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Immobilization of deoxyribonuclease via epoxy groups of methacrylate monoliths Use of deoxyribonuclease bioreactor in reverse transcription-polymerase chain reaction

Mojca Benčina[a], Katja Benčina[b], Aleš Štrancar[b], Aleš Podgornik[b,*]

[a] *Laboratory of Biotechnology, National Institute of Chemistry, Hajdrihova 19, P.O.B. 600, SI-1001 Ljubljana, Slovenia*
[b] *Bia separations d.o.o., Teslova 30, SI-1001 Ljubljana, Slovenia*

Abstract

A deoxyribonuclease bioreactor was prepared by immobilization of deoxyribonuclease I through epoxy groups inherently present on poly (glycidyl methacrylate-co-ethylene dimethacrylate) monoliths. Columns with various levels of DNase activity were prepared varying immobilization temperature, pH, time and method. The apparent Michaelis–Menten constant, K_m^{app}, and turnover number, k_3^{app}, for immobilized DNase determined by on-line frontal analysis method were, respectively, 0.28 g of DNA l^{-1} and 16 dA_{260nm} min^{-1} mg^{-1} of immobilized DNase. The highest activity of immobilized DNase was detected at 1 mM calcium ions concentration and mirrored properties of free enzyme; however, reaction temperature in the range from 25 to 37 °C has no significant effect on activity of immobilized DNase in contrary to free enzyme. The CIM DNase bioreactor was used for elimination of DNA contaminants in RNA samples prior to reverse transcription followed by PCR.

Keywords: Immobilized deoxyribonuclease I (DNase); Poly (glycidyl methacrylate-co-ethylene dimethacrylate) monolith; Convective interaction media (CIM); Reverse transcription with polymerase chain reaction

1. Introduction

Enzymes immobilized on solid phase matrices have found applications in biotechnology, molecular biology, molecular diagnostic and can serve as industrial catalysts and as specific reagents for analytical procedures [1–3]. Specificity of enzymes is the key factor exploited for purification and separation of molecules with similar chemical and physical characteristics present in biological samples. Compared to free enzymes, there are several benefits from using immobilized enzymes. Immobilized enzymes are easily removed from reaction mixture and consequently pose better control and rapid termination of the reaction; as well there is no contamination with residual enzyme. The immobilized enzymes could be reused and have enhanced stability compared to free enzymes.

In molecular biology, especially in diagnostics, methods such as reverse transcription linked to PCR (RT-PCR) demand removal of DNA from samples in order to ensure accuracy. Usually, an enzyme deoxyribonuclease I (DNase) is applied to RNA samples to remove traces of DNA impurities that could affect results of messenger RNA (mRNA) quantification and complementary DNA (cDNA) synthesis [4–7]. In some applications of RT-PCR, DNase has to be removed in order not to interfere with PCR reaction and DNase exclusion represents additional risk for contamination. In order to avoid introducing DNase into the analysing sample, the use of immobilized DNase on solid support is recommended [8]. DNase immobilized on nylon and polystyrene was used to study accessible DNA regions in chromatin structure [9]. DNase was immobilized also on magnetic bead cellulose particle and magnetic poly(hydroxyethyl methacrylate-co-ethylene dimethacrylate) microspheres [10], Sepharose [11] and porous glass [12].

* Corresponding author. Tel.: +386 1 426 56 49; fax: +386 1 426 56 50.
E-mail address: ales.podgornik@monoliths.com (A. Podgornik).

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Supports for bioreactors or chromatography that are employed for purification of macromolecules such as DNA have to meet specific requirements. The average hydrodynamic diameter of DNA is between 150 and 250 nm for 5 to 10 kilo base pairs (kbp) plasmids (M_r 3.25–6.5 × 10^6), and potentially >1 μm for high-molecular-mass DNA [13]. Because of the DNA size, very few supports enable efficient mass transfer of DNA through support without damaging macromolecules and at the same time efficient contact between ligand on support and analysing macromolecules is established.

In columns packed with enzyme-modified porous beads, the substrate has to diffuse into pores of the support in order to interact with the active site of the immobilised enzyme. The mass transfer of substrate towards immobilised enzyme is controlled by diffusion and depends on particle and pore size, flow rate and diffusion coefficient of the substrate solution. Monolithic macroporous poly (glycidyl methacrylate-co-ethylene dimethacrylate) (GMA-EDMA) columns offer new possibilities for the separation or analysis of large biomolecules and can operate at higher flow rates [14]. A large porosity, high mechanical and chemical stability, a well-defined pore-size distribution and low backpressure provide excellent conditions for chromatography especially for high-molecular-mass analytes [15]. The surface of methacrylate monolith contains epoxy groups, which can be easily modified into other groups, or used as such for immobilization [16,17]. Many different high mass ligands were immobilized so far like carbonic anhydrase [18], heparin and collagen [19], annexin [20], concanavaline A, invertase and protein A [21], glucose oxidase [21,22], immunoglobulines [23], polynucleotide phosphorylase [24], lignin peroxidase [25], citrate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, lactate dehydrogenase [2], trypsin [21,26–28] and recently also human recombinant acetylcholinesterase [29].

