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Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*

(bag cells/amino acid sequence/neurosecretory peptide/molluskan neuropeptide/microsequence analysis)

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ABSTRACT **Egg-laying hormone (ELH), a neuropeptide synthesized by the bag cell neurons, induces egg laying and its correlated behavior in *Aplysia californica*. In the present study, ELH has been purified to homogeneity and its primary structure has been determined. We find this molecule to have 36 amino acid residues with a M_r of 4385 and a calculated isoelectric point of 9.7. Direct microsequence analysis revealed a single amino acid sequence that is in agreement with the amino acid composition determined after acid hydrolysis of ELH: H-Ile-Ser-Ile-Asn-Gln-Asp-Leu-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Arg-Glu-Arg-Gln-Arg-Tyr-Leu-Ala-Asp-Leu-Arg-Gln-Arg-Leu-Leu-Glu-Lys-OH. Enzyme data indicate that the COOH-terminal lysine may be modified but its exact nature remains to be determined. There is no similarity between the amino acid sequence of ELH and that of presently known vertebrate neuropeptides. The two-step purification procedure, starting with a homogenate of bag cell clusters, consisted of cation exchange chromatography on SP C25 (Sephadex) followed by gel filtration on Bio-Gel P-6. Our purification results in a 100-fold enrichment of ELH from bag cell homogenates and a 36% recovery of purified radiolabeled marker ELH. Analysis of purified ELH radiolabeled with [^{35}S]methionine or [^{3}H]leucine on isoelectric focusing gels and on 8 M urea/sodium dodecyl sulfate gels showed only a single peak containing 90% of the radiolabel. Radiolabeled ELH migrated with a pI of 9.0–9.2 and an apparent M_r of 3500–5700. ELH retained egg-laying bioactivity when eluted from this segment of the gel. We find that 2.5 nmol of pure ELH consistently induces egg laying at 20°C.**

In molluscs, a clear example of neurohormone-induced behavior is the egg laying in *Aplysia californica* evoked by a neuropeptide egg-laying hormone (ELH) (1, 2). ELH is synthesized by two symmetrical populations of about 400 neurosecretory bag cells each, clustered around the base of the pleurovisceral connectives at the rostral edge of the abdominal ganglion (3). When a homogenate of bag cells, containing ELH, is injected into the hemocoel of a sexually mature animal, a pattern of egg-laying behavior is triggered which results in egg laying within 65 min at 14°C (1, 4). Egg-laying behavior typically consists of locomotion to a stationary position on a vertical surface, puckering of mouth musculature, inhibition of feeding, and increased frequency of head weaving movement, culminating in the extrusion and winding of the egg string into a compact knot attached to the vertical substrate (5, 6).

Bag cell neurons are normally electrically silent but can be triggered to produce a synchronous afterdischarge of action potentials lasting 20–60 min when electrically stimulated (7). Recent findings of gap junctions in bag cell clusters support the premise that this synchrony of activity is due to electrical coupling between bag cell neurons (8). *In vitro* experiments have demonstrated the release of radiolabeled and bioactive ELH only when bag cells afterdischarge (9). *In vivo*, such an afterdischarge, whether induced or spontaneous, is always followed by egg laying (10, 11).

Previous studies have characterized ELH as a basic peptide of M_r approximately 6000 with an isoelectric point of 9.0–9.3 (12, 13). We have purified ELH by a simple two-step procedure to yield a homogeneous product with biological activity. We find the molecule to be a polypeptide of 36 amino acids with a calculated M_r of 4385 and migrating with a pI of 9.0–9.2. We now report its amino acid sequence.

MATERIALS AND METHODS

Egg-Laying Bioassay. Material, at various stages of purification, was checked for its ability to induce egg laying by injection through the foot into the hemocoel of a sexually mature *A. californica* maintained at 20°C as described (9). A test was considered to be positive if eggs were sighted within 90 min of injection. Injection volumes ranged from 1 to 3 ml and usually consisted of an aliquot of test material in low-ionic strength buffer mixed with an equal volume of filtered seawater.

Bag Cell Clusters, Radiolabeling, and Homogenization. *A. californica* (>270 g) were dissected within 2 days after collection from the Palos Verdes Peninsula during October and November of 1977 and 1978. There is some correlation between body weight and reproductive tract weight, and the latter, in particular, is an indicator of sexual maturity (1). Our starting material of bag cell clusters was obtained from sexually mature animals. Bag cell clusters were cut free from the rest of the abdominal ganglia in ice-cold filtered seawater and stored at −20°C.

