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Identification of the Crustacean Hyperglycemic Hormone (CHH) and CHH-like Peptides in the Crayfish *Procambarus clarkii* and Localization of Functionally Important Regions of the CHH

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Hsin-Ju Wu, Wei-Shiun Tsai, Shao-Yen Huang, Yan-Jhou Chen, Ying-Hsin Chen, Yu-Ru Hsieh, and Chi-Ying Lee (2012) Identification of the crustacean hyperglycemic hormone (CHH) and CHH-like peptides in the crayfish *Procambarus clarkii* and localization of functionally important regions of the CHH. *Zoological Studies* **51**(3): 288-297. Four anti-peptide antibodies were raised each against a synthetic peptide corresponding in sequence to a short stretch of the crustacean hyperglycemic hormone (CHH) or CHH-like (CHH-L) peptides of the crayfish *Procambarus clarkii*. CHH and CHH-L are alternatively spliced products that share an identical sequence for the 1st 40 residues from the amino-terminus of the peptides. When used in Western blot analyses of tissue proteins, anti-CHH (1-10) recognized an immunoreactive protein band in both sinus gland (SGs) and thoracic ganglia (TGs), whereas anti-D-CHH (1-10) recognized an immunoreactive protein band only in SGs, but not in TGs; anti-CHH (59-72) recognized an immunoreactive protein band in SGs but not in TGs, and conversely, anti-CHH-L (58-72) recognized an immunoreactive protein band in TGs but not in SGs. Tissue homogenates were fractionated using high-performance liquid chromatography (HPLC). The immunoreactivity of the collected HPLC fractions was determined by an enzyme-linked immunosorbent assay and Western blotting, and the immunoreactive fractions were subjected to mass determination. A pair of stereoisomers, CHH and D-Phe^3 CHH, both with a mass of 8386.4 and respectively immunoreactive to anti-CHH (1-10) and anti-D-CHH (1-10), was identified in SGs; Western blot analyses showed that they were immunoreactive to anti-CHH (59-72), but not to anti-CHH-L (58-72). A CHH-L, with a mass of 8343.6 and immunoreactive to anti-CHH (1-10) but not to anti-D-CHH (1-10), was identified in TGs; Western blot analyses showed that it was immunoreactive to anti-CHH-L (58-72), but not to anti-CHH (59-72), and sequencing analysis of the peptide fragments generated by enzyme digestion of the immunoreactive protein revealed 3 sequences, which are contained within a CHH-L encoded by a previously identified transcript. Furthermore, anti-peptide antibodies were tested for the effects of blocking CHH-induced hyperglycemia. Results showed that anti-CHH (59-72) and anti-CHH (1-10) individually abolished CHH-induced hyperglycemia, whereas neither control treatments, pre-immune sera, nor anti-CHH-L (58-72) significantly affected CHH-induced hyperglycemia. In summary, these data reiterate the observations that CHH and CHH-L are preferentially expressed in different tissues; they also suggest that enzymes involved in L-to-D isomerization of CHH are expressed in tissue-specific manners. Finally, the data suggest the N- and C-terminal regions of CHH are important for its biological activity.
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Key words: Neuroendocrine, Neuropeptide, Anti-peptide antibody, Crustacean.

Crustacean hyperglycemic hormone (CHH) belongs to the CHH peptide family (Keller 1992, Chen et al. 2005, Montagné et al. 2010). CHH was originally identified in a crustacean neuroendocrine system, the X-organ/sinus gland complex in the eyestalk (for reviews see Keller 1992, Soyez 1997).

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It is considered to be a stress hormone and was implicated in eliciting stress-induced hyperglycemia through its effects on regulating carbohydrate metabolism (Santos and Keller 1993, Webster 1996, Chang et al. 1998 1999, Zou et al. 2003, Lorenzon et al. 2004). Additional data suggested that several other physiological processes may also be regulated by CHH (Chang et al. 1990, Yasuda et al. 1994, Spanings-Pierrot et al. 2000, Serrano et al. 2003).

The existence of multiple molecular forms of CHH family peptides is a well-documented phenomenon (for reviews see Soyez 1997, Chan et al. 2003, Chen et al. 2005). CHHs that differ from each other in the stereo configuration of the 3rd residue (a phenylalanine) were purified from sinus glands (SGs) of several astacideans (Soyez et al. 1994 1998, Yasuda et al. 1994, Aguilar et al. 1995, Bulau et al. 2003). Available evidence indicates that D-Phe^3 CHH is post-translationally derived from all L-residues of CHH (Ollivaux and Soyez 2000, Soyez et al. 2000).

