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Functional aspects of cHH C-terminal amidation in crayfish species

Alessandro Mosco [a], Paolo Edomi [a], Corrado Guarnaccia [b], Simonetta Lorenzon [c], Sándor Pongor [b], Enrico A. Ferrero [a], Piero G. Giulianini [a,*]

[a] *BRAIN Center, Department of Biology, University of Trieste, Via Giorgeri 7, I-34127 Trieste, Italy*
[b] *ICGEB, International Centre for Genetic Engineering and Biotechnology, AREA Science Park, Padriciano 99, I-34012 Trieste, Italy*
[c] *OGS, Biological Oceanography Department, Via Piccard 54, I-34014 S. Croce (TS), Italy*

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Abstract

The crustacean hyperglycemic hormone is the most abundant neuropeptide present in the eyestalk of Crustacea and its main role is to control the glucose level in the hemolymph. Our study was aimed at assessing the importance of C-terminal amidation for its biological activity. Two recombinant peptides were produced, Asl-rcHH-Gly with a free carboxyl terminus and Asl-rcHH-amide with an amidated C-terminus. Homologous bioassays performed on the astacid crayfish *Astacus leptodactylus* showed that the amidated peptide had a stronger hyperglycemic effect compared to the non-amidated peptide. To assess the relevance of amidation also in other decapods and how much the differences in the cHH amino acid sequence can affect the functionality of the peptides, we carried out heterologous bioassays on the cambarid *Procambarus clarkii* and palaemonid *Palaemon elegans*. The Asl-rcHH-amide elicited a good response in *P. clarkii* and in *P. elegans*. The injection of Asl-rcHH-Gly evoked a weak response in both species. These results prove the importance of C-terminal amidation for the biological activity of cHH in crayfish as well as the role of the peptide primary sequence for the species-specificity hormone-receptor recognition.
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1. Introduction

The crustacean hyperglycemic hormone (cHH) is a member of a family of neuropeptides that are produced and released in the X-organ sinus gland complex, located in the eyestalks of crustaceans. Besides cHH, the molt inhibiting hormone (MIH), the gonad/vitellogenesis inhibiting hormone (GIH/VIH) and the mandibular organ inhibiting hormone belong to this family. The main role of cHH is to regulate the hemolymph glucose level, but it takes part in the control of other functions too as the secretion of digestive enzymes, the lipidic metabolism, stress responses, osmoregulation, molting and reproduction (for recent reviews see [1–3]).

The cHH is the most abundant of the neuropeptides found in the eyestalks, 2–4 μg per sinus gland (SG) in *Carcinus maenas* versus 0.15 μg/SG for the GIH in *Homarus americanus* and 0.3–0.5 μg/SG for the MIH in *C. maenas* [4,5]. The cHH release into hemolymph is pulsatile and follows a circadian rhythm to keep a basal hemolymph concentration that in *C. maenas* is of about 25–50 fmol/mL [6].

The cHH family is divided into two subfamilies on the basis of the primary structure: the cHH subfamily is made up of hormones whose prepropeptides consist of a signal peptide, a cHH precursor related peptide and a mature peptide of 72 amino acids, and the MIH/GIH subfamily, which lacks the cHH precursor related peptide and possesses a Gly residue at position 12. The mature cHH has six cysteine residues that form three disulfide bridges and possesses the C-terminus amidated and the N-terminus blocked by pyroglutamate in crayfish, lobsters and shore crabs [7–9] or the N-terminus is free like in shrimps [10]. Carboxyl-terminus amidation is a relevant modification that gives biological activity to a large number of peptides in vertebrates and invertebrates like oxytocin, vasopressin, locust adipokinetic hormone, many shrimp

* Corresponding author. Tel.: +39 0405583560; fax: +39 040575079.
E-mail address: giuliani@univ.trieste.it (P.G. Giulianini).

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antimicrobial peptides and crab allatostatin [11,12]. The essential role of amidation in conferring hyperglycemic activity to cHHs was ascertained till now only for the cHH of *Marsupenaeus* (*Penaeus*) *japonicus* where the hyperglycemic activity of the recombinant Pej-SGP-I-amide was comparable in vivo to that of the native peptide, while the rPej-SGP-I-OH and rPej-SGP-I-Gly showed a reduced activity by one order of magnitude [13]. The importance of the C-terminal amide moiety was supposed also for the cHH of *Macrobrachium rosenbergii* [14], but in this study the recombinant amidated peptide was compared to a non-eyestalk form, Mar-rcHH-L. This peptide ensues from an alternative splicing, so that its sequence differs considerably starting from Arg^{40}, and showed no hyperglycemic activity. While the lack of any effect on the glucose hemolymph level was reported for Mar-rcHH-L, no attempt was made to compare the activity of the Mar-rcHH-amide to a non-amidated recombinant peptide. The cHH is a rather conservative molecule with an overall similarity of cHH sequences ranging from 40% to 99%, the highest homology found between species of the same infraorder [15]. The secondary structure predicted by the PHD server [16] for the cHH of *M. rosenbergii*, *C. maenas* and *Procambarus clarkii* resulted in an all-helix structure at the C-terminus [17]. This indicates that there may be a good similarity in the tertiary structure and therefore the restricted or absent cross reactivity, reported as group or infraorder specific [18], that was observed in heterologous bioassays, is due to small differences in primary structures.