For radiolabeling ELH, Millipore-filtered (0.22 μm) natural seawater with a final concentration of 10 mM glucose was buffered to pH 8.0 at 25°C by 20 mM Na Hepes and supplemented with 150 μg each of K penicillin G and streptomycin sulfate per ml. L-[4,5-^{3}H]Leucine [250 μCi (1 Ci = 3.7×10^{10} becquerels); Amersham; specific activity, 100 Ci/mmol] or [^{35}S]methionine (350 μCi; New England Nuclear; specific activity, 450 Ci/mmol) was taken to dryness under forced air in a boiling water bath and redissolved in the above medium. Freshly dissected abdominal ganglia were incubated in radiolabeled medium for 16 hr at room temperature (20°C) and then subjected to a series of rinses in filtered seawater lasting 2 hr. The bag cell clusters were dissected out and kept frozen at −20°C until used. To obtain radiolabeled ELH, 6 pairs of labeled clusters were ground with 34 pairs of unlabeled tissue.

In a typical preparation, 40 pairs of frozen bag cell clusters

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Abbreviations: ELH, egg-laying hormone; >PhNCS, phenylthiohydantoin; IEF, isoelectric focusing; $NaDodSO_4$, sodium dodecyl sulfate.

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were homogenized by hand in ice-cold 10 mM Na phosphate pH 6.0 (at 25°C; standard buffer) with phenylmethylsulfonyl fluoride. The homogenate was centrifuged in a Sorvall RC 2B at 12,000 × *g* for 20 min and the supernatant was dialyzed (Spectrapor 3 membrane, cutoff at M_r 3500) against two changes of 200 vol of standard buffer for 2 hr at 4°C. The retained material was then ready for purification.

Total protein in samples was measured by absorbance at 215 nm with a Beckman DB-G spectrophotometer. The sensitivity was 0.052 A_{215} units/10 μg of bovine serum albumin (used to generate a standard curve) per ml.

Amino Acid Composition. For analysis of amino acid content, ELH was hydrolyzed in two ways: in 6 M HCl under reduced pressure at 110°C for 24 hr, and in 3 M mercaptoethanesulfonic acid under reduced pressure at 110°C for 24 hr. Peptide fragments were subjected to the HCl hydrolysis only. All amino analyses were performed on a Durrum D500 amino acid analyzer.

Radiolabeled ELH was cleaved by 24-hr incubation in 6-fold (wt/wt) excess cyanogen bromide in 70% formic acid under nitrogen at 20°C. The reaction was stopped by dilution with distilled water, and the sample was frozen and lyophilized. In one experiment, the reaction products, a mixture of whole hormone and its cleavage products, were subjected directly to sequence determination in tandem. In a second experiment, the reaction products were redissolved in distilled water and separated on Bio-Gel P-6 in 10 mM Na phosphate, pH 7.5/50 mM NaCl. The radioactive peak migrating with a much higher K_{av} than ELH was again chromatographed on Bio-Gel P-2 and then on Bio-Gel P-4, both equilibrated in 1 M acetic acid. After these two additional steps of gel filtration, the major peak of radiolabel was subjected to microsequence analysis and to 6 M HCl hydrolysis and was found to be fragment 14–36 of ELH.

Sequence Determination. Automated Edman degradation was performed on the intact hormone and on the two peptides generated by cyanogen bromide cleavage by using a sequenator program similar to that described (14). The program included a 30-min coupling step using 0.3 M Quadrol buffer, a single 5-min cleavage step, and automated conversion of the anilinothiazolinones to phenylthiohydantoin (>PhNCS) amino acids by treatment with 25% aqueous trifluoroacetic acid for 45 min. The cup and conversion flask were maintained at 52°C.

The spinning cup sequenator used for the Edman degradation was designed and built at Caltech and will be described in detail elsewhere. It incorporates many of the design features described by Wittmann-Liebold (15) and Hunkapiller and Hood (14), with further refinements in the delivery head assembly and reagent/solvent reservoir system, and is equipped with a straight-edge cup from Beckman.

Polybrene (Aldrich, 6 mg) and glycylglycine (100 nmol) dissolved in 0.5 ml of distilled water were loaded into the sequenator cup and subjected to five complete degradation cycles. The peptide to be sequenced was then loaded and the sequencing program commenced with the coupling stage. >PhNCS amino acids were identified by high-performance liquid chromatography (HPLC) on a DuPont Zorbax CN column. Details on identification of >PhNCS amino acids and standard chromatograms are described elsewhere (16).

