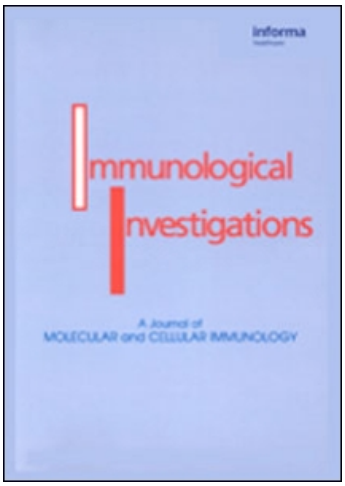

Immunological Investigations
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597256

Immunological Potential of Helix vulgaris and Rapana venosa Hemocyanins

Pavlina Dolashka-Angelova [a]; Tsetanka Stefanova [b]; Evangelia Livaniou [c]; Lyudmila Velkova [a]; Persefoni Klimentzou [c]; Stefan Stevanovic [d]; B. Salvato [e]; Hristo Neychev [b]; Wolfgang Voelter [f]
[a] Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria [b] Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria [c] Institute of Radioisotopes & Radiodiagnostic Products (RRP Institute), NCSR "Demokritos", Neapoleos & Patriarchou Grigoriou Aghia Paraskevi, Athens, Greece [d] Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, Tübingen, Germany [e] Department of Biology, University of Padova via G. Colombo 3, Padova, Italy [f] Interfacultary Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Straße 4, Tübingen, Germany

Online Publication Date: 01 November 2008

To cite this Article Dolashka-Angelova, Pavlina, Stefanova, Tsetanka, Livaniou, Evangelia, Velkova, Lyudmila, Klimentzou, Persefoni, Stevanovic, Stefan, Salvato, B., Neychev, Hristo and Voelter, Wolfgang(2008)'Immunological Potential of Helix vulgaris and Rapana venosa Hemocyanins',Immunological Investigations,37:8,822 — 840

To link to this Article: DOI: 10.1080/08820130802403366

URL: http://dx.doi.org/10.1080/08820130802403366

Immunological Investigations, 37:822–840, 2008
ISSN: 0882-0139 print / 1532-4311 online
DOI: 10.1080/08820130802403366

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Immunological Potential of *Helix vulgaris* and *Rapana venosa* Hemocyanins

Pavlina Dolashka-Angelova,[1] Tsetanka Stefanova,[2] Evangelia Livaniou,[3] Lyudmila Velkova,[1] Persefoni Klimentzou,[3] Stefan Stevanovic,[4] B. Salvato,[5] Hristo Neychev,[2] and Wolfgang Voelter[6]

[1]Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, Sofia 1113, Bulgaria
[2]Institute of Microbiology, Bulgarian Academy of Sciences, 26 Academic G. Bonchev, 1113 Sofia, Bulgaria
[3]Institute of Radioisotopes & Radiodiagnostic Products (RRP Institute), NCSR "Demokritos", Neapoleos & Patriarchou Grigoriou Aghia Paraskevi, Athens, 153 10, Greece
[4]Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076, Tübingen, Germany
[5]Department of Biology, University of Padova via G. Colombo 3, 35131 Padova, Italy
[6]Interfacultary Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Straße 4, D-72076 Tübingen, Germany

A new hemocyanin was isolated from the hemolymph of garden snails *Helix vulgaris*, composed of two isoforms, HvH1 and HvH2 separated on an ion exchange column DEAE-Sepharose 6CL. Structural and immunological properties of *Helix vulgaris* hemocyanin were studied in comparison with molluscan Hcs *Rapana venosa* and *Megathura crenulata*. The possibility of using HvH and RvH as carriers of small molecules (haptens) in immunizing protocols was studied in comparison with KLH, which is a widely used, highly immunogenic carrier protein. By using HvH as a carrier of the well-known hapten TNBS (2,4,6-trinitrobenzene sulfonic acid), an increasing with time production of hapten-specific TFN-γ was detected in splenocyte cultures of mice, which lasted longer than in case of KLH and RvH carriers. Also, use of HvH or RvH as a carrier of the hapten ProTα[101–109] (i.e., the synthetic C-terminal fragment of the poorly immunogenic protein prothymosin alpha) showed that antisera of higher titres than that of the control conjugate (ProTα[101–109]-KLH) were obtained immediately after the second bleeding. HvH and RvH may prove to be useful for the development of new antiviral, antibacterial and antitumor vaccines, since they seem to launch strong and specific immune response against the conjugated antigens.

Address correspondence to Pavlina Dolashka-Angelova, Acad. G. Bonchev Street, bl. 9, 1113 Sofia, Bulgaria; E-mail: pda54@yahoo.com

Keywords *Helix vulgaris*, Hemocyanin, Hapten carrier, Electron microscopy, Antibodies.

INTRODUCTION

Hemocyanins (Hcs) are oligomeric blue copper-containing respiratory proteins that play a role as dioxygen carriers in the hemolymph of several species of molluscs and arthropods (Salvato and Beltramini, 1990; Van Holde and Miller, 1995). The organization and size of the molluscan Hc molecules are quite different, with molecular masses varying between 3.5 to 4.5 $\times$ 10^6 Da (cephalopods and chitons) and 9 $\times$ 10^6 Da (gastropods). The average mass of a gastropod subunit is about 4.5 $\times$ 10^5 Da, or just about 1/20 of the common native molecular mass of around 9 $\times$ 10^6 Da. These oligomers contain 10 (decamer), 20 (didecamer) or more subunits with a molecular mass of about 350 or 450 kDa each. The subunits of molluscan hemocyanins have different structures. Their subunits are made up from 7 or 8 functional units (Fus) of about 50 kDa which are different in their sequences and genomic pattern (Ballweber et al., 2002; Bergmann et al. 2007; Lieb et al., 2001).

Molluscan Hcs are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition (Lommerse et al., 1997). It was found that hemocyanins accelerate the maturation of dendritic cells resulting in an enhanced T-cytotoxic anti-tumor activity (Wuhrer et al., 2004). Dendritic cells are responsible for antigen presentation including also tumor antigens. Biomedical interest in the high molecular mass hemocyanin of the marine mollusc, the giant keyhole limpet *Megathura crenulata* (keyhole limpet hemocyanin, KLH), dates back to more than 30 years, when it was first discovered to possess remarkable immunostimulatory properties in experimental animals and man (Arris and Markl, 1999; Herscowitz et al., 1972).

