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Can peptides be orally-delivered in crustaceans? The case study of the Crustacean Hyperglycaemic Hormone in *Procambarus clarkii*

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ABSTRACT

The oral administration of substances in crustaceans is a growing field of research, and is considered the most effective way to convey elements in their body instead injections or other invasive techniques. It presents huge potentialities, in fact the production of emulsions that preserves the substance to be conveyed until it reaches the target organ finds application in the development of new vaccines, in the convey of substances for aquaculture purposes and in the administration of stressors to contrast the spreading of invasive crustacean species. The red swamp crayfish (*Procambarus clarkii*, Girard 1852) is one of the most spread invasive species worldwide, but it is also considered among the most economically important freshwater crustacean species. For these reasons it is considered as a paradigmatic species.

The chemical synthesis of the D-Crustacean Hyperglycemic Hormone isoform (D-Phe-CHH) from *P. clarkii* is here presented, with its species-specificity, verified through the injection of D-Phe-CHH in both the red swamp crayfish and the threatened European species *Austropotamobius italicus* (Faxon, 1984). The D-Phe-CHH has been successfully oral-delivered in *P. clarkii* by using a water/oil/water microemulsion entrapped in a jellified matrix. After the administration of CHH, the glycaemia levels significantly increased in eyestalk-less animals at hour 6, and in intact crayfish at hour 5, from the ingestion.

These encouraging findings pave the way for the use of the double emulsion method to oral administer different peptides to both handle physiological aspects of *P. clarkii* life cycle for aquaculture purposes and to create species-specific peptides-based mix to hamper the spreading of invasive crayfish populations.

Statement of relevance: Through the injection of the *de novo* synthetized D-Phe-Crustacean Hyperglycaemic Hormone isoform in the haemolymphatic circulation of *P. clarkii*, we evaluated its action in triggering hyperglycaemia.

The peptide has been then successfully orally delivered in *P. clarkii* by w/o/w microemulsion and its bioactivity has been demonstrated. The formulated emulsion to convey peptides in crustaceans represents a very promising technique to modulate growth and reproduction in aquaculture plans.

1. Introduction

The oral administration is a way of dispensing substances, widely used in pharmacology and toxicology, to introduce drugs that have systemic effects and reach different organs and tissues via the bloodstream. In the last two decades the biotechnology industry has produced several therapeutic peptides and proteins, on commercial scale (Truong-Le et al., 2015), and the delivery systems include the use of polymeric biodegradable microspheres and nanoparticles designated to reduce the number of administrations and the amount of protein required to achieve the wanted biological effect.

In crustaceans, few studies reported the oral immuno-stimulations of DNA vaccines as technique for containing the White Spot Syndrome Virus (WSSV) infections in shrimps (Citarasu et al., 2006; Lightner, 2003; Ning et al., 2009; Witteveldt et al., 2004). Among the most used methods to orally-convey enveloped dsDNA virus, there is the direct contact of shrimps to attenuated bacteria (Ning et al., 2009), or the administration of virus vector tailored expressing the VP28 gene (Musthaq and Kwang, 2011). Chitosan nanoparticles have been also used to oral deliver VP28 gene (Rajeshkumar et al., 2009). Only a study reported the silencing of a peptide hormone (the Gonad Inhibiting Hormone)

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by feeding *Penaeus monodon* with GIH dsRNA-enriched *Artemia* sp. (Treerattrakool et al., 2013), but no studies reported so far the oral delivery of peptides and proteins. In fact, in crustacean aquaculture, the most widely diffused technique to induce vitellogenesis and ovarian maturation is the traditional eyestalk ablation or X-organ Sinus Gland removal (Jo et al., 1999; Millamena and Quinitio, 2000). Such method is ethically debatable and the application of new alternatives are being under evaluation and trials (Das et al., 2015; Girish et al., 2015). The oral administration of suitable species-specific vitellogenesis-inducer could replace this controversial technique. As far the control or the containment of alien crayfish species is concerned, the trapping is the most used technique, which is costly and sometimes ineffective to manage the invasion and the spread of such species. In this context and upon experimental trials, a mix of peptides belonging to the Crustacean Hyperglycaemic Hormone (CHH) superfamily could be administered in order to affect molt (Molt Inhibiting Hormone), reproduction (Gonad Inhibiting Hormone), osmotic regulation, and aggressiveness (CHH) in a species-specific manner.

The oral administration of peptides thus could be an option for aquaculture purposes, and for new eradication methods to the long-term control of invasive crayfish species.

In crustacean crucial aspects of the life cycle are under the control of peptide hormones such as the Crustacean Hyperglycaemic Hormone superfamily. It is, in fact, already known that the CHH controls many fundamental physiological functions such as glucose mobilization from glycogen depots during stress responses, moulting, reproduction and osmoregulation (Brown and Cunningham, 1939; Chung and Webster, 2003; De Kleijn, 1994; Huberman and Aguilar, 1989; Katayama et al., 2013; Lorenzon et al., 2005; Scharrer, 1952; Turner et al., 2013), and aggression (Aquiloni et al., 2012) and anxiety (Fossat et al., 2014), as well.

With the aim of testing the possibility of conveying peptide hormone in decapods, we focused our attention on the CHH for its pleiotropic effects, among which, the rising glycaemia is the most easily detectable. In addition, the recombinant *Litopenaeus vannamei* CHH has been proved to increase immunological responses against pathogenic bacteria, and to increase the survivor of white shrimp (Wanlem et al., 2011). The CHH has been reported involved in triggering oocyte maturation and to be able of triggering the onset of vitellogenesis, in fact high levels of such peptide have been reported in haemolymph of *Homarus americanus* females entering gonadal maturation (De Kleijn et al., 1998).