Isoelectric Focusing Gels (IEF). IEF gels were prepared and run as reported (9). On extrusion, gels were immediately sliced into 1.4-mm segments. To locate peaks of radioactivity, each slice was added to 5 ml of Wilson's cocktail (17) for assay in a Beckman LS scintillation counter.

Bioactivity was determined by incubation of gel segments in two changes of standard buffer and injecting the combined perfusates (total volume, 2 ml) into receptive test animals. To obtain the pH gradient, an equivalent gel was run concurrently and sliced into 2.8-mm segments, each of which was extracted in 1 ml of 100 mM KCl for several hours; pH was then measured directly with a Beckman Futura 39505 electrode and a Beckman Model 76 pH meter.

When 8 M urea IEF gels were used, normal IEF gels were made to 8 M urea and samples were brought to a final concentration of 3 M urea. Electrode buffers and other conditions were identical to those of normal IEF gels. Gradients of pH were similar in normal and urea IEF gels, and in both cases, cytochrome *c* was used in every gel as a reference marker.

Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Electrophoresis. $NaDodSO_4$/8 M urea slab gels were made according to the method of Swank and Munkres (18) with a final concentration of 12.5% acrylamide and a bisacrylamide/acrylamide ratio of 1:10. Standard mixtures of myoglobin (17,200), cytochrome *c* (12,300), α-bungarotoxin (7800), bovine pancreatic trypsin inhibitor (6160), insulin (5700), and glucagon (3500) were applied to wells on either side of the sample and run simultaneously. Gels were run at 15°C, to minimize diffusion, at a constant voltage of 65 V for 12–18 hr. The length of the gel containing the sample was then cut out and sliced, crosswise, into 1.4-mm segments for assay of radioactivity as described for IEF gels. The flanking slabs were stained and destained to reveal standards.

RESULTS

Hormone Purification. All operations were carried out at 4°C.

Cation exchange chromatography (SP C25 Sephadex). A dialyzed homogenate of bag cell clusters was diluted with standard buffer to a final volume of 30 ml and applied to a 6.0 × 1.5 cm column of SP C25, equilibrated to the standard buffer. After a rinse with 50 ml of standard buffer, a linear gradient made with 50 ml of standard buffer and 50 ml of 20 mM Na phosphate, pH 6.5/250 mM NaCl was applied. Two major peaks of protein were eluted at approximately 65 and 144 mM NaCl (Fig. 1). Biological activity was observed only in the first peak. Excluded material and rinse fractions also did not show any biological activity. A flow rate of 30 ml/hr was generated by gravity, and a Gilson microfractionator collected 80 fractions (1.1 ml).

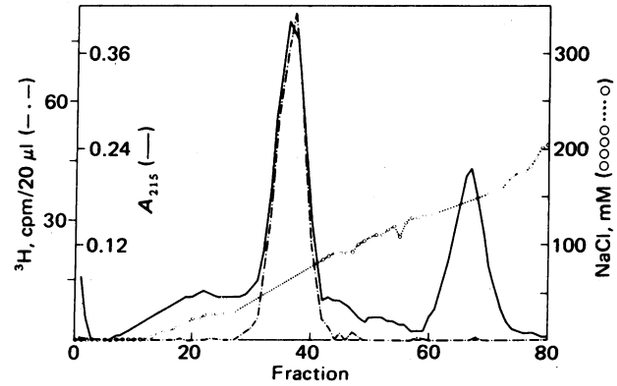

FIG. 1. Sephadex SP C25 chromatography of supernatant of bag cell extract after dialysis in standard buffer. Purified [^{3}H]leucine-labeled ELH was added as a marker to the supernatant. The column (6.0 × 1.5 cm) was equilibrated with standard buffer, and a gradient of 50 ml each of standard buffer and 20 mM Na phosphate, pH 6.5/250 mM NaCl was applied. The fraction volume was 1.1 ml and flow rate was 30 ml/hr. A background of 20 cpm was subtracted. Bioactivity coincided with the labeled marker ELH peak. Salt concentration was measured by conductivity on a conductivity meter (type CDM 2d, Radiometer, Copenhagen).

