SYNTHESIS OF [^{3}H-PYROGLUTAMYL^1]-ADIPOKINETIC HORMONE-I

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SUMMARY

L-[3,4-^{3}H(N)]-Glutamine was cyclized to [^{3}H]-pyroglutamic acid and coupled with [des-pGlu^1]-AKH-I to give [^{3}H-pyroglutamyl^1]-adipokinetic hormone-I with a specific activity of 46 Ci/mmole. Results relating to stability of this product during storage are reported.

Key Words: [^{3}H]-adipokinetic hormone-I, [^{3}H]-pyroglutamic acid

INTRODUCTION

The adipokinetic hormones constitute a family of terminally blocked insect peptides which are stored in the corpora cardiaca and released into the haemolymph to perform a wide range of biological functions (1). Two related peptide hormones involved in lipid metabolism, AKH-I and AKH-II, co-occur in the locust (2). For an investigation of AKH-I (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2) at the receptor level, radiolabelled AKH-I of high specific activity was required. [^{3}H-Leu^2]- and [^{3}H-Pro^6]-AKH-I with specific activities of 115 and 12 Ci/mmole respectively have previously been prepared by the catalytic tritiation of precursors containing dehydroleucine and dehydroproline (3,4) and an analog, containing tritiated tyrosine, with a specific activity of 57 Ci/mmole has been reported (5). Minnifield and Hayes (6) have described the partial purification of a receptor for the adipokinetic hormones from the face fly, *Musca autumnalis*, using tritiated and radioiodinated peptides. In the present study we undertook the synthesis of [^{3}H-pyroglutamyl^1]-AKH-I ([^{3}H-pGlu^1]-AKH-I).

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RESULTS and DISCUSSION

Progress in the study of AKH receptors requires the availability of a biologically active radiolabelled ligand of sufficiently high specific activity to enable receptor-binding analysis (7). Since pyroglutamic acid occupies the N-terminal position in many insect neuropeptides, we undertook to develop a general approach which would be potentially useful for the preparation of a number of radiolabelled peptides. In the present case, pyroglutamic acid could be coupled to Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 ([des-pGlu^1]-AKH-I), without the necessity of protecting groups which would require a subsequent deprotection step. Procedures were first developed with unlabelled materials beginning with glutamine, since tritium-labelled glutamine with high specific activity is available commercially. Treatment of glutamine on the 15 μg scale with trifluoroacetic acid (TFA) at 70° (8) and HPLC purification of the product provided pyroglutamic acid in high yield (92-100%). Subsequent coupling to [des-pGlu^1]-AKH-I using PyBOP gave AKH-I, characterized by HPLC retention time and bioassay, in yields of 74-97%.

Similar treatment of L-[3,4-^{3}H(N)]-glutamine and purification of the product by HPLC yielded [^{3}H]-pyroglutamic acid required for the preparation of the desired peptide. This amino acid was then coupled to [des-pGlu^1]-AKH-I. HPLC analysis of aliquots of the product indicated a 74% yield (88 μg, 3.5 mCi) of [^{3}H-pGlu^1]-AKH-I with a specific activity of 46.0 Ci/mmole. Separation of the remainder of the sample and collection of the peak corresponding to the retention volume of AKH-I gave [^{3}H-pGlu^1]-AKH-I (Fig. 1).

The labelled peptide was initially stored under nitrogen at -5° in the HPLC solvent used for elution. When an aliquot was examined after five weeks, two major radiolabelled peaks were observed in addition to the slightly later eluting peak corresponding to AKH-I. The remainder of the sample was then chromatographed and three fractions collected (Fig. 2). Material in fraction 1, corresponding to a retention time earlier than that of AKH-I, exhibited biological activity at a level similar to that for AKH-I indicating that the structural change was not extensive. Fraction 2 was inactive even at 2 and 4 ng/insect while fraction 3, eluting at the retention time of AKH-I and containing 34% of the radioactivity of the original sample, exhibited the level of bioactivity expected for the quantity of