As far as the other neuropeptides of the family are concerned, the relevance of amidation for the biological activity of VIH was assessed in *H. americanus* by means of an in vitro heterologous bioassay using ovarian fragments from *M. japonicus*. The results obtained showed that rHoa-VIH-amide was able to reduce significantly vitellogenin mRNA levels in the ovary while rHoa-VIH-OH had no effect [19]. Besides amidation, also the correct amino acid sequence at the C-terminus is important to the peptide functionality. Mutational analysis carried out on MIH of *M. japonicus* proved that the functional site is located in the region containing the C-terminal ends of the N- and C-terminal alpha-helices [20]. The relevant role of the C tail was established also in a cHH related peptide like the ion transport peptide (ITP) of *Schistocerca gregaria*. The secreted peptides Kc1ITP-GKK and Kc1ITP-G from *Drosophila* Kc1 transfected cells had reduced stimulatory activity compared to a synthetic ITP due to incomplete processing of the C-terminal sequence while the Kc1ITP-amide displayed an activity comparable to the reference ITP. A complete loss of function was reported for truncated mutants ($Kc1ITP_{71}$) lacking LGKK at the C-terminus [21].

To assess the role that amidation has for the biological activity of cHH, we have tested an Asl-rcHH on two crayfish species, *Astacus leptodactylus* and *P. clarkii*, which are commonly bred for commercial purposes, and a shrimp, *Palaemon elegans*. In this paper we show that amidation is of great importance in conferring biological activity to Asl-cHH and that heterologous bioassays can be successfully used to assess how much the differences in the primary structure of the different cHHs are relevant to their functionality.

2. Materials and methods

2.1. cDNA cloning in the expression plasmid

A previously cloned cDNA [22] encoding the mature Asl-cHH was amplified with the specific oligonucleotide primers ALE1 (5′-ATGGTA*GGTCTC*AGCGCCCAGGTGTTCGAT-CAGGCGTGTA-3′ in italics the BsaI site) and ALE2AMID (5′-ATGGTA *GGTCTC*ATA**TCA**GCCGACCATCTG-GACTCCGGAGA-3′ in italics the BsaI site, in bold the stop codon and underlined the three nucleotide residues encoding the amidating donor residue glycine). The amplified PCR products were cloned into the pPR2 (IBA) vector, which provides the N-terminal Strep-tag II, using the BsaI cloning sites inserted in the ALE1 and ALE2AMID primers, generating the Asl-cHH-pPR2 expression vector.

2.2. Expression of the recombinant peptide

Escherichia coli BL21(DE3)pLysS (Promega) competent cells were transformed with the Asl-cHH-pPR2 vector. Cells from a single colony were grown overnight at 37 °C in LB medium containing 100 μg/mL ampicillin. Three hundred milliliters of the same medium was inoculated with 6 mL of the preculture and incubated at 37 °C. When the optical density at 600 nm was equal to 0.5, isopropyl-β-D-thiogalactoside was added to the culture to a final concentration of 0.5 mM. After 3 h of incubation, the cells were harvested by centrifugation.

2.3. Refolding and purification of Asl-rcHH

Pelleted bacterial cells were resuspended in 3 mL of lysis buffer [100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, pH 8], sonicated and, after addition of 2 μL lysozyme (50 mg/mL), the suspension was incubated at room temperature for 20 min. The suspension was centrifuged for 20 min at 4 °C and 12,000 ×g, the sediment resuspended in 1 mL of lysis buffer and sonicated. This step was repeated twice. After the last centrifugation step, the sediment was resuspended in 1 mL of washing buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8), sonicated and centrifuged for 20 min at 4 °C and 12,000 ×g. The pellet was resuspended to a final volume of 1.5 mL with solubilization buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 8 M urea, 1 mM DTT, pH 8) and incubated for 2 h at room temperature. After a centrifugation step of 20 min at 15 °C and 20,000 ×g, the supernatant was collected. The peptide refolding was done by size exclusion chromatography [23]. A 5 mL Hitrap™ Desalting (GE Healthcare) column was equilibrated with refolding buffer [100 mM Tris, 0.5 M L-arginine, 10 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% (w/v) PEG 8000, 5 mM L-glutathione reduced form, 0.5 mM L-glutathione oxidized form, pH 8.5]. The solubilized peptide was loaded onto the column and the same redox buffer used to equilibrate the column was used to elute the recombinant peptide. The refolded Asl-rcHH-Gly was purified on a 1 mL Strep-Tactin® Superflow® cartridge (IBA) following the IBA protocol. The

elution buffer was exchanged with another buffer (1 M Tris–HCl, pH 7.0) by a final step with a Hitrap™ Desalting (GE Healthcare) column.