Furthermore, a CHH structural variant, CHH-like (CHH-L) peptide, was purified from pericardial organs of 3 brachyurans (Dircksen et al. 2001, Chung and Zmora 2008, Tsai et al. 2008). CHH and CHH-L are products of alternatively spliced transcripts (Dircksen et al. 2001, Chen et al. 2004, Chung and Zmora 2008, Tsai et al. 2008) and functionally differ in that CHH-L neither exhibits hyperglycemic activity nor suppresses secretion of molting hormones by the molting gland (Dircksen et al. 2001, Ohira et al. 2006, Chang et al. 2010). Although transcripts encoding CHH-L were found in several other extra-eyestalk tissues, including the thoracic ganglia (TGs), brain, and gut (Chen et al. 2004, Lee et al. 2007, Tsai et al. 2008, Zheng et al. 2010) and CHH-immunoreactivity was present in subesophageal ganglia and TGs (Chang et al. 1999), native peptides have not been identified in any of those tissues. In addition, whether CHH-L peptides synthesized in extra-eyestalk tissues, similar to the sinus-gland CHH, also contain D-amino acid residues has not been investigated.

Structural analyses of CHH and CHH-L peptides of the mud crab (*Scylla olivacea*) revealed that both peptides are α-helix-rich; comparative modeling of the 2 peptides suggested that they are similarly folded, and that functional differences between the 2 peptides are probably due to critical residues located in their C-termini (Chang et al. 2010). The latter suggestion is compatible with data implying the importance of the C-terminus of CHH to its biological activity (Marco et al. 2000, Katayama et al. 2002, Chang et al. 2010).

In the crayfish *Procambarus clarkii* Girard 1852, a pair of CHH stereoisomers, which differ from each other only in the configuration of the 3rd residue, was purified from SGs (Yasuda et al. 1994). In addition, transcripts encoding CHH-L peptides were found in TGs (GenBank accession nos.: AF474408 and JF311403), but the encoded peptides have not yet been identified. In the present study, anti-peptide antibodies were raised each against a peptide with a sequence corresponding to a stretch of the crayfish CHH or CHH-L peptides. These antibodies were used to purify and identify the native peptides synthesized in tissues. Furthermore, they were utilized in an initial attempt to map out functionally important regions of CHH.

MATERIALS AND METHODS

Animals

Animals (*Procambarus clarkii*) were purchased from a local fisherman and reared as previously described (Zou et al. 2003).

Production of anti-peptide antibodies

Sequence data available from a public repository were used to design synthetic peptides. In *P. clarkii*, alternatively spliced transcripts (GenBank accession nos.: AB027291 and AF474408) of a CHH precursor gene were respectively found in eyestalk ganglia and TGs. Based on analyses of sequence diversity and predicted antigenicity (Hopp and Woods 1981) of the mature peptides, termed CHH and CHH-L in the present study (Fig. 1) and encoded by the respective transcripts, peptides with sequences corresponding to the C-terminal region of each structural variant (CHH: Ile-Asp-Val-Val-Asp-Glu-Tyr-Ile-Ser-Gly-Val-Gln-Thr-Val-NH_2; CHH-L: Leu-Asn-Leu-Asp-Leu-Tyr-Lys-Gln-Leu-Ser-Glu-Ile-Ile-Arg-Gly) were synthesized, N-terminally conjugated to keyhole limpet hemocyanin, and used to produce the respective variant-specific antibodies, anti-CHH (59-72) and anti-CHH-L (58-72). In addition, a peptide (pGlu-Val-Phe-Asp-Gln-Ala-Cys-Lys-Gly-Ile) was synthesized based on the sequence common to the 1st 10 residues of CHH and CHH-L (Fig. 1) and C-terminally conjugated to bovine serum albumin (BSA), to produce the anti-CHH (1-10) antibody. Another peptide of the same

sequence but with a D-Phe^3 residue (pGlu-Val-*D*-Phe-Asp-Gln-Ala-Cys-Lys-Gly-Ile) was synthesized and BSA-conjugated to produce the anti-D-CHH (1-10) antibody. Pre-immune and anti-peptide immune sera were produced in New Zealand white rabbits using a previously described protocol (Tsai et al. 2008).

Cross-reactivity of anti-CHH (1-10) and anti-D-CHH (1-10) against the D-Phe^3-CHH and CHH was respectively quantified by an indirect enzyme-linked immunosorbent assay (ELISA) (Lee et al. 2001) using the purified D-Phe^3-CHH and CHH (Fig. 3) as antigens. The cross-reactivity of anti-CHH (1-10) against the D-Phe^3-CHH, expressed as a percentage of the mean optical density (O.D.) obtained when anti-CHH (1-10) was tested against D-Phe^3-CHH (30 ng or 3.6 pmoles/well) over the O.D. obtained against an equal amount of CHH, was 2.56% ± 0.12% (*n* = 4), whereas the cross-reactivity of anti-D-CHH (1-10) against CHH was 3.82% ± 0.31% (*n* = 4).

