AUTOTOMY PROMOTING FACTOR (APF) IN SOUTH WEST PACIFIC SEA STARS

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Résumé *: l'agent provoquant l'autotomie (APF) est un peptide ou une substance peptidique qui se trouve dans les fluides biologiques des étoiles de mer ébouillantées, et a été pour la première fois décelé dans une espèce du Pacifique Nord,* Pycnopodia helianthoides. *Quand l'APF est injecté à des individus non-ébouillantés, il provoque l'autotomie des bras et le ramollissement des tissus conjonctifs du corps. Dans ce travail, nous décrivons la détection et la purification partielle de l'APF de trois étoiles de mer néozélandaises de différentes familles : Sclerasterias mollis, Coscinasterias calamaria and* Astrostole scabra. *Des extraits de chaque espèce ayant subit un choc thermique sont testés sur* S. mollis, *provoquant l'autotomie complète de 1 à 5 bras sur une période de 1 à 60 minutes. Le processus d'autotomie est comparable à ce qui a été reporté pour* P. helianthoides, *mais est plus lent. Les APF des trois espèces sont croisés dans les essais. L'APF brute ou partiellement purifiée des espèces néozélandaises est un mélange complexe de peptides, protéines et d'autres molécules ; contrairement à l'APF isolée de* P. helianthoides, *celles des espèces néozélandaises sont assez instables. L'activité de l'APF est associée à une fraction de petit poids moléculaire de 1100-1200 daltons. Ceci est cohérent avec un peptide formé de 10-12 acides aminés.*

Quand L'APF des trois espèces est injectée à S. mollis, *elle provoque la ponte à la fois chez les mâles et les femelles, même lorsque l'autotomie n'a pas lieu. A cet égard, l'APF semble agir de façon synergique avec un neuropeptide beaucoup plus gros, le GSS (gonad stimulating substance) ou bien agit de la même façon que cette substance.*

Abstract *: autotomy promoting factor (APF) is a peptide or peptide-like substance found in the body fluids of scalded sea stars, and was first detected in the North Pacific species,* Pycnopodia helianthoides. *When injected into non-scalded individuals, APF elicits arm autotomy and softening of the body wall connective tissues. In this study we report the detection and partial purification of APF from three unrelated New Zealand sea stars :* Sclerasterias mollis, Coscinasterias calamaria *and* Astrostole scabra. *Heat shock extracts from all three species were bioassayed in* S. mollis, *resulting in complete autotomy of 1-5 arms over a period of 1-60 min. The autotomy process is similar to that reported for* P. helianthoides, *but occurs on a slower time scale. APF from all three species is cross reactive when bioassayed. Crude and partially purified APF from the New Zealand species is a complex mixture of peptides, proteins and other molecules ; unlike APF from* P. helianthoides, *the APF from New Zealand species is quite unstable. APF activity is associated with a low m.wt. species of 1100-1200 daltons. This is consistent with a peptide species of 10-12 amino acid residues in length.*

When injected into S. mollis, *APF from all three sources also elicits a spawning response in both male and female animals, even when autotomy does not occur. In this respect, APF appears to act synergistically with, or mimic the effects of, the much larger neuropeptide, GSS (Gonad Stimulating Substance).*

Introduction

Members of the Echinodermata are unique in their possession of collagenous connective tissues that are capable of rapid, nervously-mediated changes in their tensile strength (for a review see Wilkie, 1984), which are involved in many aspects of echinoderm biology the

most striking of which is autotomy or self-mutilation. When the tensile strength of key collagenous tissues decreases rapidly and irreversibly, the result can be arm autotomy (in asteroids and ophiuroids), evisceration (in holothurians), or fission, in those species of sea star which undergo asexual proliferation by binary fission.

Many sea stars will rapidly autotomise arms in response to disturbance or damage, and autotomy can be induced by rough handling, exposure to air, wounding, ligation, amputation of the tube feet, electrical shock and chemical stimuli (Emson and Wilkie, 1980).

Little is known about the biochemical basis for autotomy, and few workers have studied the subject since Chaet (1962) provided the first evidence that endogenous chemical factors promote autotomy in sea stars. During work on invertebrate thermal toxins, it was found that *Asterias forbesi* would autotomise arms when injected with the fluid exuded by scalded animals, but not when injected with fluid from non-scalded donors. The factor responsible was a heat-stable, dialysable molecule derived from the lining of the coelomic cavity. Mladenov *et al.* (1989) worked with the large western North American sea star, *Pycnopodia helianthoides* and reported a partial purification of APF from this source as a heat-labile, water-soluble substance derived from the body wall. On the basis of its susceptibility to inactivation by proteases, and its elution on gel permeation columns, these workers proposed that APF is a peptide with an M_+ of about 1200 daltons.