2.4. Amidating reaction

For the amidating reaction, 100 μg of the purified Asl-rcHH-Gly (400 μL) was mixed with 800 μL of a peptidylglycine α-amidating enzyme (Wako) solution (750 U/mL enzyme, 100 mM Tris, 0.3 M NaCl, pH 9.3) and with 800 μL of a reaction solution [2.5 mM *N*-ethylmaleimide, 2.5 μM ascorbic acid, 50 μM $CuSO_4$, 0.25% (v/v) Thesit and 0.05% (w/v) catalase]. This amidating solution was incubated at 37 °C for 24 h and the reaction stopped by the addition of 20 μL trifluoroacetic acid (TFA). The Asl-rcHH-amide was purified by a Gilson HPLC system equipped with a microbore column Zorbax SB300 CN 2.1×150 mm from Agilent Technologies, Inc. (DE, USA) thermostated at 25 °C. Eluent A was 0.1% TFA in water, eluent B was 0.1% TFA in acetonitrile. The separation was done using a gradient of 0–30% B in 5 min, 30%–56% B in 30 min and 56%–90% B in 2 min at 0.3 mL/min.

2.5. Mass spectral analysis

RP-HPLC purified fractions of the peptides were mixed 1:1 with alpha-Cyano-4-hydroxy cinnamic acid matrix (5 mg/mL in 70% acetonitrile). One microliter of the mixture was spotted on the MALDI plate and analyzed on a MALDI-TOF/TOF 4800 Proteomics Analyzer (Applied Biosystems) in positive linear ion mode.

2.6. Circular dichroism spectral analysis

Samples for circular dichroism spectroscopy were prepared by dissolving the RP-HPLC purified and lyophilized peptides in 5 mM Tris–HCl pH 7. Peptide concentration (21.6 and 6 μM for CHH-Val-Gly and CHH-Val-NH_2 respectively) was determined by UV absorbance at 280 nm using calculated ε values of 14,815 M^{-1} cm^{-1} for the peptide oxidized form. The extinction coefficient was computed using the ProtParam programme on the ExPASy server [24]. CD spectra were recorded on a Jasco J-810 spectropolarimeter in the range of 190–250 nm using 0.1 cm quartz cuvettes. For each spectrum, five scans were acquired at 10 nm min^{-1}, and the mean residue ellipticity (MRE, deg cm^2 $dmol^{-1}$ $residue^{-1}$) was calculated from the baseline-corrected spectrum. A quantitative estimation of secondary structure content was carried out using CDSSTR run from the DichroWeb server [25].

2.7. Native cHH extraction

Sinus glands from 20 eyestalks were collected and 100 μL of extraction solution (90% MetOH, 9% acetic acid, 1% H_2O) was added to them. After sonication, the sample was centrifuged for 10 min at 4 °C and 12,000 ×g and the supernatant collected. The pellet was resuspended in 100 μL of the extraction solution and centrifuged again. The two supernatants were mixed and left to evaporate overnight at room temperature. The dry extract was dissolved in 50 μL MilliQ water.

2.8. Biological assays for Asl-rcHH activity in vivo

A. leptodactylus, imported from Turkey, were obtained from a local dealer; *P. clarkii* specimens were kindly provided by David Mazzoni; shrimps, *P. elegans*, were caught by cages in the Gulf of Trieste (Northern Adriatic Sea) and supplied by commercial fishermen. Crayfish and shrimps were kept in 120 L tanks, in fresh or saltwater accordingly to the species, as previously described [22,26]. The animals were bilaterally eyestalk ablated 48 h before the start of the experiment. Sinus gland extracts of each species were diluted in phosphate-buffered saline and a volume of 100 μL or 50 μL, corresponding to one eyestalk equivalent, was injected into crayfish or shrimp respectively. Asl-rcHH-amide (1.7 pmol/g and 3.3 pmol/g of live weight) and Asl-rcHH-Gly (1.7 pmol/g and 16.7 pmol/g of live weight) samples were diluted in phosphate-buffered saline and 100 μL of this solution was injected into crayfish or 50 μL into shrimps. Animals were bled at 0, 30, 60, 120 and 240 min after injection and the hemolymph glucose level was quantified by One Touch glucose test kit (Lifescan). Values are expressed as mean±standard deviation. Mann–Whitney *U*-test statistics were performed using GraphPad InStat version 3.0b for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