Due to monolith advantages above other conventional supports in affinity chromatography [3] and DNA separation [30], one could reasonably assume that efficiency of DNA removal from RNA samples using immobilized DNase might be competitive to the degradation with free enzyme. In this work, we describe immobilization of DNase on convective interaction media (CIM) epoxy methacrylate monolith and its kinetic properties. The kinetic parameters of immobilized DNase were determined and compared with kinetic parameters of free DNase. DNase bioreactor was tested in RT-PCR for removal of DNA contaminants from RNA sample that was consequently used in RT-PCR.

2. Experimental

2.1. Chemicals

Deoxyribonuclease I (DNase) from bovine pancreas, highly polymerized calf thymus DNA, 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), lambda DNA *Hin*DIII/*Eco*RI 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

Convective interaction media epoxy groups containing poly (glycidyl methacrylate-co-ethylene dimethacrylate) monolithic columns (BIA Separations, Ljubljana, Slovenia) were used for immobilization of DNase. Column consisted of a single CIM disk, porous monolithic polymer with a diameter of 12 mm and thickness of 3 mm (monolith volume 0.34 ml), placed in an appropriate housing [31], which was connected to an HPLC system. Experiments were performed on monolithic disk with median pore size of approximately 6 μm, determined using mercury porosymeter PASCAL 440 (ThermoQuest, Rodano, Italy).

2.3. Immobilization of DNase

DNase solution was prepared by dissolving enzyme (2 mg ml^{-1}) in 50 mM Tris buffer pH 7 and 9 or 50 mM acetate buffer pH 5 both containing 1 mM $CaCl_2$. Before immobilization, the monolith was equilibrated with proper buffer without DNaze. Two types of DNase immobilization, static and dynamic, were tested [28]. By static method, an equilibrated monolith was immersed in 2 ml of immobilization solution and incubated for either 3 or 24 h at temperature 22 or 37 °C [28]. By dynamic method, the enzyme solution was pushed through the housing containing a monolith using a syringe, at the beginning of the immobilization and after each 15 min for a period of time up to 2 h. When immobilization was completed, the enzyme-modified CIM columns were washed first with 40 mM Tris buffer pH 8.0, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 M NaCl, to remove residual non-bound enzyme from the pores, followed by 40 mM Tris buffer pH 8.0, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ buffer.

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

2.4. Kinetic measurements

The modified Kunitz hyperchromicity assay [32] was used to determine DNase biological activity. The DNase activity on DNA is manifested as an increase in absorption at 260 nm.

Enzyme activity was expressed as an increase of absorbance at 260 nm min^{-1} at the assay conditions described in Sections 2.4.1 and 2.4.2.

2.4.1. Activity of free DNase

DNase (giving a final concentration from 0.1 mg l^{-1} (3.2 nmol l^{-1}) to 0.96 mg l^{-1} (31.0 nmol l^{-1})) was applied into solution containing calf thymus DNA (0.005–0.1 g l^{-1}) in 40 mM Tris buffer pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at 25 °C in final volume 1 ml. The subsequent increase in absorbance at 260 nm was monitored continuously for 10 min with DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The DNase activity (v^{app} in Eq. (1)) was determined as an increase in substrate absorbance at 260 nm min^{-1} at the beginning of reaction in the linear part. Several DNase concentrations were applied to determine its kinetics parameters more accurately. Changing the substrate concentration enabled calculation of kinetics parameters using Michaelis–Menten equation:

$$v^{app} = V_{max}^{app} \frac{[S]}{K_m^{app} + [S]} \quad (1)$$

where v^{app} is the apparent enzyme activity given as $dA_{260nm}\,min^{-1}$, V_{max}^{app} the apparent maximal enzyme activity, K_m^{app} the apparent Michaelis–Menten constant and [S] is the substrate concentration. Apparent values are used since absolute value cannot be determined due to the nature of the polymeric substrate, the unknown number and type of different substrate binding sites, and the unclear relationship between the absorbance signal and the actual catalytic events.

Specific activity (turnover number k_3^{app}) was calculated from enzyme activity divided by amount of dissolved enzyme.

Effect of calcium ions and temperature on biological activity and stability of DNase was determined by applying DNase (0.00048 g l^{-1}) into solution containing calf thymus DNA (0.04 g l^{-1}) in 40 mM Tris buffer pH 8.0, 1 mM $MgCl_2$, $CaCl_2$ (concentration from 0 to 2 mM) at indicated temperature (25, 30 and 37 °C) in final volume 1 ml.

2.4.2. Activity of immobilized DNase—on line frontal analysis

An HPLC system (Knauer, Berlin, Germany) built with Pump 64 (Knauer), a variable-wavelength UV–vis detector (Knauer) with a 10 or 2 mm optical path set to 260 nm, set response time of 0.1 s, connected by means of 0.25 mm i.d. capillary tubes and HPLC hardware/software (Knauer) was used for kinetic studies of immobilized DNase.

