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Purification of pre-miR-29 by arginine-affinity chromatography

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ABSTRACT

Recently, differential expression of microRNAs, in patients with Alzheimer's disease (AD) suggests that they might have key regulatory roles in this neurodegenerative disease. Taking into account this fact, several studies demonstrated that the miR-29 is significantly decreased in AD patients, also displaying abnormally high levels of β-site APP-cleaving enzyme 1. Thus, RNA biochemical or structural studies often require a RNA sample that is chemically pure and biologically active. The present work describes a new affinity chromatography method using an arginine support to specifically purify pre-miR-29 from other *Rhodovulum sulfidophilum* small RNA species. Nevertheless, in order to achieve higher efficiency and selectivity, it is essential to characterize the behavior of pre-miR-29 binding/elution. Thus, three different strategies based on increased sodium chloride (280–500 mM), arginine (25 mM) or decreased ammonium sulfate (2–0.1 M) stepwise gradients are described to purify pre-miR-29. In this way, it was proved that well-defined binding/elution conditions are crucial to enhance the purification performance. As a matter of fact, by employing elution strategies using sodium chloride or arginine, an improvement in the final pre-miR-29 yields (96.5 and 56.7%, respectively) was obtained. Moreover, the quality control analysis revealed high integrity in pre-miR-29 preparations as well as high purity (90 and 98%, respectively), demonstrated by the scarce detection of proteins. This improved method takes advantage of its simplicity, significant cost reduction, due to the elimination of some complex operations, and speed for large-scale purification of pre-miRNAs suitable for biochemical and structural studies.

1. Introduction

MicroRNAs (miRNAs) constitute a class of small non-coding regulatory RNAs that act as key regulators of gene expression through a highly conserved intracellular mechanism termed RNA interference, involving the recognition and translational control of specific messenger RNA (mRNA) [1]. MiRNAs are initially transcribed as pri-miRNAs that are processed by Drosha into pre-miRNAs stem-loop. Pre-miRNAs are then exported to the cytoplasm, where their loops are removed by Dicer resulting into mature miRNAs. Finally, miRNA is loaded onto the RNA-induced silencing complex (RISC) toward the target mRNA, resulting primarily in degradation of mRNA transcripts or repression of protein translation, depending on the degree of sequence complementarity [2].

Several studies, over the last decade, have been published concerning the expression of miRNAs in patients with Alzheimer's disease (AD), suggesting that these can play an important role in the regulation of neurodegeneration. One major hallmark of AD is the generation and subsequent accumulation of β-amyloid (Aβ) through sequential cleavage of the β-amyloid precursor protein (APP) by β-site APP-cleaving enzyme 1 (BACE1) and γ-secretase [3,4]. The regulation of the expression levels of proteins involved in this Aβ generation process has demonstrated to be important in AD. Hebert and collaborators showed that the miR-29 is potentially involved in the regulation of APP and BACE1/γ-secretase expression because *in vitro* studies revealed that miR-29 cluster was significantly decreased in AD patients displaying abnormally high BACE1 protein levels [5]. These findings raised the possibility to use miR-29 as a potential therapeutic weapon for AD therapy.

For this reason, RNA represents an important target of a wide collection of laboratory analyses, relevant, particularly, in the

Abbreviations: miRNA, microRNA; RNA, ribonucleic acid; mRNA, messenger RNA; RISC, RNA-induced silencing complex; AD, Alzheimer's disease; Aβ, β-amyloid; APP, β-amyloid precursor protein; BACE1, β-site APP-cleaving enzyme 1; SPE, silica-based solid-phase extraction; PCR, polymerase chain reaction; cDNA, complementary DNA; AC, affinity chromatography; RNases, RNA degrading enzymes; tRNA, transfer RNA; sRNA, small RNA.

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diagnostic of several disorders, as well as in basic research. As a result, RNA purification is a critical first step of a multitude of analytical techniques including real-time quantitative polymerase chain reaction (PCR), microarray analysis, Northern blotting, *in situ* hybridization, *in vitro* translation and cDNA library construction [6]. To provide relevant and reliable results, molecular biology techniques used for such purposes require pure and intact molecules of purified RNA. Consequently, there is a growing demand for commercially available kits (e.g., Exiqon's, Qiagen, Ambion, Promega and Stratagene), which are designed to isolate miRNA molecules from different biological sources with high quality, intactness and purity for further analytical application or scientific need [6]. Most of the existing kits use either silica-based solid-phase extraction (SPE) technology or a magnetic-bead based extraction technology whereby small RNAs (sRNAs) are separated from large RNAs through their capacity to bind to the column [7,8]. Furthermore, there are some chromatographic methods reported in the literature for the purification of the sRNA molecules from high-molecular-weight RNA [9–11] in attempting to overcome the limitations on RNA purification. In these methods, diverse natural or *in vitro* selected RNA aptamers sequences are inserted in RNA molecules [8], which subsequently have to be released from the chromatographic support by competitive elution or cleaved off by a protease, which can affect the overall topology of the recombinant RNA construct [12].

