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Characterization of deoxyribonuclease I immobilized on magnetic hydrophilic polymer particles

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Abstract

Magnetic bead cellulose particles and magnetic poly(HEMA–co–EDMA) microspheres with immobilized DNase I were used for degradation of chromosomal and plasmid DNAs. Magnetic bead particles were prepared from viscose and magnetite powder. Magnetic poly(HEMA–co–EDMA) microspheres were prepared by dispersion copolymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate in the presence of magnetite. Divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+}) were used for the activation of DNase I. A comparison of free and immobilized enzyme (magnetic bead particles) activities was carried out in dependence on pH and activating cation. The maximum of the activity of immobilized DNase I was shifted to lower pH compared with free DNase I. DNase I immobilized on magnetic bead cellulose was used 20 times in the degradation of chromosomal DNA. Its residual activity was influenced by the nature of activating divalent cation. The immobilized enzyme with decreased activity was reactivated by Co^{2+} ions.

Keywords: Magnetic bead cellulose particles; Magnetic poly(HEMA–co–EDMA); Deoxyribonuclease I

1. Introduction

Enzymes immobilized on solid carriers have found wide application in molecular biotechnology and special analytical procedures [1,2]. In comparison with free enzymes, carriers with immobilized enzymes have numerous advantages as has been summarized in the literature [3]. An important application of immobilized enzymes is their potential use in special molecular diagnostic procedures [1,4,5]. They can digest selected target macromolecules at a constant rate and can be easily removed from the reaction mixture (which results in the rapid termination of the reaction). Quick and simple separation of

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immobilized enzyme from reaction mixture can be achieved when magnetic carriers are used [6,7].

DNase I hydrolyses double-stranded and single-stranded DNA depending on the metal ion used as activator [8,9]. DNase I is often employed in the following applications: introduction of single or double stranded nicks into DNA chains [10] and study of gene expression using reverse transcription polymerase chain reaction (RT-PCR) [11]. Immobilized DNase I was used to remove the endogenous DNA from *Escherichia coli* cell-free extracts [12]. Micrococcal endonuclease immobilized on nylon and polystyrene supports was used for digestion of pBR322 plasmid DNA [3]. For polystyrene, hydrophobicity should be modified before use thus requiring additional steps for hydrophilization of the carrier surface. DNase I covalently immobilized on agarose matrix has also been used for removal of contaminating genomic DNA from total RNA in the process of cDNA amplification by PCR and for affinity chromatography of actin [13–15]. Recently, DNase I was adsorbed on the copper-iminodiacetic acid chelate bound to non-porous poly(methyl methacrylate–*co*–glycidyl methacrylate) particles [16].

For the above-mentioned reason we used newly designed spherical magnetic particles such as bead cellulose and non-porous poly(HEMA–co–EDMA) for immobilization. These matrices possess interesting properties such as chemical stability, low non-specific protein adsorption and bio-compatibility [17,18]. Previously we used RNase A immobilized on magnetic bead cellulose particles [19] and magnetic non-porous poly(HEMA–co–EDMA) microspheres [18] for digestion of high-molecular mass RNA.

The aim of this work was to compare the activity of free and immobilized DNase I in degradation of chromosomal and plasmid DNAs depending on pH and the presence of activating divalent cations. Repeated application of immobilized DNase I was also studied depending on the presence of activating divalent cations.

2. Materials and methods

2.1. Chemicals

Agarose was purchased from Lachema (Brno, Czech Republic), ethidium bromide, 2,4,6-trichloro-1,3,5-triazine (TCT) and DNase I from Sigma (St. Louis, USA), DNA from Reanal (Budapest, Hungary), and tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) from Serva (Heidelberg, Germany). Perloza MT 200-spherical cellulose particles swollen in water were obtained from Lovochemie (Lovosice, Czech Republic). Cubic magnetite (ferrous-ferric oxide Fe_3O_4, 200 nm) was prepared in the Institute of Inorganic Chemistry, Academy of Sciences of the Czech Republic (Prague, Czech Republic). Other chemicals and solvents were of analytical grade and from commercial sources.

2.2. Equipment

Magnetic particles were separated using a Dynal MPC-M magnetic particle concentrator (Dynal, Oslo, Norway). Agarose gels were run in a gel electrophoresis apparatus from Bio-Rad Labs (Richmond, USA). Spectrophotometric measurements were carried out on UV spectrophotometer Spectronic Genesis V (Spectronic Instruments, Rochester, USA).