In addition, the use of KLH in the field of immunochemistry as a hapten carrier for small molecules, such as chemicals, drugs, hormones, peptides, polysaccharides, lipids and oligonucleotides, against which it has often proved to be difficult or impossible to raise polyclonal antibodies, as well as its use as a tumor vaccine carrier in the field of immunotherapy have been well established (Lamm et al., 1996). Its direct immunotherapeutic potential in bladder cancer (Lamm et al., 1996), as well as its ability to serve as an effective tumor antigen carrier in antitumor vaccines, rapidly extended the applications of this protein within the broad field of immunobiology.

Though KLH is widely applied as a therapeutic agent in medicine, the market requirements for its biochemical and pharmaceutical application in experimental and clinical medicine are still not completely met. This indicates the need for further research focusing on the elucidation of the immunological and therapeutic effect of other molluscan (terrestrial or freshwater) hemocyanins as well (Moltedo et al. 2006).

Several arthropodan and molluscan hemocyanins were studied in our laboratory (Dolashka-Angelova et al., 2003a, 2005; Schütz et al., 2001). Two hemocyanin structural subunits were isolated from the mollusc *Rapana venosa* and their structural properties, partial amino acid sequences and carbohydrate structures were investigated (Beck, 2007; Dolashka-Angelova et al., 2003b, 2007; Sandra et al., 2007).

The aim of this study was to isolate a new hemocyanin from the garden snail *Helix vulgaris* (HvH), to elucidate its primary, secondary and tertiary structure and to evaluate its immunological potential, in comparison with other molluscan hemocyanins.

MATERIALS AND METHODS

Chemicals

RvH Hemocyanin. Hemocyanin *Rapana venosa* (RvH) was obtained from the marine snails and after dialysis of RvH two structural subunits were isolated (Dolashka-Angelova et al., 2003b).

Haptens. (Hapten A) Picryl sulfonic acid (2,4,6-trinitrobenzene sulfonic acid, TNBS) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. It was recrystallized once from 1 N HCl after dissolving 10 g TNBS in 20 ml of hot HCl.(Hapten B) synthetic peptide ProTα[101–109].

Bovine Albumin. Crystallized bovine plasma albumin (BSA) was purchased from Armour Pharmaceutical Company, Kankakee, Illinois.

Buffers Cacodylate Buffer. 0.28 M in cacodylic acid. Cacodylate saline refers to the same buffer diluted 1:10 in 0.15 M NaCl.

Isolation of the Native *Helix vulgaris* Hemocyanin

Helix vulgaris hemolymph was collected from the leg of garden snails 25 g and centrifuged at 10,000 RPM for 15 min to remove hemocytes. The hemocyanin was sedimented in a Beckman L-80 ultracentrifuge using an UZ rotor Ti 45 and a speed of 24,000 RPM, Beckman L-80 Ultracentrifuge, for 4 h at 5°C. The blue pellet of native hemocyanin was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$.

Isolation of the Isoforms from *Helix vulgaris* Hemocyanin

Dissociation of the native Hc molecule was achieved by dialysis for 24 h against 0.13M glycin buffer, pH 8.9, at room temperature (25°C). Briefly, purification of the functional subunit was achieved by subjecting the crude hemocyanin to a DEAE-Sepharose 6CL (12×2.5 cm), equilibrated with 50 mM Tris–HCl buffer, containing 10 mM EDTA and eluted with a linear gradient of 0–0.5 M NaCl. Electrophoretically pure isoforms were separated by ion

exchange FPLC chromatography, on a Resource™ Q column, using a stepwise 50 mM NaCl gradient (0–1.0 M) in Tris-HCl buffer, pH 7.5. Isolated samples were applied to a 7.5% gel, native polyamide gel electrophoresis. All gels were stained by Coomassie blue R-250, and the protein used as standard for molecular mass determination was ferritin (440 kDa and aldolase 250 kDa).

Amino Acid Sequence Determination

Each fraction was additionally purified on an HPLC Nucleosil RP C18 column using 0.1% TFA in H_2O as loading buffer and 0.085% TFA, 80% acetonitrile in H_2O as eluting solution (eluent B) before amino acid sequencing. The following conditions were used: 10% B for 10 min followed by 10–100% B within 70 min at a flow rate of 1 ml min^{-1}. Peak fractions were dried and after dissolving in 40% methanol, 1% formic acid, they were subjected to automated Edman N-terminal sequencing (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

Preparation of Conjugates

Ten ml picryl sulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS), used as a hapten A, were dissolved after recrystallization in 10 ml 0.28 M cacodylate buffer pH 7.2. 370 mg hemocyanins (native *Helix vulgaris*, KLH and *Rapana venosa* hemocyanins), dissolved in 10 ml saline (0.9%NaCl), were added to the solution under stirring (Rittenberg et al., 1988). The experiment was carried out in absence of light. The solution was centrifuged for 90 min at 36,000 RPM and low temperature after 1 h stirring at room temperature. The supernatant was removed and the modified hemocyanins were dissolved in 10 ml saline (0.9% NaCl).

The insoluble moiety was removed by additional centrifugation at 1500 RPM for about 5 min. The pure supernatant was eliminated from the non-reacted TNBS mixture by means of Sephadex column G-50 (3 × 43 CM), equilibrated with cacodylate buffer. 12 ml fractions were collected, containing 17.8 mg/ml hemocyanin. Non-reacted TNBS was removed by dialysis against saline (0.9% NaCl) for about 48 hours. The hemocyanin concentration of each fraction was estimated from absorbance measurements of the hemocyanins at 350 nm. The final purification of the hemocyanins was done at Sephadex column G-75, eluted by cacodylate buffer. The fractions so obtained were concentrated and used as a test for antibody determination . Crystallized bovine plasma albumin (BSA) was treated as hemocyanins with TNBS. This material was used as test antigen for determining anti-hapten antibodies.

Experimental Animals and Immunization

TNBS was chemically conjugated to hemocyanins *Helix vulgaris*, *Rapana venosa* and KLH, and bovine serum albumin (BSA). C57BL/6 mice were

immunized subcutaneously in the back with TNP-RvH, TNP-HvH or TNP-KLH (100 μg/mouse) in complete Freund's adjuvant (1:1, v/v emulsion). Control groups included untreated animals, mice injected with PBS/CFA alone, as well as mice immunized with TNP-Hc in the absence of CFA (16–18 mice per treatment). The animals were treated according to standard conditions, accepted by the Bulgarian Veterinary Health Service in the animal house of the Institute of Microbiology, Sofia. Mice were bled on days 7, 14 and 21 post- immunization via the retro-orbital vein and serum samples were immediately frozen at –70°C until analysis.