In this paper we report the first detection and partial purification of APF from three unrelated New Zealand sea star species : *Sclerasterias mollis, Coscinasterias calamaria,* and *Astrostole scabra.*

Materials and methods

Animals : *S. mollis* and *C. calamaria* were obtained by dredging from the R/V "Munida", from a depth of 60 m off the Otago Peninsula (vicinity 170°50'E, 45°50'S) and *A. scabra* was obtained by diving (5-10 m) from the rocky coastline at Karitane (170°38'E, 45°38'S) and maintained in holding tanks with continueous running seawater (aanual temp. range 5-18°C).

Crude extracts : crude extracts were prepared by scalding the donor animal (76°C, 4 min) in a polythene bag, cooling on ice (10 min) and collecting the fluid which leaches from the animal through autotomised arms and tears in the body wall. The crude extract was centrifuged to clarify it (4000 x g, 10 min, 4°C). This yields a clear, straw to pale yellow liquid, pH 6.7-7.2, with a protein content ranging from 3-4 mg/ml (*S. mollis*/*C. calamaria*) to 6-9 mg/ml for *A. scabra.* The volume of extract ranges from 15-150 mls, depending on the size of the donor animal.

Protein : protein was assayed using the Bicinchoninic acid (BCA) method of Smith *et al.* (1985).

Bioassay : currently there is no effective *in vitro* assay for APF and APF activity was detected by bioassaying live sea stars. Crude and partially purified extracts were injected into experimental animals as described by Mladenov *et al.* (1989) and the onset of autotomy observed and timed. The criterion for a positive bioassay was defined as the loss of one or more arms within two hours of injection. After assay, the sea stars were returned to a recovery tank, and both autotomising and control animals had fully recovered body tone and mobility within 4 hr.

Ultrafiltration : ultrafiltration was carried out at 4°C using a stirred cell of 80 ml capacity. This allowed volume reduction/concentration of the order of 15-fold.43 mm dia ultrafilters of 5000, 1000, and 500 NMWCO were used in a sequential batchwise process.

Gel permeation chromatography : chromatography on Sephadex G-25F and G-50F columns (80cm x 1cm) was carried out on an Atto LPLC system equipped with a UV detector (280/254nm). The solvent was 0.05M NaCl, pumped at al flow rate of 10ml/hr. The columns were calibrated with Blue Dextran (2 x 10^6), Carbonic Anhydrase (31,000), Myoglobin

(17,200), Lysozyme (14,300), Cytochrome c (10,800), Aprotinin (6,500), Bacitracin (1,400) and FMN (450).

Reverse Phase HPLC : reverse Phase HPLC was carried out on a Shimadzu LC-6AD liquid chromatograph equipped with a SPD-6AV UV/vis detector, a RF-535 fluorescence detector and an SIL-9A autoinjector. The column used was a Shimadzu Shimpack CLC-ODS (5µm, C18 bonded phase, 15 cm x 6 mm) and the solvent system was aqueous TFA-CH_3CN at 1.0 ml/min.

Results :

Unlike *P. helianthoides*, where complete autotomy was achieved in less than one minute (Mladenov*et al.*, 1989), the New Zealand species are an order of magnitude slower in reacting, taking on average 15-30 minutes to achieve autotomy. Fig. 1 shows a time course of rapid APF-induced autotomy in *S. mollis*. In 1B, after one minute, the animal at upper left has autotomised one arm, which remains attached to the disc by the pyloric duct, and in 1C, after two minutes is completely separated from the animal and moving away accross the plate. After 15 minutes, the second experimental animal has autotomised one arm (1D), which has completely separated by 26 minutes (1E) and two arms have autotomised by 56 minutes (1F). The lower animal was the control, injected with sea water. Fig. 2 shows a similar, but slower onset, time course where the experimental animal autotomised all five arms within 40 minutes of injection with crude APF.

Fig. 3 demonstrates species cross-reactivity of APF from *A. scabra* and *C. calamaria* bioassayed in *S. mollis* : 3A and 3B show the effect of *A. scabra* crude APF after 18 and 30 min., 3C and 3D the effect of *C. calamaria* crude extract after 33 and 40 min. respectively. All three sepcies are cross reactive to one another (data not shown).

Fig. 4 shows the purification regimes attempted so far, which have resulted in only a partial purification of crude APF, and this accompanied by a considerable loss of bioactivity.