2.3. Methods

2.3.1. Preparation of carriers

Magnetic bead cellulose was prepared from viscose and magnetite powder according to the published procedure [20]. Magnetic poly(HEMA–co–EDMA) hydrogel microspheres were prepared by cellulose acetate butyrate-stabilized and dibenzoyl peroxide-initiated dispersion copolymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate in the presence of magnetite [18]. The size of microspheres was 1.2 μm with polydispersity index (weight-to-number average diameter of the particles) of 1.07.

2.3.2. Immobilization technique

Enzyme (DNase I) was coupled to both types of particles by the trichlorotriazine method. Experimental conditions for immobilization of DNase I on magnetic bead cellulose were based on the previously published procedure [21]. Carriers with particle size of 63–125 and 150–400 μm and containing 19.9 and 9.4 mg of enzyme/ml of carrier, denoted as (MBC1) or (MBC2), respectively, were used for bacterial or plasmid DNAs degradation. Immobiliza-

tion protocol for coupling of DNase I on magnetic poly(HEMA–co–EDMA) microspheres briefly was as follows. A mixture of 0.1 g dry magnetic poly-(HEMA–co–EDMA) microspheres and 20 ml of acetone–water (95:5, v/v) solution was stirred for 5 min, the particles left to sediment for 10 min, the supernatant sucked off and the whole process repeated three times. Volume of acetone–water was adjusted to 5.6 ml and the mixture left to stand for 24 h at laboratory temperature. Next 0.031 ml of 2.5 wt.% aqueous NaOH solution was added (NaOH/TCT, 1 mol/mol), the mixture was stirred for 1 h at laboratory temperature and cooled to 3 °C. Then 0.02 ml of TCT/acetone solution was added (3.73 mg TCT; 0.2 mmol TCT/g of polymer) and the mixture was stirred for 45 min. The particles were repeatedly (three times each) washed with ice-cold acetone and ice-cold water, and redispersed in a solution of 10.12 mg DNase I in 5 ml of 0.05 *M* phosphate buffer containing 0.15 *M* NaCl, pH 8.0, in a stirred reactor equipped with a combined glass electrode and a capillary for the addition of 0.1 *M* aqueous NaOH to keep pH within the range 7.95–8.05. The immobilization proceeded for 4 h at laboratory temperature, the particles were separated by centrifugation, lyophilized and stored at 4 °C. Magnetic poly-(HEMA–co–EDMA) microspheres contained 13.9 mg of enzyme/g of carrier. The amount of enzyme bound to the matrix was determined from the difference of DNase I concentration in the reaction solution before and after the coupling, using UV absorption at 280 nm.

2.3.3. Spectrophotometrical measurement of DNase I activity

The effect of DNase I action on DNA is manifested by the increase in ultraviolet light absorption [22]. Activity of the immobilized enzyme was assayed after incubation of an appropriate amount of bound enzyme with 0.3 ml of DNA (1 mg/ml) in a buffer (total volume of reaction mixture was 3 ml) at 30 °C in a thermostated shaker for 1 h, followed by measurement of UV absorbance at 260 nm [22]. Relative change of UV absorbance due to the action of immobilized DNase I on DNA was expressed as A_r (%) and calculated from

$$A_r\,(\%) = (A_f - A_0)/A_{0*}100$$

where A_0 and A_f are mean absorbances of digestion mixture at the beginning ($\tau = 0$) and at the end of reaction ($\tau = 60$ min), respectively, always calculated from two independent measurements.

2.3.4. Influence of pH on the enzymatic activity

The effect of pH on the enzymatic activity was estimated after 1-h incubation of the immobilized enzyme with DNA dissolved in an appropriate buffer at 37 °C. The following buffers were used to estimate activity of the enzyme immobilized on magnetic bead cellulose: (i) acetate buffer (0.2 *M* acetic acid + 0.2 *M* sodium acetate), pH 4.0–6.0; (ii) Tris–HCl buffer (0.05 *M* Tris–HCl), pH 6.5–9.6. Tris–HCl buffer (pH 7.8) was used for testing activity of the enzyme immobilized on magnetic poly(HEMA–co–EDMA) microspheres.

