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Automated microanalysis using magnetic beads with commercial capillary electrophoretic instrumentation

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Abstract

The potential of a new microanalytical method using magnetic beads (MBs) and commercial capillary electrophoresis (CE) instrumentation for performing enzymatic and inhibition assays, as well as for analysis of biological molecules such as antigens, substrates, etc., has been explored. A small quantity of magnetic beads containing immobilized biomolecules was injected into a neutral hydrophilic-coated fused-silica capillary. The short plug (2–3 mm) of beads was held fixed by a magnet placed in the cartridge of the CE system, without the use of frits. The beads could be replaced after each run, eliminating the need to regenerate the solid support. Two protocols were used for analysis: sequential injection (SI) and SI followed by isotachophoretic (ITP) focusing. Alkaline phosphatase (AP) and HIV-protease were used to demonstrate the SI procedure for enzymatic and inhibition assays. The second protocol, SI/ITP, was employed to quantitate an antigen (mouse mAB) using antibodies (sheep IgG towards mouse AB) immobilized on the beads. The MB–CE method, requiring only femtomole (fmol) quantities of material, can potentially be employed in diagnostic and forensic assays, kinetic studies and searching for inhibitors, ligands, receptors, etc. © 1997 Elsevier Science B.V.

Keywords: Magnetic beads; Enzymatic analysis; Immunoassays; Enzymes; Antigens

1. Introduction

Miniaturization of biochemical assays is of increasing interest, and, as a result, various microanalytical techniques based on a capillary format have recently appeared. For example, electrophoretically mediated microanalysis (EMMA) that allows enzyme assays to be carried out in capillary electrophoresis (CE) columns has been presented [1]. This method utilizes differences in the mobility of enzymes, substrates and products for electrophoretically mixing reagents and detecting enzymatically derived products. In another approach, the combination of CE and on-line post-column microreaction was described for separating enzymes and monitoring their activity [2]. In both methods, all components of the enzymatic system were in the liquid phase. Alternatively, enzymes were immobilized on the inner wall of a capillary enzyme reactor [3,4]. Additionally, the capillary format has been utilized for enzyme immunoassay with flow-injection analysis for digoxin as a model analyte [5].

On-line sample preconcentration in CE is another emerging development in microanalysis [6]. The use of multiple capillary bundles coupled to a single CE capillary was demonstrated for use as an analyte concentrator [7]. Reversed-phase packing material [8], as well as an impregnated membrane encased in

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a polytetrafluoroethylene (PTFE) cartridge [9] for sample preconcentration, have also been reported. Solid supports such as reversed-phase packing into the capillaries often requires frits to maintain the particles in the column. Preparation of these capillaries, as well as the membrane preconcentrator, can be a laborious procedure. Furthermore, the frits may generate bubbles [10]. We therefore sought a different approach.

The unique properties of magnetic beads (MBs) have led to their wide use as a solid support in a variety of applications, including immunoassays, cell sorting, support for immobilized enzymes and bioaffinity adsorbents [11,12]. In these applications, MBs were used in a batch mode. The use of MBs in flow injection immunoassays has also been reported [13].

In the present investigation, an automated miniaturized assay format, based on placing the MBs in the capillary and using a commercial CE instrument has been explored. MBs were injected by low pressure, kept in place by a magnet during the analysis, and could be removed from the capillary under high-pressure wash conditions. The approach is demonstrated using sequential injection (SI) and SI followed by isotachophoretic (ITP) focusing for enzymatic assays, inhibition analyses and immunotrapping with antigen determination. The use of MBs, which does not require frits and provides an automatable approach for the replacement of the solid-phase, is shown to have important potential in microscale analysis.