Analysis of both crude (Fig. 5) and partially purified (Fig. 6) APF indicated an extremely complex mixture of proteins and peptides which, although altered in profile by serial ultrafiltration (Fig. 6), in not markedly reduced in complexity. APF activity is associated with an early eluting peak (approx. 10% CH_3CN), indicative of a polar molecule.

Conventional (Fig. 7) and HPLC (Fig. 8) gel permeation chromatography confirms the previous work of Mladenov *et al.* (1989) that APF activity is associated with a small molecule of M_r about 1000 daltons. The most active fractions derived from HP-GPC on a Phenomenex SEC-2000 column calibrated with a range of proteins and peptides M_r 66,000—>555 corresponded to a species with an M_r of 1100 daltons, which is in good agreement with the previous estimate of 1200 daltons (Mladenov *et al.*, 1989).

Discussion

APF is now clearly established in both Northern and Southern hemisphere sea-star species, and appears to have very similar physical and physiological properties regardless of source.

Given that many physiological lines of evidence implicate the nervous system in the control of echinoderm connective tissues (Motokawa, 1988 ; Wilkie, 1988), it is not unreasonable to postulate that APF may well be a neurochemical factor, probably a neuropeptide. There is still a dearth of information on echinoderm neurochemicals, and neuropeptides in particular, with only one-gonad stimulating substance (GSS) being thoroughly studies (Kantani, 1979) and partially sequenced (Shirai *et al.*, 1987) ; whilst FMRFamide-like activity has been detected recently in *Asterias rubens* (Elphick *et al.*, 1989a) though as yet the role of this compound in echinoderms has not been determined, and the same group has sequenced three novel peptides from the radial nerves of the same species (Elphick *et al.*, 1989b) whose role is unknown.

The complete purification and structural elucidation of APF will be one of the first steps required in understanding the biochemical and physiological basis of autotomy in its various forms.

Note added in proof

Preliminary experiments indicate that unlike the APF from *A. forbesi* (Chaet, 1962) the APF from the three New Zealand species described has an autotomy (evisceration) effect when injected into specimens of the holothurian *Stichopus mollis*, indicating that the effects of APF may not be confined to the Asteroidea.

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Figure 1 : **Time course of APF-induced autotomy in *S. mollis*.**
The experimental animals (upper left and upper right) were injected with crude *S. mollis* APF (1ml/arm) at zero time. The lower animal was the control, injected with seawater at zero time. Panel A : zero time, Panel B : 59 seconds after injection. Panel C : 2 minutes. Panel D : 15 minutes. Panel E : 26 minutes. Panel F : 56 minutes.