Gel filtration (Bio-Gel P-6). After cation exchange chromatography, fractions of the biologically active peak (Fig. 1, fractions 32–42) were pooled, lyophilized to a final volume of 0.8 ml, and applied onto a 50 × 1.0 cm Bio-Gel P-6 column equilibrated with 10 mM Na phosphate, pH 7.5/100 mM NaCl. Three protein peaks were seen in the A_{215} profile (Fig. 2). A small peak of material was excluded followed by the major peak of protein with a K_{av} of 0.2 (fractions 36–43) and another peak eluting with a K_{av} of 0.9 (fractions 70–75). Biological activity was only found in the major, middle peak, and this peak contained only ELH.

When radiolabeled pure marker ELH was copurified with an unlabeled bag cell cluster homogenate, the radioactive peak of ELH comigrated only with biologically active fractions in both steps of purification (Figs. 1 and 2).

In early experiments, a four-step procedure was used to purify ELH, which included ammonium sulfate precipitation and anion exchange chromatography followed by the cation exchange chromatography and gel filtration steps described above. ELH purified by this lengthy procedure was subjected to partial sequence analysis and was found to be homogeneous as judged by the presence of only one NH_2-terminal sequence. Later, the simpler two-step procedure was found to yield ELH which, when sequenced, was identical in purity and content to ELH obtained by the four-step method. Radiolabeled material purified by either procedure produced only one radioactive peak on IEF gels and on rechromatography on Bio-Gel P-6.

Amino Acid Sequence and Composition of ELH. The amino acid sequence of ELH and high-performance liquid chromatography data from a sequenator run with 4 nmol of intact hormone are shown in Fig. 3. Similar results were obtained from runs with 2 nmol of the cyanogen bromide-cleaved fragments. In Table 1, amino acid composition of ELH after acid hydrolysis is compared with that obtained from sequence data of the intact hormone. Because there is a decrease in yield of subsequent amino acids as one progresses toward the COOH terminus during sequence determination, ELH_{14-36} was first isolated and then examined to confirm the COOH-terminal half of the molecule. Fragment 14–36 was obtained by cyanogen bromide cleavage and was separated from undigested hormone and from fragment 1–13 by gel filtration. This COOH-terminal fragment was subjected to sequence determination and separately hydrolyzed for amino acid composition (Table 1) For

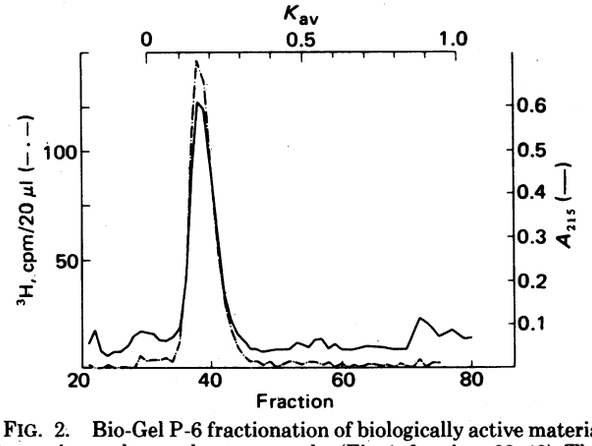

FIG. 2. Bio-Gel P-6 fractionation of biologically active material after cation exchange chromatography (Fig. 1, fractions 32–42). This material was pooled, concentrated to 0.6–0.8 ml, and applied to a 50 × 1 cm P-6 column equilibrated with 10 mM Na phosphate, pH 7.5/100 mM NaCl. Each fraction was 0.6 ml and flow rate was 4 ml/hr. Radioactivity was from [^{3}H]leucine-labeled marker ELH.

