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A New Method for Rapid DNA Extraction from *Artemia* (Branchiopoda, Crustacea)

R. Manaffar, R. Maleki, S. Zare, N. Agh, S. Soltanian, B. Sehatnia,
P. Sorgeloos, P. Bossier and G. Van Stappen

Abstract*—Artemia* is one of the most conspicuous invertebrates associated with aquaculture. It can be considered as a model organism, offering numerous advantages for comprehensive and multidisciplinary studies using morphologic or molecular methods. Since DNA extraction is an important step of any molecular experiment, a new and a rapid method of DNA extraction from adult *Artemia* was described in this study. Besides, the efficiency of this technique was compared with two widely used alternative techniques, namely Chelex® 100 resin and SDS-chloroform methods. Data analysis revealed that the new method is the easiest and the most cost effective method among the other methods which allows a quick and efficient extraction of DNA from the adult animal.

***Keywords*—**APD, *Artemia*, DNA extraction, Molecular experiments

I. INTRODUCTION

IT has been years that the brine shrimp *Artemia*, mainly because of its versatile use and its nutritional value, is considered as one of the most widely used live foods in larviculture [1]. In addition this animal is also a known model for genetic and evolutionary studies [2]. Molecular experiments on *Artemia*, not only as scientific approaches for evolutionary analysis, but also as tools in the commercial industry of *Artemia* hold promise to be widely used methods [3]-[4].The isolation and purification of DNA from biological samples for subsequent molecular analysis is generally the most important and undervalued step in many biological and biomedical applications [5]. DNA extraction comprises three basic steps: The first step is cell breaking, commonly referred to as cell disruption, in order to expose the DNA within, using different methods such as grinding, sonicating, thermal shock or chemical reagents.

R. Manaffar, Author is with Artemia and Aquatic Animals Research Institute, Urmia University, Iran. (Corresponding author, phone: 984413440295, P. O. Box 165, Raminmanaffar@gmail.com

R. Maleki, is with Food and Chemical Analysis Research Laboratory, Jahad-e-Daneshgahi, Urmia University, Iran.

S. Zare, is with Department of Biology, Faculty of Science, Urmia University, PO Box 165, Urmia, Iran

N. Agh, is with Artemia and Aquatic Animals Research Institute, Urmia University, Iran.

S. Soltanian, is with Aquatic Animals Health & Disease Department, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

B. Sehatnia, is with Artemia and Aquatic Animals Research Institute, Urmia University, Iran.

P. Sorgeloos, is with Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, B-9000 Gent, Belgium.

P. Bossier, is with Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, B-9000 Gent, Belgium.

G. Van Stappen, is with Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Rozier 44, B-9000 Gent, Belgium

The second step is removing membrane lipids by adding a detergent. The last step is the precipitation of DNA by an alcohol, usually ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salts, PCR inhibitors (e.g. enzymes or ions). A refinement of the technique includes adding a chelating agent to sequester divalent cations such as Mg^{2+} and Ca^{2+}. This stops DNAase enzymes for further DNA degradation. Moreover, cellular and histone proteins that are bound to DNA can be removed prior to its precipitation, for instance either by adding protease or sodium and ammonium acetate. The objective of these procedures is to achieve a yield of the highest purity as quick as possible.

Contamination during DNA extraction mainly due to the repetition of sample transferring between tubes is the main defect of conventional methods. Therefore it is necessary to set separate vials for each individual sample and to apply a long extraction protocol. Although the traditional DNA purification methods such as organic extraction and chelex extraction have demonstrated good ability to remove the interfering contaminants, they are not amenable to the trends in genetic analysis towards miniaturization and automation because most of these purification procedures require a large amount of DNA samples, reagent volumes, multiple steps and time-consuming labour [6]. In this paper, a new surfactant (Ammonium Pyrrolidin Dithiocarbamate, APD complex) was developed for effective DNA extraction under an easy protocol.

Elimination of materials by surfactants is one of the most favorable methods of purification, due to their excellent binding ability for a variety of biomolecules and ions. Chelating resins or surfactants have been used in ion-exchange columns, trace metal removal, metal analysis and water testing in environmental and agricultural laboratories. In clinical applications and biomedical research, these materials can be used to remove or assay cations in whole blood or urine, to remove contaminants from buffers and stock solutions, and to prepare samples for nuclear magnetic resonance spectroscopy. Recently the solid phases modified with immobilized organic compounds have been attracting great interest because of the high enrichment capacity and operational simplicity for solid phase extraction of metal ions [7]-[8]. It is well understood that, in an acidic medium, sodium dodecyl sulfate (SDS) as an anionic surfactant is sorbent on the alumina to form surfactant aggregates. In fact, hydrophobic chelating agents can