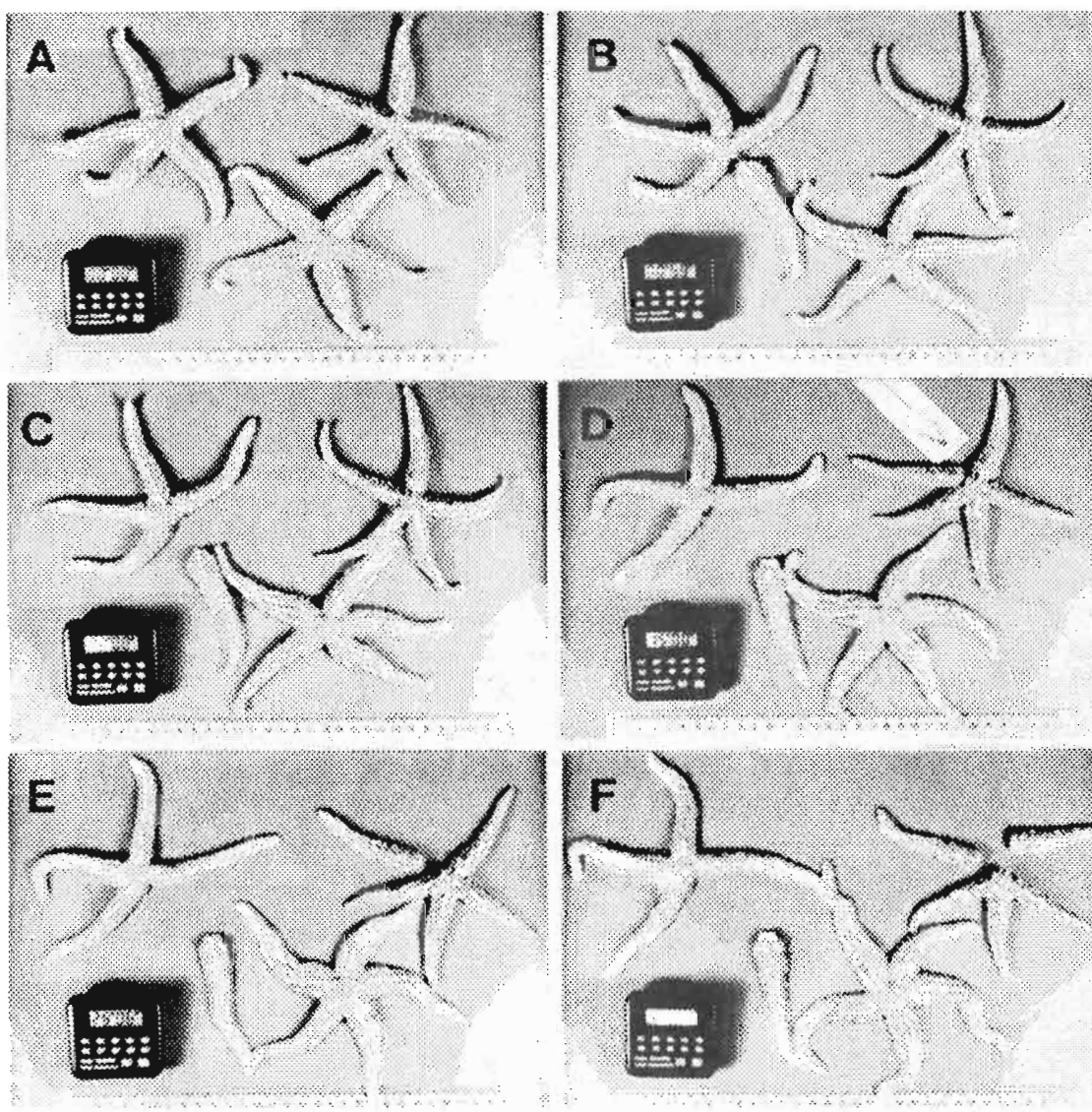


Figure 2 : **Time course showing APF-induced total autotomy in *S. mollis*.**
Experimental animal right, control on left. Panel A : T=C. Panel B : T=26.5min. Panel C : T=29 min. Panel D: T=34 min. Panel E : T=39 min. Panel F : T=41 min. Experimental details as for Figure 1.

