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Preparation and characterisation of ribonuclease monolithic bioreactor

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Abstract

In gene therapy and DNA vaccination, RNA removal from DNA preparations is vital and is typically achieved by the addition of ribonuclease into the sample. Removal of ribonuclease from DNA samples requires an additional purification step. An alternative is the implementation of immobilized ribonuclease. In our work, ribonuclease was covalently coupled onto the surface of methacrylate monoliths via epoxy or imidazole carbamate groups. Various immobilization conditions were tested by changing immobilization pH. Ribonuclease immobilized on the monolith via imidazole carbamate groups at pH 9 was found to be six times more active than the ribonuclease immobilized on the monolith via epoxy groups. Under optimal immobilization conditions the Michaelis–Menten constant, K_m, for cytidine-2,3-cyclic monophosphate, and turnover number, k_3 were 0.52 mM and 4.6 s^{-1}, respectively, and mirrored properties of free enzyme. Enzyme reactor was found to efficiently eliminate RNA contaminants from DNA samples. It was active for several weeks of operation and processed 300 column volumes of sample. Required residence time to eliminate RNA was estimated to be around 0.5 min enabling flow rates above 1 column volume per min.
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1. Introduction

Demand for RNA free DNA and proteins samples increases with the increased production of DNA vaccines and biological drugs. Usually, for RNA removal enzyme ribonuclease A (ribonuclease) is applied, which has to be removed afterwards. An alternative is to use ribonuclease immobilized on solid support. This approach offers additional advantages such as reuse, better control, rapid termination of the reaction and sometimes also enhanced stability compared to the free enzymes. Despite mentioned advantages there are only few reports of ribonuclease immobilization on various supports such as on magnetic poly(2-hydroxyethyl methacrylate) microspheres [1], Sepharose linked antibodies [2], concanavalin A-Sepharose [3] and on the inner walls of short fused-silica capillaries through glutaraldehyde attachment [4]. Lack of ribonuclease immobilization on chromatographic supports is probably a consequence of particular required support features.

Supports for the immobilization of enzymes treating macromolecular substrate, such as DNA or RNA, have to exhibit high mechanical and chemical stability to achieve the required throughput, preferably large porosity, an appropriately large well-defined pore-size distribution and low backpressure [5]. Because of the large RNA size, very few supports enable high throughput without damaging macromolecules in combination with efficient mass transfer between immobilized ligand and macromolecular substrate present in the mobile phase [6]. One such group is monoliths. They are cast as homogenous matrix and differ from conventional supports in respect to hydrodynamic properties. In conventional columns packed with particulate porous supports, the substrate has to diffuse into the pores in order to interact with the active sites of the immobilized enzyme which determines the overall kinetics. In contrary, monoliths exhibit negligible diffusion resistance [7]. Among them, methacrylate monoliths seem to be especially suitable for ligand immobilization due to inherently present epoxy groups [7]. Many different high mass ligands were immobilized so far [7,8], most of them via epoxy groups [9–11], but recently also due to imidazole carbamate groups, where immobilization reaction seems to be faster [6,12].

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Due to monolith advantages over porous particulate supports in affinity chromatography [13], taking into account the efficient DNA separation using methacrylate monoliths [14,15] even on industrial level [16,17], one could reasonably assume that ribonuclease immobilized on such type of support might be competitive to the RNA degradation with free enzyme. In this work we describe immobilization of ribonuclease on methacrylate-based convective interaction media (CIM; BIA Separations, Ljubljana, Slovenia) monoliths via epoxy and imidazole carbamate groups. The kinetic parameters and stability of immobilized ribonuclease were determined, and the applicability of RNA removal was investigated.

2. Experimental

2.1. Chemicals

Ribonuclease A (ribonuclease; EC 3.1.27.5), cytidin-2,3-cyclic monophosphate and BCA protein assay were obtained from Sigma (Taufkirchen, Germany); mixture of deoxyadenosine, deoxyguanosine, deoxythymidine and deoxycytidine 5′-triphosphates (dNTPs) from Amersham Bioscience (Uppsala, Sweden); AmpliTaq polymerase and AmpliTag buffer from Applied Biosystems (Foster City, CA, USA); avian myeloblastosis virus reverse transcriptase (AMV-RT), AMV-RT buffer, ribonuclease inhibitor from Promega (Madison, WI, USA); 100 base pairs DNA ladder marker from NewEngland Biolabs (Hertfordshire, UK); TRIzol reagent from Invitrogen (Paisley, UK). Other chemicals and solvents were of analytical grade and from commercial sources.