Western blot analyses of tissue proteins

To probe for immunoreactive proteins, tissues (SGs and TGs) were separately homogenized, and tissue homogenates were separated by Tricine-sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Schagger and Von Jagow (1987), electroblotted onto polyvinylidene difluoride membranes, and probed using each anti-peptide antiserum (at a 1:2000 dilution). Procedures for Western blotting were as described by Tsai et al. (2008). As controls, Western blot analyses were performed with the primary antiserum pre-adsorbed by the respective synthetic peptide (10 or 100 μg/ml diluted antiserum) that was used for antiserum production. For additional controls, instead of using the primary antiserum, tissue proteins were also probed using the respective pre-immune serum, which detected no immunoreactive protein in the analyzed tissues (data not shown).

Purification and identification of the CHH and CHH-L peptides

Tissues (SGs and TGs) dissected from *P. clarkii* were separately homogenized (100 tissues per batch) in 0.1 N HCl containing 0.2 mM phenylmethylsulfonyl fluoride at 85°C, centrifuged (at 10,000 xg and 4°C for 20 min), and filtered. The homogenates were fractionated using an ÄKTA Purifier (Amersham Pharmacia Biotechnology, Uppsala, Sweden) high-performance liquid chromatographic (HPLC) system as previously described (Lee et al. 2001). For SGs, homogenates were fractionated on a C_{18} reversed-phase column (Nucleosil, 5 μm, 250.0 × 4.6 mm, MetaChem Technologies, Torrance, CA, USA) eluted with a gradient of acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Fractions were collected and analyzed by an ELISA (Lee et al. 2001) using the immunoglobulin G (IgG) fraction purified from anti-CHH (1-10) or anti-D-CHH (1-10) by established procedures (Zou et al. 2003). For TGs, homogenates were fractionated by a 2-step chromatographic fractionation protocol, first on an ion-exchange column followed by a 2nd C_{18} reversed-phase column, as previously described (Chang et al. 2010). Fractions collected from the 1st fractionation step were analyzed by an ELISA; the immunoreactive fractions were combined

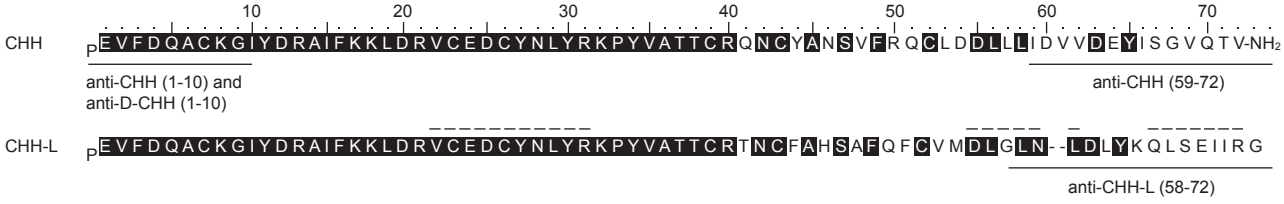

Fig. 1. Alignment of the sequences of crustacean hyperglycemic hormone (CHH) and CHH-like (CHH-L) peptides in the crayfish *Procambarus clarkii*. Two alternatively spliced transcripts of a CHH precursor gene were found in eyestalk ganglia and thoracic ganglia, respectively (accession nos.: AB027291 and AF474408). Only sequences of the encoded mature peptides, termed CHH and CHH-L in the present study, are presented. The N- and C-terminal ends of CHH are shown, according to Yasuda et al. (1994), to be pyroglutamated and amidated, which agree with the mass spectrometric data obtained in the present study (Table 1). The N-terminal end of CHH-L was shown to be pyroglutamated, according to previously characterized CHH-L peptides (Dircksen et al. 2001, Chang et al. 2010). The sequence of the synthetic peptides for raising antiserum is underlined, and the sequence of the fragments generated by enzyme digestion (see RESULTS) is indicated by a dashed overline. An additional CHH-L-type transcript (accession no.: JF311403) was found in thoracic ganglia encoding a variant of the CHH-like peptide, with substitution of R^{65} for Q^{65}.

and further fractionated on a C_{18} reversed-phase column, and the collected fractions were again analyzed by an ELISA. HPLC-purified immunoreactive proteins were quantified using a commercially available protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's procedures.