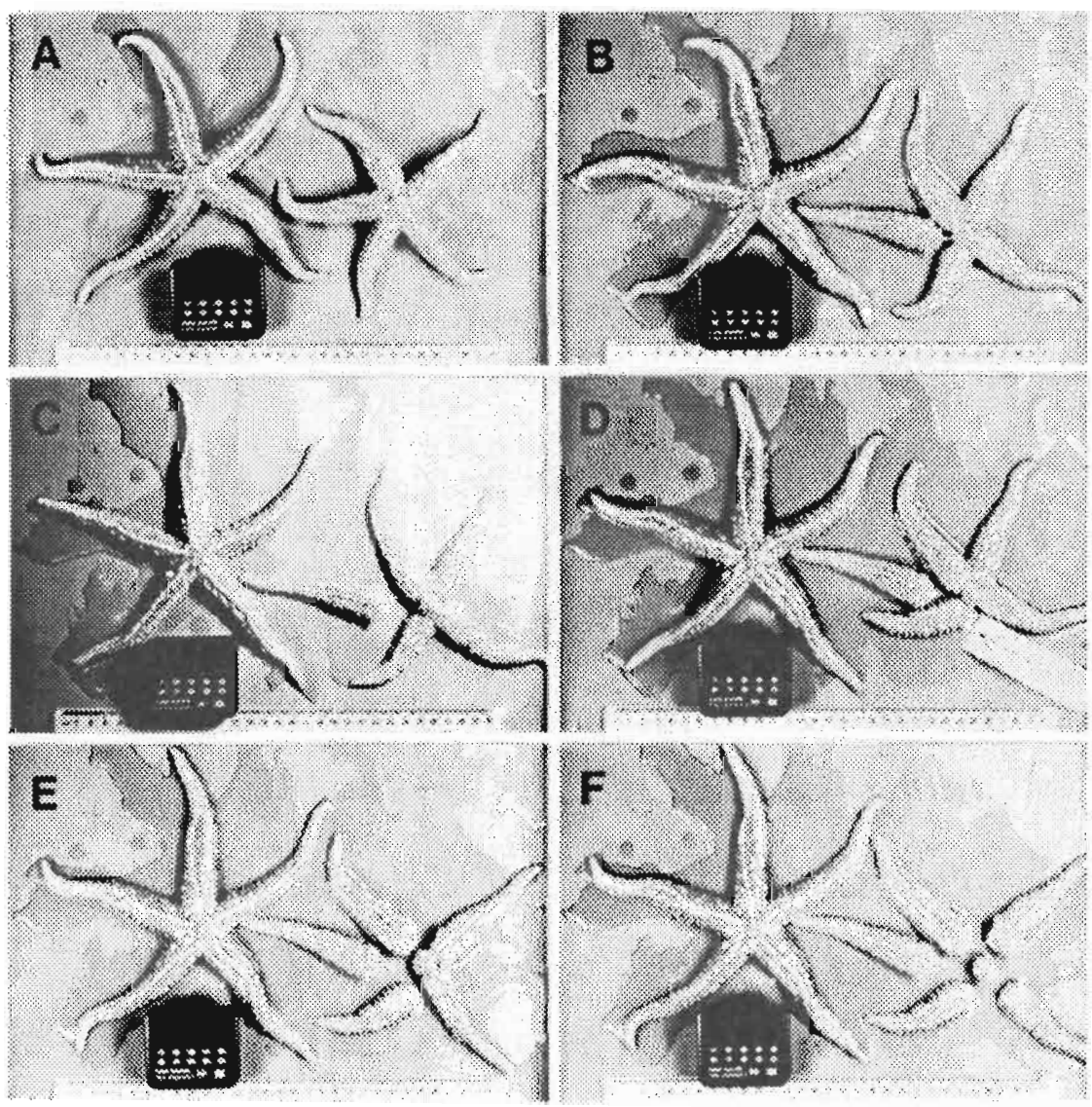


Figure 3 : **Species crossreactivity of APF.**
Panels A&B : *A. scabra* APF bioassayed in *S. mollis* ; 18.5 and 30 min. after injection.
Panels C&D : *C. calamaria* APF bioassayed in *S. mollis* ; 33 and 40 min. after injection. Conditions as for Figures 1 & 2.