3.1. Expression of Asl-rcHH

After induction, bacterial cells were harvested by centrifugation, resuspended in lysis buffer and sonicated. The soluble and insoluble fractions were separated by centrifugation and the presence of the Asl-rcHH-Gly checked by Tricine SDS-PAGE. A band of higher intensity in Coomassie staining and of the expected molecular mass of about 10 kDa was present in the pellet fraction (Fig. 1, lane 3); thus the recombinant peptide was purified from the inclusion bodies using a buffer containing urea 8 M and, then, refolded.

3.2. Refolding and purification

The refolding reaction was performed by a size exclusion chromatography method. The folded Asl-rcHH-Gly was then purified through an affinity chromatography on a Strep-Tactin Superflow cartridge. The sample purity was checked by Tricine SDS-PAGE, where only one band of about 10 kDa was observed (Fig. 1, lane 4). Mass spectrometry of the purified Asl-rcHH-Gly showed the peak at *m/z* 9804.38 $(M+H)^+$, a value well in agreement with the calculated average molecular mass (Mr) of 9804.25 Da, that is composed by the contribution of the Strep-tag and of the 73 residues of the hormone, taking into account the formation of three disulfide bridges. The purified Asl-rcHH-Gly was treated with a peptidylglycine α-amidating enzyme in order to obtain the amidated Asl-rcHH. The product of the amidating reaction was analyzed by MALDI-TOF and a

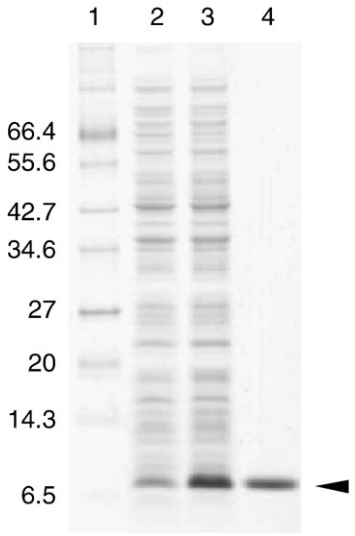

Fig. 1. SDS-Tricine PAGE (10% acrylamide) of extracts from non-induced and induced cells stained by Coomassie blue. Lane 1: molecular weight markers, sizes in kDa; lane 2: extract from 0.5 mL culture of non-induced cells; lane 3: extract from 0.5 mL culture of cells induced with isopropyl-β-D-thiogalactoside; lane 4: the purified peptide after affinity chromatography on a Strep-Tactin® Superflow® cartridge (IBA). The arrow indicates the position of Asl-rcHH-Gly.

peak at *m/z* 9745.93 $(M+H)^+$ was observed, the value in agreement with the calculated molecular mass (Mr) of 9746.03 Da. The Asl-rcHH-amide was purified by RP-HPLC using a linear gradient of 30%–56% acetonitrile, lyophilized and resuspended in MilliQ water (Fig. 2).

3.3. Conformational analyses

CD spectra (Fig. 3) were recorded to assess if amidation influenced the secondary structure of the recombinant peptides, Asl-rcHH-Gly and Asl-rcHH-amide. Both spectra present the typical features of alpha-helical structures (positive band at 190 nm and negative bands at 208 and 222 nm). The calculated percentages of secondary structures found in the two peptides were somehow different (Table 1), with the amidated rcHH showing a greater fraction of alpha-helical content.

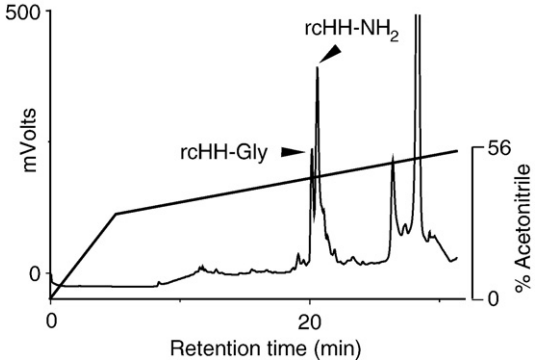

Fig. 2. RP-HPLC elution profile of Asl-rcHH-amide. The separation was performed with a Zorbax SB300 CN column (2.1 × 150 mm) using a gradient of 0–30% acetonitrile in 0.1% TFA in 5 min and 30%–56% acetonitrile in 0.1% TFA in 30 min at 0.3 mL/min. The arrows indicate the Asl-rcHH-Gly and Asl-rcHH-NH_2 peaks.