Cell Cultures

Mice were sacrificed 7, 14 and 21 days after immunization, spleens were aseptically removed and single cell suspensions were obtained. Spleen cells were suspended in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO, USA) and antibiotics (Serva Feinbiochemica GmbH&Co., Heidelberg, Germany). The viability of the cells was over 95% by trypan blue exclusion test. Cells were adjusted to a concentration of 2.5×10^6/ml and were plated in a volume of 1 ml in 24-well cell culture plates (Corning Inc., Corning, NY, USA). Splenocytes were cultured for 3 days in the presence or absence of 50 μg/ml BSA (control) or TNP-BSA in a humidified CO_2 incubator. Supernatants were collected and frozen at –70°C until analysis for IFN-γ and antibodies.

Measurement of Anti-Hapten Antibodies and TNP-Specific IFN-γ Production

Hapten-specific antibodies were assessed in splenocyte supernatants and in sera by ELISA. Briefly, ELISA microtiter plates were coated overnight with TNP-BSA (1 μg/well) in PBS. The plates were then washed four times with PBS-0.05% Tween 20, blocked with PBS-1% BSA and washed again. Splenocyte supernatants were added undiluted, while serum samples were initially diluted 1:100, and then twofold serial dilutions were prepared. All samples were assayed in duplicate. Then goat anti-mouse IgG or IgM peroxidase conjugates were added (Calbiochem, Cambridge, MA, USA) and the plates were developed using o-phenylenediamine dihydrochloride in the substrate solution. The reaction was stopped with 4N H_2SO_4 and the plates were read at 492 nm in an ELISA reader.

TNP-specific IFN-γ production of splenocytes was measured by an ELISA kit from PeproTech Inc. according to the manufacturer's instructions. The sensitivity of the assay was 16 $pg.ml^{-1}$.

Immunization of Rabbits

New Zealand white rabbits were intradermally or subcutaneously injected (Vaitukaitis et al., 1981) with the hapten B - synthetic peptide ProTα[101–109]

(in-house prepared by following the Fmoc solid phase peptide synthesis strategy), conjugated to keyhole limpet hemocyanin (KLH, commercially available product – Pierce 77600), *Helix vulgaris* hemocyanin (HvH, isolated as above described) or *Rapana venosa* hemocyanin (RvH, isolated as described in Chemicals). Conjugation of ProTα**[101–109]** to the hemocyanins was performed according to the glutaraldehyde method (Avrameas, et al., 1969), as previously reported for the N-terminal fragment [1–14] of thymosin beta 4 (Livaniou et al., 1992). Two mg of the carrier protein (i.e., KLH, HvH or RvH), 0.5 mg of the synthetic peptide, ProTα[101–109], and 40 μl of a glutaraldehyde solution (Sigma G5882) were used in the conjugation reaction. The following day the reaction mixture was adjusted to a final volume of 10 ml with 0.9% NaCl and divided into 0.5 ml aliquots.

For each injection, one 0.5-ml aliquot was emulsified with an equal volume of Freund's Adjuvant and administered to the rabbit; thus, 100 μg of the carrier protein and 25 μg of the peptide were given to each animal per each injection. Complete Freund's Adjuvant (Difco, 263810) was used in the first injection and Incomplete Freund's Adjuvant (Difco, 263910) was used in the boosters. The animals were boosted initially after six weeks and subsequently every four weeks. Blood was collected two weeks after each booster injection. Antisera were obtained with low speed centrifugation of whole blood and stored at –35°C. Care of the animals was in accordance to the corresponding European legislation.

Antiserum ELISA-Titre Curves

ELISA microwells were coated with ProTα, dissolved in 0.1 M citrate buffer, pH 5.0 (100 ng/ml, 100 μl/microwell, and overnight incubation at 37°C). The following day, the solution was discarded and the microwells were washed twice with 0.01 M PBS, pH 7.4. Blocking was performed with 2% BSA in PBS (200 μl/microwell, 1 h at room temperature). The blocking solution was discarded and the microwells were washed three times with PBS containing 0.05% Tween20 (PBS-T). The microwells were then incubated with serial dilutions of the primary rabbit antiserum [anti-ProTα[101–109]/KLH (3^{rd} bleeding), anti-ProTα[101–109]/HvH (1^{st} – 6^{th} bleedings), or anti-ProTα[101–109]/RvH (1^{st} – 4^{th} bleedings), diluted 1:1,000 – 1:64,000 in diluting buffer i.e. PBS-T containing 0.2% BSA; 100 μl/microwell, 2 h at 37°C).

Afterwards, the solutions were discarded and the microwells were washed three times with PBS-T. The microwells were then incubated with a solution of goat anti-rabbit IgG/HRP (Sigma), diluted 1:3,000 in diluting buffer (100 μl/microwell, 2 h at 37°C). The solutions were then discarded and the microwells were washed three times with PBS-T. The microwells were finally incubated with ABTS/H_2O_2 (100 μl/microwell, 30 min at room temperature), the optical absorbance at 405 nm was measured and the corresponding titre curves were plotted. Various blank microwells were included in each run, in which (i) the

ProTα coating solution was replaced with the coating citrate buffer, (ii) the primary antiserum was replaced with the corresponding pre-immune rabbit serum.

Antiserum ELISA-Displacement Curves

ELISA microwells were coated, blocked and washed as described above. Then, the microwells were incubated with a solution of the primary rabbit antiserum [anti-ProTα[101–109]/KLH (3^{rd} bleeding), anti-ProTα [101–109]/HvH (5^{th} bleeding), or anti-ProTα[101–109]/RvH (2^{nd} bleeding) diluted 1:2,000, 1:4,000, or 1:2,500, respectively, 50 µl/microwell), concomitantly with a series of standard solutions of ProTα in diluting buffer (100 µg/ml – 100 ng/ml, 50 µl/microwell; 2 h at 37°C). After incubation, the microwells were washed and treated as described above. Finally, after measuring the optical absorbance at 405 nm, the displacement curves were plotted.