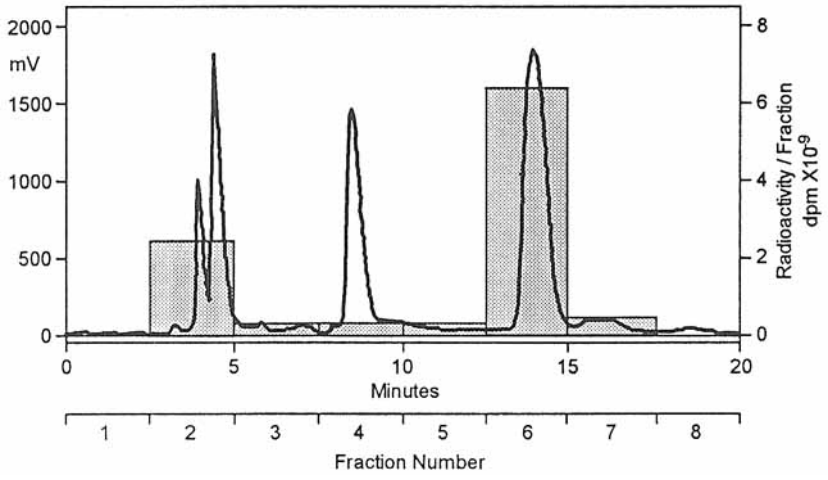

Figure 1. HPLC purification of [^{3}H-pGlu^1]-AKH-I and [^{3}H]-radioactivity profile based on 2.5 min fractions. Column: Phenomenex Bondclone 10 C_{18} (300 x 3.9 mm i.d.). Elution: 25% acetonitrile in 10 mM TFA at 1 ml/min. Detection: 220 nm. Retention times: [des-pGlu^1]-AKH-I, 8.4 min; AKH-I, 13.9 min.

peptide remaining. No apparent decomposition was observed when unlabelled AKH-I was stored in the same manner. As it seemed probable that the observed changes were due to radiation-induced self-decomposition and that the solvent might contribute to the decomposition, the repurified peptide (1 mCi) was stored in distilled water. Analysis (data not shown) after storage at -80° for 3 months indicated that degradation had continued and only 30% of the radioactivity of the original sample remained as [^{3}H-pGlu^1]-AKH-I. Thus, changing from HPLC solvent to water did not alter the degradation rate so further attempts to improve the stability of [^{3}H-pGlu^1]-AKH-I were made using the solvent system employed for peptide purification. Since others have observed more degradation at temperatures below freezing than at slightly above, attributed to the concentration of radioactive substance around ice crystals (9,10), repurified [^{3}H-pGlu^1]-AKH-I was stored for 6 months at 4° in 0.1% TFA-acetonitrile. Again, substantial degradation occurred (Table 1). HPLC and radioanalysis following further storage of repurified peptide for 3 months and 1 year at a broad range of temperatures indicated that

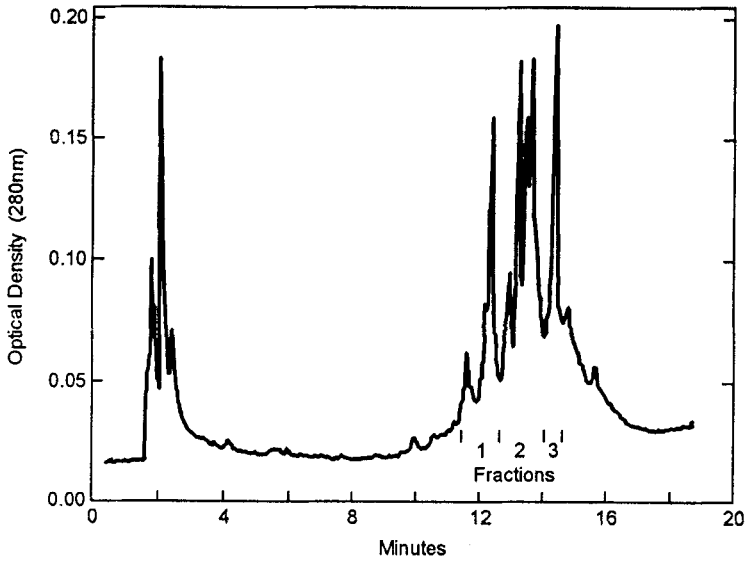

Figure 2. HPLC repurification of [^{3}H-pGlu^1]-AKH-I. Column: Whatman PartiSphere C_{18} (120 x 3.9 mm i.d.). Elution: linear gradient of 12-48% acetonitrile in 0.1% TFA over 20 min at 1 ml/min. AKH-I retention time: 14.2 min. Three fractions were collected as indicated for lipid mobilization assay.