The reagent solution stream carrying substrate, calf thymus DNA 0.006–0.08 g l^{-1} in 40 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at 25 °C (if not otherwise stated) was pumped through immobilized DNase column with flow rates from 0.1 to 10 ml min^{-1} (corresponding to residence time from 0.0197 to 1.97 min calculated by dividing column void volume (0.197 ml) with the flow rate) and substrate absorbance at wavelength of 260 nm was monitored. When the substrate solution at a certain concentration was pumped through the enzyme reactor at fixed flow rate, immobilized DNase has been hydrolyzing DNA what was observed as an increase of the absorbance at the column outlet, which was constant in time. Absorbance values at the outlet were plotted against the residence time. The DNase activity (v^{app}) was determined as the slope of the linear increase in absorbance of DNA at low residence time [33]. Changing the substrate concentration enabled calculation of kinetics parameters V_{max}^{app} and K_m^{app} using Michaelis–Menten equation (Eq. (1)).

Specific activity (turnover number k_3^{app}) was calculated from enzyme activity divided by amount of immobilized enzyme.

Effect of calcium ions and temperature on biological activity and stability of DNase was determined accordingly: reagent solution stream carrying substrate calf thymus DNA (0.02 g l^{-1}) in 40 mM Tris buffer pH 8.0, 1 mM $MgCl_2$, $CaCl_2$ (concentration from 0 to 2 mM) was pumped through DNase monolithic column with flow rates from 0.1 to 10 ml min^{-1} (corresponding residence time from 0.0197 to 1.97 min) at indicated temperature (25, 30 and 37 °C).

2.5. Isolation of RNA and DNA from Aspergillus niger

The filamentous fungi *Aspergillus niger* A158 used for isolation of RNA and DNA was obtained from Microbial Culture Collection of National Institute of Chemistry. Sterile minimal growth medium [34] containing per litre: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, pH 6.0, and 0.2 ml trace metal solution [35] was inoculated with *A. niger* spores at concentration of 10^6 conidiospores ml^{-1} and the mycelium was grown in 500 ml baffled Erlenmeyer flasks with 100 ml medium at 30 °C in a rotary shaker at 100 rpm (RVI-403, Tehnica, Železniki, Slovenia). The mycelia of filamentous fungi *A. niger* was harvested 1 day after spore inoculation using vacuum filtration, washed with ice cold 50 mM Tris buffer pH 7.5 containing 10 mM ethylenediaminetetraacetic acid (EDTA) and pulverized under liquid nitrogen. 0.2 and 0.5 g pulverized mycelia were used for DNA and RNA isolation, respectively.

2.5.1. RNA isolation

For reverse transcription, total RNA was isolated from 0.5 g of grinded *A. niger* mycelium using TRIzol method according to the manufacturer's protocol (Invitrogen). The RNA was extracted from crushed mycelium with the addition of 0.8 ml of TRIzol reagent followed by gentle mixing for 1 min, addition of 0.2 ml of chloroform and additional mixing. To separate the cell remains from dissolved RNA, the mixture was centrifuged at $9000 \times g$ for 10 min at room temperature. The dissolved RNA was precipitated by 2.5 volume of ethanol (100%, v/v) and washed with 70% (v/v) ethanol. The precipitated RNA was dissolved in 0.5 ml TE buffer containing 50 mM Tris, 1 mM EDTA buffer pH 7.4. Additional step of chloroform treatment and

ethanol precipitation was used to remove traces of TRIzol reagent.

2.5.2. DNA isolation

Genomic DNA from *A. niger* mycelium used as a control for PCR was isolated by phenol extraction and ethanol precipitation according to Benčina et al. [34]. 0.2 g pulverized mycelia was resuspended in 1 ml RNB phenol buffer containing 0.375 ml of phenol, 0.25 ml of triisopropylnaphtalene (0.02 g/ml), 0.25 ml *p*-aminosalicylic acid (0.12 g/ml) and 0.125 ml 5 × RNB buffer (1 M Tris pH 8.5, 0.25 M ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.25 M NaCl) heated up to 55 °C. After 1 min of mixing, 0.2 ml of chloroform was added and mixed again. The water phase containing dissolved DNA was separated from cell debris with centrifugation at 10 000 × *g* for 10 min at 4 °C. The phenol chloroform extraction (0.5 ml of each) was repeated followed by centrifugation and to remove phenol, additional extraction with 1 ml chloroform was performed. The clear cell extract containing genomic DNA, total RNA and non-precipitated proteins was obtained by centrifugation at 11 000 × *g* for 10 min. DNA was precipitate with 0.7 ml isopropanol (100%, v/v), precipitated DNA was washed with 70% (v/v) ethanol and dissolved in 0.5 ml of TE buffer.

2.6. Reverse transcription coupled with polymerase chain reaction (RT-PCR)

The reverse transcription followed by PCR was performed according to Benčina [36] and Benčina and Legiša [37]. Total RNA (2 μg) and primer PEPC2 [5′-TATCACGGTGAGAGATACGAGC] were used. Reverse transcription was performed in 10 μl reaction volume containing 5 U Avian Myeloblastosis Virus reverse transcriptase, 60 pmol of primers, AMV-RT buffer (50 mM Tris buffer pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT), 0.5 mM dNTPs and 20 U ribonuclease inhibitor at 42 °C.