RESULTS AND DISCUSSION

Isolation of the Native Hemocyanin and its Isoforms from the Hemolymph of the Garden Snails *Helix vulgaris*

Native *Helix vulgaris* Hc was purified from the hemolymph of garden snails. After sedimentation of the hemocyanin in Beckman L-80 ultracentrifuge using an UZ rotor Ti 45, speed 24,000 RPM, for 4 h at 5°C, the blue pellet of native hemocyanin was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$. About 100 mg of the hemocyanin were dialysed against 0.13 M Gly buffer and dissociated fractions were applied on the DEAE Sepharose CL-6B column. By eluting the column with a linear gradient of 0.2–0.4M NaCl in 0.5 l 50 mM Tris–HCl buffer, pH 7.5, two different polypeptide chains (HvH1, and HvH2) were isolated of *Helix vulgaris* hemocyanin. Three different fractions were separated as shown in Figure 1.

The first peak fraction corresponds to the non-dissociated Hc (Fig. 1, insert line 3), while the second peak fraction contains HvH1 and the third one is HvH2 (Fig. 1, insert: lines 3 and 4, respectively). While *Helix pomatia* α_D-hemocyanin itself consists of two closely related subunits α and α', *Helix pomatia* β-hemocyanin is composed of two kinds of subunits: α_N (formerly β_S, now known to be immunologically identical to α_D) and β_C (Lambert et al., 1995). As shown by PAGE, the isolated isoforms (HvH1 and HvH2) of HvH have molecular masses of about 450 kDa (Fig. 1, insert: lines 3 and 4).

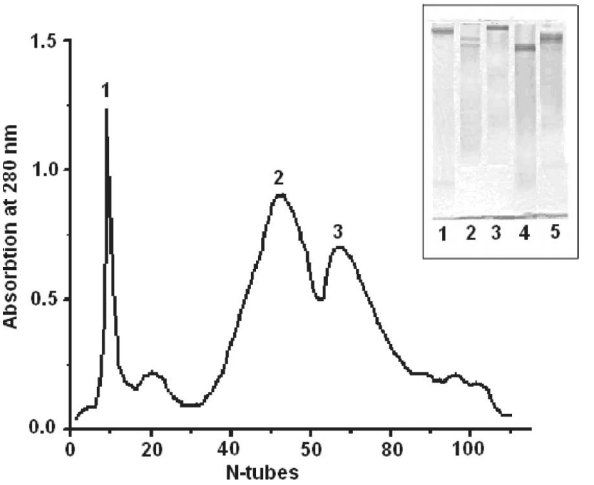

Figure 1: DEAE-Sepharose chromatography of *Helix vulgaris* hemocyanin. 100 mg of the native hemocyanin after overnight dialysis against of 0.13M Gly/NaOH buffer, pH 9.6, were brought on a DEAE-Sepharose 6CL column (12 × 2.5 cm) and eluted with a linear 0.2–0.4M NaCl gradient in 50 mM Tris–HCl, pH 7.5. (Insert) 8% PAGE: lane 1 – native HvH, lane 2 – fraction 1; lane 3- fraction 2; line 4 – fraction 3 and line 5 - standard Ferritin.

N-Terminal Sequence Determination

Purified isoforms HvH1, HvH2 and β-HvH were dissolved in 40% methanol, 1% formic acid and subjected to automated Edman degradation, as described in Materials and Methods. Two different sequences were identified for HvH1 (**ER**VRKNVDKLT**K**DE**L**YDL**QQ**) and HvH2 (**SL**VRKNVDKLT**Q**DE**V**YDL**F**Q) with very high identity, 83% (Table 1). Only five positions, 1 (E/S), 2 (R/L), 12 (K/Q), 15 (L/V) and 19 (Q/F) are occupied by different amino acid residues. By comparing the N-terminal sequences of both isoforms of *H. vulgaris*, with those of the two functional units Hpd and Hpg of *Helix pomatia* hemocyanin (HpH, P12031, P56823), *Rapana venosa* RvH1 (Dolashka et al., 1996), *Haliotis tuberculata* (CAC20588.1), *Octopus dofleini* (Od, AAK28276), *Nautilus pompilius* (NpH, Bergmann et al., 2006), and *Aplysia californica* (Ac, CAD88977) hemocyanins, some significant differences can be observed, but the conserved positions 3–8 (-VRKNVD-), 10–11 (-LT-), 14 (-E-) and 18 (-L-) for these hemocyanins fit with the sequences of both isoforms of *H. vulgaris* Hc (Table 1). The overall sequence identity is around 55%.

Helix vulgaris Hemocyanin as Potential Hapten Carrier (Hapten A: 2,4,6-trinitrobenzene Sulfonic Acid)

It is known that molluscan Hcs are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition (Lommerse et al., 1997). Therefore, we compared our preliminary studies on

Table 1: Alignment of N-terminal sequences of the isolated isoforms HvH1 and HvH2 from *Helix vulgaris* hemocyanin with N-terminal sequences of functional units d and g of *Helix pomatia* hemocyanin (HpH, P12031, P56823), *Rapana venosa* (RvH1, Dolashka et al., 1996), *Haliotis tuberculata* (HtH, CAC20588.1), *Octopus dofleini* (Od, AAK28276), *Nautilus pompilius* (NpH, Bergmann et al., 2006), *Aplysia californica* (Ac, CAD88977).

Hemocyanins	1																5										10										15										20				
HvH			E	R	**V**	**R**	**K**	**N**	**V**	**D**	**K**	**L**	**T**	K	D	**E**	L	Y	D	**L**	Q	Q
HvH1			E	R	**V**	**R**	**K**	**N**	**V**	**D**	**K**	**L**	**T**	K	D	**E**	L	Y	D	**L**	Q	Q
HvH2			S	L	**V**	**R**	**K**	**N**	**V**	**D**	**K**	**L**	**T**	Q	D	**E**	V	Y	D	**L**	F	Q
Hp d	V	A	S	H	**V**	**R**	**K**	**D**	L	**D**	T	**L**	**T**	A	G	**E**	I	E	S	**L**	R	S
Hp g	A	G	V	G	**V**	**R**	**K**	**D**	**V**	T	R	**L**	**T**	V	S	**E**	T	E	N	**L**	R	E
RvH1			S	L	L	**R**	**K**	**N**	**V**	**D**	T	**L**	**T**	E	Q	**E**	I	L	R	**L**	Q	N
HtH1		D	N	V	**V**	**R**	**K**	**D**	**V**	S	H	**L**	**T**	V	D	**E**	Q	A		**L**	H	G
Od			N	L	**T**	**R**	**K**	**D**	V	**D**	A	**L**	**S**	E	D	**E**	V	L	N	**L**	Q	V
NpH1			L	L	**V**	**R**	**K**	**D**	V	**D**	S	**L**	**T**	T	A	**E**	V	L	A	**L**	Q	E
Ac	N	A	A	L	**V**	**R**	**K**	**S**	V	**D**	Q	**L**	**T**	S	E	**E**	I	L	N	**L**	Q	K