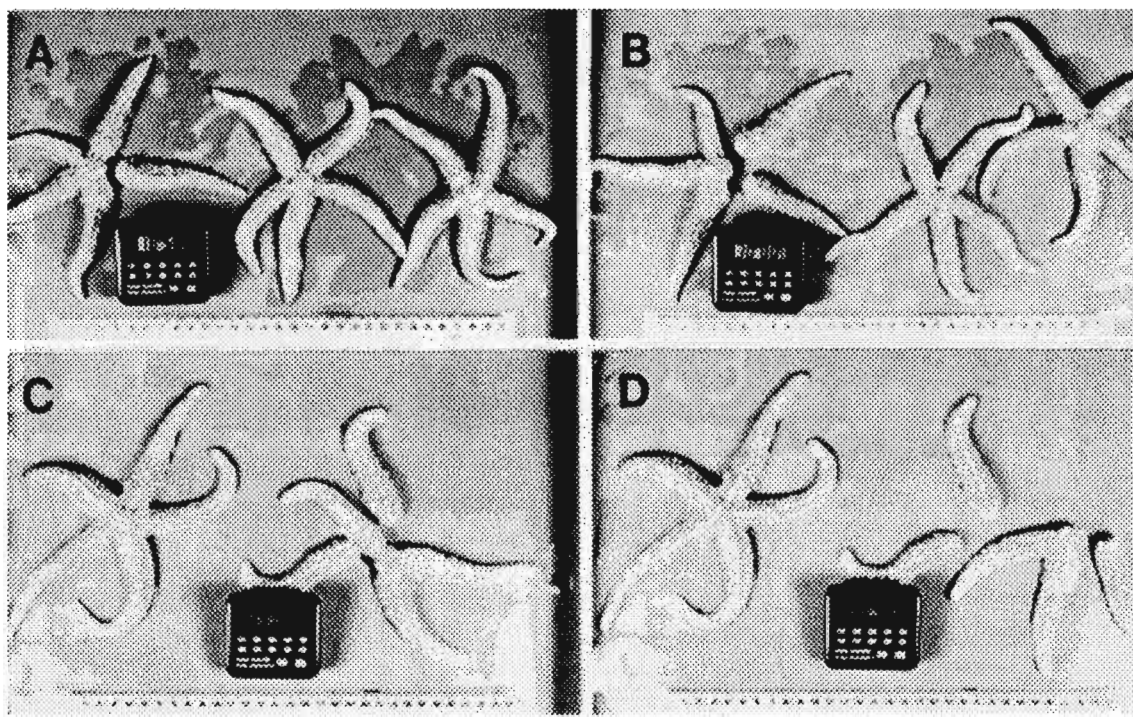


Figure 4 : Preparation and processing of APF

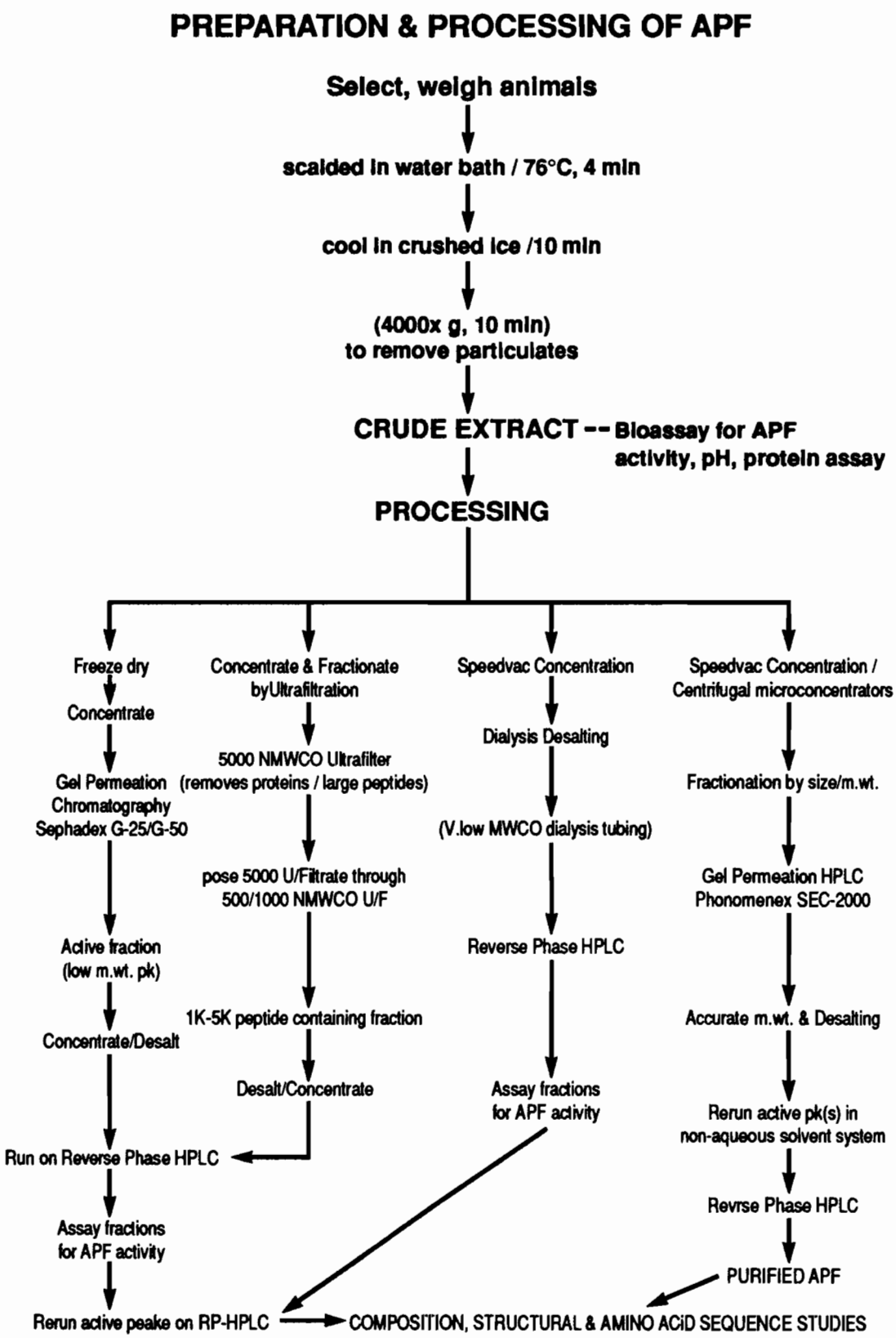


Figure 5 : Reverse phase HPLC fractionation of desalted, crude APF ex *S. Mollis*

Column : Shim-pack CLC-ODS (C_{18}), 6 mm x 15 cm, 5 µ
Flow : 1 ml/mn
Detector : UV, 220 nm
Gradient : 0-50% CH_3CN in 0.1% aq. TFA over 70 in
Sample : 1ml prep load (5mg) of *S. mollis* crude extract, desalted thru 1000 NMWCO Ultrafilter

