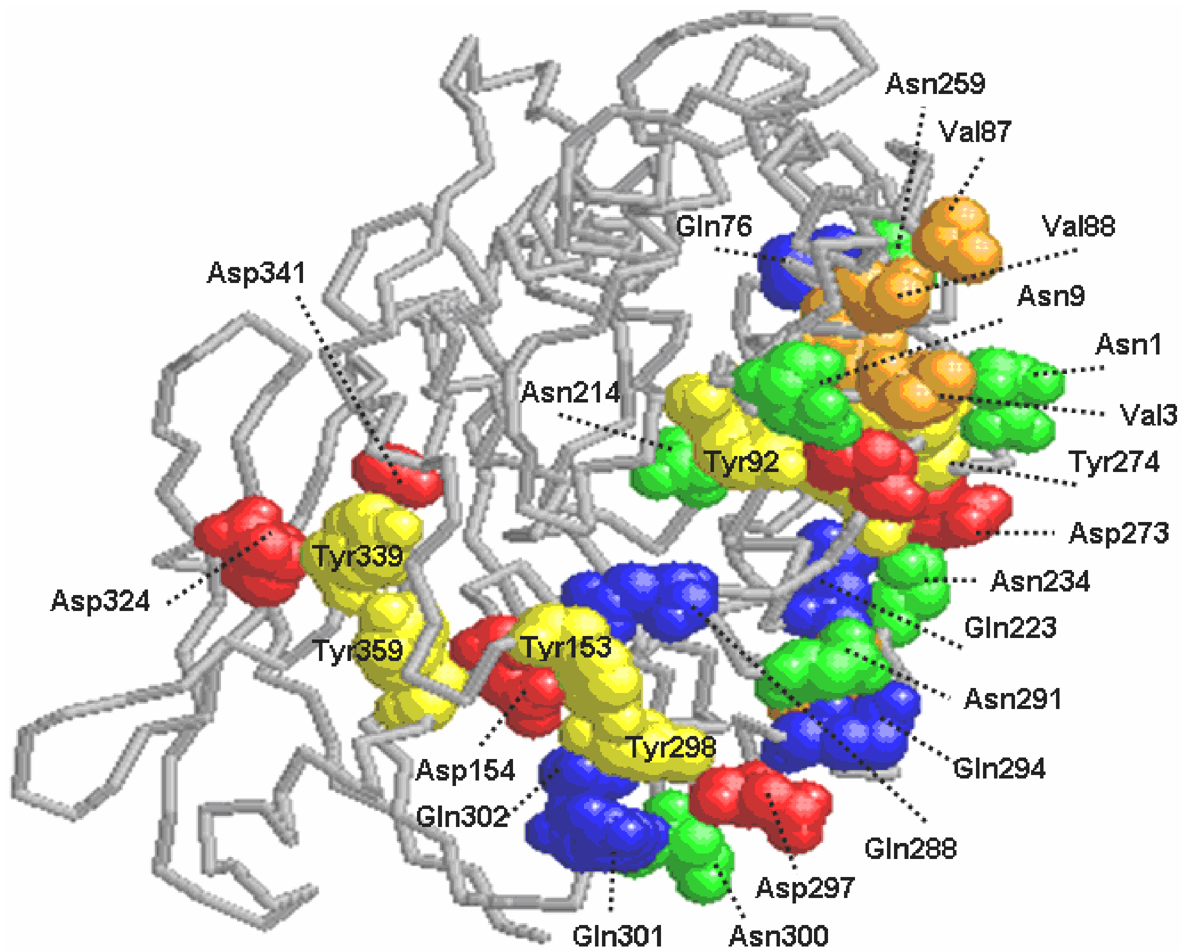

Fig. 4 3D model of functional unit RvH2-e. Amino acid residues included in the loops are shown in blue and green (Dolashka *et al.*, 2010)

Glycosylation of hemocyanins

The relevance of specific glycosylation patterns as a differentiating factor for immunostimulatory properties has already been raised in studies on the hemocyanin isoforms KLH1 and KLH2 of *M. crenulata* and *R. venosa,* (Geyer *et al.*, 2005; Sandra *et. al.*, 2007). Molluscan hemocyanin genes contain several potential N-glycosylation sites, and some of those have been already effectively demonstrated to be glycosylated using a series of analytical strategies and MALDI-TOF-MS and tandem mass spectrometry (Lieb *et al.* 2008; Dolashka-Angelova *et al.,* 2009; Dolashka *et al.,* 2010; De Smet *et al.*, 2011). The identification of the oligosaccharide structure of several molluscan hemocyanins revealed a highly heterogeneous mixture of glycans of the compositions Hex0–9 HexNAc2–4 Hex0–3 Pent0–3 Fuc0–3. A novel type of N-glycan with an internal fucose residue connecting one GalNAc(b1-2) and one hexuronic acid, was detected which also occurs in subunits RvH1 and RvH2 (Sandra *et. al.,* 2007; Dolashka-Angelova *et al.,* 2009; Dolashka *et al.,* 2010).

