Molecular Ecology and Biotechnology

I.A.M.C.-CNR di Capo Granitola

Procedures for high quality RNA extraction from *Paracentrotus lividus* (LAMARCK, 1816) embryos and gonadal tissue

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1. Introduction

1.1 CISAS project

The extraction protocol described below is part of the CISAS project, aimed at the realization, within CNR, of an "International Centre of advanced study in environment, ecosystem and human health".
The project aims at investigating environmental pollution and its connection with the ecosystem and human health, starting from a significant number of case studies (SIN's of Augusta, Milazzo and Crotone), true natural laboratories suitable for modern multidisciplinary investigation.
The primary objective of this project is to understand processes and mechanisms for the transfer of conventional (heavy metals, POPs, radionuclides, etc.) and emerging contaminants (eg., PBDE, antibiotics, pharmaceutical compounds of new generation, antitumorals intensively used in the studied areas and discharged at sea generally without strict control, etc.) from the environment (as a complex of atmosphere, soil, sediment, inland waters and sea matrices) to ecosystem and to humans using an integrated analysis of the biochemical and biophysical mechanisms, determining and regulating the interactions between pollutants, ecosystem (overall and at different species/populations levels) and human health.
The most important scientific and technological impacts of this project are: knowledge improvement on the stability and dynamics of traditional and emerging pollutants in all the environmental compartments (terrestrial and marine); development of dispersal models of contaminants in the different environmental compartments and estimate of inventories at small and large scale; improvement of knowledge about transfer mechanisms within the marine and terrestrial ecosystems; development of understanding about mechanisms and interactions at biochemical and molecular level of contaminants in model species (terrestrial and marine); improvement of knowledge on ecotoxicology and ecotoxicodynamics of selected groups of pollutants; development of epidemiological knowledge in the studied areas; model species with ecotoxicological approaches focused on early-warning approaches; improvement of knowledge about mechanisms of transfer of contaminants from the environment to humans by food, drinking, breathing; building of integrated and innovative systems for monitoring in real time all the environmental matrices; development of conceptual models oriented to a modern management of landscapes and ecosystems.

1.2 Ecosystem and contaminants WP3

The project is organised around six work packages (WP) closely interlinked and strongly interconnected; in particular, the group "Molecular Ecology and Biotechnology" has carried out his activities within Work Package 3 (WP3), that is focused on pollutants toxicity and related molecular response mechanisms in marine ecosystem.
Biological and toxicological responses to different contaminants, including emerging ones, are investigated in the three study areas (Priolo-Augusta, Crotone, Milazzo), with the aim to highlight possible relationships with human diseases.
In order to analyse toxic effects at various levels of biological organization, a systemic ecotoxicologycal approach was employed. Additionally, cellular mechanisms involved in pollutants toxicity are characterized, so as to provide information useful for natural ecosystems protection and management.
In particular, it was evaluated the alteration of transcriptional expression of genes in selected marine model organisms (as for example *Paracentrotus lividus, Danio rerio*, *Mytilus galloprovincialis, etc.)* associated to chemical "defensome" and the transcriptome and epigenetic modifications in response to selected pollutants.

1.3 The use of model organisms to unveil novel toxicity mechanisms

The possible mechanisms involved in emergent diseases are investigated in model systems (i.e. *Paracentrotus lividus, Dicentrarchus labrax, Octopus vulgaris,* etc.) [1- 4]. The strategy is based on the widely recognized and accepted knowledge of the evolutionary conservation of the most molecular mechanisms involved in basal functions, in both embryonal and adult tissues (like stress response, cell cycle regulation, embryo development and morphogenesis, etc.) [5]. Moreover, the overlapping between many molecular mechanisms involved in tumoral and endocrine diseases and development/differentiation is well known. Thus, embryonal/larval stages of selected species (easy to manage and manipulate, and well characterized at molecular level) are well suitable both for the preliminary detection of phenotypical effects (i.e. teratogenicity, etc.) [6] of the exposure to pollutants and for further analyses focused to unveil molecular mechanisms involved [7, 8]. Furthermore, transcriptomic analyses (carried out by means of Next Generation Sequencing) are provide global transcriptional profiles, suitable to detect variations involving any RNA species, including ncRNAs (non-coding RNA), induced by environmental pollutant found in the three study areas [9, 10].
To carry out these analyses it is necessary to proceed with the extraction of the total RNA from samples used for the purpose; in particular, in this report we describe a RNA extraction protocol from *Paracentrotus lividus* embryos and gonadal tissue.

2. Sea urchin as a model organism

Echinoids have been considered ideal models for monitoring marine environmental hazards [11], because they are often key herbivore species, having a major role in structuring and controlling macroalgal assemblages, thereby shaping the benthic seascape, and also playing an important role in coastal food webs throughout the world [12–16]. They have traditionally been used as model organisms to study reproduction and early cell differentiation, sperm-oocyte interactions and apoptosis [17–19]. These organisms have been also proposed as valuable bioindicators for detecting environmental perturbations [20-23].
Among the echinoderms, the sea urchin *Paracentrotus lividus* is considered a suitable organism to study the ecotoxicological responses to xenobiotics and the physiological reactions to physical stressors [24–29].
The species represents a useful test organism for several reasons: it is an important component of benthic communities in the Mediterranean Sea and Atlantic Ocean; extraction and maintenance of gametes are easy; the embryos grow rapidly and synchronously (pluteus stage is reached 48 hours post-fertilization) and embryos are transparent and suitable for microscope detection of sub-lethal effects of pollutants on development; they have a sufficiently long reproductive season (from October to May).