2. Experimental

2.1. Equipment

All experiments were carried out on a Beckman P/ACE 5100 CE instrument (Fullerton, CA, USA). The cartridge was modified by placing two round rare earth cobalt magnets [0.25 in. (6.35 mm) diameter, 0.2 in. (5 mm) thickness; Edmund Scientific, Barrington, NJ, USA], to contact the CE capillary in the mandrel. Capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). To prevent electroosmotic flow (EOF) and adsorption of the proteins to the walls, 27 cm×75 μm I.D. capillaries were coated with polyvinyl alcohol [14]. The effective column length was 20 cm.

2.2. Reagents

Monoclonal mouse antibodies against human growth hormone, biotinylated alkaline phosphatase (AP) and a phosphatase substrate kit, containing *p*-nitrophenyl phosphate (PNPP) and diethanolamine at pH 9.8, were purchased from Pierce (Rockford, IL, USA). HIV-protease was obtained from Pharmacia–UpJohn (Piscataway, NJ, USA). As a substrate of HIV-protease, a synthetic biotinylated (N-terminus) peptide was labeled with fluorescein at the C-terminal lysine (see Fig. 1). Other reagents were from Sigma (St. Louis, MO, USA).

2.3. Magnetic beads

Supermagnetic polystyrene microspheres (Dynabeads M-280, Dynal, Great Neck, NY, USA) of uniform size of 2.8 μm (R.S.D. max 3%) diameter were used in all experiments. Dynabeads precoated with sheep anti-mouse immunoglobulin G (IgG) were used for the immunotrapping experiments, and Dynabeads precoated with streptavidin were used for the enzymatic assay experiment.

2.4. Procedure

2.4.1. Pressure

All experimental steps such as injections, rinsings, reagent transfers, etc., were performed under low pressure conditions. The only step utilizing high pressure was removal of the beads from the capillary after analysis. Throughout this paper, low and high pressure refer to standard pressure conditions of the Beckman CE instrument, i.e., 3.5 kPa (0.5 p.s.i.) and 138 kPa (20 p.s.i.), respectively.

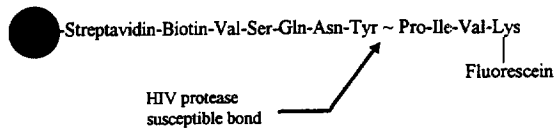

Fig. 1. Structure of the fluorescently-labeled substrate of HIV-protease attached to MBs.

2.4.2. Injection of MBs

In all experiments, a uniform suspension of MBs with concentration $2 \cdot 10^8$ beads/ml (3 mg/ml) was injected into the capillary for 1 min, using low pressure, leading to an MB plug of ~2–3 mm. To assure a uniform suspension, the beads were agitated by autopipette mixing directly in the inlet vial immediately prior to the injection.

Details of the procedures for enzymatic assays and antigen analyses are discussed in Section 3.1 Section 3.2.

3. Results and Discussion

This study expands the usefulness of CE instrumentation for enzymatic and immuno-microanalysis for sample volumes in the nanoliter range. The microanalytical procedures are based on the use of magnetic beads as a solid support. The beads can be easily loaded or removed from the capillary in an automated CE instrument, where various conditions (e.g., pressure and electric field) can be easily employed. The automated and microscale format of enzymatic assays has potential for use in screening and diagnostic applications.

3.1. Enzymatic analysis

3.1.1. Alkaline phosphatase

MBs with immobilized alkaline phosphatase and PNPP as substrate were used for these experiments. Streptavidin-coated MBs were first incubated with biotinylated AP. After injection into the capillary, the coated beads were washed with diethanolamine buffer supplied in the phosphatase substrate kit, and the capillary was then filled with the substrate solution at low pressure for 3 min, see Fig. 2a. Next, the system was incubated for up to 30 min while the enzymatic reaction took place. Following incubation, low pressure was applied, and the product, *p*-nitrophenol, formed when the substrate contacted the immobilized AP, was detected at 405 nm. For a given set of experiments, the same plug of beads was reused in each run.