Conserved amino acid residues are shaded in dark gray. Amino acids differences in both isoforms HvH1 and HvH2 are in box.

oligosaccharide structure of HvH with the carbohydrate structure of RvH and KLH (Beck et al., 2007; Dolashka-Angelova et al. 2004; Kurokawa et al., 2002; Sandra et al., 2007; Wuhrer et al., 2004). *Helix vulgaris, Rapana venosa* and *Megathura crenulata* hemocyanins showed different oligosaccharide structures, which has recently received particular interest for their immunostimulatory properties. Structural studies of HvH hemocyanin revealed that it is very heterogeneously glycosylated carrying preponderantly methylated high mannose-type glycans. The same glycans were identified in KLH but a specific branch with a terminal Gal β-1,3-linked to GalNAc, was sequenced. Xylosylated glycans were detected in both HvH and KLH, as well as in RvH.

Several novel types of N-glycans, with an internal Fucose connecting one N-acetylhexuronic acid (HexNAc) and one hexuronic acid (HexA), were identified in the two structural subunits of RvH (Sandra et al., 2007). It is important to mention that the methylated glycans in HvH and KLH do not show a strange migration behaviour, i.e. they do not migrate as negatively charged glycans. Hence, the presence of hexuronic acid, instead of methyl-hexose, is unlikely. Structures with methylated sugars were identified also in *Helix pomatia* Hc (Gielens et al., 2004; Lommerse et al., 1997).

The immunostimulatory properties of several hemocyanins are well known and their biomacromolecules are widely applied as hapten carriers and different antiviral, antibacterial or antitumor vaccines some of which undergo clinical trials. Hemocyanin was selected to serve as a carrier in these studies because it is highly immunogenic. Quantities of antibody measurable by precipitin test are produced following immunization of rabbits with a single injection of 5 mg or more of hemocyanin. Picryl (trinitrophenyl) radicals are also of

well-known antigenicity and picryl sulfonic acid will conjugate to free amino groups of proteins at neutral pH 9 offering the possibility of reaction without major configurational change in the protein due to pH extremes.

Our preliminary studies on the immunological properties of molluscan *Rapana venosa*, *Helix vulgaris* and keyhole limpet as well as arthropodan *Carcinus aestuarii* hemocyanins have shown that they are potentially applicable in medicine. Comparative study on the effect of the immunization of BDF1 mice with the above mentioned hemocyanins and selected fragments of them on the proliferation of spleen lymphocytes in response to T and B cell mitogens has shown that the *Carcinus* hemocyanin has produced a stimulating effect on the reactivity of the animal spleen lymphocytes to the mitogens.

Serum IL-2 production was higher in animals immunized with *Helix vulgaris* and *Carcinus* hemocyanins and with native KLH (Toshkova et al., 2006). It was published that the addition of KLH to dendritic cells that had been incubated with tumor cells increased IL-12 (an accelerating T-cell differentiation factor) secretion (Schnurr et al., 2001); therefore, the IL-2 levels in the culture of lymphocytes and in the animal serum were parallel analyzed after immunization with these Hcs. The increased production of IL-2 observed was a proof of dendritic cell maturation after immunization of the mice with native *Carcinus eastuarii* and KLH as well as their selected fragments (Toshkova et al., 2006).

Although a few other hemocyanins, besides KLH, have been used as antigen carriers, comparative studies of hemocyanins isolated from various sources and differing in chemical structure in terms of their ability to serve as carrier proteins have not been conducted. Therefore, the capacity of *Helix vulgaris* and *Rapana venosa* hemocyanins to function as carriers of non-immunogenic molecules was studied in comparison with KLH. In experiments with hapten A, we studied whether the differences in the chemical structure of hemocynins influence the time course and intensity of primary immune response to the hapten. To follow the development of primary immune response, immunogenicity of trinitrophenyl-hemocyanin (TNP-Hc) complexes was characterized by the production of antibodies and hapten-specific IFN- γ at different time points after a single immunization of mice with these conjugates.

In experiments with hapten A, C57BL/6 mice were immunized with 100 μg HvH, RvH or KLH chemically conjugated to TNP in complete Freund's adjuvant. TNP conjugated to bovine serum albumin, which is well known as a carrier of haptens, was used for in vitro stimulation of splenocyte cultures.

IFN-γ production in splenocyte cultures from mice immunized with TNP-Hc plus CFA in the presence of TNP-BSA is shown in Figure 2. In addition, no IFN-γ was detected (sensitivity of the assay was 16 pg.ml^{-1}) in any supernatant fluids obtained from the control groups stimulated in vitro with TNP-BSA at any time point tested. Moreover, no IFN-γ was detectable in splenocyte supernatants from mice primed with TNP-Hc/CFA in the absence of in vitro stimulation or in the presence of 50 μg/ml BSA. Together, the results indicate

that IFN-γ production detected was TNP-specific since it required both adequate priming (including CFA) with TNP-Hc and additional in vitro stimulation of splenocytes with TNP-BSA. As shown in Figure 2, production of IFN-γ developed faster but also ceased earlier when KLH was used as a hapten carrier. Although TNP-specific IFN-γ response with other hemocyanin carriers was lower on day 7 post-immunization, it was increased in time and lasted longer. When HvH was used as a hapten carrier, significant IFN-γ production was detected even on day 21 post-immunization.

To follow the time course of hapten-specific antibody response in splenocyte cultures after a single immunization of mice with modified TNP-HvH, TNP-RvH and TNP-KLH, splenocytes obtained from spleens on days 7, 14 and 21 post-immunization were cultured for 3 days in the presence or absence of TNP-BSA. In the absence of TNP-BSA in the cultures, no anti-hapten antibody production was detectable. Priming of mice with TNP-Hc in the absence of CFA was not effective in inducing significant anti-hapten antibody production in splenocyte cultures. When spleen cells of mice administered TNP-Hc plus CFA were cultured with TNP-BSA, significant IgM and IgG antibody response was measured (Figs. 3 A, B). Some differences were noticed in the time-course of antibody production; particularly a long-lasting anti-TNP response was detected in mice immunized with TNP-HvH conjugate, where 21 days after immunization with TNP-HvH, higher amounts of TNP-specific IgM and IgG antibodies were detected in comparison with TNP-KLH.