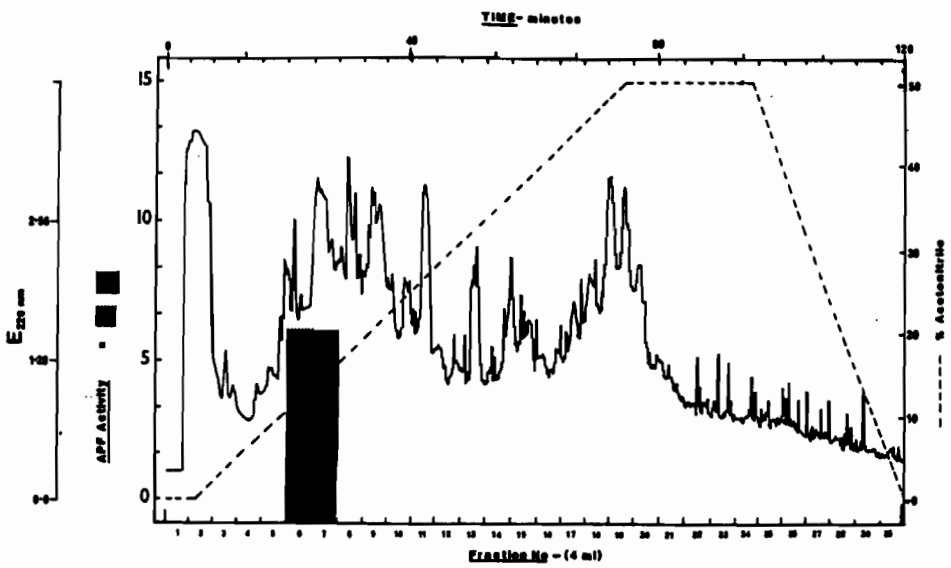


Figure 6 : Reverse phase HPLC fractionation of 1K-5K ultrafiltrate fraction

Column : Shim-pack ODS
Flow : 1 ml/mn
Detector : UV, 220 nm
Gradient : 0-50% CH_3CN 5-75 min, 50% CH_3CN 75-95 min, 50% - 0% CH_3CN 95 - 120 min
Solvent : 0.1% TFA
Sample : 1ml prep load (5mg) of *S. mollis* crude extract, desalted thru 1000 NMWCO Ultrafilter

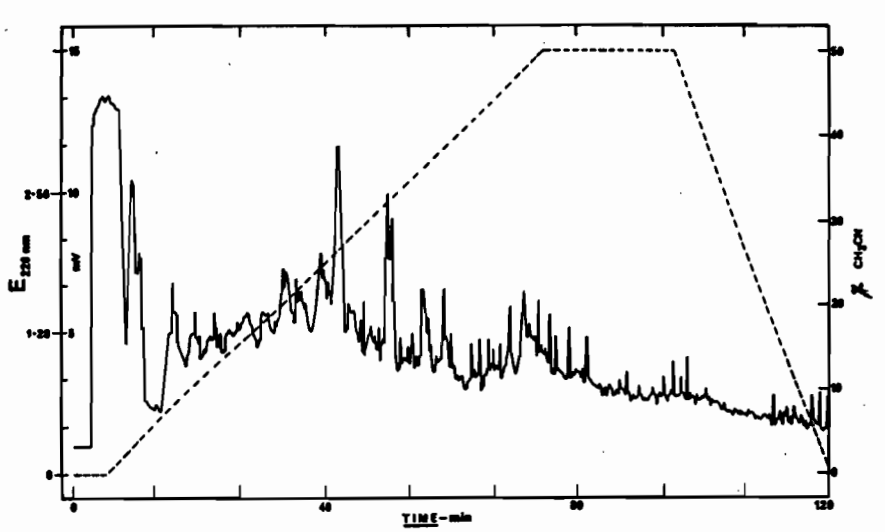


Figure 7 : Fractionation of Sea star crude extracts by gel filtration chromatography

Column : Sephadex G-50F, 80 cm x 1 cm
Flow : 8 ml/hr
Buffer : 0.05M Na Cl, pH 6.7
Sample : 7 - 20 mg (protein) crude extract
A : *S. mollis*, 7mg ; B : *C. calamaria*, 6-5mg ; C : *A. scabra*, 21mg