2.3.5. Isolation of plasmid DNA

Plasmid DNA pUC19 (2686 bp) was isolated from *E. coli* JM109 (pUC19) cells by alkaline lysis [10] and dissolved in TE buffer (100 m*M* Tris–HCl and 10 m*M* EDTA, pH 8.0). The primary sample containing plasmid DNA and bacterial RNA impurities was used to test the activity of both free and immobilized DNase I.

2.3.6. Gel electrophoresis

Gel electrophoresis was carried out in 1% agarose at 1.5 V/cm for 16 h using TBE buffer (45 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0). Gels were stained with ethidium bromide (0.5 μg/ml). The identity of nucleic acids was checked by gel electrophoresis and UV spectrophotometry. The absorbance ratio $A_{260\ nm}/A_{280\ nm}$ was used as a test of nucleic acid purity [23].

2.3.7. Biological activity

Biological activity of immobilized DNase I was checked by introduction of single- or double stranded nicks into plasmid DNA depending on the metal ion used as an activator. While Mg^{2+} ions were used for introduction of single-stranded (ss) nicks into plasmid DNA molecule, Co^{2+} and Mn^{2+} ions were used for double-stranded (ds) nicks [8,9]. Plasmid DNAs were treated in Tris–HCl buffer (pH 7.8, 37 °C) complemented with the required cation for the appropriate time. Agarose gel electrophoresis was

used to check differentiation between various structural forms of plasmid DNA carrying ss or ds nicks.

3. Results and discussion

Hydroxyl groups containing supports obtained by sol–gel transition of viscose [20] or by the dispersion copolymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate in the presence of iron oxide [18,24] were chemically modified to introduce chlorotriazine groups on their surface which were used for immobilization of protein DNase I. The advantage of chemical attachment of enzyme to the support consists in the stability of the bond between DNase I and the support. It is in contrast to simple adsorption of proteins onto particles with immobilized metal ions, where leakage of enzyme from the carrier is a problem [16].

3.1. pH Dependence of enzymatic activity

Protein activity may vary with pH and thus the effect of pH on the activity of free DNase I and DNase I immobilized on magnetic cellulose (MBC1) was studied. At first the influence of pH on the activity of free DNase I was determined. The acetate and Tris–HCl buffers were used for pH range 4.0–6.0 and 6.5–9.6, respectively. Results of the degradation of chromosomal DNA are given in Figs. 1 and 2. The maximum of DNase I activity was shown to be at pH 6.0 for Ca^{2+}, Mg^{2+} and Mn^{2+} ions in acetate buffer whereas it was shifted to lower value pH 5.5 for Co^{2+} ions. However, the acetate buffer contained besides divalent also monovalent Na^{+} ions and DNA degradation occurred in pH range 5.0–6.0 in the presence of Na^{+} ions only. Observed enzymatic activity could be thus a result of synergic effect of mono and divalent ion activation. A greater influence of Na^{+} ions was estimated for pH 6.0 than for pH 5.0. The maximum of DNase I activity was shifted to pH 5.0 after mathematical elimination of the effect of Na^{+} ions. No DNA cleavage was detected after the addition of EDTA. This fact confirms the early published finding [8,9] that DNase I is activated by metal ions. DNase I (Fig. 2) immobilized on magnetic bead cellulose (MBC1) had a maximum activity at a lower pH value (5.0 for

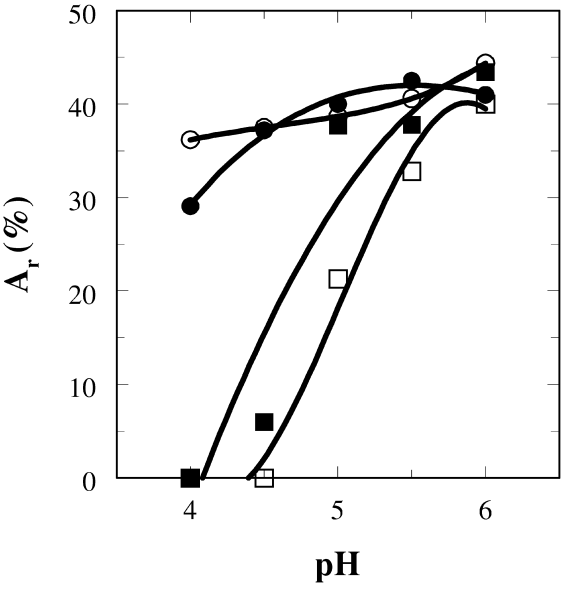

Fig. 1. pH Dependence of activity A_r (%) of free DNase I in acetate buffer. Activated by □ Ca^{2+}, ■ Mg^{2+}, ○ Mn^{2+}, and ● Co^{2+} ions (10 m*M*).