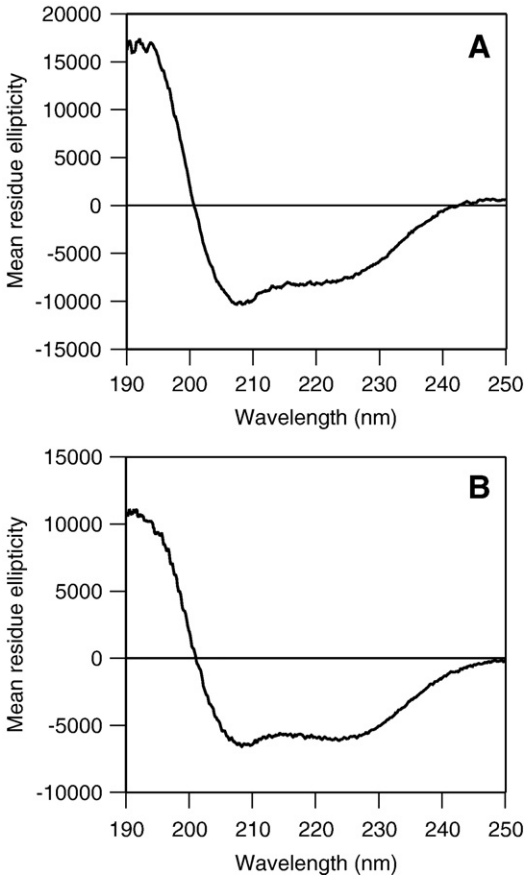

Fig. 3. CD spectra of Asl-rcHH-amide (A) and Asl-rcHH-Gly (B). The recombinant peptides were dissolved in 5 mM Tris–HCl pH 7 and the CD spectra were recorded in the range of 190–250 nm using 0.1 cm quartz cuvettes.

3.4. Homologous bioassays

The biological assays on *A. leptodactylus* were performed as time-course experiments to assess how the biological activity of the recombinant peptides compares to the wild type hormone and as dose-related treatments to evaluate the entity of a dose–response effect on the glucose hemolymph level (Fig. 4). The injection of one Asl sinus gland equivalent (SGE) extract into crayfish induced an increment of glucose that reached a maximum of 30.7±30 mg/dL at 2 h and the maintenance of high glucose levels until 4 h after injection, when a slight decrease was observed. The injection of 1.7 pmol/g live weight of Asl-rcHH-amide induced a quicker response than that induced by the sinus

Table 1
Secondary structure composition of the recombinant peptides Asl-rcHH-Gly and Asl-rcHH-amide

Peptide	Helix	Strand	Turns	Unordered	Total
Asl-rcHH-Gly	0.21	0.23	0.17	0.38	0.99
Asl-rcHH-amide	0.35	0.18	0.16	0.30	0.99

The deconvolution results from CD spectra indicate the percentage of secondary structure present.

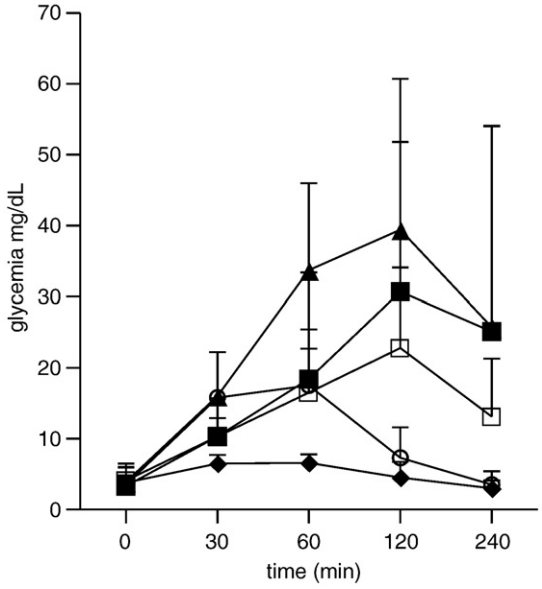

Fig. 4. Time course of hemolymph glucose in eyestalk-ablated *A. leptodactylus*. Comparison of hyperglycemic activity of 1 Asl SGE ■ ($n=9$); 1.7 pmol/g live weight Asl-rcHH-amide ○ ($n=4$); 3.3 pmol/g live weight Asl-rcHH-amide ▲ ($n=7$); 1.7 pmol/g live weight Asl-rcHH-Gly ◆ ($n=8$); 16.7 pmol/g live weight Asl-rcHH-Gly □ ($n=8$). Values are expressed as mean±standard deviation.

