SYNTHESIS OF A NEW NEUROPEPTIDE, THE HEAD ACTIVATOR FROM HYDRA

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1. Introduction

Recently a neuropeptide, the head activator, was isolated from hydra, from sea anemones and from mammalian brain and intestine [1–3]. In hydra this peptide acts as a head-inducing morphogen by stimulating head-specific growth and differentiation processes [1,4]. The head activator was found to be an undecapeptide with the amino acid sequence:

pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe [2,3]

Since enzymes and micromethods were used to determine the sequence, it seemed desirable to confirm the structure of the head activator by synthesis. We show here that only one of the synthetic peptides was chemically and biologically indistinguishable from the native head activator from hydra.

2. Materials and methods

2.1. *Amino acids*

All amino acids were protected at their α-amino function by α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz) [5], the side chain protecting groups were benzyloxycarbonyl (Z) for lysine, tertiary butyl (Bu^t) for tyrosine and serine, 4,4′-dimethoxybenzylhydryl (Mbh) for glutamine.

2.2. *Carrier*

As support 0.5% divinylbenzene crosslinked chloromethylated polystyrene gels were used [6], 1.5 mmol Ddz–Phe were bound/g polymer.

2.3. *Synthesis*

The Ddz-protecting groups were removed with 5% trifluoracetic acid (TFA) in dichlormethane within 15 min and monitored at 282 nm to check whether the cleavage of the protecting group was complete. After deprotonation with 10% triethylamine in dichlormethane and washing of the polymer support, the coupling was mediated with dicyclohexylcarbodiimide (DCC) in dichlormethane using a 12-fold excess of Ddz amino acids and 6-fold of DCC over the growing chain. The coupling step was repeated at least once or more often, if required. The efficiency of the coupling reaction was monitored by hydrolysing ~10 beads of the polymer (in 12 N HCl/propionic acid 1:1 at 160°C for 30 min), followed by amino acid analysis in a Biotronic amino acid analyser. Yields were calculated in relation to the phenylalanine originally coupled to the resin. For most of the sequences to be synthesised the last amino acid was pGlu. We decided to incorporate Ddz–Gln(–Mbh) and make use of the tendency of the terminal Gln (Mbh) to cyclise to the pyroglatamyl derivative during cleavage of the 4,4′-dimethoxybenzhydryl group by heating in trifluoroacetic acid [7].

2.4. *Deprotection and cleavage from the carrier*

The peptide was cleaved from the carrier using HBr–trifluoroacetic acid subsequent to reflux boiling of the polymer in trifluoroacetic acid for 15 min. After evaporation of the acidic filtrates, the cleaved peptide was precipitated and washed with ether and dried.

2.5. *Chromatography on Sephadex LH-20*

The crude synthetic material was purified on Sephadex LH-20 using at first pure methanol as eluent and later water or water trifluorethanol mixtures. The homogeneity of the synthetic products was checked by thin layer chromatography, HPLC and amino acid analysis.

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2.6. *High-pressure liquid chromatography (HPLC)*

Prepacked reverse phase octylsilylsilica columns (LiChrosorb RP-8, column size 250 × 4 mm, particle size 7 μm) were from Merck. As mobile phase either water–methanol or water–acetonitrile mixtures were used.

2.7. *End-group analysis*

The peptide was dansylated and hydrolysed. The dansylated amino acids were separated by thin-layer chromatography [8].

2.8. *Enzymic digestions*

Trypsin TPCK was from Worthington and used at 10 μg/ml, carboxypeptidase Y and aminopeptidase M were from Boehringer, Mannheim, and used at 100 μg and 10 μg/ml, respectively. An endopeptidase from *Astacus fluviatilis*, called low molecular mass protease, (LM_rP), was purified by and a kind gift from R. Zwilling, Zoophysiology Department, University of Heidelberg [9]. It was used at 1 μg/ml.

2.9. *Biological assays*

The synthetic peptides were assayed in the normal head-activator assay [10] for their effect on stimulating head and bud formation in hydra [10]. The effect on cellular mitosis and nerve-cell differentiation was measured as in [11,12].

3. Results

3.1. *Synthesis and chemical characterisation*

The following peptides were synthesised:

(1) pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile –Leu–Phe

(2) Tyr–Gln–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile –Leu–Phe

(3) pGlu–Pro–Pro–Gly–Gly–Ser–Lys –Pro–Val Ile –Leu–Phe

Peptide 1 was synthesised as the most likely structure for the head activator, peptide 2 for antibody production and peptide 3 to resolve an uncertainty in our sequence analysis. The efficiencies of coupling based on the starting load were 95–99% for all amino acids, except for isoleucine in peptides 1 and 2, where the efficiency was 86%, and for the tyrosine of peptide 2 which was 89%.