Masses of the immunoreactive proteins in the HPLC fractions were determined using matrix-assisted laser desorption/ionization quadrupole-time-of-flight (MALDI Q-TOF) mass spectrometry (MS) at the Core Facilities for Proteomics Research, Institute of Biological Chemistry, Academia Sinica (Taipei, Taiwan). In addition, peptide fragments of the immunoreactive proteins generated by enzyme digestion were sequenced using liquid chromatography – quadrupole-mass spectrometry (LC-Q-MS/MS, LCQ DECA XP Plus, ThermoFinnigan) at the Proteomics Research Core Laboratory, National Cheng Kung University (Tainan, Taiwan). Sample preparation and instrument settings were as previously described (Chang et al. 2010).

Immunoreactive proteins in the HPLC fractions were also probed by anti-CHH (59-72) or anti-CHH-L (58-72) using Western blot analyses (Tsai et al. 2008).

Hyperglycemia assay

Eyestalks of adult animals (with a mean body weight of 35.8 ± 2.7 g) were ablated and heat-cauterized, and then the animals were starved for 2 d. Animals were divided into different treatment groups (n = 6/group); each group received an injection of 0.01 M phosphate-buffered saline (PBS), or the IgG fraction (3 ng/animal) of anti-CHH (1-10) or anti-CHH (59-72), followed by a 2nd injection of the HPLC-purified CHH (20 pmoles/animals dissolved in PBS), 30 min after the 1st one. Animals of a separate group received PBS for both injections. As additional controls, groups of animals were pretreated with an injection of the IgG fraction of anti-CHH-L (58-72) or the preimmune serum bled before immunization (see "Production of anti-peptide antibodies"), followed by a 2nd injection of CHH as mentioned above. The injection volume was 50 μl for each injection. Immediately before the 2nd injection (taken as time 0 hr) and at designated time points thereafter, hemolymph was withdrawn from each animal. Preparation of hemolymph samples was previously described; hemolymph glucose levels were quantified using a glucose (GO) assay kit (GAGO20-1KT, Sigma, St. Louis, MO, USA) (Lee et al. 2000). Effects of treatments were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test (SPSS Manager, SPSS, Chicago, IL, USA). For each individual animal, differences in glucose concentrations between time 0 hr and subsequent sampling time points (1, 2, or 3 hr) were calculated; differences were averaged for each treatment group at a given time point and reported as increases in glucose concentration.

RESULTS

Western blot analyses of tissue proteins using anti-CHH (1-10) revealed an immunoreactive protein band that migrated slightly ahead of the 11-kDa standard in both SGs and TGs (Fig. 2A); the immunoreactive bands were completely abolished when the blots were probed with the pre-adsorbed antibody (Fig. 2A). To examine whether the immunoreactive proteins contained D-Phe[3] residues, tissue proteins were probed with anti-D-CHH (1-10); the immunoreactive band was absent in TGs but present in SGs, the immunoreactivity of which was abolished by preadsorption of the antibody (Fig. 2B).

An immunoreactive band of similar mobility was present in SGs but not in TGs, when tissue proteins were probed with anti-CHH (59-72) (Fig. 2C); conversely, it was present in TGs but not in SGs when tissue proteins were probed with anti-CHH-L (58-72) (Fig. 2D). The immunoreactive bands were abolished when the blots were probed with antibodies pre-adsorbed by the respective synthetic peptide (Fig. 2C, D).

Tissue homogenates were fractionated using HPLC. Collected fractions were screened for immunoreactivity by an ELISA using anti-CHH (1-10) or anti-D-CHH (1-10), and the immunoreactive fractions were subject to an MS analyses for mass determination. An ELISA analysis of SG-derived fractions showed that when using anti-CHH (1-10) as the primary antibody, the major immunoreactive proteins were eluted at 40.3% ACN, and, when using anti-D-CHH (1-10), the major immunoreactivity was present in the fraction eluted at 40.7% ACN (Fig. 3). Molecular masses of substances in the 2 immunoreactive fractions were determined to be 8386.4 (Table 1), which agrees with the theoretical value (8386.7), predicted for an N- and C-terminally blocked CHH with 3 highly conserved disulfide bridges (Table 1).

