A radioimmunoassay for the *Hydra* head activator

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A highly sensitive and specific radioimmunoassay for the head activator has been developed which utilises tritiated head activator. The assay is sensitive in the range of 40–200 fmol. Immunochemical studies showed that the antiserum recognised the intact molecule better than any of the fragments or derivatives produced by enzymatic treatment or chemical synthesis.

Radioimmunoassay *Hydra head activator* *Tritiated head activator* *Neuropeptide* *HPLC*

1. INTRODUCTION

The head activator (HA) is a biologically active peptide which was isolated and sequenced from different coelenterates such as hydra, sea anemones and also from mammalian brain and intestine [1,2]. In these various species and tissues the same sequence pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe was found. In hydra this peptide acts as head inducing morphogen at 10^{-13} M, in mammals it affects stimulation of exocrine pancreas secretion at 10^{-10} M [3].

Up to now the HA was assayed in the normal HA assay measuring the stimulation of head and bud formation in hydra [4]. Although being very sensitive, both assays are extremely laborious and time consuming. This work describes the establishment of a radioimmunoassay (RIA), which enables us to determine the concentration of immunoreactive HA within a relatively short time (2 days) and with a very high degree of precision.

2. MATERIALS AND METHODS

2.1. *Preparation of the antigenic conjugate*

Trifluoroethanol (200 μl) were added to 5.5 mg HA (Bachem) to solubilise the hydrophobic peptide. This solution was slowly diluted with 1.5 ml water, and 22.5 mg of keyhole limpet hemocyanin (Calbiochem) were added. After an intermittent addition of 1 ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (20 mg/ml, Calbiochem) over 1 h [5] the mixture was stirred overnight at 22°C and dialysed afterwards against 3 changes of 1 l of 0.15 M NaCl.

2.2. *Immunisation*

Specific pathogenic free hybrid rabbits (2.5–3 kg) were immunised with 500 μl antigenic conjugate containing an equivalent of 110 μg peptide and an equal volume of Freund's complete adjuvant. One half of this mixture was injected directly into the lymphnodes of the knees. The lymphnodes had been made visible before with Evans blue (200 μl/knee) and exposed under anaesthesia (phentanyl, 2 ml/kg). The rest of the mixture was injected intradermally over 10 sites into the backs of the rabbits. The rabbits were boosted at monthly intervals with 500 μl of conjugate and serum was obtained 5 days after each injection. For a first analysis of antibody titer the sera were assayed in an enzyme-linked immunosorbent assay (unpublished).

2.3. *Preparation of the tracer*

[3,4-dehydropro^{2,3}]HA (Bachem) was catalytically tritiated by Amersham. The tritiated product

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was heterogenous and therefore purified according to the following procedure: HA specific antiserum, after purification on protein A–Sepharose, was bound to 1.5 g CNBr-activated Sepharose 4B as recommended by the manufacturer (Pharmacia Fine Chemicals) and packed into a 1.2 × 6 cm column. The radioactive material was applied to this column in 0.1 M Na_2HPO_4 (pH 7.0) and after extensive washing with the same buffer the adsorbed peptide was eluted with 1 N acetic acid. The eluate (18% of the applied radioactivity) was collected in 2 M Tris–HCl (pH 8.6) and desalted on Sep-Pak C18 (Waters), which was equilibrated with water. After washing the cartridge with 10 ml water and 5 ml 20% methanol, the HA was eluted with 80% methanol. This purified tritiated peptide was analysed for homogeneity on HPLC using a column C_8 (Li Chrosorb, particle size 7 μm, column size 250 × 4 mm) and a 10 min gradient from 20–40% acetonitrile/0.1% trifluoroacetic acid. The specific activity was found to be 40–60 Ci/mmol.

2.4. *Preparation of fragments*

Peptides 2 and 5 (table 1) were produced by incubating 10 nmol of HA with TPCK-trypsin (50 μg/ml, Worthington) for 2 h in 0.05 M NH_4Ac, pH 7.6 at 37°C. Peptides 3 and 4 were generated by digestion of the HA with *Astacus* endopeptidase [6] in 0.01 M Tris/0.1 M NaCl pH 7.5 at 37°C for 18 h (10 μg/ml). The fragments were resolved on HPLC (conditions as in section 2.3) and analysed for the amino acid composition after hydrolysis and dansylation according to [7].