After cleavage from the carrier the peptides were purified by chromatography on Sephadex LH-20 using 100% methanol as eluent first, followed by chromatography in distilled water or in 1% trifluorethanol. Peptide 1 was obtained in an overall yield of 18%, peptide 2 in 47% and peptide 3 in 33%. The amino acid composition of the three main products is given in table 1. As side products in minor yield we also isolated truncated sequences with one amino acid missing and peptides with substitutions of L- by D-amino acids due to racemisation. Thus by using the procedure developed to extract the head activator from natural sources [2,3], we isolated a biologically active peptide from synthesis 3 which lacked the proline at the carboxyterminal part of the molecule, i.e., had the sequence:

pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–
Leu–Phe

This peptide represented a minor product of synthesis 3 and was obtained in a yield corresponding to ~2% of the total synthesised material.

Characterisation of the peptides by HPLC showed that the three main peptides chromatographed on RP-8 LiChrosorb columns as homogeneous, symmetric peaks (fig.1a–c) indicating that the degree of purity was satisfactory. Only the peptide with the sequence:

pGlu–Pro–Pro–Gly–Gly–Ser– Lys Val–Ile–
Leu–Phe

showed the same retention times on HPLC under various conditions as the native head activator from hydra (table 2). Co-chromatography of the two peptides, head activator and synthetic peptide, injected simultaneously, gave a uniform peak.

3.2. *Enzymic digestions*

The head activator was characterised and sequenced by means of several enzymes [2,3]. Thus it was shown that trypsin cleaved the molecule into two fragments. By applying these enzymatic digestions to the synthetic products, we found that only peptides (1) and (2) were split by trypsin, yielding fragments with the right composition and sequence, whereas peptide (3)

Table 1
Amino acid analysis of the purified peptides

Amino acid	Peptide (1)	Peptide (2)	Peptide (3)
Ser	1.2	1.1	1.2
Glu	0.9	0.8	1.2
Pro	2.2	2.0	2.6
Gly	2.0	1.7	1.7
Val	0.9	0.8	0.7
Ile	0.9	1.1	0.7
Leu	1.1	1.3	1.1
Tyr	–	1.1	1.1
Phe	1.1	1.1	1.0
Lys	1.1	0.9	1.1

was resistant which is in accordance with the enzyme specificity which cannot cleave after lysine, if lysine is followed by proline. The head activator was degraded by a low molecular mass protease (LM_1P), isolated from *Astacus fluviatilis* [9]. This enzyme cleaved out glycine and in addition yielded two fragments, the amino-terminal fragment of which could be digested sequentially with amino peptidase M. All three synthetic peptides, like the head activator, were cleaved by LM_1P. The resulting fragment with a free amino-terminus was digested with amino peptidase M. In accordance with the enzyme specificity in peptides (1) and (2) all the amino acids appeared sequentially. In peptide (3) lysine and proline were cleaved out as dipeptide and could only be detected as individual amino acids after acidic hydrolysis. The head activator after involuntary conversion of pyroglutamic acid to glutamic acid was almost completely digested (except glutamic acid) by carboxypeptidase Y as was its biologically active synthetic counterpart.

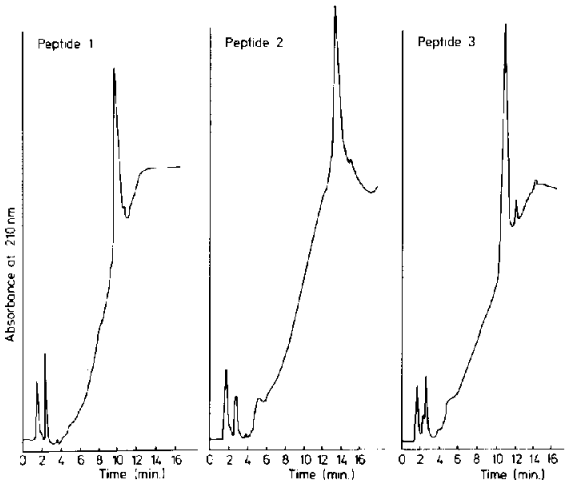

Fig.1. Chromatography of the three purified peptides on RP-8 HPLC columns (column size 250 × 4 mm particle size 7 μm, flow rate 1 ml/min). Mobile phase: gradient from 40–60% methanol in 5 mM ammonium bicarbonate: (a) peptide (1); (b) peptide (2); (c) peptide (3).