Two different chiral isomers have been reported so far (Bulau et al., 2003; Ollivaux et al., 2002), but the reason of their existence is still poorly understood. Two recent studies led on *Pontastacus leptodactylus* (Eschscholtz, 1823) analyse the effects, both at glycaemic and transcriptomic levels, of the injection of both isoforms, namely L- and D-Phe-CHH (Manfrin et al., 2013; Mosco et al., 2012), revealing different outcomes played by the two isomers. Up to now, it seems that the D-enantiomer plays a more detectable effect, at the hepatopancreatic transcriptomic level, in inducing peptidases transcription and suppressing glycolysis-related transcripts with a consequent increase of glycaemia levels. Starting from these previous works, we directly synthesized the D-Phe-CHH isoform and we assess the feasibility of its synthesis, its species-specificity activity and its diffusion throughout oral baits.

These findings pave the way for the planning of new neurohormone-based baits to hinder invasive crayfish populations without affecting the safeguard of protected crayfish species, and the conservation of other species. Furthermore, such method will possibly help in creating new strategies of stimulation of crustacean maturity for aquaculture intentions, thus definitively abandoning the eyestalk ablation procedure, as example.

Table 1
Sequences of the two fragments CHH 1–38 and CHH 39–72. D-Phe3 residue is presented in bold.

	Peptide sequence
CHH 1–38	QVFDQACKGIYDRAIFKKLDRVCEDCYNLYRKPYVATT
CHH 39–72	CRQNCYANSVFRQCLDDLLLIDVVDEYISGVQTV-NH2

2. Methods

2.1. Chemical synthesis of the CHH peptide

The sequence of the translated protein of the CHH was achieved from one of the CHHs present within the eyestalk transcriptome of *P. clarkii* (Manfrin et al., 2015), in particular the one corresponding to the transcript Prcla_ES_334596_1_21 in the Transcriptome Shotgun Assembly deposited at DDBJ/EMBL/GenBank under the accession GARH01000000.

The synthesis of D-Phe-CHH 1–72 was predicted to be very difficult, due to the length of the peptide (72 AA), the presence of 6 cysteines, 9 aspartic acid residues and a very hydrophobic region at C-terminus. For this reason we decided to split the synthesis in two fragments CHH 1–38 and CHH 39–72, (Table 1) and use the native chemical ligation at Cys39 to obtain the full-length sequence according to Mosco et al. (2012).

Briefly the two fragments were synthesized at 0.1 mmol scale by solid phase method using Fmoc/tBu chemistry using double coupling reaction with PyBOP as coupling reagent and in order to minimize the Cys racemization, Fmoc-Cys(Trt)-OPfp ester was used instead of Fmoc-Cys(Trt)-OH.

The cleavage and side chain de-protection of CHH 39–72 peptide amide resin was performed for 4 h with 20 ml/g resin of TIPS/H2O/DODT/phenol/TFA (3/3/8/5/81% v/v). The crude CHH 39–72 was subjected to *S*-sulfonation for 3 h and then purified by semi-preparative RP-HPLC (Jupiter C4, 10 × 250 mm column, Phenomenex) using 1%/min gradient slope from 25 mM triethylammoniumacetate (TEAA) in water (mobile phase A) to 25 mM TEAA in 80% acetonitrile (MeCN) (mobile phase B). The pure fractions (according to ESI-MS analysis on amaZonSL ion trap mass spectrometer, Bruker Daltonics) were pooled and freeze-dried.

The cleavage of the protected CHH 1–38 peptide was performed with 33% HFIP in DCM. The thioester formation of CHH 1–38 peptide was performed according to procedure of von Eggelkraut-Gottanka (von Eggelkraut-Gottanka et al., 2003). The crude thioester peptide was fully deprotected for 3 h with H2O/TIPS/Phenol/TFA (2.5/2.5/5/90% v/v, 20 ml/g resin), precipitated/washed with diethyl ether and purified by semi-preparative RP HPLC (Jupiter C4, 10 × 250 mm, Phenomenex) with 1%/min gradient slope from 0.1% TFA in water (mobile phase A) to 0.1% TFA in acetonitrile (mobile phase B). The pure fractions (according to ESI-MS) were pooled and freeze-dried.

2.2. Native chemical ligation

The native chemical ligation reaction of the thioester peptide CHH 1–38 with *S*-sulfonated CHH 39–72 peptide was performed dissolving the two peptide fragments at concentration of about 5–10 mg/ml in ligation buffer [0.5 M HEPES, 6 M Gu.HCl, 50 mM TCEP, 20 mM EDTA, 50 mM mercaptophenylacetic acid (MPAA), 1% Zwittergent 3–14] at pH 7.0 and room temperature (Mosco et al., 2012). The reaction was monitored by analytical LC-MS on a Phenomenex Gemini C18, 4.6 × 150 mm column linked to an amaZonSL ion trap mass spectrometer, Bruker Daltonics, using a 2%/min gradient from 0.1%TFA in water to 0.1% TFA in acetonitrile at 0.5 ml/min.

2.3. Purification and oxidative folding

The linear reduced D-Phe-CHH 1–72 peptide was purified from the native chemical ligation (NCL) mixture by RP-HPLC using the classical acidic (0.1% TFA) mobile phase system on a semi-preparative column