incorporate into the immobilized micelles of alumina. Therefore, it is possible that a typical complex agent is immobilized on SDS-coated alumina phase. This system is used for separation or pre-concentration of various cations which can be combined with an immobilized hydrophobic agent. Surfactant-coated alumina modified with various organic compounds has been used for the pre-concentration of trace amounts of some heavy metals from aqueous samples [9]. In the present study, SDS aggregates were formed on alumina surface by columbic attraction. Then, Ammonium Pyrrolidin Dithiocarbamate (APD) (Fig.1) was immobilized on SDS-coated alumina. Finally, the produced modified alumina was used for removing the interfering metal ions in the DNA extraction and electrophoresis process. It is important to note that APD has been used as spectrophotometric reagent for quantitative determination of heavy metal ions [10]-[12].

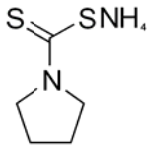

Fig. 1 Structure of Ammonium Pyrrolidin Dithiocarbamate (APD)

II. MATERIAL AND METHOD

A. Sample preparation

The DNA was extracted using four different methods: APD complex (two different incubation conditions), SDS-chloroform and Chelex® 100 resin. Hence fresh laboratory cultured individuals of adult *Artemia urmiana* Günther 1899, were washed with tap water and rinsed with distilled water to remove all debris. Totally, 24 *Artemia* individuals (including six individuals for each method) were randomly collected for each method of DNA extraction. In all methods, the extraction was carried out according to the protocol which has been described in literature. The extraction of DNA by APD complex was followed out by two different incubation periods and temperatures (more detailed in DNA extraction section).

B. DNA Extraction

1. DNA extraction by APD complex

To perform the APD extraction, the extraction complex was prepared beforehand. Purified alumina particles (Y-type) weighing 1.5 g were suspended in 75 ml of distilled water and mixed with 80 mg of SDS. Then, 10 mg dithiocarbamate was dissolved in 25 ml distilled water containing 20 mg of SDS and this solution was added to the prepared alumina-SDS suspension. Subsequently, the pH was adjusted to 5-6 using HCl or NaOH. Afterwards, 200 µl of this suspension was transferred to a 2 ml vial that already contained 1 fresh *Artemia*, and 14 µl of proteinase K solution (20 mg/ml) was added to the suspension. In order to accelerate the protein-stripping process, the tubes were mixed well. From this step onward the samples were treated at two different temperatures (55°or 65°C, respectively the ADP_1 and ADP_2 method) and incubation time (1 hour or 2 hour). Hence, six *Artemia* individuals were separately treated in each incubation group, to evaluate the efficiency of the ADP method itself. In both groups, the vials were shaken by vortex every 10 min. The matrix solution was boiled for 10 min. After that, the tubes were chilled out on ice and centrifuged at 2700 g for 15 min. The supernatant was carefully transferred to a new tube. The DNA was precipitated by 100% EtOH at -20°C for 1 hour. at the ratio of 1:2 respectively. The pellet was dissolved in 25 µl of distilled water or TE buffer (pH 8.0).

2. DNA extraction by SDS-chloroform

The extraction of DNA in this method was carried out according to Sambrook [13]. Hence 800 µl of SDS buffer (tris-HCl 10mM, EDTA 0.5mM, NaCl 75mM and SDS 0.5%) and 10 µl of proteinase K were added to vials containing one *Artemia* adult. The vials were incubated in a water bath at 55-60°C for 60 min (intermittently vortexing every 10 min) and centrifuged at 2700 g for 30 min. The aqueous phase was taken and an equal volume of saturated phenol (pH=7.4) was added and subsequently centrifuged at 2700 g for 15 min. The aqueous phase was transferred into a new tube. Subsequently a phenol and chloroform-isoamyl alcohol solution was added to the tube up to half the volume of the supernatant. The complex of chloroform-isoamyl alcohol had already been prepared by mixing 24 volumes of chloroform with 1 volume of isoamyl alcohol. The vials were centrifuged at 2700 g for 15 min. The supernatant was transferred to a new tube and was mixed with an equal volume of chloroform-isoamyl alcohol complex. All samples were centrifuged at 2700 g for 15 min. Twofold EtOH 100% (-20°C) was added to each vial and stored for 1 hour at -20°C. Then, the samples were centrifuged at 20000 g for 15 min. Subsequently, the EtOH was poured off and the DNA pellet was dried at 37°C in an incubator for 2 hours. Finally, the pellet was dissolved in 25 µl of distilled water or TAE buffer (pH 8.0) and stored at -20°C [13].