Based on the obtained information about carbohydrate structure of hemocyanins the antiviral properties of hemocyanins against Herpes simplex virus type 1, was suggested. One potential site for N-glycosylation in FU RvH2-e at Asn-127 was shown to be glycosylated and this carbohydrate chain is likely to interact with specific regions of glycoproteins of HSV, through van der Waals interactions in general or with certain amino acid residues in particular. As is shown in figure 4 several groups of these residues can be identified on the surface of RvH2-e which may interact with a carbohydrate chains of glycoprotein gp120 of HSV. The surface of glycoprotein gp120 was very well analysed (Sander *et al.,* 2002).

Development and identification of polyclonal antibodies against viruses

Hemocyanins are commonly used to develop polyclonal antibodies e.g. also against predicted B cell epitopes in HIV-1 accessory protein Vpr. The synthesized B-cell peptide epitopes were subsequently conjugated with keyhole limpet hemocyanin (KLH) and then used to immunize rabbits. The antibody titers and specifities were determined by ELISA, Western-blotting, and immunoprecipitation analyses, respectively. Two B cell epitopes of Vpr were successfully predicted by bio-informatical methods and polyclonal antibodies against those peptides could be successfully prepared (Sun *et al.,* 2012).

KLH was used to analyse the protective potential of polyclonal IgG antibodies specific to the ectodomain (eM2) of M2 protein of influenza A virus (IAV) against lethal influenza infection of mice. Two fractions, KLH alone and the ectodomain eM2, conjugated to KLH, were administered with Freund's adjuvant intraperitoneally (i.p.) to BALB/c mice. Analysis of the preparation of anti-KLH-eM2 IgGs by ELISA revealed that it contained about 25% of anti-eM2 IgGs and 75% of anti-KLH IgGs and after infection with 3 LD50 IAV, a 100% survival of mice was observed with 320 µg anti-eM2IgGs (Király *et al.*, 2011).

Recently, molluscan hemocyanins receive increasing interest in vaccine development. Several carrier proteins were used to prepare vaccines against highly variable HIV-1. Two synthesized peptides, JY1 (V3 region of HIV-1 gp120, subtype D) and JY1-MAP8, were coupled to carrier proteins such as KLH, hepatitis B surface antigen (HbsAg) and meningococcal P64k protein and used for immunization in mice. It was found that JY1-MAP8 conjugates were more immunogenic than JY1-MAP8 alone. Furthermore, conjugates to HBsAg and KLH were more immunogenic than those to P64k. The analysis showed that conjugate-based immunogens are more prompt to elicit immunogenicity and cross-reactivity and hemocyanins are promising candidates the development of HIV vaccines (Cruz *et al*., 2009).

We found that hemocyanins are a new class of natural antiviral compounds against different viruses. However, arthropodan hemocyanins are active against those own viruses of arthropods, such as WSSV and TSV, while the molluscan hemocyanins are active against viruses of human, such as RSV and EBV. Maybe the mechanisms of antiviral activity of both species are different and therefore, we consider them as an appropriate for further investigation as antiviral agents.

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References

Arancibia S, Salazar F, Inés Becker M. Hemocyanins in the Immunotherapy of Superficial Bladder Cancer – From Basic Science to Robotic Surgery. Review 221-242, 2012.

Balzarini J. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. Nat. Rev. Microbiol. 5: 583-597, 2007.

Chongsatja P, Bourchookarn A, Lo CF, Thongboonkerd V, Krittanai C. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. Proteomics 7: 3592–3601, 2007.

Cruz LJ, Cabrales A, Iglesias E, Aguilar JC, González LJ, Reyes O. Enhanced immunogenicity and cross-reactivity of HIV-1 V3-peptide and multiple antigen peptides conjugated to distinct carrier proteins. Int Immunopharmacol. 9: 12, 1452-1459, 2009.