degradation continued to occur and that temperature apparently had little effect on the rate of decomposition (Table 1). The presence of N_2 or the addition of 2% ethyl alcohol to the solvent did not significantly affect the rate of degradation during the period that the peptide was stored. In the current study when unlabelled AKH-I was stored at -20° for 3 months under the conditions described in Table 1, no loss of biological activity was detected in tests for the mobilization of lipid in male adult locusts performed with serial dilutions of the hormone. The degradation of [^{3}H-pGlu^1]-AKH-I reported here proceeded at a rate similar to that observed for a [^{3}H]-labelled substance P analog having a specific activity of 27 Ci/mmole (11). When stored in water-ethanol (1:1) at -25°, only 17% of this peptide remained after 10 months.

Several groups have reported, without mention of stability problems, the preparation of tritium-labelled AKH-I and related peptides by chemical synthesis (3-5) or by incubating locust corpora cardiaca with [^{3}H]-labelled tryptophan, phenylalanine and leucine and isolating the AKH by HPLC (12).

Table 1. Effect of storage condition on stability of [^{3}H-pGlu^1]-AKH-I. Samples stored for (a) 6 months, and then (b) a further 3 months and (c) 12 months after repurification (see Experimental).

		Percent remaining (HPLC determination)		
	Storage Temp., °C	0.1% TFA-CH_3CN (1:1)	0.1% TFA-CH_3CN 1:1), N_2	0.1% TFA-CH_3CN (1:1), 2% EtOH, N_2
a)	4	57.8	56.7	63.5
b)	4	44.9	50.5	58.9
	-20	51.0	57.0	54.0
	-80	47.2	47.3	60.8
c)	4	9.4	9.0	13.0
	-20	8.1	9.0	9.8
	-80	7.8	8.0	9.0

However, Professor W. Mordue (University of Aberdeen, personal communication) found that a preparation of [^{3}H]-AKH-I was unstable even when stored in liquid nitrogen. Recently, Ziegler *et al.* (13) observed that a [^{3}H]-labelled *Manduca sexta* adipokinetic hormone with a specific activity of 27 Ci/mmole was initially stable when stored at -80° but subsequently started to degrade. Thus, it appears that the radiolabelled AKH instability observed in the present study is not unique. Stability may depend on the location of the label in the molecule so, in cases such as the present where it is a problem, the only recourse may be to use the labelled peptide as soon as possible after preparation. Older, partially degraded preparations can be repurified by HPLC (13).

In conclusion, [^{3}H-pGlu^1]-AKH-I of high specific activity can be conveniently prepared by coupling [^{3}H]-pyroglutamic acid to [des-pGlu ^{1}]-AKH-I. The methodology is simple and can be applied to the labelling of any peptide with a N-terminal pyroglutamyl moiety. Experience with [^{3}H-pGlu^1]-AKH-I suggests that the peptide should be prepared in small batches and used promptly.

EXPERIMENTAL

L-[3,4-^{3}H(N)]-Glutamine (48.4 Ci/mmol) was purchased from Du Pont Canada Inc. and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was obtained from Novabiochem USA. Unlabelled AKH-I was supplied by Peninsula Laboratories Inc. Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 was synthesized by the Core Facility for Protein/DNA Chemistry, Queen's University, Kingston, ON. HPLC analyses and separations concerned with the synthesis were performed with a Waters system controlled by a Waters 840 data and chromatography control station and equipped with a Model 481 variable wavelength detector operated at 220 nm. Separations were achieved on a Bondclone 10 C_{18} column (300 x 3.9 mm; Phenomenex). Radioactivity was quantitated using a LS-5801 counter (Beckman Instruments). Aliquots of HPLC fractions were added to liquid scintillation cocktail (Ready Gel; Beckman). Repurification of the radiolabelled peptide and studies of its stability were conducted with a Scientific Systems HPLC system equipped with a Milton Roy UV monitor operated at 280 nm. Separations were performed on a PartiSphere C_{18} column (120 x 3.9 mm; Whatman) with a linear gradient of 12-48% acetonitrile in 0.1% TFA over 20 min at 1 ml/min. Radioactivity was measured with a Minaxi Tricarb 4000 counter (Packard) using liquid scintillation cocktail for aqueous samples (Amersham).