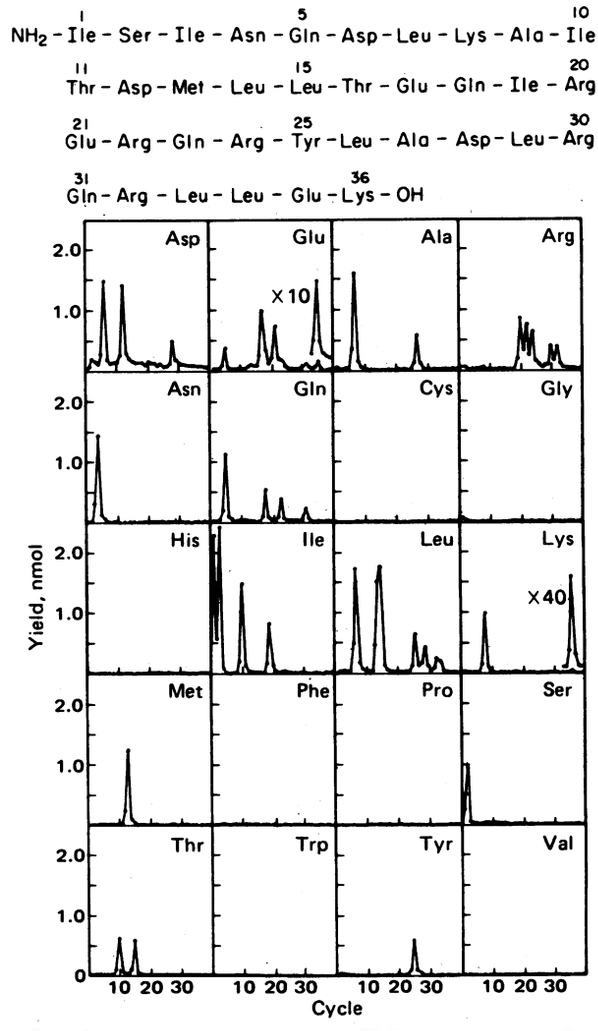

FIG. 3. Amino acid sequence of ELH and yields of >PhNCS amino acids from an NH_2-terminal sequenator analysis of 4 nmol of the neuropeptide. Aliquots of each cycle were analyzed by high-performance liquid chromatography; peak heights were converted to nmol for each derivative by using values for standard mixtures of >PhNCS amino acids and the yields were normalized to a 100% injection.

both whole ELH and ELH_{14-36}, sequence content and amino acid composition results match well. The COOH-terminal sequence was also confirmed by isolation of a tetrapeptide (Lys, Glu, Leu_2), from a tryptic digest of ELH succinylated in 5 M guanidinium hydrochloride, that corresponds to the terminal Leu-Leu-Glu-Lys sequence.

Isoelectric Focusing and Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Analyses. When [^{3}H]leucine-labeled purified ELH was analyzed on normal IEF gels, 90% of total radioactivity was recovered as a single peak in gel segments corresponding to a pI of 9.0–9.2 (Fig. 4). Bioassayable activity was eluted only from these regions of an equivalent gel. These results are identical to those of Arch and colleagues (13) in their characterization of ELH. The profiles of [^{3}H]ELH, biological activity and marker cytochrome *c* were similar on both 8 M urea and normal IEF gels.

For M_r analysis of polypeptides, radiolabeled ELH was run on a 12.5% $NaDodSO_4$/8 M Urea gel; 90% of total radioactivity comigrated with insulin (M_r, 5700) and slightly ahead of a marker of lower molecular weight, glucagon (M_r 3500) (Fig. 5).

ELH Recovery and Content. Table 2 summarizes the re-

Table 1. Comparison of amino acid contents of ELH and ELH_{14-36} determined from microsequence analysis and from acid hydrolysis

	ELH			ELH_{14-36}*	
	a	*b*	*c*	*a*	*b*
Asx	4	3.8	4.0	1	1.3
Thr	2	1.7	1.9	1	0.7
Ser	1	0.7	0.7	—	—
Glx	7	7.2	7.4	6	6.4
Ala	2	2.1	2.4	1	1.1
Met	1	1.0	1.3	—	—
Ile	4	4.2	3.0	1	1.2
Leu	7	7.7	7.1	6	6.4
Tyr	1	0.9	1.1	1	0.5
Lys	2	1.9	2.2	1	1.1
Arg	5	4.5	4.8	5	5.0

Column *a* lists amino acid composition derived from microsequence analysis. Column *b* lists molar ratios of amino acids obtained after 6 M HCl hydrolysis of the peptide. Column *c* lists molar ratios of amino acids obtained after hydrolysis by mercaptoethanesulfonic acid.
* ELH_{14-36} was obtained from cyanogen bromide cleavage of intact ELH followed by gel filtration.

covery of ELH during the two-step purification. When 117.5 μg of purified [^{3}H]leucine-labeled ELH was added to a dialyzed homogenate of 35 pairs of unlabeled bag cell clusters, the specific activity of this mixture was 628 cpm/mg of total protein. Upon elution with a salt gradient, 45% of counts applied to the SP C25 column was recovered in the protein peak with biological activity (Fig. 1) which had a specific activity of 46.8 cpm/μg; 24% of the total radiolabel input was excluded by the cation exchanger. Because the excluded material never exhibited any biological activity, these excluded counts may represent inactive fragments of ELH after proteolytic degradation as a result of extraction. An alternate possibility is that ELH has a high affinity for some other cell product and the complex formed by their interaction neutralized the basic nature of free ELH and prevented the retention of ELH by the cation exchanger. If this were the case, the complexed ELH also would have no biological activity.