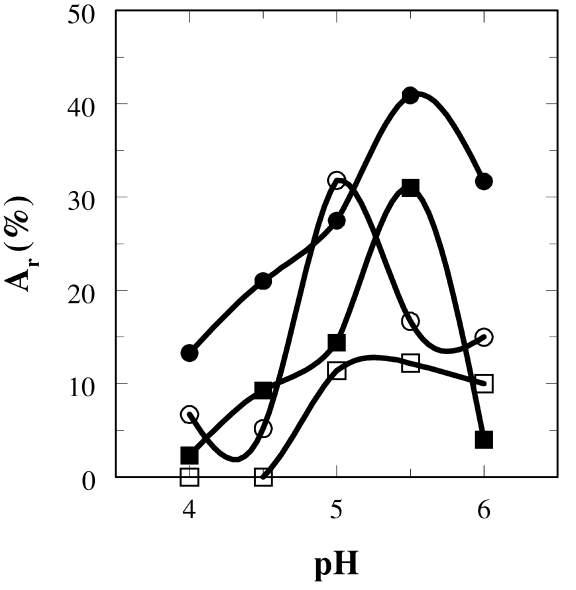

Fig. 2. pH Dependence of activity A_r (%) of DNase I immobilized on magnetic bead cellulose MBC1 in acetate buffer. Activated by □ Ca^{2+}, ■ Mg^{2+}, ○ Mn^{2+}, and ● Co^{2+} ions (10 m*M*).

Ca^{2+}, Mn^{2+} ions and 5.5 for Co^{2+}, Mg^{2+} ions) than free enzyme. A shift of the pH maximum was observed for immobilized enzymes in many other systems [25].

Tris–HCl based buffers were used for determination of free and immobilized DNase I activity in pH range 6.5–9.6. The results are given in Figs. 3 and 4. A plateau occurred in pH range 6.5–7.6 if Mg^{2+} and Ca^{2+} ions were used as activators. An indistinctive maximum was observed at pH 8.9, when enzyme was activated by Mg^{2+} ions. The highest absorbances found for Mn^{2+} ions (pH>8.0) and Co^{2+} ions (pH>6.6) were probably caused by chemical changes of these ions, as precipitation in measured samples occurred at both the above-mentioned pH values. No synergic effect of Na^{+} ions was found in Tris–HCl based buffers because any DNA cleavage was found without the addition of divalent ions.

3.2. Repeated use of immobilized DNase I

The determination of activity of DNase I immobilized on magnetic bead cellulose (MBC1) was repeated 20 times. The same ion was used as an activator for the immobilized enzyme. A different course of relationship between activity and number of repeated cycles was obtained for Ca^{2+} and Mg^{2+} ions on one site, and for Mn^{2+} and Co^{2+} ions on the other site. A pronounced decrease in the activities was found for immobilized DNase I activated by Ca^{2+} and Mg^{2+} ions. Totals of 3.4, 20.5, 72.2 and 91.2% of original activities were achieved using Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} ions, respectively. Each immobilized enzyme was reactivated after 20 cycles by Co^{2+} ions and original activity was reached.

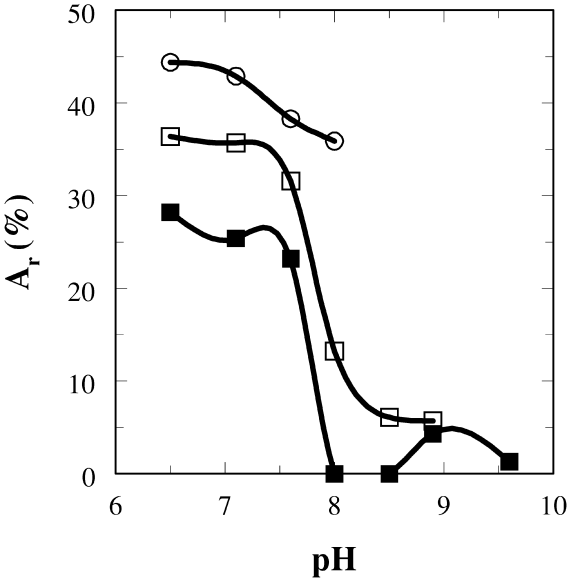

Fig. 3. pH Dependence of activity A_r (%) of free DNase I in Tris–HCl buffer. Activated by □ Ca^{2+}, ■ Mg^{2+} and ○ Mn^{2+} ions (10 m*M*).