2.5. *Radioimmunoassay*

The incubation buffer for the RIA was 40 mM sodium phosphate (pH 7.4) containing 2.5 mg/ml bovine serum albumin, 6 mg/ml NaCl and 1 mg/ml sodium azide. Peptide standard or unknown sample were incubated at 4°C for 4 h with the antiserum (1:1500) in a total volume of 450 μl. After a further incubation of 4 h with the tracer (1200 cpm), 30 μl goat anti rabbit serum were added, together with 20 μl normal rabbit serum (1:500 final dilution), for precipitation. After 18 h the assay tubes were centrifuged for 30 min at 4°C at 2000 × *g* and 350 μl of the supernatant were counted in a Kontron MR 300 beta counter.

2.6. *Extraction of HA from tissues*

Tissues were homogenised in a 9-fold excess (w/w) of ice-cold methanol. After keeping this homogenate for 30 min at 60°C, a supernatant was obtained by centrifugation at 2000 × *g* for 20 min at 4°C. This supernatant was condensed to 0.5 ml and applied to a Sep-Pak C_{18} cartridge as in section 2.3. The 80% methanol fraction was condensed and either directly used for the RIA or after a further purification on HPLC.

Purification on HPLC was carried out on a semi-preparative reverse phase column (C_8, 5 μm particle size, 250 × 10 mm) with a gradient as in section 2.3. The retention time of the HA was determined with a small amount of tritiated peptide. Column fractions were evaporated and resuspended in 1 ml incubation buffer for the RIA.

Table 1

Cross-reactivity studies of head activator antiserum with head activator fragments and related peptides (Dhp: dehydroproline)

Peptide	Structure	% Immunoreactivity
1	pGlu–Pro –Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe	100
2	pGlu–Pro –Pro–Gly–Gly–Ser–Lys	50
3	pGlu–Pro –Pro–Gly	5
4	Ser–Lys–Val–Ile–Leu–Phe	10
5	Val–Ile–Leu–Phe	<0.1
6	Glu–Pro –Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe	1
7	Glu–Dhp–Dhp–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe	50
8	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	<0.1

3. RESULTS AND DISCUSSION

The sensitivity of a RIA is highly dependent on the quality of the tracer used. Since the binding efficiency of the unpurified tritiated tracer was very low (15% under the conditions in section 2.5), we decided to purify this crude tritiated product on an affinity column having the same immunoglobulins bound as used in the RIA. This was shown to be useful, since only 18% of the applied radioactivity bound to the fixed antibodies. It is obvious, that during the tritiation many side products were produced which are not recognised by the antibodies, although they elute to a great part at the same place on HPLC as the HA does. After this purification step the binding capacity in the RIA raised from 15–45%.

The production of antibodies against the HA is difficult, probably due to the weak immunogenicity of its amino acids. However, after application of the immunogenic conjugate directly into the lymphnodes one rabbit produced an antiserum which after 3 boosters reacted with tritiated HA. As found in the antiserum dilution curve, this serum 12/4 bound 45% of the tracer (1200 cpm added) at a dilution of 1:1500. This value was already obtained after 4 h and did not change with longer incubation times. A typical displacement curve with affinity purified tracer and synthetic HA is presented in fig.1. It is apparent that the assay is sensitive in 40–200 fmol range.

The specificity of the antiserum was characterised by means of carboxy-terminal and amino-terminal fragments, which have been produced by enzymatic digestion of synthetic HA either with trypsin (peptides 2 and 5) or with *Astacus* endopeptidase (peptides 3 and 4). The intact HA (peptide 1) was found to be most effective in displacing the tritiated peptide (table 1). With bradykinin (peptide 8), which has some sequence homology at the amino-terminal end with the HA, the cross-reactivity was $<0.1\%$. When carboxy- or amino-terminal ends of the HA were missing, the ability to bind to the antiserum decreased more or less drastically. Peptide 2 missing the carboxy-terminal end had a cross-reactivity of 50%, whereas peptide 4 missing the amino-terminal end was only 10% as effective as the intact HA. Further shortening of the peptide chains resulted in a more significant decrease of immunoreactivity.