Fig. 2a shows the chromatogram absorbance profile at one substrate concentration, while Fig. 2b presents the amount of *p*-nitrophenol at five different

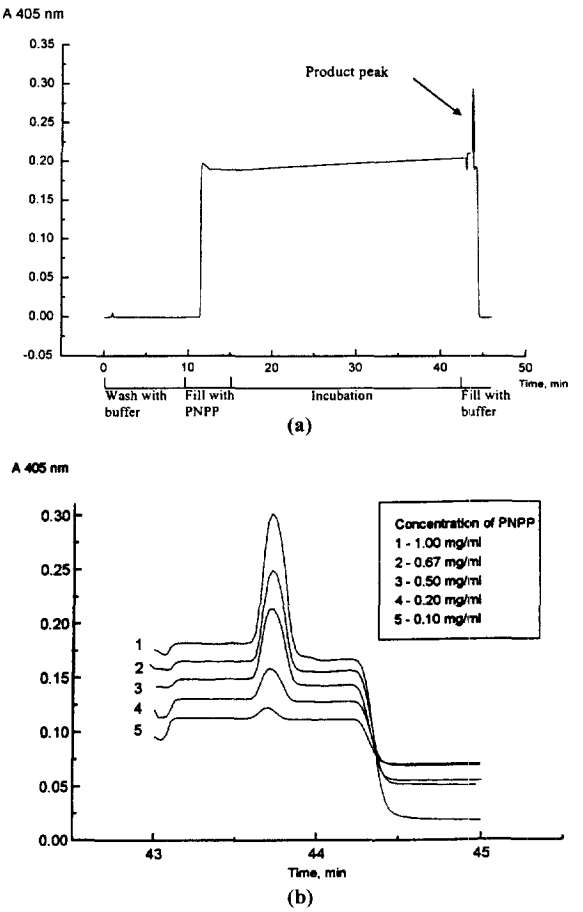

Fig. 2. Alkaline phosphatase enzymatic reaction on MBs in the capillary. (Enzyme is immobilized on MBs). (a) The absorbance profile of the enzymatic assay. Steps after loading beads in the capillary: (1) beads are washed with diethanolamine buffer for 10 min; (2) capillary is filled with substrate, *p*-nitrophenyl phosphate (PNPP), 1 mg/ml, for 3 min; (3) system is incubated for 30 min; (4) low pressure is applied for 2 min to detect *p*-nitrophenol at 405 nm. (Absorbance axis is in arbitrary units). Column conditions: see Section 2. (b) Enzymatic assay at five substrate concentrations; conditions as in (a).

substrate concentrations using the SI procedure. The change in baseline in Fig. 2b on the two sides of the product peak may be due to small leakage of the immobilized AP from the magnetic beads during loading of the substrate on the column. Product formed in the column during incubation would raise the baseline on the front side of the peak over that on the backside. Five identical runs at a PNPP con-

centration 1 mg/ml were analyzed for reproducibility with an R.S.D. of the product peak area of 9%.

To demonstrate the validity of this approach for microscale enzymatic assays, we determined the Michaelis constant K_M from the data in Fig. 2b using the Lineweaver–Burk equation, a modification of the well-known Michaelis–Menten equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} \quad (1)$$

where V=reaction velocity (i.e., product peak area/time), V_{max}=maximum reaction velocity and $[S]$=substrate concentration. Fig. 3 shows a linear plot of $1/V$ vs. $1/[S]$ with the y-intercept equal to $1/V_{max}$ and the x-intercept equal to $1/K_M$. The K_M value of 0.9 mM at pH 9.8 is in good agreement with the literature of 2 mM at pH 10 for intestinal AP [15].

3.1.2. HIV-protease

Instead of the above immobilization of an enzyme, for HIV-protease, a substrate (see Fig. 1) was attached to the MBs, and the beads were replaced after each run. The separation of reaction product from substrate prior to LIF detection and quantitation makes the approach a highly sensitive assay, employing fmol amounts of material.