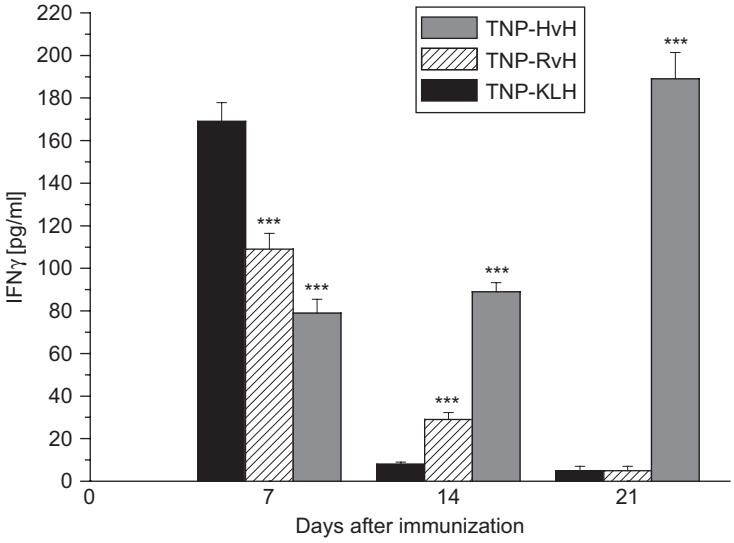

Figure 2: TNP-specific IFN-γ production in splenocyte cultures 7, 14 and 21 days after the immunization of mice with: TNP-HvH (▭), TNP-RvH (▨) and TNP-KLH (■). Splenocytes from mice were cultured for 3 days in the presence of TNP-BSA (50 μg/ml). ***– $p < 0.001$, Student's *t*-test. Data represent results from 3 independent experiments.

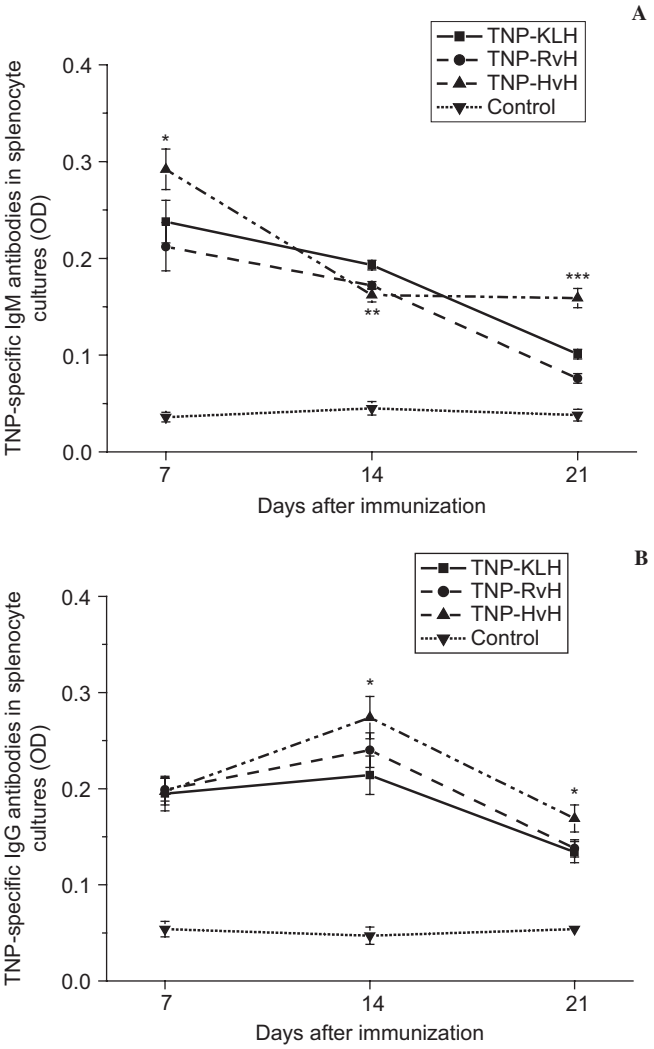

Figure 3: Production of TNP-specific antibodies in splenocyte cultures. Splenocytes from mice immunized with TNP-KLH (—■—), TNP-HvH (—▲—), and TNP-RvH (—●—) plus CFA were cultured for 3 days in the presence of TNP-BSA (50 μg/ml). Controls were immunized with PBS/CFA (—▼—). (A) Production of TNP-specific IgM antibodies; (B) Production of TNP-specific IgG antibodies. Antibody response was measured by ELISA. *– $p < 0.05$, **– $p < 0.01$, ***- $p < 0.001$, Student's *t*-test. Data represent results from 3 independent experiments.

To examine the effects of different hemocyanin carriers on the development of anti-hapten antibody response, mice received a single immunization with TNP-Hc in the presence or absence of CFA. Sera were tested for the level of TNP-specific IgM and IgG antibodies by ELISA using TNP-BSA coated plates on days 7, 14, and 21 post-immunization. In groups immunized with TNP-Hc in the absence of CFA, a weak anti-hapten IgG antibody response

was noticed only on day 14 post-immunization (titers did not exceed 1:200). Serum antibody titers of mice, immunized with TNP-KLH, TNP-RvH and TNP-HvH together with CFA are presented in Figure 4. As shown, serum anti-TNP IgM antibody response developed more slowly when HvH was used as a carrier; however, both RvH and HvH developed higher antibody titers of this class on day 14, compared to KLH (Fig. 4A). Anti-hapten IgG antibody titers launched by RvH were higher than those obtained with KLH at all time points. Using HvH as a carrier, TNP-specific antibody response developed later, but on day 14 and 21 it was higher than that with KLH (Fig. 4B).