3. Materials and methods

RNA extraction protocol has been applied to gonadal tissue of adult sea urchins exposed in mesocosm to different amounts of selected pollutants as well as their mixtures, or exposed to contaminated sea water from each study areas or from adult sea urchins collected in control areas and transferred in each study area (SIN's of Augusta, Milazzo and Crotone) in order to assess their biological effects and validate data obtained in mesocosm experiments.
Sea urchins were weighted and dissected immediately after collection to establish the initial mean gonadal indices and to discriminate and separate male and female gonads. The gonads samples were taken with a plastic spoon (Fig. 1 and 2) and directly put into microcentrifuge tube to carry out extraction total RNA.
The biological effects of different amounts of specifics pollutants were also evaluated on *Paracentrotus lividus* embryos at different developmental stages; in particular, the analysis were carried out both on embryos derived from adult sea urchin exposed or not to pollutants and on embryos exposed directly to pollutants. The embryos were collected at the blastula/gastrula (18 h – 24 h) stages and from these total RNA was extracted according to the following extraction protocol.

***Figure* 1**

***Figure* 2**

3.1 RNA extraction protocol

In this protocol, total RNA is isolated from embryos and gonadal tissue by organic extraction with the phenolic TRIzol reagent (Invitrogen™), a monophasic solution of phenol, guanidine isothiocyanate and other components which allows for simultaneous processing of a large number of samples, and is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi [30], and chloroform.
When working with RNA it is essential to avoid contamination with RNase, therefore it is necessary use RNase-free virgin plastic ware, RNase-free solutions, don't handle tubes or reagents with ungloved hands and use filter tips.

The manufacturer's instructions recommend 1 mL of TRIzol Reagent for 50 mg of tissue. The procedure carried out were modified from the manufacturer's instructions since we experienced that better results were obtained using TRIzol at ratio 1.5/2: 1 sample. This procedure is carried out when adipose tissues are processed such as gonadal tissue).

3.2 Lyse samples and separate phases

The following protocol is written for 50 mg of gonadal tissue.
The steps of RNA extraction protocol from *Paracentrotus lividus* embryos are similar to those described below except for the first step where the volume of TRIzol Reagent added was equal to the starting sample volume (100 µl of TRIzol for a volume of 100 µl of embryos); consequently, the volume of chloroform and isopropanol added changes according to the volume of TRIzol Reagent used for lysis.

1) To 50 mg of gonadal tissue in a 1,5 mL microcentrifuge tube add 1,5 mL of TRIzol reagent and shake the tube 5 times;
2) At this point proceed to homogenize with a micro-syringe by aspirating and rejecting the sample several times inside the tube, in order to lyse completely the tissue. *(Note: at this step the sample must be completely homogeneous and clear. The samples can be stored at 4°C overnight or at – 20°C for up to a year*)
3) If samples have a high fat content, like in this case, centrifuge the lysate for 2 minutes at 12,000 × *g* at 4–10°C.
4) Incubate at room temperature for 5 minutes to permit complete dissociation of the nucleoproteins complex
5) Add 0.3 mL of chloroform per 1, 5 mL of TRIzol Reagent used for lysis and shake vigorously by hand
6) Incubate for 2–3 minutes and centrifuge the sample for 15 minutes at 12,000 × *g* at 4°C
7) After the centrifugation mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase
8) Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out *(Note: in this step avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase*)
9) Proceed directly to isolate RNA

3.3 Isolate RNA

10) Add 0.75 mL of isopropanol to the aqueous phase per 1, 5 mL of TRIzol Reagent used for lysis
11) Incubate at room temperature for 10 minutes and centrifuge at 12,000 *x g* for 10 minutes at 4°C; in this way total RNA precipitate forms a white gel-like pellet at the bottom of the tube
12) Remove the supernatant with a micropipette and wash the pellet with 1- 1.5 mLof 75% ethanol *(Note: at this step the The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C)*
13) Vortex the sample briebly, then centrifuge for 5 minutes at 7500 × *g* at 4°C
14) Remove the supernatant with a micropipette and than centrifuge briefly and carefully remove the last of the supernatant with a micropipette

15) Air dry the pellet for 10 minutes and resuspend the pellet in 100 µl RNase-free water by pipetting up and down *(Note: the volume of RNase-free water depends on the size of the pellet)*

16) Finally, the amount of total RNA extracted was estimated using the appropriate Qubit RNA assay kit and the samples stored at -80°C for further assays.

For each sample, the total RNA pattern was checked by non-denaturing agarose gel electrophoresis. The results are shown in figure 3. All tissue samples revealed good band patterns (the ratio of the 28S to the 18S rRNA band), which indicates that less degradation of RNA occurred during the extraction process and the quality of RNA obtained was good.

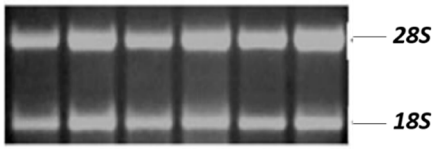

Figure 3: Gel electrophoresis of total P. lividus RNA purified from different samples

Acknowledgments

- Project: “International Centre of advanced study in environment, ecosystem and human health CISAS”. CUP: B62F15001070005

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