3. DNA extraction by Chelex® 100 resin

The DNA in this method was extracted according to Walsh and Estoup [14]-[15]. Therefore 200 µl of Chelex® 100 6% and 14 µl of proteinase K (20 mg/ml) were poured in 1.5ml vials each containing one *Artemia* individual. The samples were incubated at 55°C for 2 hours with intermittent vortexing every 10 min. Then, the vials were boiled for 10 min and subsequently cooled down for 30 min at room temperature (25°C). The supernatant was directly used for PCR for quality and quantity assessment.

C. Determination of DNA concentration and purity

In order to determine the concentration and purity of the extracted DNA, the absorbance of UV light was measured using a spectrophotometer for all samples. Routinely, measuring the intensity of absorbance of the DNA solution at wavelengths of 260 nm and 280 nm is used as an indicator of DNA purity. In fact, DNA absorbs UV light at 260 and 280 nm, while aromatic proteins absorb UV light at 280 nm. Normally, a pure sample of DNA has the 260/280 ratio at 1.8 and is relatively free from protein contamination, whereas

DNA samples contaminated with protein have a 260/280 absorbance ratio lower than 1.8. The extracted DNA was diluted in double distilled water and the absorbance was measured spectrophotometrically at both 260 and 280 nm.

To evaluate PCR amplification of the extracted DNA, proper dilutions (0.5 to 1 ug) were used. The primer combination used for this purpose were, for the forward primer: 5'-ccc-tag-gag-tca-acg-gag-tct-tga-gg -3', and for the reverse primer: 5'-gca-gca-act-acc-aca-gtg-tta-g-3'. These primers have been already designed especially for partial amplification of 18s rDNA gene. The fragments were visualized with a UV transilluminator after staining with ethidium bromide [16].

III. RESULTS

A. Determination of DNA concentration and purity

The efficiency of the DNA extraction and purification procedure and the suitability for subsequent PCR amplification is illustrated in Table I and Fig. 2 and 3 The ADP_1 method (1 h incubation at 56°C) was considered as the most rapid able to yield DNA faster than the other protocols. The highest concentration of DNA was obtained with the APD_2 and the Chelex methods (Table I). In contrast, the highest purity of DNA was found when using the SDS-chloroform and the APD_1 methods.

The inverse relationship between quality (measured as A260/280 absorbance ratio) and quantity of the extracted DNA is shown in fig. 2.

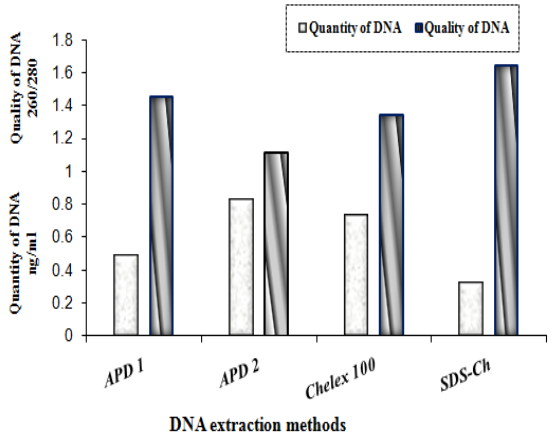

Fig. 2 Relationship between purity and concentration of the DNA extracted by various methods

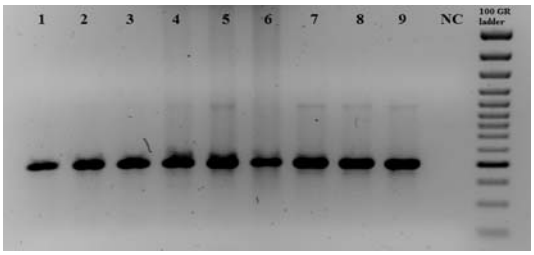

Fig. 3 Electrophoretic profile of PCR product of the 500 bp fragment of the partial 18S rDNA gene using the APD_1 method (lanes 1-2), APD_2 method (lanes 3-4), Chelex® 100 resin method (lanes 5-6), SDS-chloroform method (lanes 7-9)

TABLE I
EFFICIENCY (AS DETERMINED BY SPECTROPHOTOMETRY AT 260 nm AND 280 nm) AND SPEED (OF THE TOTAL PROCEDURE) OF THE DNA EXTRACTION METHODS. VALUES ARE MEAN±STANDARD DEVIATION OF SIX MEASURMENTS.