Bioassay of AKH-I. Determination of lipid mobilizing activity was performed on 10 day old adult *Locusta migratoria* as described by Carlisle and Loughton (14). Groups of five locusts were injected with known amounts of synthetic AKH-I (Peninsula Laboratories) or with aliquots of fractions obtained from HPLC repurification of [^{3}H]-AKH-I. The threshold dose for synthetic AKH-I was 0.2 ng/insect and maximum lipid mobilization was achieved at 2.0 ng/insect.

[3,4-^{3}H(N)]-Pyroglutamic Acid. L-[3,4-^{3}H(N)]-Glutamine (5 mCi), supplied in ethanol-water (2:98, 5 ml), was transferred in several portions to a small glass tube (1 ml) and dried in a Speed Vac Concentrator (Savant Instruments). The radiolabelled sample tube was then transferred to a Pico-Tag reaction vial (Waters) containing TFA (400 µl) and, after partial evacuation, the closed vial was heated at 70° for 2 h. The product, [^{3}H]-pyroglutamic acid, was purified by HPLC on the Bondclone C_{18} column using 3% acetonitrile in 10 mM TFA at 1 ml/min. Fractions (1.5 ml) were collected in

polypropylene micro test tubes (2 ml) and aliquots (1 μl) removed for radioanalysis. The fraction corresponding to the peak with the retention time of pyroglutamic acid was dried (Speed Vac Concentrator).

[^{3}H-Pyroglutamyl^1]-Adipokinetic Hormone-I. The radiolabelled pyroglutamic acid was coupled to [des-pGlu^1]-AKH-I using PyBOP essentially as described by Coste *et al.* (15). [Des-pGlu^1]-AKH-I (126 nmol, 1.2 equiv) was added to the labelled pyroglutamic acid sample (105 nmol, 1 equiv; 5.08 mCi) and the mixture was dried *in vacuo* (Pico-Tag Work Station). To the residue was added PyBOP (116 nmol, 1.1 equiv) and N,N-diisopropylethylamine (237 nmol, 2.2 equiv) in anhydrous dimethylformamide (45 μl). After mixing, the solution was left 2 h at room temperature and then dried (Speed Vac Concentrator). To assess yield and specific activity of the product, two analytical samples were chromatographed on the Bondclone C_{18} column using 25% acetonitrile in 10 mM TFA at 1 ml/min. Fractions (2.5 ml) were collected and aliquots were removed for radioanalysis. The remainder of the product was then chromatographed in the same manner. The fraction containing the radiolabelled AKH-I was stored initially at -5° under nitrogen in HPLC solvent (7.6 ml) used for elution.

Stability Studies. After five weeks of storage as described above, an aliquot of [^{3}H-pGlu^1]-AKH-I was analyzed by HPLC and scintillation spectrometry. The remainder of the sample was repurified by HPLC and aliquots were bioassayed. The fraction containing [^{3}H-pGlu^1]-AKH-I (approximately 1 mCi) was then stored at -80^0 in 10 ml double distilled water for 3 months, analyzed, and again purified by HPLC. Equal aliquots of the remaining labelled AKH-I (300 μCi) were then stored at 4° for 6 months under 3 different conditions: (1) in acetonitrile-0.1% TFA (1:1, 0.5 ml); (2) as for (1) under nitrogen; (3) as for (2) with the addition of 2% ethanol. After analysis, the remaining [^{3}H]-AKH-I in the samples was combined, repurified by HPLC, subdivided and again stored under the above 3 conditions (each aliquot in 0.1 ml solvent) at either 4°, -20°, or -80° for 1 year with analysis at 3 months and 1 year to determine the effect of temperature on stability.

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