The biotinylated substrate, labeled with fluorescein at the C-terminus, was attached off-line to the MBs

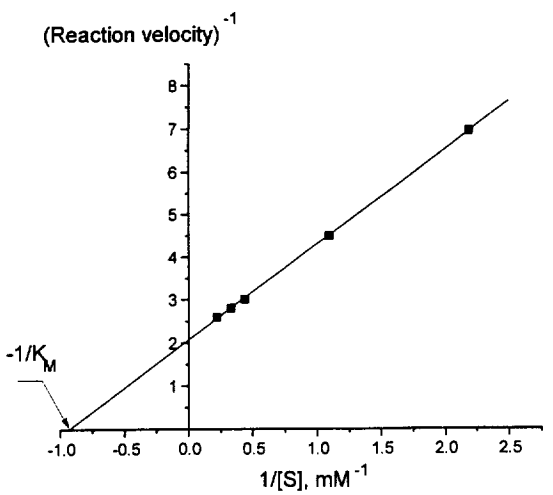

Fig. 3. Lineweaver–Burk plot for alkaline phosphatase enzymatic reaction using the MBs; [S]: substrate (PNPP) concentration; K_M=1.05 mM. (Reaction velocity^{-1} axis is in arbitrary units).

through the biotin–streptavidin linkage. After washing with 20 mM MES buffer (pH 6) containing 0.1% Triton X-100, the coated beads were loaded into the capillary and rinsed with the same buffer at low pressure. Then, a 12 μM solution of HIV-protease in the buffer was injected for 30 s with or without the protease inhibitor, acetyl-pepstatin, and incubated for 5 min with the 2–3 mm plug of beads. Low pressure was applied to transport the fluorescent digestion product to the detection window for LIF analysis. After each run, the beads were removed from the capillary by high pressure and a new plug of MBs loaded.

3.1.2.1. Determination of enzymatic activity

The enzymatic reaction for HIV-protease was examined using the SI procedure at different incubation times. The experimental profiles and control runs (without enzyme or 0 min incubation time) are presented in Fig. 4a. The broad earlier migrating peak is the proteolytically cleaved fluorescent product, and the sharp, later migrating peak represents the MBs passing the LIF detector region. The resulting plot of product peak area vs. incubation time for the enzymatic reaction is shown in Fig. 4b. For an HIV-protease concentration of 12 μM, a linear increase in product formation is observed, up to 5 min incubation, indicating that during this time period, substrate amount is not a limiting factor in the enzymatic reaction. A plateau in fluorescent product amount was reached after 10 min incubation. Next, the influence of HIV-protease concentration on the enzymatic reaction was studied. As seen in Fig. 4c, a linear relationship was obtained between the concentration of HIV-protease and the amount of fluorescent peptide released after the digestion of substrate on the beads during 5 min of incubation.

3.1.2.2. Inhibition analysis

Besides enzymatic assays, the inhibition of HIV-protease can be easily studied with the same system. The inhibitor, acetyl-pepstatin, was selected for this purpose. Using the SI procedure, the yields of the fluorescent product for different inhibitor concentrations were measured, relative to the blank experiment, and the results are shown in Fig. 5. The IC_{50} value (50% inhibition) of 1.93 μM for acetyl-pepstatin is in good agreement with literature results,