Method	DNA concentration (ng/µl)	$A_{260/280}$	Required time (Minute)
APD_1 (1 h incubation at 65°C)	485.6±293.6	1.45±0.14	150
APD_2 (2 h incubation at 55°C)	824.6±170.4	1.14±0.08	180
Chelex® 100 resin	729.4±533.0	1.34±0.16	180
SDS-chloroform	314.3±30.1	1.63±0.10	400

B. PCR assays

In the present study, the amplification of all replicate aliquots of DNA extracted by the new extraction methods (APD_1 and APD_2) was always successful when using primer combinations allowing amplification of the 18S rDNA gene as it was the case for the DNA extracted with the other methods (Fig.3).

IV. DISCUSSION

A. The importance of DNA extraction in molecular biology

The need of suitable extraction methods to obtain highly purified nucleic acids without inhibitors has been demonstrated [17].

Indeed the development of a quick, simple, cost-effective method resulting in a high purity yield has shown a parallel progress for several model organisms including human forensic material [14]-[18]-[19], mouse [20] and *Arabidopsis* and crop plants [21], but their wider application for molecular ecology is timely [22]. Molecular genetics surveys of aquatic invertebrates (cladocerans, anostracans, rotifers, copepods, ostracods, etc.) have yielded valuable insights into the evolutionary forces shaping their genetic structure and microevolution [23]-[25], and are helping to characterize the cryptic biodiversity of these groups [26]. Accordingly, PCR methods have been successfully applied for *Artemia*

biodiversity analysis [2] thanks to their high specificity and sensitivity as well as to their rapidity, but they are limited by the sample size of planktonic animals. Thus, DNA isolation methods for different species and types of tissue must provide sufficient yield of DNA. DNA extraction and concentration are the first critical steps in molecular analytical methodologies. Therefore any method must provide sufficient DNA free of inhibitory compounds hindering the amplification reaction such as enzymes and ions.

In order to achieve the highest yield and quality of DNA, several methods of DNA extraction as well as ready-to-use kits have been invented. Methods such as Chelex®100, SDS-chloroform and CTAB are well known now for many applications and are being used accordingly. The efficiency, speed and the fact that neither expensive facilities, nor toxic chemicals are required, make the ADP protocol an attractive alternative to the existing methods of DNA extraction. Indeed extraction methods are evaluated based on their yield and the quality of results. For instance a very simple and efficient method of DNA extraction was described by [27] which takes advantage of DNA sedimentation induced by millimolar concentrations of $ZnCl_2$. This method works with plasmid DNA and oligonucleotide concentrations as low as 100 ng/ml and 10 µg/ml. The Chelex®100 method is also known as very simple, fast and inexpensive, not necessitating hazardous chemicals, and requires fewer tube manipulations as compared to traditional organic extraction methods. The Chelex (resin particles) can bind heavy metal cations and removes some PCR inhibitors. However, this method has some disadvantages too. Chelex® 100 extraction methods are performed in a harsh environment (pH between 10-11 and temperature approximately 100°C) that may not be suitable for highly degraded or low level DNA samples. Also this method may show potential degradation of isolated DNA samples when stored for a long time. As already explained, the SDS-chloroform method is one of the most known and effective methods of DNA extraction. This method is especially known to yield high quality DNA, as also illustrated here. However it requires several reagents and especially numerous manipulations making this method less attractive.

B. Advantages of APD

In this study DNA extraction by APD complex was indicated as the cheapest DNA extraction method, not only for one handling step but also for the value of materials itself. In chelex method 1 euro is sufficient to perform 15 DNA extractions (each 200 ul) whereas in APD complex the same value is sufficient to prepare 1000 ml extraction solution which makes possible to perform more than 4000 extraction at top speed. The SDS-chloroform method did not enter in this category due to the much more required materials.

The comparison of the extraction methods studied, *i.e*. The APD methods, the Chelex® 100 resin and the SDS-chloroform methods, has shown a different efficiency in DNA and purity. As already stated, the highest quality of DNA was obtained by the SDS-chloroform method, also yielding the lowest quantity. In fact in the other methods DNA quantity and quality was inversely related, probably due to the fact that a large fraction of the DNA is being lost during the elimination of interferences/inhibitors and ions. A method which can extract DNA in high quality and quantity would be considered optimal. Our results show that the APD DNA extraction method could successfully produce DNA of acceptable purity. In the present study, repeated PCR amplification using different molecular markers such as 12S-16S region [4], having different fragment sizes, such as mtDNA and 18S rDNA revealed no defect in either amplification or artifact patterns (results of mtDNA not shown). When compared to alternative, standard methods, the newly presented procedure for DNA preparation is thus rapid, cheap, simple and reproducible, providing a more efficient protocol for identification of genetic variability.

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