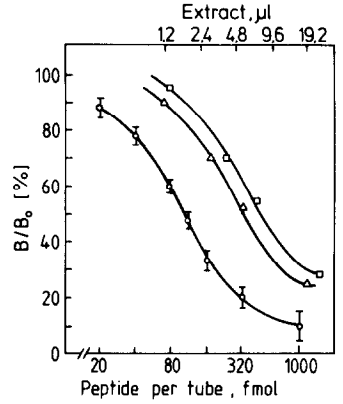

Fig.1. Comparison of the displacement curves produced by HA extracted from 4×10^4 hydras before (△) and after HPLC (□) to that of synthetic HA (○). The extracts were prepared as in section 2.6 and dissolved in 1 ml RIA buffer. Each tube contained 1200 cpm of tritiated HA and serum 12/4 at a dilution of 1:1500.

After substitution of pGlu by Glu (peptide 6), the binding capacity was $<1\%$. From these results we can conclude, that the antiserum 12/4 binds preferentially to the amino-terminal portion of the HA and less to the carboxy-terminal portion. This is not surprising since the amino-terminal end of the HA remained free during the preparation of the antigenic conjugate by the carbodiimide method so that it represents most probably the major immunogenic determinant of the peptide in this conjugate. The extremely bad displacing capacity of peptide 6 having Glu instead of pGlu at the amino-terminal end indicates the special role of pGlu for the overall structure of the HA.

As a first application of the RIA we determined the amount of HA-like immunoreactivity present in rat intestines and hydra. The preparation of HA from these tissues included methanol extraction and reverse-phase chromatography. With a 9-fold excess of methanol during the extraction most of the proteins were precipitated. After this very potent purification step the methanol soluble HA was desalted on Sep-Pak and was directly assayed in the RIA. With this extraction procedure we isolated 60 pmol immunoreactive material/rat intestine and 0.4 fmol/hydra. For a further purification we applied this material to HPLC with gradient elution. From both extracts we could detect one major peak of immunoreactivity, which ap-

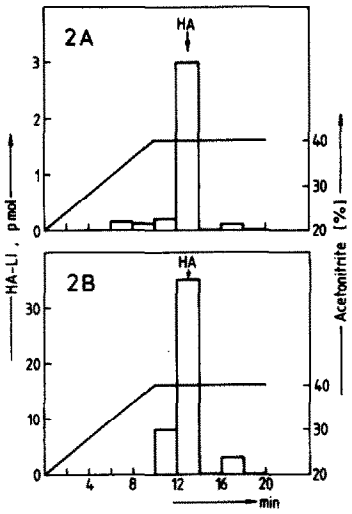

Fig.2. Reverse phase HPLC of peptide extracts of 10^4 hydras (A) and one rat intestine (B) on a C_8 column (250 × 10 mm) with acetonitrile/trifluoroacetic acid as mobile phase. The flow rate was 4 ml/min, the fraction size 8 ml. The column was calibrated with tritiated head activator (⟶). The content of head activator-like immunoreactivity (HA-LI) was measured.

peared exactly with the same retention time as the synthetic HA does (fig.2). From the hydra extract 84% of the applied immunoreactivity appeared at the HA position, from the intestinal extract 77% had the same retention time. The competition lines of these HPLC purified extracts were parallel to the HA line (fig.1, not shown for the intestinal extract). With this additional purification on HPLC we could demonstrate, that the major immunoreactivity found in the RIA with this extraction procedure was most probably due to HA as such. The nature of the residual immunoreactive products remains to be determined.

From the comparison of the biological activity of synthetic HA with the biological activity contained in one hydra we expected one hydra to contain <1 fmol. The results obtained by the RIA measuring the immuno-like reactivity indicate a good correspondence with these values from the bioassay. From the bioassay we also concluded, that one rat intestine contained 10^5–10^6 hydra equivalents of HA [8], which is also in good agreement to the results obtained by the RIA. The good correspondence of these values means that the RIA is now a reliable method for quantitative HA determinations.

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