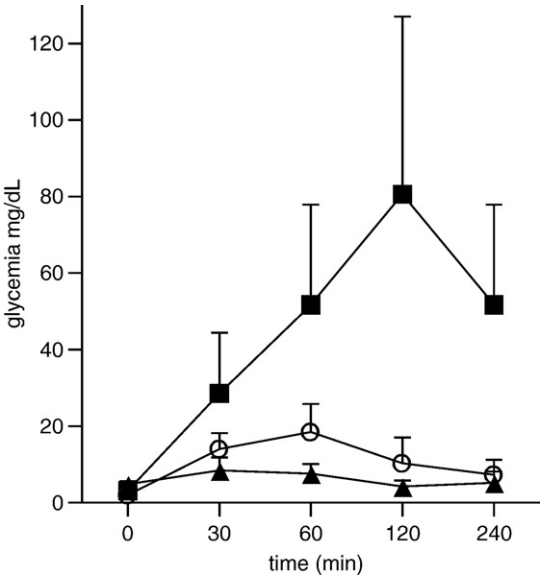

Fig. 5. Time course of hemolymph glucose in eyestalk-ablated *P. clarkii*. Comparison of hyperglycemic activity of 1 Prc SGE ■ ($n=7$); 3.3 pmol/g live weight Asl-rcHH-amide ○ ($n=4$); 16.7 pmol/g live weight Asl-rcHH-Gly ▲ ($n=5$). Values are expressed as mean±standard deviation.

gland extract, with the maximum peak of 17.5±7.9 mg/dL glucose after 1 h and the glycemia returning to its basal level, 3.5±1.9 mg/dL, within 4 h. Injection of 3.3 pmol/g live weight Asl-rcHH-amide induced a significantly stronger response ($p<0.05$) compared to the injection of 1.7 pmol/g live weight and a peak of glucose of 39.4 mg/dL±12.4 was observed at 2 h, moreover the glucose titer falling slowly and not reaching the initial level at 4 h. The dose–response effect of the Asl-rcHH-Gly was completely different. At a dose of 1.7 pmol/g live weight its ability to raise glucose levels was significantly lower ($p<0.05$) compared to the same amount of amidated peptide, while the time course was similar with a peak of 6.6±1.2 mg/dL glucose measured after 1 h. Only a ten fold amount of Asl-rcHH-Gly, 16.7 pmol/g live weight, induced a relevant increase of hemolymph glucose of 22.8±11.3 mg/dL, that was still significantly lower ($p<0.05$) than that elicited by 3.3 pmol/g live weight of Asl-rcHH-amide. The time course was comparable to that of 3.3 pmol/g live weight Asl-rcHH-amide, with the maximum peak at 2 h and a hyperglycemic effect still detectable at 4 h.

3.5. Heterologous bioassays

To test the biological activity of recombinant Asl-cHH on species different from Astacidea, we performed a set of bioassays on the crayfish *P. clarkii* (Cambaridae) and the shrimp *P. elegans* (Palaemonidae). When injected with one Prc SGE, *P. clarkii* showed a glucose concentration of 80.6±46.5 mg/dL after 2 h that slowly decreased to 51.7±26.2 mg/dL at 4 h (Fig. 5). An amount of 3.3 pmol/g live weight of Asl-rcHH-amide induced a weaker response if compared to *A. leptodactylus* with a glucose peak of 18.5±7.3 mg/dL observed at 1 h. Four hours after the injection, a hyperglycemic effect was still present. On the contrary the injection of 16.7 pmol/g live weight of Asl-rcHH-Gly induced an extremely weak response with a maximum of 8.4±3.4 mg/dL at 30 min, a lower value compared to that elicited by the amidated peptide. Asl-rcHH-amide induced a hyperglycemic effect also in *P. elegans* (Fig. 6). In this species the injection of one Pae SGE induced a glucose peak of 22.8±3.55 mg/dL after 2 h, with the hemolymph glucose returning to basal levels at 4 h. The injection of 3.3 pmol/g live weight of Asl-rcHH-amide induced an increase of glucose of 16.4±5.8 mg/dL after 1 h, while after the injection