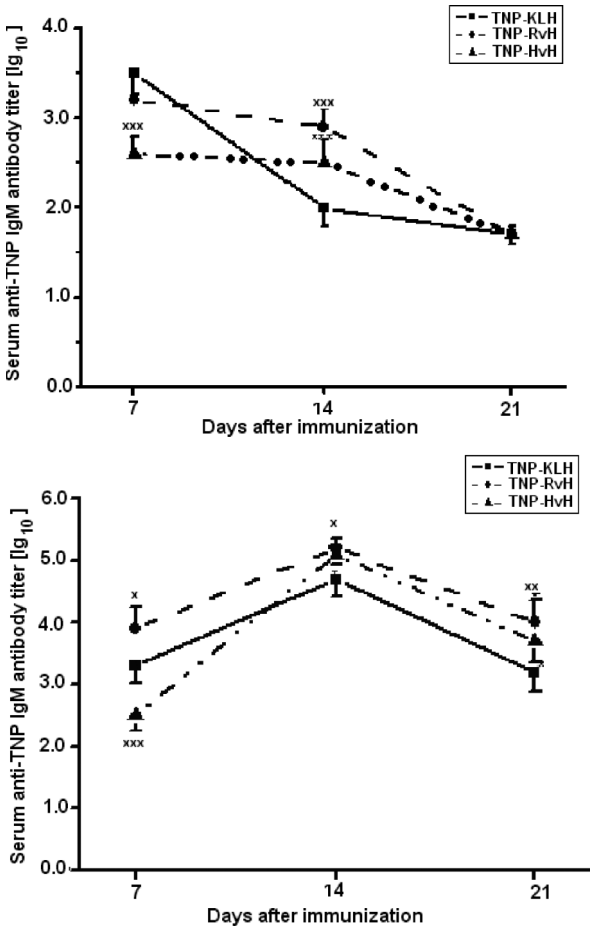

Figure 4: Serum anti-TNP antibody titers. Individual sera from mice immunized with TNP-HvH (—▲—), TNP-RvH (— ● —) and TNP-KLH (—■—) were diluted initially 1/100 and then serially $\log_2$. (A) Serum anti-TNP IgM antibody titer; (B) Serum anti-TNP IgG antibody titer. Serum titers were assessed by ELISA. Microplates were coated with 1 μg TNP-BSA/well. *- $p < 0.05$, **- $p < 0.01$, ***- $p < 0.001$, Student's *t*-test. Data represent results from 2 independent experiments.

Helix vulgaris Hemocyanin as Potential Hapten Carrier (Hapten B: Synthetic Fragment of the Poorly Immunogenic Polypeptide Prothymosin Alpha)

Prothymosin alpha (ProTα) is an acidic polypeptide (MW = ~11 kDa, pI = 3.55), widely distributed and highly conserved in mammalian tissues and cells, which participates in important intracellular and extracellular functions (Freire et al., 2002; Hannappel et al., 2003). The ProTα molecule shows some unusual structural features, since it consists mainly of aspartic and glutamic acid residues (more than 50 %), whereas few hydrophobic and no aromatic or sulfur amino acids are present in its sequence (Gast et al., 1995); under physiological conditions, ProTα is monomeric and adopts a random coil conformation. Antibodies for ProTα are in most cases low in titre and often broad in specificity (Enkemann et al., 2000). The absence of secondary structure as well as its evolutionary high conservation within mammalian species may possibly explain this well-known, poor immunogenicity (Freire et al., 2002) of the polypeptide.

Our efforts to develop antibodies against non-conjugated bovine ProTα in rodents, i.e. rabbits and Balb-c mice, have failed many times, while many other groups have also reported unsuccessful trials (Sukhacheva et al., 2002). To the contrary, at least in our hands, antibodies could be raised against ProTα, or synthetic fragments of ProTα, after they had been conjugated to the well-known, highly immunogenic carrier protein keyhole limpet (*Megathura crenulata*) haemocyanin (KLH) (Costopoulou et al., 1998; Klimentzou et al., 2007). Development of antibodies against ProTα-KLH conjugates can be explained by assuming that the KLH molecule offers the T-cell epitopes necessary for stimulating humoral immunity in the host animal. On the other hand, Sukhacheva et al. (2002) reported for the first time the development of high titre anti-ProTα antibodies by using ProTα fused, via molecular biology techniques, to green fluorescence protein as immunogen (Sukhacheva et al., 2002), which verifies that the nature of the ProTα-immunogen can greatly affect the quality of the anti-ProTα antibodies developed.

Therefore, we studied the effect of *Helix vulgaris* and *Rapana venosa* hemocyanins as carriers of a synthetic fragment of ProTα on the development of antibodies for ProTα, in comparison with the well established carrier protein, *Keyhole limpet* hemocyanin. More specifically, in the frame of the present work, the synthetic C-terminal fragment ProTα[101–109] was conjugated to commercially available KLH (control conjugate) as well as to the above mentioned hemocyanins isolated from *Helix vulgaris* and *Rapana venosa* and the conjugates were used for immunizing rabbits. Ultimate goal of this work was to investigate whether it would be feasible to develop anti-ProTα antibodies of high titre and affinity by changing the carrier protein used in the immunization procedure.

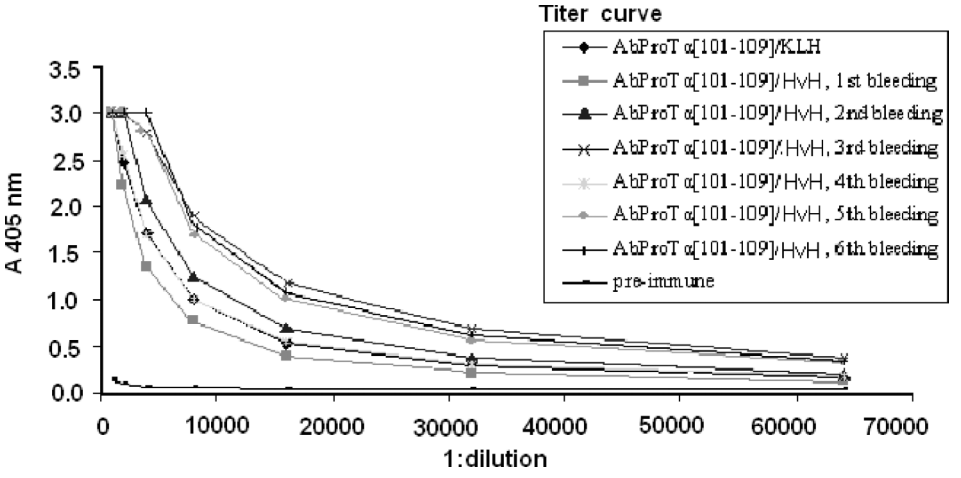

Figure 5: ELISA-titre curves of the rabbit antiserum raised against ProTα(101–109)/HvH. Different bleedings have been tested, obtained after the 1st (—■—), 2nd (—▲—), 3rd (—x—), 4th (—*—), 5th (—●—) and 6th (—l—) booster injection. Rabbit antiserum raised against ProTα(101–109)/KLH was used as control (—✦—, 3rd bleeding). The titre-curves shown here are indicative of the results obtained in 4 independent experiments.