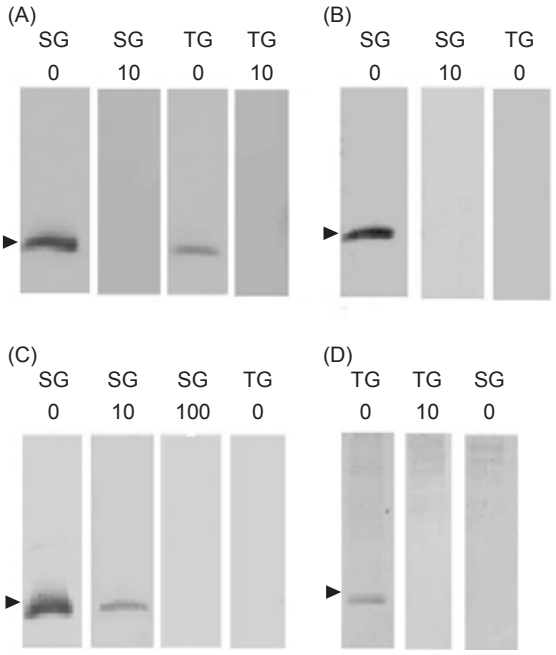

Fig. 2. Western blot analyses of tissue protein using anti-peptide antibodies. Sinus gland (SG) and thoracic ganglion (TG) homogenates (10 μg proteins/lane) were electrophoresed, blotted, and probed by anti-peptide antibodies. The primary antibodies were (A) anti-crustacean hyperglycemic hormone (CHH) (1-10), (B) anti-D-CHH (1-10), (C) anti-CHH (59-72), and (D) anti-CHH-L (58-72); they were used as un-adsorbed (0 μg/ml as specified above the lanes) or pre-adsorbed with different amounts of respective synthetic peptides (10 or 100 μg/ml as specified above the lanes) used for immunization. The position of the 11-kDa standard is indicated by an arrowhead.

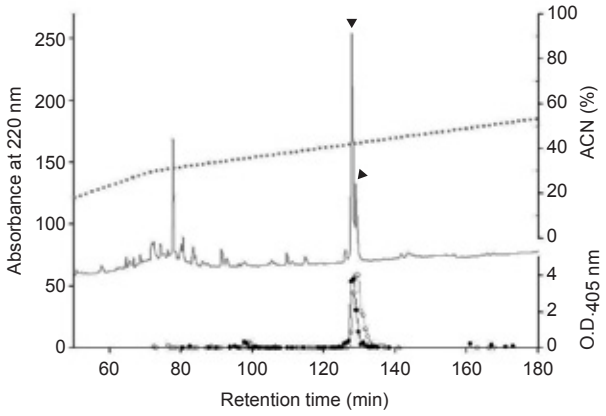

Fig. 3. Reversed-phase high-performance liquid chromatograph purification of the crustacean hyperglycemic hormone (CHH) and D-Phe^3 CHH. Homogenates of the sinus gland were fractionated using a reversed-phase C_{18} column eluted with a gradient of 0%-60% acetonitrile (ACN) containing 0.1% trifluoroacetic acid, and the eluate was monitored for ultraviolet (UV) absorbance at 220 nm (continuous trace). The dotted line represents the percentage of ACN. The immunoreactivity of the collected fractions was determined by an ELISA using (●) anti-CHH (1-10) or (○) anti-D-CHH (1-10) as the primary antibody. Arrowheads point to the UV peak of major immunoreactivity, eluted at 40.3% and 40.7% ACN.

Table 1. Matrix-assisted laser desorption/ionization quadrupole-time-of-flight mass spectrometric analyses of immunoreactive proteins purified from crayfish *Procambarus clarkii* tissues using high-performance liquid chromatography

Tissue[a]	Peptide identified	Mass (Da)		Antibodies to which each peptide positively reacted[b]
		Theoretical	Observed	
SG	CHH	8386.7	8386.4	anti-CHH (1-10)
				anti-CHH (59-72)
SG	D-Phe^3 CHH	8386.7	8386.4	anti-D-CHH (1-10)
				anti-CHH (59-72)
TG	CHH-L	8401.9[c]	8343.6	anti-CHH (1-10)
		8429.9[c]		anti-CHH-L (58-72)

[a]SG, sinus gland; TG, thoracic ganglia. [b]Based on results obtained by ELISA and Western blot analyses (Figs. 3-5). [c]Theoretical values predicted for 2 variants of crustacean hyperglycemic hormone (CHH)-like peptides, each with a presumptive N-terminal pyroglutamation and formation of 3 disulfide bonds; the accession numbers for the encoding transcripts are AF474408 and JF311403 (Fig. 1).