Complementary DNA (cDNA) product (3 μl) or genomic DNA (10 ng) was transferred to 20 μl PCR reaction mixture containing 0.5 U AmpliTaq polymerase, GeneAmp buffer (50 mM KCl, 10 mM Tris pH 8.3) 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 200 nM primers PEPC2 and PEPC1 [5′-CTATCTGGGTCTCAAGAACACC]. Thirty cycles of amplification were carried out with the following thermal profile: 94 °C 1 min, 66 °C 1 min, 72 °C 1 min. PCR was performed in a Perkin Elmer thermal cycler GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA).

2.7. Gel electrophoresis

Highly polymerized DNA and DNase treated DNA (25 μl) and RT-PCR and PCR products (10 μl) were fractionated using horizontal agarose gel electrophoresis (Bio-Rad, Richmond, USA) of appropriate density [38]. The fractionated nucleic acid was visualized by staining the gel with DNA sensitive stain, ethidium bromide 0.05 μl ml^{-1}. At the same time, either lambda DNA *Hin*DIII/*Eco*RI marker or *PepC* markers were fractionated on the same gel as the DNA samples. In RT-PCR and PCR experiments, PepC markers were prepared with specific amplification of a plasmid carrying *PepC* gene with an intron and a plasmid carrying *PepC* transcript without intron. Products of amplification were 270 base pairs and 230 base pairs long for *PepC* with and without intron, respectively. The resulting fractionated nucleic acids were visualized and photographed under short-wavelength UV light (Bio-Rad).

3. Results and discussion

For accurate detection of RNA transcripts by RT-PCR, elimination of DNA is requited. To remove DNA, RNA samples are regularly treated with free DNase, which should be removed previous to the RT-PCR [4–7]. In order to avoid additional step of sample manipulation and consequently possible contamination of samples, DNase immobilized on solid support could be applied instead of free one.

3.1. Immobilization of DNase

DNase was immobilized on methacrylate monoliths by covalent linking of either free nucleophilic primary or secondary amines, sulphydryl groups or hydroxyl groups (NH_2, SH groups or OH but only those of tyrosine) [39,40] of amino acid side chain to epoxy groups inherently present on monolith resulting in either support–CH_2–CH(OH)–CH_2–(nuc-enzyme) or support–CH_2–CH–(nuc-enzyme)–CH_2–OH structure. Due to the size of DNA, which is for large DNA molecules >1μm [13], we immobilized DNase on DEAE monoliths having pore size approximately 6 μm, which is larger than commercial ones with the pore size of 1.5 μm. Such monoliths were used for purification of genomic DNA without any significant reduction in DNA binding capacity and DNA damage, indicating that also larger channels could ensure sufficient contacts between DNA and surface [30]. In addition, the support could be used at high flow rates providing efficient mass transfer accompanied with low backpressure [14] and no blocking of the column with DNA is expected.

An influence of immobilization temperature, pH and immobilization time for DNase immobilization on epoxy methacrylate monoliths was tested. According to Wheatley and Schmidt [39], the addition of affinity ligand with nucleophilic group is conducted under basic conditions, which is above 9 for amino and above 11 for hydroxyl groups. At near neutral pH, an increase in the reaction rate between the nucleophilic groups and epoxy groups could be enhanced by high salt concentration like ammonium sulphate and potassium phosphate. The pH optimum for DNase is between 7 and 8.2 [41,42]. We immobilized DNase at pH 5, 7 and 9

Table 1
Effect of temperature, immobilization time and pH of buffer on DNase immobilization on methacrylate monolith via epoxy groups

Temperature (°C)	Time (h)	pH value	Method	DNase activity (dA_{260nm} min^{-1})	Amount (mg DNase g^{-1} support)	Specific activity (dA_{260nm} min^{-1} mg^{-1})
37	3	5	Static	0.1	4.3	0.15
37	24	5	Static	1.9	9.4	1.26
37	3	7	Static	0.1	1.9	0.33
22	24	7	Static	1.2	3.4	2.21
22	0,5	7	Dynamic	0.9	2.9	1.94
22	2	7	Dynamic	1.2	3.5	1.96
37	24	7	Static	1.32 (s_D 0.01)	5.00 (s_D 0.25)	1.65
37	24	9	Static	0	5.6	0

DNase (2 mg ml^{-1}) in 50 mM Tris pH 7 or 9, or 50 mM acetate buffer pH 5 containing 1 mM $CaCl_2$ was immobilized on a CIM epoxy monolithic columns 12 mm × 3 mm (0.16 g). The amount of enzyme bound to the monolith was established by difference in DNase present in a solution before and after immobilization. The activity of immobilized DNase (v^{app}) was determined by on-line frontal analysis at 25 °C as described in Section 2.4.2; DNA concentration was 0.02 g l^{-1} in 40 mM Tris buffer, pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and detection wavelength 260 nm. Specific activity is expressed as DNase activity per mg of DNase.