Commonly, miRNA-based therapy applications rely on the use of synthetic miRNAs obtained by *in vitro* transcription and chemical synthesis. Although these methods can be very efficient, they are still expensive to be produced in large scale, limited with respect to the length of oligonucleotides that can be synthesized and labor intensive [12]. In addition, issues associated with the presence of contaminants arising from synthesis still restrict the implementation of these oligonucleotides onto clinical applications, mostly because the presence of impurities may lead to non-targeted gene silencing and are often associated with a decrease in therapeutic effectiveness. Therefore, the requirement for the production of highly purified and clinically suitable miRNAs in large quantity arises as one of the most important challenges in the development of therapeutic strategies based on this technology.

The pursue of highly selective, reliable and economical processes capable of preparing therapeutic biomolecules with the strict quality assurance has been accompanied by an improvement in the downstream strategies that are based on affinity chromatography (AC) [13]. Thus, the development of an affinity-based methodology using amino acids as specific ligands have recently led to an improvement in purification technologies, since this technique exploits natural biological processes such as molecular recognition for the selective purification of target biomolecules [14–18].

Hence, the main goal of this work is to develop new methodologies for the RNA isolation that enable the pre-miR-29 recovery with high integrity and purity, in view of the application in molecular biology procedures, namely for gene expression analysis. To accomplish this purpose, it was explored the possibility of using arginine-AC to selectively isolate the pre-miRNA from *Rhodovulum sulfidophilum*. This bacteria was selected as recombinant host to biosynthesize the pre-miR-29, once it does not produce any detectable ribonucleases (RNases) in the culture medium, maintaining the stability of the RNA produced [19]. Thus, this fact constitutes a great advantage since RNA is an instable molecule due to its susceptibility to nucleases mediated degradation. The method based on arginine-AC can be an attractive approach for the miRNA purification process, since previous results from our research group have demonstrated the potential of this amino acid in the purification of RNA [20] and plasmid DNA [21–23], suggesting that the binding mechanism involves phenomenological interactions like biorecognition between arginine and nucleic acids. In addition, arginine was chosen because it is a conserved amino acid in the active center of the Argonaute protein [24], which is part of the RISC complex. The binding/elution behavior of pre-miR-29 under the influence of different environmental conditions, such as the composition, the ionic strength of the elution buffer, and the temperature were investigated. The study revealed several interesting characteristics of RNA molecules, including their chromatographic behavior and natural interactions that can occur between the arginine support and pre-miRNA. These results support the interest in applying amino acid-based AC to develop new pre-miRNA isolation and purification processes. Therefore, this new affinity protocol for miRNA isolation can offer advantages over other less-selective and time-consuming multistep procedures and can improve process economics. Moreover, some improvements over other chromatographic techniques are expected because RNA recovery can be achieved under mild elution conditions, suggesting the feasibility of exploiting different affinity interactions between amino acids and miRNA to develop an interesting bioseparation methodology.

2. Materials and methods

2.1. Materials

Arginine–Sepharose 4B gel was acquired from Amersham Biosciences, the guanidinium salt and all the chemicals used in the lysis buffer were obtained from Sigma-Aldrich. Other compounds used in the elution buffer were ammonium sulfate ($(NH_4)_2SO_4$) and sodium chloride (NaCl) purchased from Panreac and Tris from Merck. All solutions were freshly prepared using 0.05% diethyl pyrocarbonate (DEPC; Sigma-Aldrich) treated water and the elution buffers were filtered through a 0.20 μm pore size membrane and degassed ultrasonically. The DNA molecular weight marker was obtained from Vivantis Technologies. All the materials used in the experiments were RNase-free.

2.2. Bacterial strain and growth conditions

The pre-miR-29 used in this study was obtained from a cell culture of *R. sulfidophilum* DSM 1374 strain (BCCM/LMG, Belgium) modified with plasmid pBHSR1-RM [19] containing the sequence of pre-miR-29. Growth was carried out in shaker flasks containing 100 mL of Nutrient Broth medium (g per liter of deionized water: beef extract, 1; yeast extract, 2; peptone, 5 and sodium chloride, 5) supplemented with 30 μg/mL kanamycin in a rotary shaker at 30 °C and 250 rpm under dark-aerobic conditions. Cell growth was evaluated by measuring the optical density of the culture medium at a wavelength of 600 nm after dispersing cell flocs by vortexing for 30 s. Cells were recovered by centrifugation and stored at −20 °C.

2.3. Lysis and small RNA isolation

Cells were lyzed and sRNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method based on the protocol described by Chomczynski and co-workers [25]. Bacterial pellets were resuspended in 5 mL of denaturing cell lysis solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 4.0; 0.5% (m/v) *N*-laurosylsarcosine and 0.1 M β-mercaptoethanol) to perform lysis. After incubating on ice for 10 min, cellular debris, genomic DNA and proteins were precipitated by gently adding and mixing 5 mL of water-saturated phenol and 0.5 mL of 2 M sodium acetate (pH 4.0). The sRNA isolation was achieved by adding 1 mL of chloroform/isoamyl alcohol (49:1), and by mixing vigorously until two immiscible phases were obtained. The upper aqueous phase, which contained mostly RNA, was recovered and