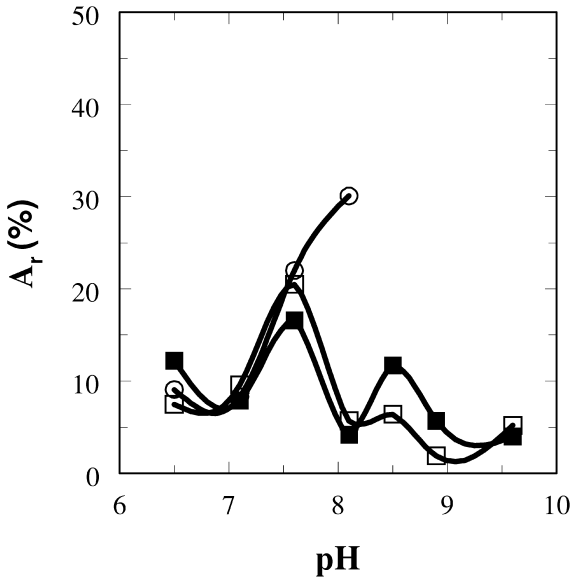

Fig. 4. pH Dependence of activity A_r (%) of DNase I immobilized on magnetic bead cellulose MBC1 in Tris–HCl buffer. Activated by □ Ca^{2+}, ■ Mg^{2+} and ○ Mn^{2+} ions (10 m*M*).

3.3. Cleavage of plasmid DNA

Agarose gel electrophoresis, the advantage of which consists in its ability to differentiate between various structural forms of plasmid DNA after introduction of single- (ss) and double-stranded (ds) nicks, was used for the analysis of cleaved plasmid depending on ions used. Tris–HCl based buffers were used in this study, because these buffers are usually used in molecular diagnostics, working with DNA. According to Melgar and Goldwait [8], DNase I hydrolyses DNA by single- and double hit mecha-

nisms depending on the type of ion used as activator. These different mechanisms led to the introduction of non-specific ss and ds nicks in the presence of Mg^{2+} or Ca^{2+} and Mn^{2+} or Co^{2+} ions, respectively. Introduction of ss nicks into plasmid DNA molecules results in the formation of a relaxed circle due to the conversion of ccc into oc plasmid DNA form. Oc form can be then converted to linearized DNA, if it is nicked at the same position in the complementary strand. The aim of designed experiments was to verify the possibility of quick differentiation of linear plasmid molecules from one of the other structural plasmid forms. As plasmid DNA was present in more bands due to different structural forms their number and intensity changed after cleavage with DNase I activated by an appropriate ion. Using DNase I immobilized on the carrier MBC2, the plasmid cleavage was achieved (despite of lower enzyme activity in the pH range used; Fig. 4). The results are given in Fig. 5. The single hit mechanism of DNA hydrolysis by DNase I can be reverted to double hit mechanism by the addition of Na^{+} ions. From this reason the increase of linear plasmid DNA band intensity was also found after activation with Mg^{2+} and Ca^{2+} ions in the presence of Na^{+} ions.

In our search for an optimal carrier with immobilized DNase I for DNA cleavage, we have studied the reaction on magnetic non-porous poly(HEMA–co–EDMA) microspheres. Since this support lacks of internal porosity, enzyme molecules are unable to be trapped in the pores and the effect of diffusion restriction is eliminated. DNase I immobilized on this carrier was also active in plasmid cleavage. However, DNA hydrolysis was slower in contrast to DNase I immobilized on MBC2. Results are given in Fig. 6.