After fractionation on Bio-Gel P-6, 80% of the applied radioactivity was recovered in fractions with a K_{av} of 0.2 (Fig. 2). The specific activity of this purified ELH was 62.7 cpm/μg, a 100-fold increase from the initial specific activity of 0.628 cpm/μg of the homogenate. A total recovery of 36% was derived from the product of the percentage recoveries of radiolabel in the ELH peaks after cation exchange and gel filtration.

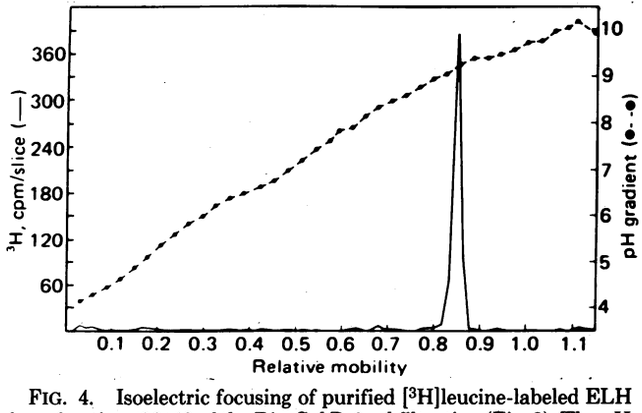

FIG. 4. Isoelectric focusing of purified [^{3}H]leucine-labeled ELH from fractions 36–43 of the Bio-Gel P-6 gel filtration (Fig. 2). The pH gradient was obtained from an equivalent gel run concurrently. Mobility is relative to the internal marker cytochrome *c*, pI 9.4–9.6.

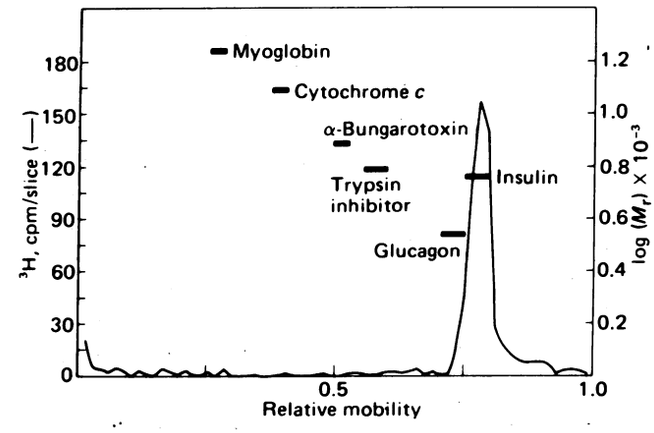

FIG. 5. NaDodSO$_4$/8 M urea/12.5% polyacrylamide gel electrophoresis of purified [^{3}H]leucine-labeled ELH from fractions 36–43 (Fig. 2). Slab gels were used and the six standards were applied to wells flanking the sample well. Mobility of Coomassie blue-stained standards and of radioactivity in gel slices were calculated relative to that of bromphenol blue.

DISCUSSION

We have established the purity and homogeneity of ELH prepared by our procedures by four types of analysis: amino acid sequence, amino acid composition, IEF, and NaDodSO$_4$/polyacrylamide gel electrophoresis. Microsequence analysis of purified ELH consistently revealed only one polypeptide chain. Moreover, it shows a good match with the amino acid composition after conventional acid hydrolysis of biologically active ELH. There is therefore no evidence of the presence of a contaminant with a blocked NH_2 terminus that may have copurified with the molecule analyzed. Gel analyses of radiolabeled ELH revealed one band of radioactivity that migrated with a pI of 9.0–9.2 on IEF gels and an apparent mass of 5700–3500 daltons on 8 M urea/NaDodSO$_4$ gels. In each case, the single peak contained 90% of the total radioactivity recovered on the gel. Biologically active material was eluted only from these same regions of equivalent gels. A conclusive demonstration of the identity of ELH will require that the peptide be synthesized and shown to be bioactive.