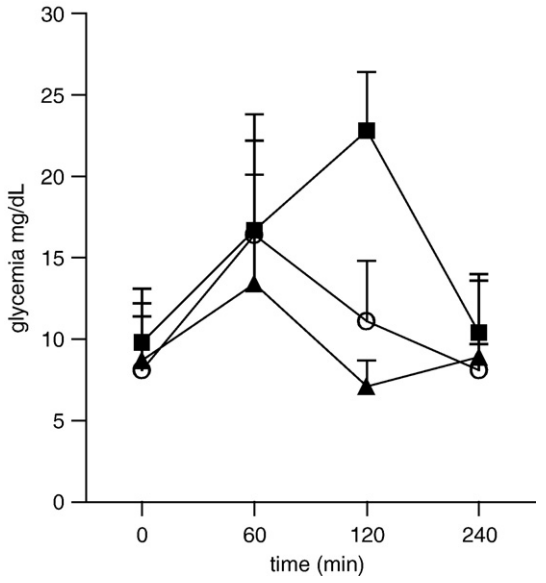

Fig. 6. Time course of hemolymph glucose in eyestalk-ablated *P. elegans*. Comparison of hyperglycemic activity of 1 Pae SGE ■ ($n=10$); 3.3 pmol/g live weight Asl-rcHH-amide ○ ($n=8$); 16.7 pmol/g live weight Asl-rcHH-Gly ▲ ($n=7$). Values are expressed as mean±standard deviation.

of 16.7 pmol/g live weight of Asl-rcHH-Gly a glucose peak of 13.4± 10.4 mg/dL was present at the same time.

4. Discussion

C-terminus amidation is a widespread post-translational process required to give biological activity to many bioactive peptides. All crustacean cHHs so far characterized by means of biochemical techniques [2,3] present an amidated C-terminal valine residue. The essential role of this modification for the biological activity of crustacean neuropeptides was assessed for pigment dispersing hormone, red pigment concentrating hormone and VIH [19,27,28]. The importance of amidation for a fully biologically active cHH has been proved so far only in the penaeid *M. japonicus* [13]. Our study confirmed this result in the crayfish *A. leptodactylus* and, by the way of heterologous bioassays, extended the finding that the C-terminus amide moiety is essential for the hormone functionality in two other decapod species, *P. clarkii* and *P. elegans*, the first belonging to the same superfamily as *A. leptodactylus* (Astacoidea), the second belonging even to a different infraorder (Caridea).

cHHs are reported to have 72 amino acid residues [2,3] and our expressed Asl-rcHH-amide consisted of an amino acid sequence made up of 72 residues identical to the endogenous hormone sequence and a short tag of 12 residues fused at the N-terminus due to the Strep-tag, with a theoretical molecular mass of 9746.03 Da, which is in good agreement with the value of 9745.93 Da resulting from MALDI-TOF analysis. Conformational analysis showed that Asl-rcHH-Gly and Asl-rcHH-amide have a high degree of helical arrangement, the latter in a higher proportion. These results agree with previously reported data for the cHH of *Procambarus bouvieri* and *M. japonicus* and MIH of *M. japonicus* [13,29,30] and strongly support the hypothesis of a correct conformation of our recombinant peptides. The short tag of our recombinant peptides did not affect their biological activity. The same was previously reported for the recombinant cHHs from *M. ensis*, where a long tag of 27 residues fused at the N-terminus did not influence their hyperglycemic activity, as well as for the rPej-SGP-I-amide, a recombinant cHH extended at the N-terminus by a short tag of four residues that conserved its biological activity [13,31]. In *A. leptodactylus* the Asl-rcHH-amide showed a hyperglycemic activity comparable to that of the native peptide. An amount of 1.7 pmol/g live weight was able to raise the glucose hemolymph concentration rapidly, with the maximum peak detected 1 h post-injection. After 4 h no hyperglycemia was observable and the glucose returned to basal level. Doubling the amount of Asl-rcHH-amide injected resulted in a stronger response with a glucose concentration nearly two times greater, confirming a dose dependent effect in inducing hyperglycemia. The time course of the response curve was different if the hyperglycemia was induced by 1.7 pmol/g live weight or 3.3 pmol/g live weight of Asl-rcHH-amide or by the native peptides. The recombinant peptide induced a maximum increase after 1 h, while the sinus gland extract induced a slower response, with a shift of the hyperglycemic peak observed at 2 h. When injected with 1.7 pmol/g live weight, the hyperglycemic effect of the peptide dropped within 2 h and at 4 h no hyperglycemia was detected. This behaviour is in good agreement with the half-life of 10 min reported for the cHH of *C. maenas* [6]. The higher dose of Asl-rcHH-amide injected not only elicited a greater effect, but also resulted in a delayed return to glucose basal level, which is due to an insufficient clearing of the peptide from the hemolymph as a result of the high concentration of the circulating hormone. A similar trend was displayed by the Asl SG extract that retained a strong hyperglycemic effect still at 4 h. The extended biological activity of the native peptides may be also due to the presence of different stereoisomers in the SG extract. Indeed, in *A. leptodactylus* and *Orconectes limosus* two isoforms were found, differing in the epimerization of Phe^3. The maximal hyperglycemic responses were similar, but differed in the time course, the D-cHHs showing an extended hyperglycemic activity [32,33].

