Analytical Biochemistry 419 (2011) 309–316

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Altered nucleic acid partitioning during phenol extraction or silica adsorption by guanidinium and potassium salts

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ARTICLE INFO

Article history:
Received 11 July 2011
Received in revised form 24 August 2011
Accepted 26 August 2011
Available online 31 August 2011

Keywords:
Nucleic acids
Partitioning
Phenol extraction
Silica adsorption
Guanidinium
Potassium
RNA isolation
Genomic DNA-free total RNA

ABSTRACT

Nucleic acids were found to partition into the phenol phase during phenol extraction in the presence of guanidinium at certain concentrations under acidic conditions. The guanidinium-concentration-dependent nucleic acid partitioning patterns were analogous to those of the nucleic acid adsorption/partitioning onto silica mediated by guanidinium, which implied that phenol and silica interact with nucleic acids through similar mechanisms. A competition effect was observed in which the nucleic acids that had partitioned into the phenol phase or onto the silica solid phase could be recovered to the aqueous phases by potassium in a molecular weight–salt concentration-dependent manner (the higher molecular weight nucleic acids needed higher concentrations of potassium to be recovered, and vice versa). Methods were developed based on these findings to isolate total RNA from *Escherichia coli*. By controlling the concentrations of guanidinium and potassium salts used before phenol extraction or silica adsorption, we can selectively recover total RNA but not the high molecular weight genomic DNA in the aqueous phases. Genomic DNA-free total RNA obtained by our methods is suitable for RT-PCR or other purposes. The methods can also be adapted to isolate small RNAs or RNA in certain molecular weight ranges by changing the salt concentrations used.

High-quality total RNA with few contaminants is a prerequisite for many RNA expression analysis techniques such as reverse transcription PCR (RT-PCR),[1] Northern blot, microarray assay, and high-throughput RNA sequencing. Currently there are many RNA extraction methods available. One category is based on phenol extraction, which is a classical and efficient nucleic acid purification method [1]. During phenol extraction, proteins and other impurities are removed to the interphase or the phenol phase, but nucleic acids remain in the aqueous phase. For RNA extraction, denaturants are needed to break the cells and denature endogenous ribonucleases (RNases) simultaneously [2–8]. Guanidinium thiocyanate, a RNase denaturant used in the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method [3,5] and the derivative reagent Trizol [4], are effective for RNA isolation from RNase-rich materials. Sodium dodecyl sulfate (SDS) is also a denaturant that is used in SDS-phenol RNA extraction methods for rapid lysis of cells and inactivation of the endogenous RNases [6,7].

Another category of RNA isolation methods is solid phase purification. These methods are based on the fact that nucleic acids in aqueous solutions can adsorb onto the solid sorbents under certain conditions. Many materials can adsorb nucleic acids, most of which belong to silica or its derivatives [9–23]. Adsorption of nucleic acids to glass powder in the presence of chaotropic agents, such as iodide or perchlorate, was used in early methods for nucleic acid extraction [9,10]. Many current commercial RNA purification kits are based on the silica adsorption of nucleic acids mediated by guanidinium [11]. Nucleic acids adsorbing onto sand, mica, or mesoporous silica materials in the presence of divalent cations (Mg^{2+}, Ca^{2+}, Ni^{2+}) have been studied with the purposes of nuclease protection [12,13], imaging by atomic force microscopy [14,15], and nucleic acid delivery for gene therapy [16–20]. However, the mechanisms of the nucleic acid adsorption are still poorly understood. The dominate driving forces for the adsorption mediated by chaotropic anions were proposed to be electrostatic shielding, dehydration, and hydrogen bond formation [23,24]. Forming salt bridges between nucleic acids and silica or mica surface was considered the reason for the divalent cation-mediated adsorption [13–15,20,21,25]. During the adsorption of nucleic acids to mesoporous silica materials, the formation of hydrogen bonds between nucleic acids and silica by adsorbed water molecules was regarded as a main factor [19].

It seems that there is no other relationship between these two categories of the methods except for their abilities for RNA isolation. However, we have found that nucleic acids could partition

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[1] *Abbreviations used:* FTs, flowthrough fractions; MOPS, 4-morpholinepropanesulfonic acid; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.08.047

into the phenol phase during phenol extraction with certain concentrations of guanidinium salts under acidic conditions. This altered phase partitioning is analogous to the guanidinium-mediated adsorption of nucleic acids onto silica, albeit in the former case nucleic acids have partitioned into the liquid phenol phase while in the latter one nucleic acids have adsorbed/partitioned onto the solid silica phase. In fact, phenol and silica both have weakly dissociated hydroxyl groups. Guanidinium cations might form bridges between the negatively charged phosphate groups of nucleic acids and the hydroxyl groups of phenol or silica, which caused the altered phase partitioning. Nevertheless, after partitioning into the phenol phase or onto the silica solid phase mediated by guanidinium, nucleic acids could be recovered into the aqueous phase by potassium, dependent on the molecular weight and salt concentration. This phenomenon resembles the competition effect between divalent and monovalent cations during the adsorption of DNA onto mica, which has been experimentally and theoretically studied [14,25,26]. Our results have extended the competition effect to guanidinium-mediated adsorption or phase partitioning, the molecular weight dependence of which is a novel finding.

Despite various methods for RNA extraction, few of them could guarantee removal of genomic DNA (gDNA) completely because of the similar properties of DNA and RNA. It is crucial to remove the residual gDNA from RNA samples when techniques such as quantitative RT-PCR and microarray assays were used [5]. The removal of gDNA can be accomplished by treatment with RNase-free DNase. However, this enzymatic treatment obviously raises the costs of time and expense, and the risk of RNA degradation [27]. Furthermore, it is difficult to monitor the efficiency of DNA digestion, which increases the uncertainties of experiments. Removing gDNA without additional DNase treatment is advantageous for obtaining unbiased results.