(Table 1). The highest amount, 5.6 mg of DNase/g of support, was immobilized at pH 9; however, no enzyme activity was detected. In contrary, free DNase was active between pH 7 and 9 when 1 mM Mg^{2+} and Ca^{2+} ions were present while at higher ionic strength, the activity was reduced reaching zero in buffer containing 0.2 M NaCl. Below pH 5 and above pH 9, no activity was detected probably due to charge change of DNase amino acid histidine 131 present in the active site. DNase immobilized at pH 5 and 7 exhibited specific activity of 1.26 and 1.65 dA_{260nm} min^{-1} mg^{-1}, respectively, indicating that even at low pH, immobilization of enzyme could be achieved (Table 1). The DNase specific activity was the highest when immobilized for 24 h at 22 °C, pH 7, probably due to better stability of the enzyme at slightly lower temperature.

We also analysed effect of immobilization time on DNase activity. After 24 h of immobilization at pH 5 and 7, 19 and 13 times higher activity was detected compared to 3 h immobilization and 2.2 and 2.6 times higher amount of enzyme was immobilized, respectively (Table 1). According to Vodopivec et al. [2], diffusion is limiting process in immobilization of proteins via epoxy groups by static method. To determine if this is the case for DNase immobilization, we have tested activity of immobilized DNase after 30 min and 2 h of immobilization performed by dynamic method [28] (Table 1). Already after 2 h, the DNase activity was as high as that obtained after 24 h of immobilization by static method indicating that to speed up immobilization the dynamic method should be used.

3.2. Kinetics of free and immobilized DNase

DNase is an endonuclease that catalyses the hydrolysis of double-stranded DNA predominantly by a single-stranded nicking mechanism under physiological conditions [41–46]. Effective double-stranded scission could result either from a mechanism where individual nicks were introduced in close proximity on both strands or by a processive nicking mechanism that creates DNA gaps instead of nicks, thus increasing the probability of double-stranded breaks. Types of products di-, tri- and oligo- nucleotides are dependent on the presence of divalent cations [41–44]. With calcium ions bound on two asparagine side chains 99 and 201 of DNase, enzyme adapts suitable conformation that stabilizes the enzyme and protects enzyme from proteolitic cleavage. Magnesium or manganese ions bind to both DNase and DNA and modify conformation to be the most appropriate for DNase. We have studied DNase activity in buffer pH 8, containing both calcium and magnesium ions using highly polymerized DNA as a substrate for free and immobilized enzyme. K_m^{app} and V_{max}^{app} were calculated using Eq. (1) and compared (Fig. 1, Table 2). Although these values do not represent a real enzyme activity due to a complex mechanism of reaction [46], a comparison of the apparent K_m^{app} and k_3^{app} (turnover number) values between free and immobilized DNase provides useful information since the mechanism is the same in both cases. We obtained for DNase k_3^{app} values 76 dA_{260nm} min^{-1} mg^{-1} (s_D 13; $n = 8$) and 16 dA_{260nm} min^{-1} mg^{-1} for free and immobilized enzyme, respectively, thus demonstrating five times reduction upon immobilization (Fig. 1). K_m^{app} values for free and immobilized DNase were 0.07 g l^{-1} (s_D 0.01; $n = 8$) and 0.28 g l^{-1}, respectively (Fig. 1). There is approximately four times increase in K_m value upon immobilization.

Table 2
Effect of immobilization method on activity of DNase [28]

	Immobilization method		
	Static	Dynamic	
	24 h[a]	30 min[a]	2 h[a]
V_{max} (dA_{260nm} min^{-1})	9	7.5	9
K_m (g l^{-1})	0.27	0.27	0.26

DNase (2 mg ml^{-1}) in 50 mM Tris pH 7 containing 1 mM $CaCl_2$ at 22 °C was immobilized on CIM epoxy monolithic columns 12 mm × 3 mm by static method for 24 h and by dynamic method for 30 min and 2 h. The reagent solution stream carrying substrate, calf thymus DNA 0.006–0.08 g l^{-1} in 40 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ was pumped through DNase monolithic column with flow rates from 0.1 to 10 ml min^{-1} at 25 °C. The maximal activity (V_{max}^{app}) of immobilized DNase was determined by on-line frontal analysis as described in Section 2.4.2.

[a] Immobilization time.