The Asl-rcHH-Gly did not show a comparable potency. At a dose of 1.7 pmol/g live weight, the glucose concentration after 1 h was significantly lower than that observed after stimulation by the same dose of the amidated peptide. The injection of 16.7 pmol/g live weight of non-amidated hormone induced an increase of hyperglycemia that was lower than that induced by a five fold lesser amount of amidated Asl-rcHH. Our results clearly show that amidation is essential in providing full biological functionality to cHH, though the non-amidated peptide retained a lower, but still significant, potency. In fact, to observe a hyperglycemic effect with a non-amidated recombinant peptide, it is necessary to inject it at high dose [13,22,31]. These data agree with the study carried out in the penaeid *M. japonicus*, where the role of amidation was proved to influence molecular conformation and confer hyperglycemic activity [13]. The ability of non-amidated peptides to induce hyperglycemia was confirmed for Mee rcHH-A and Mee rcHH-B [31], which have a VGK C-terminus, and for rPej-SGP-I-Gly [13], whose effect was similar to rPej-SGP-I-OH and by one order of magnitude lower than that of rPej-SGP-I-amide. The lower affinity for the receptor that C-terminus unprocessed peptides display is explained by a change in the molecular structure, while the presence of the amide moiety may render a peptide more hydrophobic and thus enhancing its binding to the receptor [12].

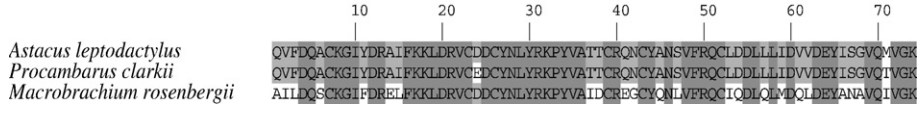

Fig. 7. Comparison of cHH amino acid deduced sequences (the relative GenBank GI numbers are indicated) from *A. leptodactylus* (GI 42766536), *P. clarkii* (GI 18766959) and *M. rosenbergii* (GI 17646172). *A. leptodactylus* and *P. clarkii* sequences have a homology of 97.3%, while the homology between *A. leptodactylus* and *M. rosenbergii* is much lower, 68.92%. Homologies were calculated using Jalview [35].

As far as heterologous bioassays are concerned, it should be considered that *A. leptodactylus* and *P. clarkii* belong to two sister families and their cHH sequences are highly homologous (Fig. 7). In the amino acid sequence of Asl-rcHH an Asp residue is present at position 24, while in *P. clarkii* a Glu residue is present. Both amino acids are acidic, the only difference being in the length of the lateral chain and therefore not so relevant. The other difference concerns the C tail, where Met^{71} of *A. leptodactylus* is substituted by a Thr residue. The Asl-rcHH-amide was able to induce a valuable hyperglycemic response in *P. clarkii* although lower than in *A. leptodactylus*. The time course was similar, with a peak present at 1 h. Instead, the injection of Asl-rcHH-Gly resulted in a very weak hyperglycemic activity, even if the amount injected was 5 times greater. The injection of one Prc SGE elicited a strong raise in the glucose level and, as well as in *A. leptodactylus*, the glucose peak was observed at 2 h followed by a slow decrease in the glucose hemolymph concentration that at 4 h was still high. To test the ability of the Asl-rcHH to induce a hyperglycemic effect on a not closely related species the heterologous bioassay with the saltwater shrimp *P. elegans* (Palaemonoidea) was performed. The amino acidic sequence of Pae-cHH is unknown, but we can speculate that it should present a good degree of homology with the known sequence of another Palaemonoidea, *M. rosenbergii* [17] and therefore should have a minor homology with the Asl-cHH (Fig. 7) [34], so that a lower activity was expected. Cross injection experiments performed using SG extracts showed that the SG extract from *A. leptodactylus* was not able to induce any response in *Palaemon serratus* [18]. On the contrary, Asl-rcHH-amide induced a detectable response with a peak after 1 h that doubled the basal level, a positive effect lower than that elicited in *A. leptodactylus*. The activity of the Asl-rcHH-Gly was even lower compared to the amidated peptide. The positive hyperglycemic effect induced by the recombinant hormone compared to the negative data reported for the Asl SG extract that did not induce hyperglycemia in *P. serratus* [18] can be easily explained by the large amount of Asl-rcHH-amide injected. This demonstrates that even in phylogenetically distant species the biological activity is maintained to a certain degree, probably due to an overall structural similarity of the neuropeptide.

This study demonstrated the relevance that amidation has in conferring biological activity to crayfish cHH. The use, for the first time, of in vivo heterologous bioassays for amidated recombinant cHH proved to be a powerful method to study the importance that the differences in the amino acid sequence can have on the functionality of a hormone.

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