Based on the amino acid sequence, ELH has a M_r of 4385. Previous studies have characterized ELH as a 6000-dalton peptide on the basis of gel filtration on Sephadex G-50 (12). However, on Bio-Gel P-6 (exclusion size, M_r 6000) ELH is in the included volume with a K_{av} of 0.2 which is compatible with a M_r of 4000–5000. On 8 M urea/NaDodSO$_4$ gels, ELH comigrates with insulin (M_r 5700) and glucagon (M_r 3500). However, the rate of migration on such gels may be greatly affected by other considerations such as charge and shape, particularly in the case of oligopeptides (18). Clearly, NaDodSO$_4$ gels and gel filtration studies at best provide approximations of the M_r for such small peptides, and previous and present data from such analyses are not incompatible with the M_r of 4385 calculated from amino acid content. It is conceivable that this mass could be increased if polysaccharides were associated with ELH, although sequence analysis shows no polysaccharides.

The amino acid sequence revealed a greater number of basic residues than acidic ones, which explains the basic nature of ELH that persists even after denaturing by 8 M urea. If the COOH-terminal residue is an unblocked lysine, calculated by the method of Edsall and Wyman (19), the pI of ELH should be 9.7, which agrees with the apparent pI of 9.0–9.2 on normal and 8 M urea IEF gels. However, the inability of carboxypeptidases A and B to cleave ELH suggests a blocked COOH ter-

Table 2. Recovery of purified, [^{3}H]leucine-labeled ELH during copurification with unlabeled bag cell extract

	Specific radioactivity, cpm/mg total protein	Total radioactivity, cpm	% recovery of initial radioactivity
Bag cell extract supernatant*	628	50,000	100
After cation exchange chromatography, fractions 32–42 (Fig. 1)	46,800	22,390	44.8
After gel filtration, fractions 35–45 (Fig. 2)	62,700	17,920	35.8

* Bag cell clusters (35 pairs) were homogenized and, after dialysis of the supernatant, 117.5 μg of [^{3}H]leucine-labeled marker ELH was added prior to purification.

minus, possibly by amidation. The majority of biologically active neuropeptides, including substance P, LHRH, TRH, oxytocin, and vasopressin, have amidated COOH termini. In many cases, biological activity of the molecules requires the presence of the terminal amide. Alternatively, cleavage of the terminal lysine residue by carboxypeptidase B may be inhibited by the presence of the penultimate glutamic acid residue or by a peptide tertiary structure that shields the lysine from the enzyme. At present, we do not have an answer to the nature of the block at the COOH terminus of ELH, which may affect the calculated pI of the molecule.

Besides the basic character of ELH, the composition of this molecule is also unusual in that it has no valine, proline, tryptophan, histidine, glycine, phenylalanine, and cysteine. The absence of cysteine means that disulfide linkages do not play a role in intramolecular or intermolecular interactions with ELH.

Our two-step procedure resulted in a 100-fold purification of ELH based on specific activities in the recovery experiment in Table 2. This suggests that ELH may make up as much as 1% of the soluble protein in bag cell clusters. On the average, 7–14 μg of hormone is recovered from a pair of bag cell clusters. Because there is a 36% recovery of marker radiolabeled ELH, the calculated initial level of the hormone is 19–39 μg per cluster pair. The actual value may be higher because we do not know how efficient our method of extraction is, and ELH contained in some of the bag cell terminals extending into the abdominal ganglion and connective tissue have not been included. Most of the clusters used were dissected from sexually mature animals during October and November, at the height of the reproductive season. This high ELH content may not be maintained all year round.

The neuropeptide, ELH, whose primary structure has been determined, is a molluskan neurohormone that is synthesized by identifiable neuron clusters. A cardioexcitatory factor, the tetrapeptide amide Phe-Met-Arg-Phe-NH_2, has been extracted from the ganglia of clams, but the precise neuronal source and its normal physiological role have still to be defined (20). The atrial gland in the large hermaphroditic duct of *Aplysia* has been shown to contain peptide factors capable of inducing egg laying in *Aplysia* (21). Two of these peptide factors have recently been purified in this laboratory and their sequence has been determined (22). The two atrial peptides are rather similar but they bear no resemblance to ELH, and their mode of action in egg laying appears to be to produce bag cell afterdischarge (22). ELH therefore remains as the only known neuropeptide directly implicated in egg laying in *Aplysia*.

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