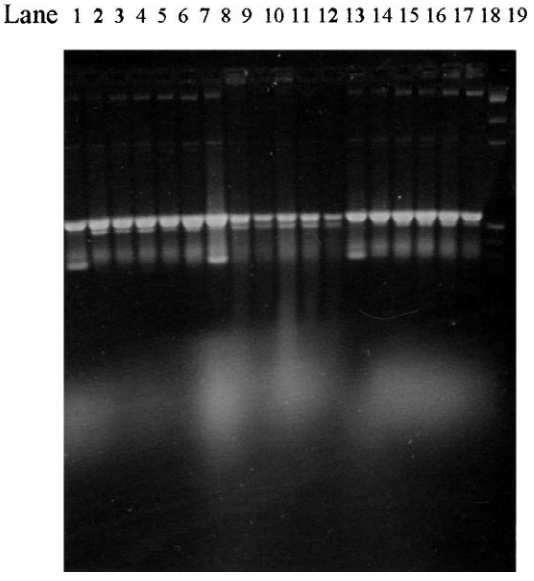

Fig. 5. Agarose gel electrophoresis of plasmid DNA pUC19 digested for 5 h by different amounts (2–10 μl) of DNase I immobilized on magnetic bead cellulose MBC2. Tris–HCl buffer (pH 7.8), 37 °C, complemented with Mg^{2+}, Mn^{2+}, and Ca^{2+} ions. Lanes: 1, 7, 13: plasmid DNA pUC19 without DNase I treatment; lanes 2–6, 8–12, and 14–18: plasmid DNA pUC19 treated with 2, 4, 6, 8, and 10 μl of immobilized DNAse I and activated by Mg^{2+} (10 m*M*), Mn^{2+} (10 m*M*), and Ca^{2+} ions (10 m*M*), respectively; lane 19: lambda DNA/Hind III standard complemented with pUC19/EcoRI.

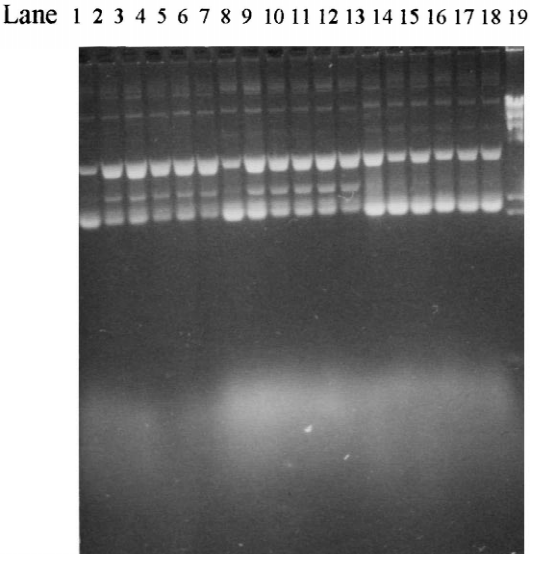

Fig. 6. Agarose gel electrophoresis of plasmid DNA pUC19 digested for 5 h by DNase I immobilized on poly(HEMA–co–EMDA) microspheres. Tris–HCl buffer (pH 7.8), 37 °C, complemented with Mg^{2+}, Mn^{2+}, and Ca^{2+} ions. Lanes: 1, 7, 13: plasmid DNA pUC19 without DNase I treatment; lanes 2–6, 8–12, and 14–18: plasmid DNA pUC19 treated with 2, 4, 6, 8 and 10 μl of immobilized DNAse I and activated by Mg^{2+} (10 m*M*), Mn^{2+} (10 m*M*), and Ca^{2+} ions (10 m*M*), respectively; lane 19: lambda DNA/Hind III standard. .

Enzymes immobilized on magnetic bead cellulose have a disadvantage in that they can not be prepared in a dry state and therefore they must be stored in an appropriate buffer. In molecular diagnostics, enzymes and other solutions are dosed using pipettes with plastic tips. Here, large particles tend to quickly sediment due to their size and density. The above mentioned fact influenced the accuracy and reproducibility of immobilized enzyme pipetting. On the other hand, magnetic poly(HEMA–co–EDMA) microspheres formed relatively stable suspension. This fact enables more accurate dosing of immobilized enzyme in comparison with magnetic bead cellulose particles. This is of great advantage from a practical point of view in routine laboratory work.

4. Conclusion

The results presented in this report show that DNase I immobilized on magnetic bead cellulose and non-porous poly(HEMA–co–EDMA) supports is suitable for degradation of chromosomal and plasmid DNAs. Maximal activity of DNase I immobilized on magnetic bead cellulose was shifted to lower pH compared with free DNase I. In our experiments DNase I immobilized on magnetic bead cellulose was used 20 times. The activity of immobilized Dnase I was dependent on the nature of activating divalent cation used. Immobilized enzyme with decreased activity can be reactivated by Co^{2+} ions.

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