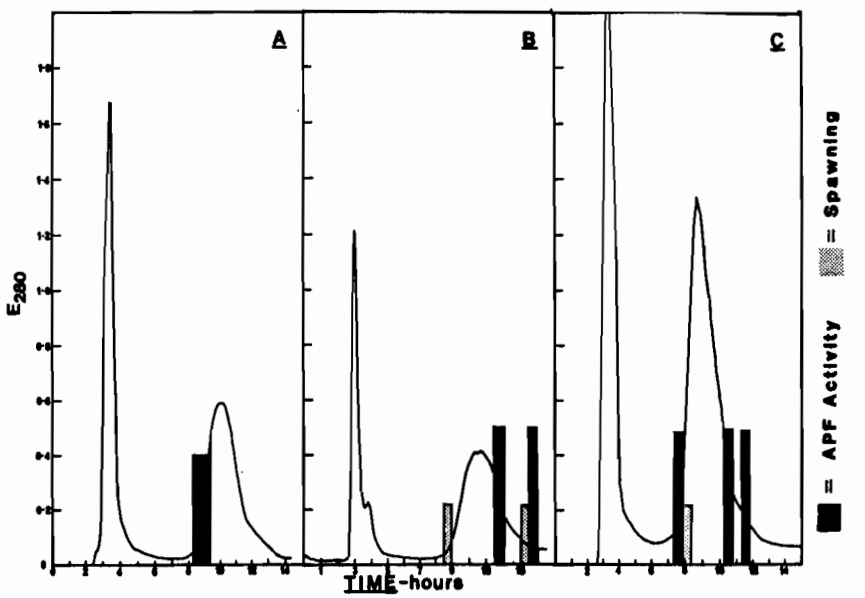
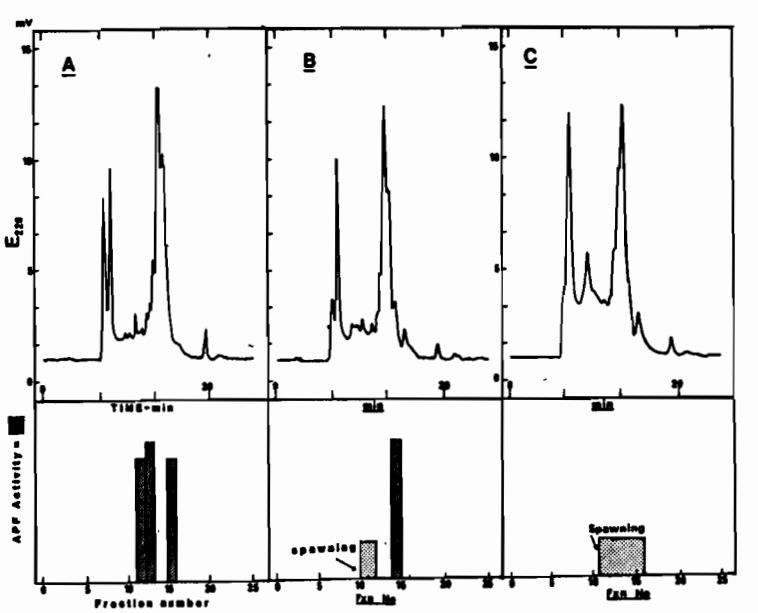


Figure 8 : Size exclusion HPLC of heat-shocked Sea star extracts

Column : PhenomenexSEC 2000
Flow : 1 ml/min
Buffer : 0.1M Phosphate, pH 6.8
A : *S. mollis*, 7mg ; B : *C. calamaria*, 6-5mg ; C : *A. scabra*, 21mg



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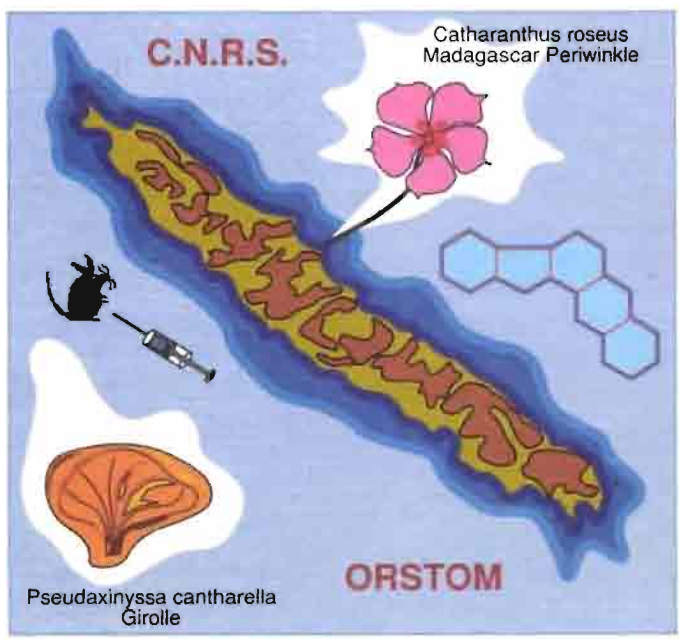

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