The ELISA analyses of the TG-derived HPLC fractions obtained using an ion-exchange column detected several immunoreactive fractions, when anti-CHH (1-10) was used as the primary antibody (data not shown); anti-D-CHH (1-10) detected no immunoreactive fraction (data not shown). The immunoreactive fractions were pooled and further fractionated using a reversed-phase C_{18} column. Analyses of the fractions showed that when using anti-CHH (1-10) as the primary antibody, major immunoreactivity was present at fractions eluted at 39.1% ACN (Fig. 4), while no immunoreactivity was detected in any fractions tested when anti-D-CHH (1-10) was used (Fig. 4). The molecular mass of the substance in the immunoreactive fraction was determined to be 8343.6, which did not agree with the theoretical value predicted for the 2 variants of CHH-L peptide (8401.9 and 8429.9, Table 1) encoded by transcripts found in TGs (Fig. 1). LC-Q-MS/MS analysis of trypsin-digested fragments of the immunoreactive protein revealed the sequence of 2 fragments (^{22}VCEDCYNLYR^{31} and ^{65}QLSEIIR^{71}) that are contained within the deduced CHH-L sequence encoded by the transcript AF474408 (Fig. 1); analysis of endoproteinase Asp-N-digested fragments revealed the sequence of 1 fragment (^{55}DLGLNL^{60}) that is contained within the deduced CHH-L sequence (Fig. 1).

The SG-derived immunoreactive proteins eluted at 40.3% and 40.7% ACN (Fig. 3) were probed with anti-CHH (59-72) or anti-CHH-L (58-72) in the Western blot analysis; anti-CHH (59-72) recognized both proteins that migrated ahead of the 11-KDa standard (Fig. 5, lanes 1 and 2), while anti-CHH-L (58-72) recognized neither protein (Fig. 5, lanes 3 and 4). These purified proteins are hereafter called CHH and D-Phe^3 CHH, respectively. Typical yields for HPLC purification of native peptides from tissues were approximately 17.4 pmol/SG for CHH and 5.8 pmol/SG for D-Phe^3 CHH. Western blot analysis of the TG-derived immunoreactive protein eluted at 39.1% ACN (Fig. 4) using anti-CHH-L (58-72) revealed an immunoreactive band that migrated ahead of the 11-KDa standard (Fig. 5, lane 5); no immunoreactive band was detected when anti-CHH (59-72) was used as the primary antibody (Fig. 5, lane 6). This purified protein is hereafter called CHH-L. The typical yield for HPLC purification of the native peptide from tissues was approximately 2.7 pmol/TG.

In an attempt to locate regions of CHH that are important to its activity, anti-peptide antibodies were also used to determine their effects on blocking CHH-induced hyperglycemia. Hemolymph glucose levels in animals pretreated with an injection of PBS significantly increased at

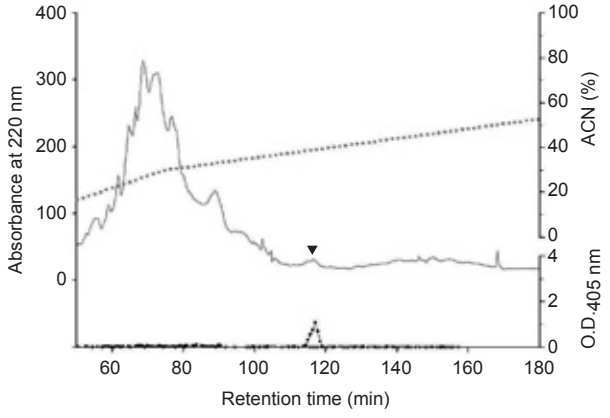

Fig. 4. Reversed-phase high-performance liquid chromatography purification of the crustacean hyperglycemic hormone-like (CHH-L). Fractions collected from an ion exchange chromatographic separation of thoracic ganglion homogenates, containing anti-CHH (1-10) immunoreactivity, were pooled and fractionated using a reversed-phase C_{18} column eluted with a gradient of 0%-60% acetonitrile (ACN) containing 0.1% trifluoroacetic acid, and the eluate was monitored for ultraviolet (UV) absorbance at 220 nm (continuous trace). The dotted line represents the percentage of ACN. The immunoreactivity of the collected fractions was determined by an ELISA using (●) anti-CHH (1-10) or (○) anti-D-CHH (1-10) as the primary antibody. The arrowhead points to the UV peak of major immunoreactivity eluted at 39.1% ACN.