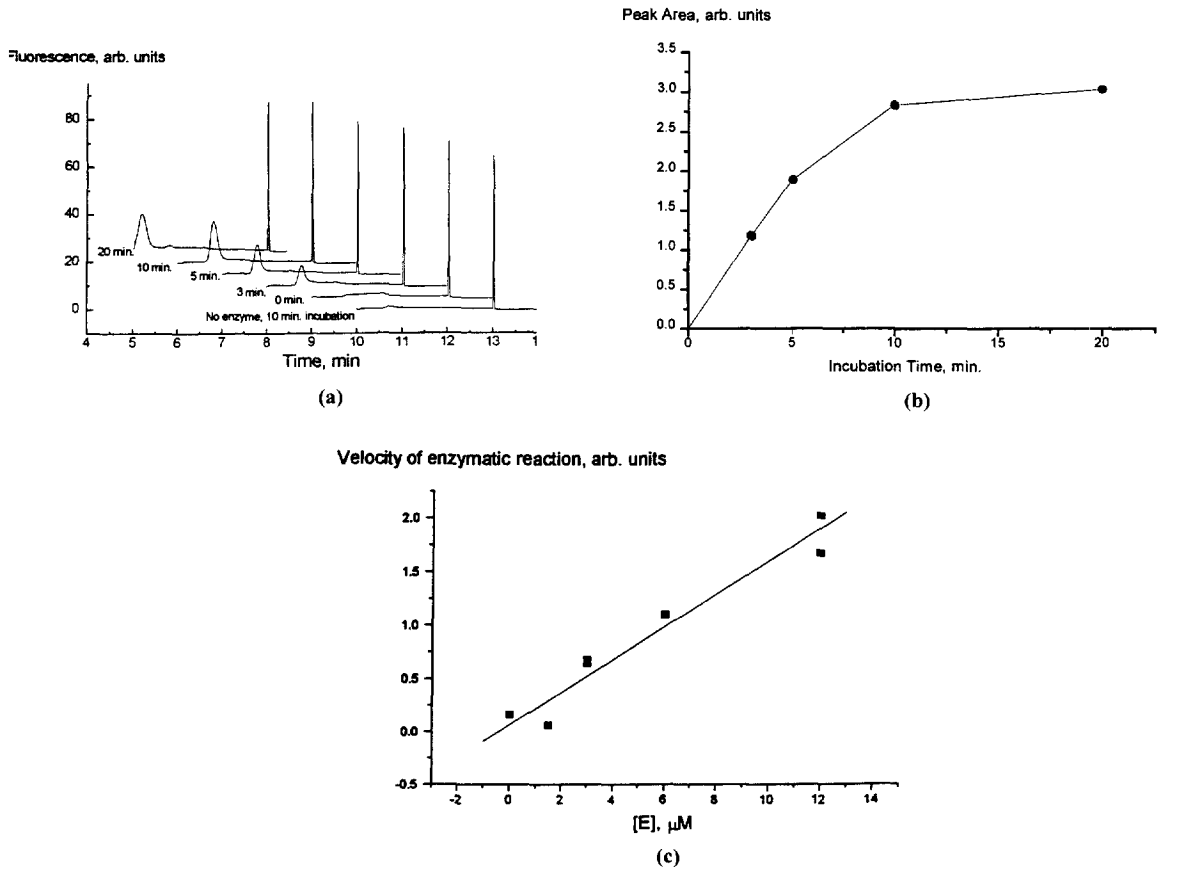

Fig. 4. Proteolysis by HIV-protease of fluorescein-labeled peptide (see Fig. 1) attached to the MBs. (a) The fluorescence profiles of the enzymatic assay for different incubation times. Conditions: HIV-protease concentration = 12 μ*M*; 20 m*M* MES buffer, pH 6, 0.1% Triton X-100; LIF detection at 520 nm. Column conditions: see Section 2. (b) Plot of the time course of the proteolysis reaction for HIV-protease from (a). (c) Plot of the velocity of the enzymatic reaction as a function of HIV-protease concentration. Conditions: see (a).