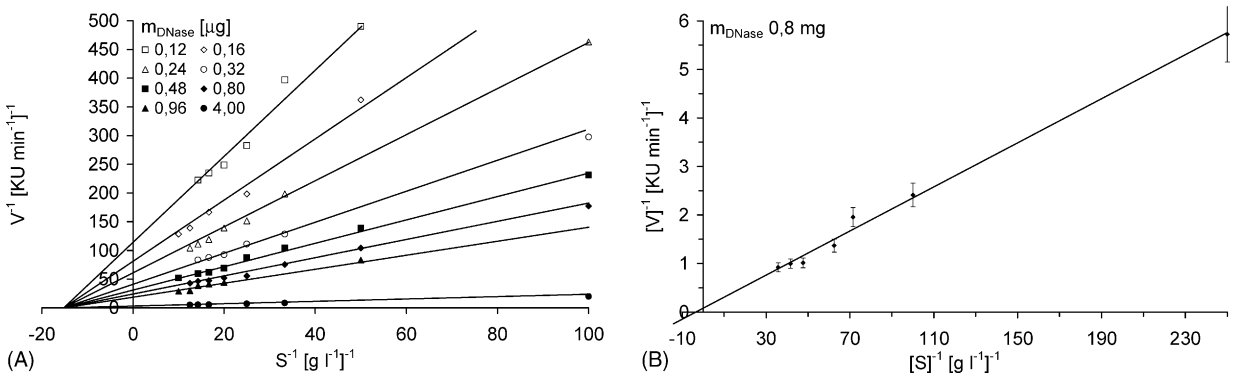

Fig. 1. A double reciprocal plot of free and immobilized DNase kinetics: reciprocal activity (v^{app}) is plotted as a function of reciprocal substrate concentration (S). (A) Free DNase (0.0001–0.00096 g l^{-1}) was applied into solution containing calf thymus DNA 0.005–0.1 g l^{-1} in 40 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at 25 °C in final volume 1 ml. The biological activity of free DNase was determined by monitoring the subsequent time dependant increase in absorbance at 260 nm as described in Section 2.4.1. (B) The reagent solution stream carrying substrate, calf thymus DNA 0.006–0.08 g l^{-1} in 40 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ was pumped through DNase monolithic column with flow rates from 0.1 to 10 ml min^{-1} and absorbance at 260 nm was monitored. The biological activity of immobilized DNase was determined by on-line frontal analysis as described in Section 2.4.2.

For majority of immobilized enzymes, an increase in K_m and a decrease in k_3 are characteristic [2]. K_m increase could be assigned to either limited interaction between substrate and enzyme or to conformational changes of the enzyme resulting in a lower potential to form substrate enzyme complex. The diffusion limitation that also contributes to an increase of K_m is brought to a minimum with monolithic supports compared to conventional supports [2,14]. A decrease in k_3^{app} for immobilized DNase could be related to restricted conformation adaptation of DNase and limited accessibility for large macromolecules. At the same time, we have to take in consideration the possibility that not all DNase is active after immobilization, which consequently artificially reduces k_3^{app} readings. However, decrease of the turnover number is still an order of magnitude smaller as for the immobilized enzymes with low molecular mass substrates [2].

Flow dependent digestion of highly polymerized DNA (size from 50 to 200 kbp) using DNase bioreactor was evaluated by horizontal agarose electrophoresis (Fig. 2). Highly polymerized DNA at 0.036 and 0.027 g l^{-1} substrate concentration was efficiently degraded when the substrate flow rate through columns with immobilized DNase was below 0.6 ml min^{-1}. Degradation of DNA was detected already at flow rate 9 ml min^{-1} compared to control II, which stands for DNA after passing through epoxy monolithic column without immobilized DNase and used

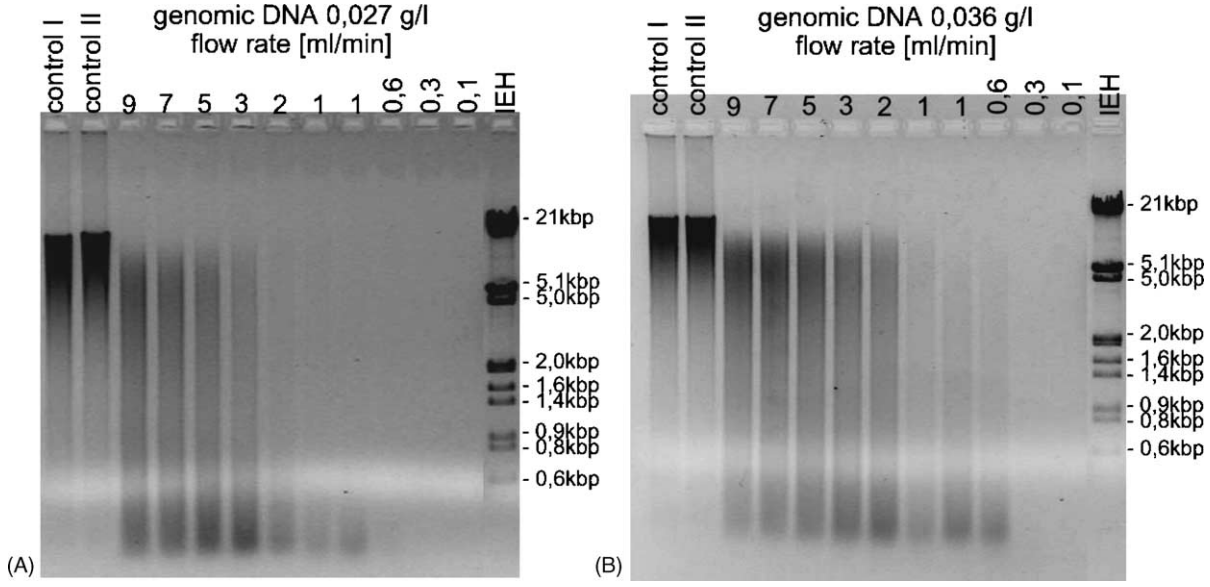

Fig. 2. Flow dependent degradation of DNA caused by immobilized DNase. The reagent solution stream carrying substrate, calf thymus DNA 0.027 g l^{-1} (A) and 0.036 g l^{-1} (B) in 40 mM Tris buffer pH 8, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at 25 °C was pumped through DNase monolithic column with flow rates from 0.1 to 10 ml min^{-1}. DNA samples (25 µl) were collected, separated on 0.8% agarose gel, voltage 100 V for 5 h, and DNA was stained with ethidium bromide. Legend: control I, DNA used in experiments; control II, DNA solution passed through HPLC system with epoxy monolithic column containing no DNase; lEH, lambda DNA *Eco*RI/*Hin*DIII size marker.

as a reference. The results reveal that DNase immobilized on monolith effectively degrades highly polymerized DNA.