3.3. *Biological activity*

The peptide of the sequence:
pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe

had the full biological activity as the head activator from hydra. For example, the head activator isolated from hydra stimulated the outgrowth of buds reaching a half-maximal stimulation (50% increase over untreated controls) at 10^{-13} M [2]. As shown in fig.2 the synthetic peptide is equipotent in this assay to native head activator. All other peptides had no effect.

Table 2
Retention times of the synthetic peptides and of the native head activator on HPLC

Conditions	Head activator	Peptide (1)	Peptide (2)	Peptide (3)
Isocratic				
50% Methanol/5 mM ammonium bicarbonate	7.6	7.6	9.2	4.2
30% Acetonitrile/ 0.1% trifluoro acetic acid	8.0	8.0	10.5	9.0
Gradient (10 min)				
40–60% Methanol/ 5 mM ammonium bicarbonate	10.5	10.5	13.5	11.3
20–40% Acetonitrile	11.2	11.2	11.9	11.6

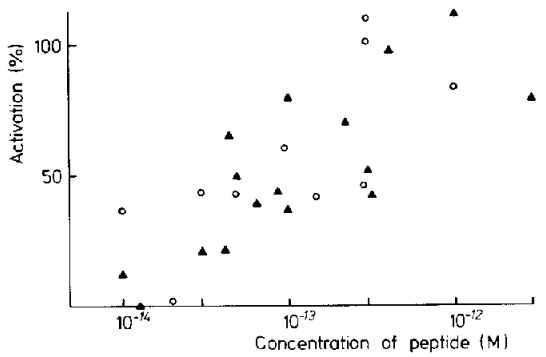

Fig.2. Stimulation of bud outgrowth in hydra by incubation in native head activator (○) and in synthetic head activator (▲). The concentration is given as moles per liter (M) and the effect as percentage increase (% activation) over untreated controls.

This outgrowth of buds is measured 3 h after addition of head activator to the medium and it is due to the mitotic action of the head activator [11]. To assay this mitotic effect directly, the mitotic index was determined after a 3 h incubation of whole hydra in 5×10^{-13} M of the synthetic head activator. The mitotic index of interstitial cells in untreated controls was 3.6 ± 0.7, incubation in synthetic head activator lead to the expected effect, namely it increased it to 5.6 ± 0.5 corresponding to a 56% increase (1500 cells were counted each). A second action of the head activator is that it influences the determination of interstitial cells to nerves [12]. This determination occurs in the early S phase of the precursor cells, requires 10-times higher head-activator concentrations and becomes visible after one cell cycle, i.e., 24 h later. To assay this, hydra were incubated in the synthetic head activator at 10^{-12} M and 10^{-10} M. In both cases the density of nerves showed the expected increase 29 h later, 2-fold at the lower and 2.5-fold at the higher concentration. Peptides (2) and (3) were completely inactive at the cellular level as were all the side products with minor changes affecting one or more amino acids.

4. Discussion

Synthesis of the head activator was to serve two purposes:

(1) To confirm the structure proposed for the native head activator;
(2) To provide more material than the few nanomoles we could isolate from nature [2,3].

The first goal we achieved with ease. Of all peptides synthesised with or without intention, only the peptide with the structure:

pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe

was biologically active. Leaving out one amino acid, e.g., one glycine or one proline, changing the amino-terminus from pyroglutamic acid to glutamic acid or to glutamine, introducing one additional amino acid, e.g., tyrosine at the amino-terminus, or inserting proline between lysine and valine, all resulted in eliminating the biological activity. In our attempt to synthesise the head activator we also produced peptides with the desired amino acid composition, but due to partial racemisation with chromatographic properties and enzyme degradabilities different from the native molecule. None of those had any biological effect on hydra. We interpret this to mean that only amino acids in the L-configuration are allowed as constituents of the head activator. This also suggests that the molecule as a whole has a very unique tertiary structure allowing no change in the sequence.

The second goal of our synthesis was to produce head activator in large quantities. The yield of the purified synthetic head activator was relatively low. We think that this was mainly due to the fact that in pure form and at high concentration the head activator has the tendency to form aggregates with itself resulting in a very diffuse chromatographic elution pattern; and in insolubility. Astonishingly, it was easier to purify in high yield the peptide with identical sequence, but with glutamine instead of pyroglutamic acid and with an additional tyrosine at the amino-terminus. This peptide was synthesised as a possible substrate for iodination to build up a radioimmuneassay. The synthetic products may provide us with a handle to investigate the action of the head activator at a more molecular level.

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