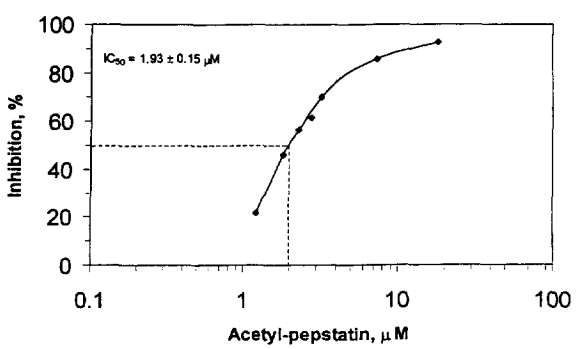

Fig. 5. Inhibition of HIV-protease as a function of the concentration of acetyl-pepstatin: $IC_{50} = 1.93 \pm 0.15$ μ*M*. 5 min incubation time. See Fig. 4a for other conditions.

where 35% of inhibition was observed for 1 μ*M* of acetyl-peptstatin at pH 7 [16]. Given the reliability of the assay, the method can be used for enzyme kinetic studies as well as for screening in the search for bioactive compounds, especially when sample amounts are limited. Note also that the method is easily automatable.

3.2. Determination of analyte by ITP focusing after on-line affinity trapping on magnetic beads

Besides enzymatic assays, the magnetic bead approach can potentially be used for affinity adsorption, followed by ITP focusing for quantitative analysis. We briefly explored this possibility using as

a model system, MBs with immobilized sheep IgG against mouse antibodies, for the detection of anti-human growth hormone monoclonal mouse antibodies (mAB). mAB will be referred to as "antigen", in order to emphasize that the method can be used for general analyte detection. For each experiment, a fresh batch of MBs was loaded, and then the beads were washed with 50 m*M* formic acid (Acid) followed by 50 m*M* ammonium formate–formic acid buffer, pH 7.6 (Buffer). Fig. 6a shows a schematic diagram of the process. An antigen sample dissolved in Buffer containing 1 mg/ml BSA was passed through the beads by low-pressure injection (Fig. 6a, step 1). The beads were then washed with Buffer at low pressure (Fig. 6a, step 2), and a plug of Acid sufficient to cover the beads was injected into the capillary for 0.5 min (Fig. 6a, step 3). Electrophoresis was performed at constant current (40 μA) with low pressure (Fig. 6a, step 4) and the eluted antigen was detected at 214 nm.

The above procedure uses a discontinuous electrolyte system to provide ITP stacking of the antibodies released from the beads. The scheme of the ITP process, shown in Fig. 6a, can be described as follows: after antigen loading on the MBs, 50 m*M* formic acid was injected into the capillary so that the plug of MBs with the trapped antigen was just covered with this low pH (~3.5) solution (step 3). The low pH caused the release of the captured antigen molecules from the MBs. Next, the electric current was turned on with the polarity as shown in the Fig. 6a, step 4. The boundary created between the ammonium formate solution (pH 7.6) and formic acid in the capillary started to move, due to the migration of ammonium ions followed by the H_3O^+ ions. Since the pH in the ammonium formate solution was high, the H_3O^+ ions could not penetrate through the moving boundary due to neutralization caused by acid–base equilibria. This process maintained a sharp moving boundary during the migration. More details of this process can be found in [17]. The antigen molecules released by the acidic pH possessed a strong positive charge and migrated quickly towards the moving boundary where they concentrated into a sharp zone. The final concentration in the antigen zone depended on the concentration and pH of the ammonium formate electrolyte [17]. In these experiments, the electric field and low pressure were simultaneously applied to overcome reversed EOF generated by the positively charged protein on the surface of the MBs under acidic conditions.

The profile of a typical run is shown in Fig. 6b, where the individual steps of Fig. 6a are shown on the time scale. Step 5 represents the high pressure removal of the MB with the appearance of a sharp peak. The peak at ~4.7 min. represents the antigen migrating past the detector. Fig. 7a presents results for different amounts of antigen from ~80–472 fmol. A plot of peak area vs. amount of antigen results in a linear calibration, see Fig. 7b. Note that in this case a UV detector has been used and that fmol amounts of material in nl volumes have been detected. Even lower quantities of material could be determined if fluorescently active antigens were used with LIF detection.