3.3. Parameters effecting enzyme activity

Upon immobilization, the kinetic constants, K_m^{app} and k_3^{app}, of DNase were changed. The observed alternations could be assigned to either steric hindrance caused by presence of solid support or by shaping an enzyme conformation upon immobilization. For DNase is well documented that divalent cations, especially calcium ions have important impact on activity as well as on stabilization of enzyme [41,46]. Calcium ions bind on two asparagines (99 and 201) and tyrosine 87 of DNase and alter enzyme conformation and simultaneously stabilize and protect enzyme against proteolitic degradation [41–44]. We were interested if immobilization alters meaning of calcium ions for DNase activity. The effect of calcium ions was studied in the range from 0 to 2 mM calcium ion concentrations at 25 °C all in the presence of 1 mM magnesium ions at pH 8. For both, free and immobilized enzyme, highest activity (v^{app}) was observed when 1 mM calcium ions were present in solution. The free DNase expressed only 28, 58, 86 and 74% of its highest activity at 0.1, 0.2, 0.5 and 2 mM calcium ions concentration. Similarly, immobilized DNase expressed 34, 44, 64 and 93% of its highest activity at 0.1, 0.2, 0.5 and 2 mM calcium ions concentration. From these results it can be concluded that immobilization has no significant impact on the calcium binding site of DNase.

Chemical reactions and enzyme-catalyzed reactions up to a point proceed at a faster velocity with an increase in temperature. Because of that, the biological activities of free and immobilized enzyme at different reaction temperatures, 25, 30 and 37 °C were measured. The free DNase showed highest activity (v^{app}) at 37 °C, decreasing down to less than 40% at 25 °C. In contrary, the activity of immobilized DNase was not significantly changed when temperature was decreased from 37 to 25 °C. Therefore, degradation of DNA can be performed using immobilized enzyme already at room temperature facilitating the whole procedure.

Some enzymes in solution are not stable and activity decreases gradually during time. The enzyme activity (v^{app}) of free DNase decreased more than 50% in 6 days. Deactivation of immobilized DNase was much slower since on second day 89% of initial activity was preserved when stored in reaction buffer containing calcium, 75% of specific activity was measured after 6 days while after a month 10% of enzyme activity still remains. When immobilized DNase was stored in pure water without calcium ions, the activity decreased dramatically, reaching after 8 days only 20% of its original activity. It seems that immobilization places the enzyme either into more stable position less accessible to inactivation in comparison to a free enzyme or during immobilization process, impurities present in the enzyme, such as proteases, are removed from close proximity of the enzyme. Prepared enzyme reactors were used continuously 36 h (6 h over 6 days) with the activity of enzyme retained 75% of their original activity.

3.4. Use of immobilized DNase for removing DNA contaminants in reverse transcription

Reverse transcription coupled with PCR (RT-PCR) is a powerful method for detecting specific mRNA. However, for the detection of RNA transcripts by RT-PCR, the DNA contaminants have to be removed prior reverse transcription [4–7]. To remove DNA contaminants, samples of RNA are often treated with DNase that is removed from the reaction mixture by phenol extraction when reaction is completed. To omit unnecessary purification steps that represent a danger for contamination of samples, immobilized DNase could be used to treat RNA samples. The DNA contaminants in total RNA isolate were removed by passing crude RNA samples through column with immobilized DNase. DNase treated, total RNA was reverse transcribed into cDNA followed by PCR using specific primers that recognize different exons of the respective gene. Schematic presentation of amplification of genomic DNA and RNA with and without treatment with DNase is illustrated in Fig. 3.

In order to demonstrate activity of immobilized DNase, DNA isolated from *A. niger* was passed through the column with immobilized DNase at the flow rate 0.1 ml min^{-1} (treated sample). As a control, same DNA was passed through epoxy methacrylate disk without DNase (untreated sample). *PepC* gene was than amplified in treated and untreated samples. PCR product was detected only in untreated sample indicating that immobilized DNase efficiently removed DNA from treated sample (Fig. 4A).