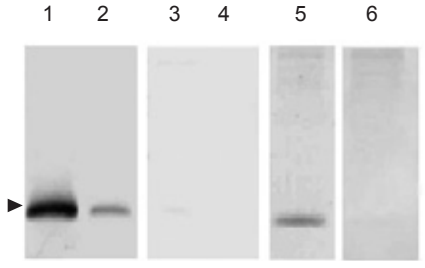

Fig. 5. Western blot analyses of high-performance liquid chromatographic (HPLC)-purified proteins. HPLC-fractionated proteins derived from sinus glands (SGs) or thoracic ganglia (TGs) of the crayfish *Procambarus clarkii* were electrophoresed, blotted, and probed with anti-CHH (59-72) or anti-CHH-L (58-92). Samples loaded were SG-derived fractions eluted at 40.3% acetonitrile (ACN) (lanes 1 and 3, 100 ng/lane) and 40.7% ACN (lanes 2 and 4, 20 ng/lane), or TG-derived fractions eluted at 39.1% ACN (lanes 5 and 6, 20 ng/lane); blots were probed by anti-CHH (59-72) (lanes 1, 2, and 6) or anti-CHH-L (58-92) (lanes 3-5). The position of the 11-kDa standard is indicated by an arrowhead.

1 and 2 hr after CHH injection (Fig. 6). Pretreating animals with either anti-CHH (59-72) or anti-CHH (1-10) completely abolished CHH-induced hyperglycemia (Fig. 6). As controls, animals were pretreated with anti-CHH-L (58-72) (Fig. 6) or the preimmune serum, obtained from rabbits subsequently used for raising anti-CHH (59-72), anti-CHH (1-10), and anti-CHH-L (58-72) (data not shown), and none had a significant effect on CHH-induced hyperglycemia.

DISCUSSION

In the present study, antibodies were produced, each of which was raised against a synthetic peptide corresponding to a stretch of CHH or CHH-L. These antibodies were used to characterize tissue proteins in Western blot analyses, and to screen for immunoreactive proteins during purification processes of the peptides. Combined data showed that SGs and TGs respectively contained CHH- and CHH-L-immunoreactive proteins.

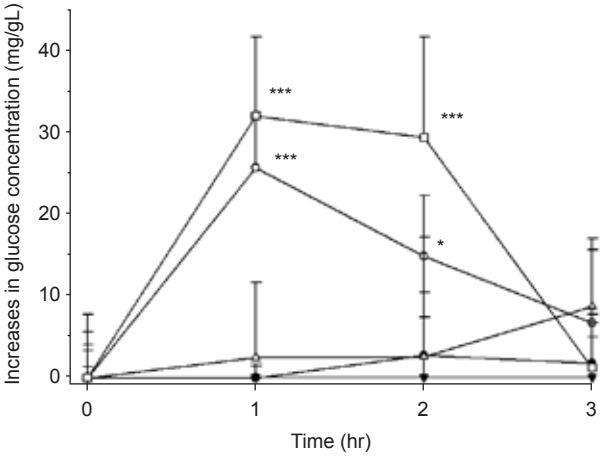

Fig. 6. Effects of anti-peptide antibodies on the crustacean hyperglycemic hormone (CHH)-induced hyperglycemia. Eyestalk-ablated animals received an injection of (○) phosphate-buffered saline (PBS), or the immunoglobulin G (IgG) fraction of the following antibodies: (Δ) anti-CHH (1-10), (▼) anti-CHH (59-72), or (□) anti-CHH-L (58-72), followed 30 min later by a 2nd injection of HPLC-purified CHH (20 pmoles/animals dissolved in PBS). Animals of a separate group (●) received PBS in both injections. Hemolymph was sampled from all animals immediately before and at designated time points after the 2nd injection, and glucose was quantified. For the sake of clarity, data are given as the mean with one-sided standard deviation (S.D.) bars (mean ± S.D., *n* = 6/group) and are reported as increases in the glucose concentration from respective values at a time of 0 hr (see "MATERIALS AND METHODS"). * and *** respectively represent values statistically different from time 0 h at the 5% and 0.1% levels.

Specifically, a CHH-L-immunoreactive protein was identified in TGs. The mass determined for the immunoreactive protein did not closely agree with those of 2 variants of CHH-like peptides encoded by transcripts found in TGs (GenBank accession nos.: AF474408 and JF311403). One of the explanations for the discrepancy in molecular mass is that the CHH-L immunoreactive protein is a degraded CHH-L with the terminal glycine removed. Supporting data includes the theoretical mass value for such a truncated CHH-L peptide encoded by the transcript (AF474408) would be 8344.7, which agrees with the observed value; Table 1). In addition, sequence of three fragments generated by trypsin or endoproteinase Asp-N digestion of the CHH-L immunoreactive protein was determined by LC-Q-MS/MS to be contained with the CHH-L peptide encoded by the transcript AF474408. Thus, although all of the CHH-L peptides previously purified from other species were intact without any C-terminal truncation (Dircksen et al. 2001, Chung and Zmora 2008, Chang et al. 2010), it is suggested that the CHH-L immunoreactive peptide identified in the present study is a truncated peptide with the C-terminal glycine being removed.