2.2. Epoxy methacrylate monoliths and carboxydiimidazole activated methacrylate monoliths

CIM disk monolithic columns bearing epoxy (epoxy-CIM) and imidazole carbamate groups (CDI-CIM) (both from BIA Separations) were used for the immobilization of ribonuclease. CIM disk monolithic column consisted of a single CIM disk with a monolith diameter of 12 mm and thickness of 3 mm (monolith volume 0.34 ml), placed in an appropriate CIM housing [18], which was connected to an HPLC system. Monolith median pore-size was of approx. 6 μm, determined using mercury porosimeter PASCAL 440 (ThermoQuest, Rodano, Italy).

2.3. Immobilization of ribonuclease

Ribonuclease solutions were prepared by dissolving enzyme ($2\,g\,l^{-1}$) in 50 mM Tris buffer of pH 7 and 9, 50 mM acetate buffer of pH 5, 50 mM sodium carbonate buffer of pH 11 and 50 mM KCl–NaOH buffer of pH 13. Testing of wide pH range is possible due to the high ribonuclease stability, exhibiting optimum between pH 7 and 9 [19]. Before immobilization, the monolith was equilibrated with proper buffer without ribonuclease. Dynamic immobilization method was used since it was previously shown to give better results than the static method [12]. CIM disk monolithic column was washed with 5 column volumes (CVs) of water and 5 CVs of immobilization buffer afterwards. A 3 ml of immobilization solution containing ribonuclease was pushed through the CIM disk monolithic column. The procedure was repeated at regular time intervals of 15 min for 2 h. After immobilization was completed, the enzyme-modified CIM disks monolithic column was washed first with 5 CVs of immobilization buffer containing 0.5 M NaCl and with 5 CVs of immobilization buffer afterwards. When not used, column was stored in 20% ethanol solution at 4 °C to prevent microbial growth.

Quantity of immobilized enzyme was determined from the concentration difference of ribonuclease in the immobilization solution before and after immobilization using protein determination kit BCA (Sigma) according to the manufacturer instructions.

2.4. Kinetic measurements

Activity of immobilized ribonuclease was determined using cytidine-2′,3′-cyclic monophosphate ($\varepsilon_{288\text{nm}}$ $1308\,M^{-1}\,cm^{-1}$) as substrate [20].

2.4.1. Activity of free ribonuclease

Ribonuclease (0.0041–$0.0162\,g\,l^{-1}$) was applied into the solution of 10 mM Tris pH 7.5, 2 mM EDTA, 0.1 M NaCl buffer containing cytidine-2,3-cyclic monophosphate at concentrations from 0.12 to 0.6 mM at 25 °C in final volume 1 ml. The subsequent increase in absorbance at 288 nm was monitored continuously for 5 min with DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The ribonuclease activity was determined as a slope of linear increase of substrate absorbance (AU/min) at the beginning of reaction. Several ribonuclease concentrations were applied to determine its kinetic parameters more accurately. Changing the substrate concentration enabled calculation of kinetic parameters using Michaelis–Menten equation. Specific activity was calculated by dividing enzyme activity with the amount of dissolved enzyme.

Effect of sodium ions on ribonuclease activity was determined by applying ribonuclease ($0.0162\,g\,l^{-1}$) into the solution containing cytidine-2,3-cyclic monophosphate (0.57 mM) in 10 mM Tris pH 7.5, 2 mM EDTA, NaCl (concentration from 0 to 0.3 M) at 25 °C in a final volume of 1 ml and measuring ribonuclease activity.

2.4.2. Activity of immobilized ribonuclease—on line frontal analysis

An HPLC system (Knauer, Berlin, Germany) built of Pump 64 (Knauer), a variable-wavelength UV–vis detector (Knauer) with a 10 mm optical path set to 288 nm, set response time of 0.1 s, connected by means of 0.25 mm I.D. capillary tubes and HPLC hardware/software (Knauer, Berlin, Germany) was used for kinetic studies of immobilized ribonuclease.

The reagent solution containing substrate cytidine-2,3-cyclic monophosphate at concentrations from 0.39 to 0.68 mM in 10 mM Tris pH 7.5, 2 mM EDTA, 0.1 M NaCl at 25 °C was pumped through the ribonuclease CIM disk monolithic column at flow rates from 0.1 to 1 ml min^{-1} (corresponding to residence time from 0.197 to 1.97 min, which was calculated by dividing

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