In RNA samples, first we have determined the presence of DNA by performing PCR experiment on RNA sample without treatment and a PCR product similar in size but less in-

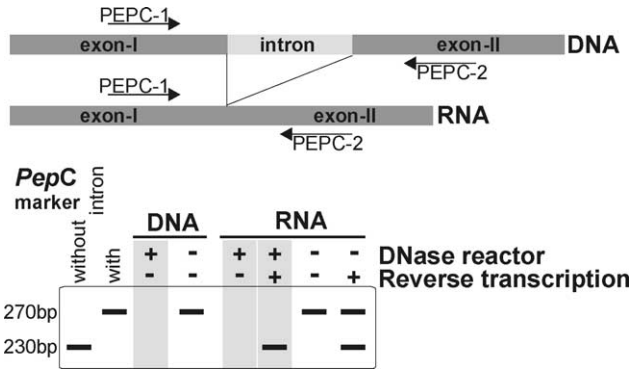

Fig. 3. Schematic presentation of either PCR products of DNA or RT-PCR products of RNA and DNA passed through column containing immobilized DNase (DNase reactor) or column without DNase activity (epoxy monolith). In order to distinguish between RNA and DNA RT-PCR products and consequently determine DNA impurities in RNA samples, primers used in PCR and RT-PCR reactions were chosen to anneal at different exons of DNA, which causes that PCR fragment of DNA is 40 base pairs larger than RT-PCR product of RNA.

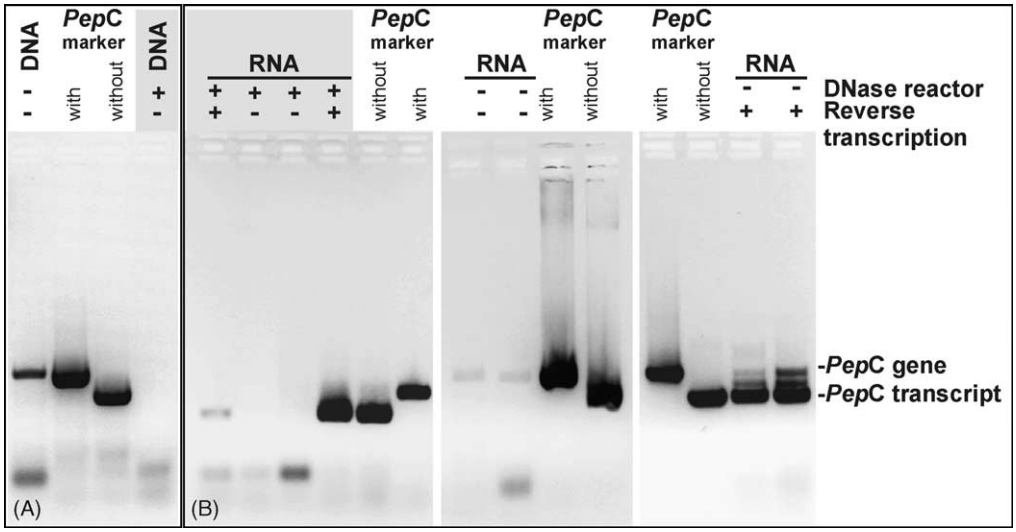

Fig. 4. Application of DNase monolithic column in DNA and RNA samples. (A) The PCR products of genomic DNA isolated from *Aspergillus niger* passed through DNase reactor and control reactor. (B) The PCR and RT-PCR products of total RNA isolated from *Aspergillus niger* passed through DNase reactor and control. Simultaneously with samples, PCR or RT-PCR was performed on plasmids containing *PepC* gene with and without intron and presence of PCR products was determined together with the PCR or RT-PCR products of the sample. Products (10 μl) were loaded on 1.6% agarose gel stained with ethidium bromide. DNase immobilized methacrylate monolithic column, with size 12 mm × 3 mm, median diameter of channels 6 μm, was used as DNase reactor. For control experiments, instead of immobilized DNase epoxy methacrylate monolithic column with identical characteristics as for DNase reactor was used. Flow rate was 0.1 ml min^{-1}.

tense compared with untreated DNA was obtained (Fig. 4B; RNA −,−). Presence of DNA was also shown when RT-PCR was performed on untreated RNA samples (Fig. 4B; RNA +,−). We obtained two distinct amplified products that matched both *PepC* markers that indicate presence of mRNA and DNA in a sample. The same RNA sample was than passed through column with immobilized DNase and RT-PCR was performed. Only one product was detected similar to *PepC* marker without intron indicating that specific RNA was reverse transcribed and amplified in RT-PCR. Amplification products corresponded to cDNA and no the genomic sequences were detected after treatment with immobilized DNase in samples for which it was previously shown to be contaminated with genomic DNA (Fig. 4B, RNA +,+).

The above experiments confirmed that DNase immobilized via epoxy groups present on methacrylate monolithic support could be used before RT-PCR to remove DNA contaminants in RNA samples. The DNase bioreactor presents useful tool for RT-PCR especially when we consider automation, high throughput and time saving as important factors in molecular biology, in particular diagnostics.

4. Conclusions

Epoxy methacrylate monolithic CIM supports are suitable for immobilization of deoxyribonuclease I. Short immobilization time, reasonably high activity and much longer stability of the immobilized enzyme in comparison to the free one are features that make such column a method of choice for the processes, where DNA removal is required, e.g. for RT-PCR. Furthermore, since the maximal activity of the immobilized enzyme is similar at elevated and room temperature, no need for accurate tuning of the temperature is required making the process removal more robust.

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