The antisera developed were parallel evaluated in titre and displacement ELISA systems. According to our preliminary results, antisera of higher titres than that of the control conjugate (ProTα[101–109]/KLH) were obtained with both, the ProTα[101–109]/HvH (Fig. 5) and the ProTα[101–109]/RvH (Fig. 6) conjugates, immediately after the second bleeding. On the other hand, typical displacement curves, similar to that obtained with the control antiserum (anti-ProTα[101–109]/KLH), were obtained with the antisera raised against ProTα[101–109]/HvH or ProTα[101–109]/RvH (Fig. 7) in the presence of standard solutions containing increasing amounts of ProTα, which indicates that both antisera are specific for ProTα. According to our preliminary data, the displacement curve obtained with the anti- ProTα[101–109]/HvH antiserum seems to have a better slope and, therefore, better sensitivity (Fig. 7), but this finding remains to be further investigated.

CONCLUSION

In conclusion, we have demonstrated that the *Helix vulgaris* hemocyanin (HvH) is a new dioxygen-binding protein found freely dissolved in the hemolymph of the garden snails *Helix vulgaris*. The didecameric molecule is built up of two type of subunits. After overnight dialysis against dissociating buffer the gastropod *Helix vulgaris* hemocyanin dissociates into two isoforms, HvH1 and HvH2, which have molecular masses about 450 kDa.

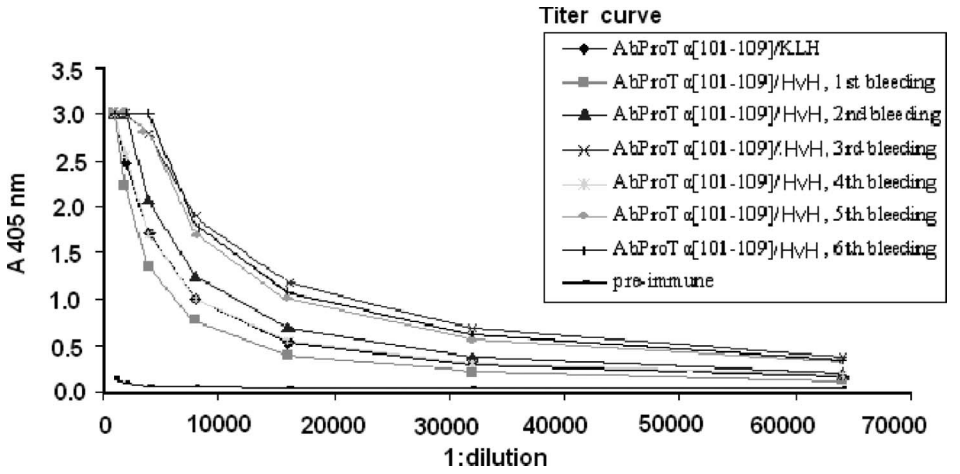

Figure 6: ELISA-titre curves of the rabbit antiserum raised against ProTα(101–109)/RvH. Different bleedings have been tested, obtained after the 1st (—■—), 2nd (—▲—), 3rd (—×—), and 4th (—*—) booster injection. Rabbit antiserum raised against ProTα(101–109)/KLH was used as control (—✦—, 3rd bleeding). The titre-curves shown here are indicative of the results obtained in 4 independent experiments.

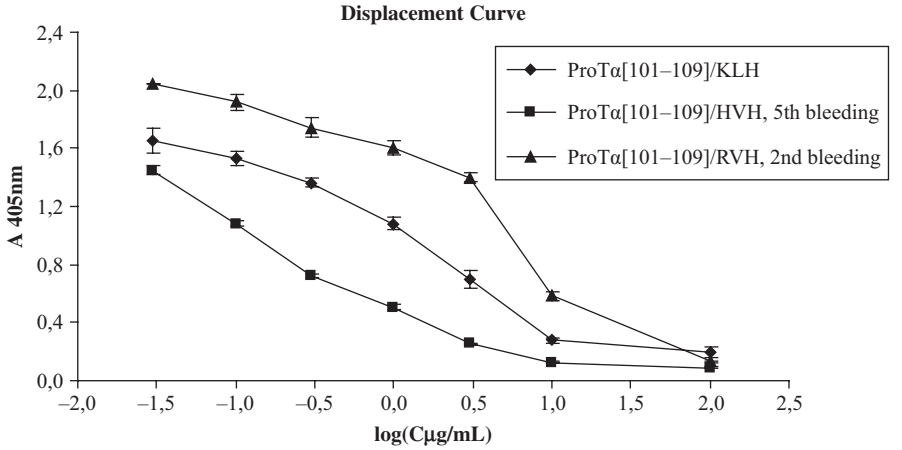

Figure 7: ELISA-displacement curves obtained with randomly selected bleedings of the rabbit antisera raised against ProTα(101–109)/HvH (—■—, 5th bleeding) or ProTα(101–109)/RvH, (—▲—, 2nd bleeding). Rabbit antiserum raised against ProTα(101–109)/KLH was used as control (—✦—, 3rd bleeding). The displacement-curves shown here are indicative of the results obtained in 4 independent experiments.

The results obtained show that hemocyanins from *Helix vulgaris* and *Rapana venosa* are effective carriers of non-immunogenic or poorly immunogenic antigens. The differences in the time-course of antibody response development could be ascribed to the variations in the chemical structure of hemocyanins from various sources. Thus, the benefit of using various hemocyanins as

antigen carriers could be the possibility to direct the timing of immune response development according to the particular needs. Although data obtained with a greater number of immunized animals as well as with more molecules that are poor immunogens are needed, in order to prove that HvH and RvH can be used as alternative to KLH carrier proteins for developing antibodies against poorly immunogenic molecules, the results of this work are considered promising and support continuation of relevant studies. On the other hand, the above results may be considered as preliminary basic information for further investigating whether the hemocyanins isolated from *Rapana venosa* and *Helix vulgaris* might be also used to develop *in vivo* immunotherapeutics (Gathuru et al., 2005).

ACKNOWLEDGMENTS

This work was supported by NATO grant LST.CLG. 978560, Bulgarian Ministry of Education (UL-601), Higher Education of the Greek Ministry of Education and Cults, CNR (Italy), DAAD. Dr. P. Dolashka-Angelova would like to thank DFG (Deutsche Forschungsgemeinschaft) and DAAD for granting a scholarship.

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