While the results in Fig. 7 are quite promising, it is important to note that in order to obtain consistent results, the concentration of suspended MBs had to be maintained constant during an experiment, and not exceed 5 mg/ml. (In our studies, a 3 mg/ml suspension was used). For a more concentrated suspension, the beads could plug the capillary during the injection. Improvements in the procedure for greater ruggedness and more complete automation would require: (1) more precise pressure control of the "injection" and "low pressure rinse" functions on the CE instrument; (2) automatic stirring of the magnetic beads to maintain the uniform suspension prior to injection; and (3) a better temperature and sample evaporation control in the sample compartment to maintain a constant concentration of suspended MBs.

4. Conclusions

The feasibility of using MBs as a solid support for analysis in a commercial CE instrument for enzymatic assays and preconcentration has been demonstrated. The procedure is rapid and automatable and utilizes fmol quantities of analytes. As necessary, the MBs can be replaced after each analysis, eliminating the need to regenerate the solid support. Among the advantages of the procedure are the potential for microscale operation (low sample and

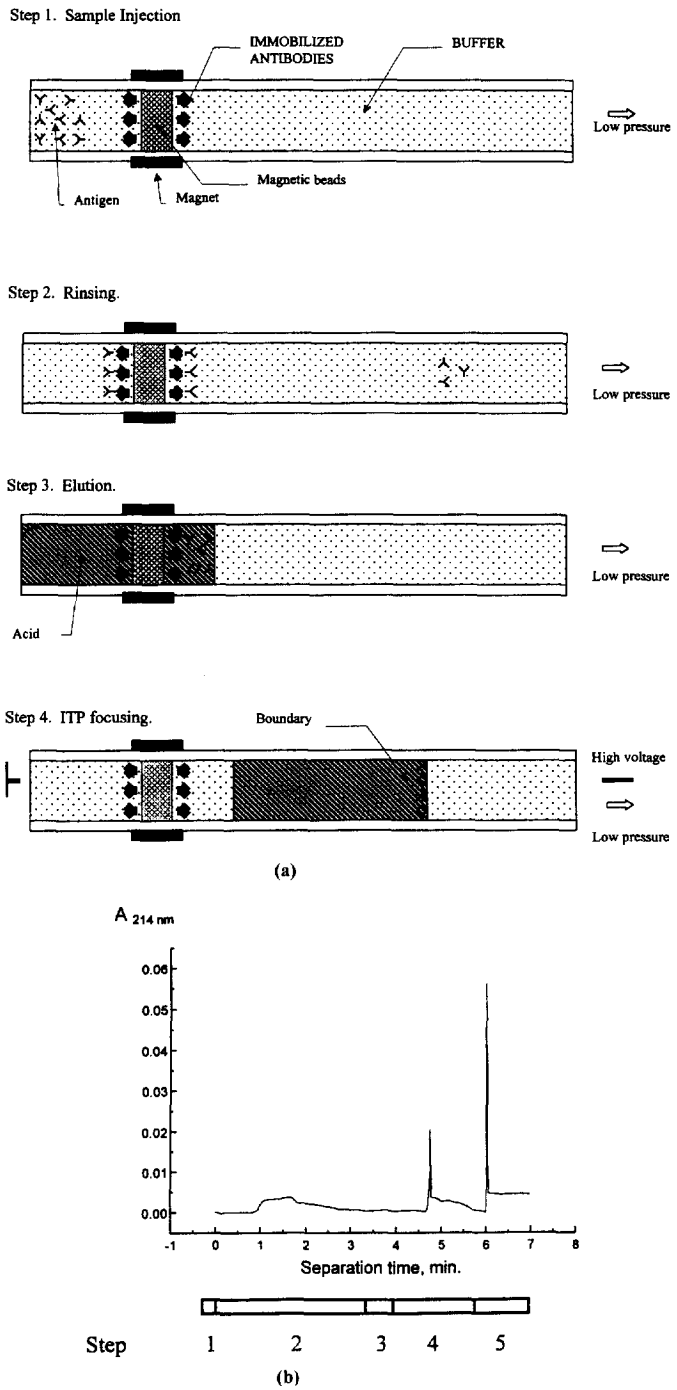

Fig. 6. Analysis of an antigen by immunotrapping and ITP focusing. (a) Conditions: 50 m*M* ammonium formate buffer, pH 7.6 (Buffer); 50 m*M* formic acid (Acid); PVA coated capillary, 27 cm (20 cm effective length)×75 μm I.D.; immobilized antibodies: sheep IgG against mouse