Guo D, Wang H, Zeng D, Li X, Fan X, Li Y. Vaccine Potential of Hemocyanin from *Oncomelania Hupensis* against *Schistosoma Japonicum.* Parasit. Internat. 60: 3, 242-246, 2011.

De Clercq E. Antivirals and antiviral strategies. Nat. Rev. Microbiol. 2: 9, 704-720, 2004.

De Smet L, Dimitrov I, Debyser G, Dolashka-Angelova P, Dolashki A, Van Beeumen J *et al*. The cDNA sequence of three hemocyanin subunits from the garden snail *Helix lucorum*. Gene 487: 2, 118-128, 2011.

Del Campo M, Arancibia S, Nova E, Salazar F, González A, Moltedo B, *et al*. Hemocyanins as immunostimulants. Revista Medica de Chile 139: 2, 236-246, 2011.

Dolashka-Angelova P, Beltramini M, Salvato B, Voelter V. Carbohydrate composition of *Carcinus aestuarii* hemocyanin. Arch. Biochem. Biophys. 389: 2, 153-158, 2001.

Dolashka-Angelova P, Schwarz H, Dolashki A, Beltramini M, Salvato B, Schick M, *et al*. Oligomeric stability of *Rapana venosa* hemocyanin (RvH) and its structural subunits. Biochim. Biophys. Acta 1646: (1-2) 77-85, 2003.

Dolashka-Angelova P, Lieb B, Velkova L, Heilen N, Sandra K, Nikolaeva-Glomb L, *et al*. Identification of glycosylated sites in Rapana hemocyanin by mass spectrometry and gene sequence, and their antiviral effect. Bioconjug. Chem. 20: 7, 1315-1322, 2009.

Dolashka P, Velkova L, Shishkov S, Kostova K, Dolashki A, Dimitrov I, *et al*. Glycan structures and antiviral effect of the structural subunit RvH2 of Rapana hemocyanin. Carboh. research 345: 16, 2361-2367, 2010.

Dolashka P, Velkova L, Iliev I, Beck A, Dolashki A, Yossifova L, *et al*. Antitumor Activity of Glycosylated Molluscan Hemocyanins *via* Guerin ascites tumor. Immunol. Investig. 40: 2, 130-149, 2011.

Geyer H, Wuhrer M, Resemann A, Geyer R. Identification and characterization of keyhole limpet hemocyanin N-glycans mediating cross-reactivity with *Schistosoma mansoni*. J Biol. Chem. 280: 49, 40731-4048, 2005.

Havanapan PO, Kanlaya R, Bourchookarn A, Krittanai C, Thongboonkerd V. C-terminal hemocyanin from hemocytes of *Penaeus vannamei* interacts with ERK1/2 and undergoes serine phosphorylation. J Proteome Res. 8: 5, 2476-2483, 2009.

Huang B, Zhang J, Xiang J. Purification and primary identification of haemocyanin in the Chinese shrimp *Fenneropenaeus chinensis* (Decapoda, Penaeoidea). Crustaceana 81: 7, 769-780, 2008.

Flegel TW, Sritunyalucksana K. Shrimp molecular responses to viral pathogens. Mar. Biotechno.l (NY).13: (4), 587-607, 2011.

Iliev I, Toshkova R, Dolashka-Angelova P, Yossifova L, Hristova R, Yaneva J *et al*. Haemocyanins from *Rapana venosa* and *Helix vulgaris* display an antitumour activity via specific activation of spleen lymphocytes. Compt. Rend. Acad Bulg. Sci. 61: 203-210, 2008.

Jaenicke E, Pairet B, Hartmann H, Decker H. Crystallization and preliminary analysis of crystals of the 24-meric hemocyanin of the emperor scorpion (*Pandinus imperator*). PLoS One.7: 3, e32548, 2012.

Király J, Varečková E, Mucha V, Kostolanský F. Evaluation of anti-influenza efficiency of polyclonal IgG antibodies specific to the ectodomain of M2 protein of influenza A virus by passive immunization of mice. Acta Virol. 55: 3, 261-265, 2011.

Kostadinova E, Dolashka P, Velkova L, Dolashki A, Stevanovic S, Voelter W. Positions of the glycans in molluscan hemocyanin, determined by fluorescence spectroscopy. J. Fluoresc. 23: 753–760, 2013.