CHH, CHH-L, or both transcripts were found in many non-eyestalk tissues of adult animals (Chen et al. 2004, Lee et al. 2007, Tsai et al. 2008, Li et al. 2010, Zheng et al. 2010). Thus far, CHH was purified and identified only in the gut of *C. maenas* (Chung et al. 1999), and CHH-L in the pericardial organs of *C. maenas* and *Scylla olivacea* (Dircksen et al. 2001, Chang et al. 2010). Transcripts encoding CHH-L peptides were reported (Chen et al. 2004, Tsai et al. 2008, Zheng et al. 2010) and CHH-immunoreactivity was detected (Chang et al. 1999) in TGs from several species; the CHH-L immunoreactive protein reported in the present study represents the 1st CHH-related peptide identified in this tissue.

The present study also identified CHH and D-Phe^3 CHH in SGs. A pair of CHH stereoisomers was reported from *Procambarus clarkii* (Yasuda et al. 1994), and other astacideans species (Soyez et al. 1994 1998, Aguilar et al. 1995, Bulau et al. 2003). D-Phe^3 CHH appeared to be more potent than its all-L counterpart in inhibiting ecdysteroid release from Y-organs (Yasuda et al. 1994) and regulating hemolymph osmolality (Serrano et al. 2003). It was proposed that D-Phe^3 CHH is derived from the all-L CHH by the action of a

putative peptide isomerase in a late step during the maturation process of the prohormone (Ollivaux and Soyez 2000, Soyez et al. 2000). On the other hand, D-Phe^3-containing CHH-L was not detected in TGs, despite the fact that the sequences of the 1st 40 residues from the N-terminus of CHH and CHH-L are identical. Considering the substrate specificity exhibited by known isomerases (Jilek and Kreil 2008, Bai et al. 2009), it was suggested that the putative isomerase activity involved in the post-translational L-to-D conversion of CHH (Ollivaux and Soyez 2000, Soyez et al. 2000) is absent from TGs.

The effects of anti-peptide antibodies in blocking CHH-induced hyperglycemia were also tested. Specifically, 2 antibodies, anti-CHH (1-10) and anti-CHH (59-72), completely abolished CHH-induced hyperglycemia. The effect of anti-CHH (59-72) on CHH-induced hyperglycemia is generally compatible with data reported for the spiny lobster *Jasus lalandii* in which a truncated CHH (1-65), lacking the last 7 C-terminal residues, did not have hyperglycemic activity when tested *in vivo* (Marco et al. 2000) and with those showing that the C-terminal amide moiety is essential for hyperglycemic activity (Katayama et al. 2002, Mosco et al. 2008, Chang et al. 2010).

Stretches of the sequence against which anti-CHH (1-10) and anti-CHH (59-72) were raised approximately cover motifs A1 and A5 of group I peptides of the CHH family, respectively (Lacombe et al. 1999). Motifs A1 and A5, located respectively at the N- and C-terminal ends, are characteristic of group-I (CHH) peptides, but are absent from most group-II (VIH/MIH) peptides, which also contain the group-specific motifs, A1' and A5' (Lacombe et al. 1999). Thus, the combined data suggest that N- and C-terminal regions of CHH are important for its hyperglycemic activity, and more broadly support the notion that these regions contain functionally critical sites for CHH family peptides (Katayama et al. 2003). However, it should be noted that the observed effects of anti-CHH (1-10) and anti-CHH (59-72) in blocking CHH-induced hyperglycemia could be due to a global steric hindrance exerted by IgG on the CHH molecule, and not necessarily to specific blockage of residues against which the antibodies were raised. The suggestion made above should be tested by additional study, for example, by employing CHH mutants generated by site-directed mutagenesis. Functionally important residues in the N- and C-terminal regions of insect ion transport peptides, also members of the CHH family, were experimentally demonstrated using mutated peptides (Wang et al. 2000, Zhao et al. 2005).

In summary, anti-peptides antibodies were produced and used to identify CHH and CHH-L peptides in tissues. Synthesis of CHH and CHH-L peptides and the post-translational event of L-to-D isomerization appeared to be tissue-specific. In addition, we provide experimental data supporting the notion that the N- and C-terminal regions of CHH are important to its function. With an increasing number of tissue sites of biosynthesis demonstrated, more efforts are needed to correlate hormone titers in blood and tissues with the physiological status of animals and devise corresponding bioassays to gain a fuller understanding of the functional roles of CHH/CHH-L peptides. Further, it is expected that identification of CHH and CHH-L receptors would eventually reveal structure/functional relationships of various structural CHH variants in greater depth.

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