The discovery of altered phase partitioning of nucleic acids by guanidinium and potassium provides an excellent tool to obtain gDNA-free total RNA. By controlling the concentrations of guanidinium and potassium salts used, we can conveniently and selectively recover total RNA but not the high molecular weight gDNA. It is also well known that alkaline lysis with SDS for plasmid preparation has a good performance to remove gDNA [28]. Thus we have combined the potassium precipitation step after SDS lysis with the altered phase partitioning step to develop methods for gDNA-free total RNA isolation from *Escherichia coli*, by either phenol extraction or silica adsorption. Using our methods, we can obtain total RNA in high yields and purity without gDNA contamination, which are suitable for directly using as RT-PCR templates or other purposes. These methods can also be adjusted to specially isolate small RNAs or RNA in certain molecular weight ranges by changing the salt concentrations. Furthermore, it is discussed why direct lysis by guanidinium gives poor yields for bacterial RNA extraction.

Materials and methods

Reagents and enzymes

All aqueous solutions were prepared with autoclaved ultrapure water. The suspension solution was 50 mM glucose and 10 mM EDTA. The lysis solution was 2% (w/v) SDS (70 mM). The acidic potassium acetate solution was 3 M potassium acetate, adjusting the pH to 5.0 with acetic acid (the final concentration of acetic acid was about 6.5 M). The stock solution of guanidinium thiocyanate was 4.5 M without adjusting the pH. Phenol used in extraction was a mixture of water-saturated phenol, chloroform, and isoamyl alcohol (125:24:1). The washing solution was 70% (v/v) ethanol. The denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sarkosyl, 0.1 M 2-mercaptoethanol) and other reagents used in the AGPC method were prepared according to the literature [3,5]. The 10× MOPS electrophoresis buffer contained 200 mM MOPS, 20 mM sodium acetate, 10 mM EDTA (pH 7.0) [28]. High range RNA ladder was a product from Fermentas. DNase-free RNase A (10 mg ml^{-1}) was prepared as described [28]. The UNIQ-10 silica-gel membrane columns were purchased from Sangon. AMV reverse transcriptase and the hot start fluorescent PCR kit (SYBR Green I) used in the real-time PCR were from BioBasic. Specific primers and random hexamer primers were synthesized by Sangon.

Bacterial strains, growth conditions, and nucleic acid isolation

Escherichia coli strains of AB1157, AB2463, AB2480, and UNC1085 were obtained from *E. coli* Genetic Stock Center (Yale University). Cells were grown in Luria Broth (LB) medium with vigorous shaking overnight. About 1.5 ml of culture was centrifuged at 10,000*g* for 1 min. The cell pellet was resuspended with 100 μl suspension solution by vortexing. After that, 100 μl 2% (w/v) SDS was added, gently mixed, and incubated at room temperature for 3 min. To extract crude nucleic acids, the lysate was directly extracted with an equal volume of phenol. After centrifugation at 10,000*g* for 5 min, the aqueous phase was carefully transferred to a fresh tube, and mixed with an equal volume of isopropanol and incubated at −20 °C for 10 min. The nucleic acids were pelleted by spinning at 10,000*g* for 10 min at 4 °C, and washed with 70% (w/v) ethanol twice. The pellet was air dried for 10 min and dissolved in 100 μl water. To analyze the altered phase partitioning of nucleic acids during phenol extraction in the presence of guanidinium salts, aliquots of 100 μl crude nucleic acids were supplemented with serial concentrations of guanidinium thiocyanate or chloride (0 to 3.3 M, adjusted to desired pH values with acetic acid and 10 mM sodium acetate). The mixtures were extracted with phenol. The aqueous phases were pipetted out and the nucleic acids in them were precipitated by isopropanol. The sodium acetate (100 mM, pH 5.2) was supplemented for the tubes with low salt concentrations during precipitation to obtain adequate pellet (few nucleic acids would be precipitated by isopropanol if the salt concentrations in the solution were low). The pellet was then dissolved in 20 μl water after washing with 70% (w/v) ethanol. Similar adsorption experiments were carried out by transferring the nucleic acids with serial concentrations of guanidinium thiocyanate or chloride (0 to 4 M) to UNIQ-10 silica-gel membrane columns. The columns were spun at 8000*g* for 2 min. The nucleic acids in the aqueous flowthrough fractions (FTs) were precipitated and washed as described above. Then, the pellet was dissolved in 20 μl water. After the columns were washed with 500 μl 70% (w/v) ethanol and centrifuged at 10,000*g* for 1 min, the nucleic acids partitioned onto silica were eluted by 20 μl of water. To explore the recovery of the nucleic acids from the phenol or silica solid phase to the aqueous phase by potassium acetate, 1.5 M guanidinium thiocyanate and various concentrations of potassium acetate (0 to 1 M) at pH 5.0 were added to aliquots of 100 μl crude nucleic acids. The mixtures were either extracted with phenol or transferred to UNIQ-10 silica-gel membrane columns. The nucleic acids in the aqueous phase of phenol extraction or the FTs of the silica adsorption were precipitated, washed, and dissolved in 20 μl water. The nucleic acids partitioned onto silica were washed with 70% (w/v) ethanol and eluted with 20 μl of water. To compare the efficiencies and yields of various methods, 1.5 ml aliquots of *E. coli* culture were treated with the procedures listed in Table 1, and extracted with an equal volume of phenol. The nucleic acids in the aqueous phase were then precipitated with isopropanol, washed with ethanol, and dissolved in 50 μl water.