Lei K, Li F, Zhang M, Yang H, Luo T, Xu X. Difference between hemocyanin subunits from shrimp *Penaeus japonicus* in anti-WSSV defense. Dev. Comp. Immunol. 32: 7, 808-813, 2008.

Lieb B, Todt C. Hemocyanin in mollusks - a molecular survey and new data on hemocyanin genes in *Solenogastres* and *Caudofoveata*. Mol. Phylogenet. Evol. 49: 1, 382-385, 2008.

Lin JC. Antiviral Therapy for Epstein-Barr Virus-Associated Diseases. Tzu. Chi. Med. J. 17: 1, 379–385, 2005.

Manubens A, Salazar F, Haussmann D, Figueroa J, Del Campo M, Martínez Pinto J, *et al*. Concholepas hemocyanin biosynthesis takes place in the hepatopancreas, with hemocytes being involved in its metabolism. Cell Tissue Res. 342:3, 423-435, 2010.

Markl J. Evolution of Molluscan Hemocyanin Structures. Biochim. Biophys. Acta 1834: 1840-1852, 2013.

Martin AM, Martin GG, Butler R, Goffredi SK. Synthesis of keyhole limpet hemocyanin by the rhogocytes of *Megathura crenulata*. Invert. Biol. 130: 4, 302–312, 2011.

Mičetić I, Losasso C, Muro PD, Tognon G, Benedetti P, Beltramini M. Solution structures of 2×6-meric and 4×6-meric hemocyanins of crustaceans *Carcinus aestuarii, Squilla mantis* and *Upogebia pusilla*. J. Struct. Biol. 171: 1–10, 2010.

Minozzi G, Parmentier HK, Nieuwland MG, Bed'hom B, Minvielle F, Gourichon D, *et al*. Antibody responses to keyhole limpet hemocyanin, lipopolysaccharide, and Newcastle Disease virusvaccine in F2 and backcrosses of white Leghorn lines selected for two different immune response traits. Poult Sci. 86: 7, 1316-1322, 2007.

Moltedo B, Faunes F, Haussmann D, De Ioannes P, De Ioannes AE, Puente J *et al*. Immunotherapeutic effect of Concholepas hemocyanin in the murine bladder cancer model: evidence for conserved antitumor properties among hemocyanins. J. Urol. 176: 2690-2695, 2006.

Nesterova N, Dolashka-Angelova P, Zagorodnya S, Moshtanska V, Baranova G, Golovan A *et al*. *In vitro* Investigation of cytotoxic action of hemocyanins on cell cultures. Antivir. Research 86: 1, A63-A63, 2010.

Nesterova N, Zagorodnya S, Moshtanska V, Dolashka P, Baranova G, Golovan A, *et al*. Antiviral activity of hemocyanin isolated from marine snail *Rapana venosa.* Antivir. Research 90: 2, p.A38, 2011.

Pagano JS, Gershburg E. Epstein–Barr virus infections:prospects for treatment. J. Antimicrob. Chemother. 56: 277–281, 2005.

Rattanarojpong T, Wang HC, Lo Chu F, Flegel TW. Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection. Proteomics 7: 3809–3814, 2007.

Rehm P, Pick C, Borner J, Markl J, Burmester T. The diversity and evolution of chelicerate hemocyanins. BMC Evol. Biol. 12: 19, 2012.

Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, *et al*. The Mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. 76: 14, 7293–7305, 2002.

Sandra K, Dolashka-Angelova P, Devreese B, Van Beeumen J. New insights in *Rapana venosa* hemocyani N-glycosylation resulting from on-line mass spectrometric analyses. Glycobiol. 17: 2, 141-156, 2007.

Sun J, Meng ZF, Xu JQ, Zhang XY, Lv JX. Development and identification of polyclonal antibodies against HIV-1 Vpr-derived polypeptides. Bing Du Xue Bao .28: 2,151-157, 2012.

Velkova L, Nikolaeva-Glomb L, Mukova L, Dolashki A, Dolashka P, Galabov AS. Antiviral Effect of Molluscan Haemocyanines. Antivir. Research 90: 2, p.A47, 2011.

Zagorodnya S, Dolashka P, Baranova, G, Golovan A, Nesterova N. Anti-EBV Activity of Hemocyanin Isolated from *Helix lucorum*. Antivir. Research 90: 2, p.A66, 2011.

Zhang X, Huang C, Qin Q. Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon.* Antivir. Res. 61: 93-99, 2004.