antibodies; antigen: mouse monoclonal antibodies, injected from 0.1 mg/ml stock solution in Buffer, containing 1 mg/ml BSA. (b) 214 nm absorbance profile for a typical run; 315 fmol of antigen were injected. (Absorbance axis is in arbitrary units). Conditions: see (a).

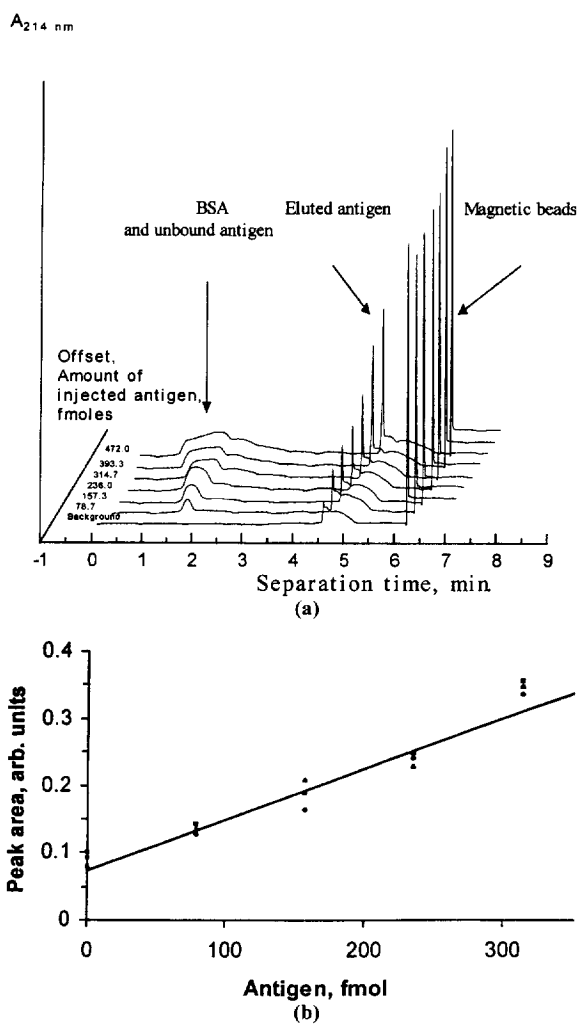

Fig. 7. (a) Separation profiles from 0–472 fmol of injected antigen. Conditions as in Fig. 6. (b) Calibration curve for the detection of mouse monoclonal antibodies. Conditions as in Fig. 6.

reagents consumption); automation; easy solid support load–unload procedures; absence of frits and short analysis time per assay. In conjunction with CE separation and ITP focusing, this approach may be advantageous for trace analysis. Although improvements with regard to using commercial CE systems are desirable, the method, nevertheless, has the potential to be used for separation, preconcentration and determination of biological molecules.

In this study, several examples of the applicability of magnetic beads for microanalysis in a capillary have been demonstrated. Alkaline phosphatase activity was measured and the Michaelis constant K_M easily determined. In the case of HIV-protease assay, the activity of this enzyme in the sample could be measured using a fluorescein- labeled substrate attached to the MBs. The presence of an inhibitor in the sample and its inhibition constant could also be determined. Finally, ITP was applied for analyte preconcentration after affinity separation and elution from MBs for trace analysis.

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