Biotechnology Letters **24:** 355–358, 2002.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*

Determination of selected xenobiotics with ferrofluid-modified trypsin

I. Šafařík[1,2,*], L. Ptáčková[1], M. Koneracká[3], M. Šafaříková[1], M. Timko[3] & P. Kopčanský[3]
[1]*Laboratory of Biochemistry and Microbiology, Institute of Landscape Ecology, Na Sádkách 7, 370 05 České Budějovice, Czech Republic*
[2]*Department of General Biology, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic*
[3]*Department of Magnetism, Institute of Experimental Physics, Watsonova 47, 04353 Košice, Slovak Republic*
**Author for correspondence (Fax: +420 38 5300249; E-mail: safarik@uek.cas.cz)*

Received 5 November 2001; Revisions requested 22 November 2001; Revisions received 2 January 2002; Accepted 4 January 2002

Key words: ferrofluid-modified trypsin, magnetic fluids, trypsin inhibitors

Abstract

Ferrofluid-modified trypsin has been used for the detection and determination of selected xenobiotics that inhibit trypsin activity. The procedure is useful especially when colored samples or samples containing suspended solid impurities are to be assayed. Ferrofluid-modified trypsin was inhibited by Ag^+ and Pb^{2+}, selected dyes (safranin, thionin), bacitracin and 4-aminobenzamidine. Enzymes immobilized on magnetic particles can form a basis of new automated assay procedures for the determination of xenobiotics.

Introduction

Various enzymes can be used to monitor the presence of inhibitory compounds in various matrices. For example, acetylcholinesterase is inhibited by various organophosphate and carbamate insecticides (Huber 1986). This inhibition forms the basis of many assay procedures used for the detection of these insecticides in various samples. Heavy metals ions can be determined using urease (Krawczyk *et al.* 2000, Preininger 1999) or horseradish peroxidase (Han *et al.* 2001). Both free and immobilized enzymes can be used for this type of assays.

Detection and determination of enzyme inhibitors may be complicated in situations when colored samples or samples containing suspended solids are to be handled. In this case immobilization of enzymes on magnetic solid supports can be advantageous (Šafařík & Šafaříková 1997). After interaction of the inhibitors with the immobilized enzyme the magnetic particles are captured using an appropriate magnetic separator, the excess of contaminating compounds and particles can be washed out and subsequently the remaining enzyme activity can be determined using the standard procedure. Additionally, enzymes immobilized on magnetic particles can form a basis of automated procedures for xenobiotics determination (Kindervater *et al.* 1990).

In order to evaluate the proposed procedure, trypsin immobilized on fine magnetite particles was used as a biologically active target structure. The decrease of trypsin activity by the tested inhibitors was evaluated.

Methods

Preparation of magnetic nanoparticles

Magnetite nanoparticles were prepared by coprecipitation of ferric and ferrous salts with alkaline solution and subsequent treatment under hydrothermal conditions. $FeSO_4 \cdot 7H_2O$ (27.8 g) and $FeCl_3 \cdot 6H_2O$ (54 g) were each dissolved in 100 ml double distilled water, thoroughly mixed and added to 8 M NH_4OH under continuous stirring at room temperature. The magnetic particles formed were finally dispersed in alkaline medium (pH 8.9) to form a pH-stabilized ferrofluid. The amount of magnetic particles in a

given volume of the solution was estimated by thermogravimetry and by measurements of magnetization curves using vibrating sample magnetometer (VSM). The particle size distribution was determined from the observations using transmission electron microscopy (TEM).

Immobilization of trypsin

Coupling agent 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (CDI, Sigma) was used to immobilize trypsin on magnetic nanoparticles (Koneracká *et al.* 1999). The reaction mixture containing trypsin, magnetic particles (ferrofluid) and CDI (dissolved and suspended in phosphate buffer, pH 7.5) in the ratio 1:2:2 (by wt) was shaken at room temperature for 24 h. After shaking, samples were placed on the top of a bar magnet where sedimentation of magnetic particles occurred within 2 min. Magnetic particles with immobilized trypsin were washed with saline. The quantity of non-bound trypsin was estimated in supernatant using the Bradford's dye binding assay.

Determination of trypsin activity

Activity of immobilized trypsin was determined spectrophotometrically using BAPNA (N-α-benzoyl-L-arginine-4-nitroanilide) as a substrate. The method of constant time was used. In the standard procedure an appropriate amount of magnetic trypsin suspension (usually 0.2 ml, corresponding to the sedimented volume 0.1 ml) was pipetted into a test tube and after magnetic capture (using a test tube magnetic separator MPC-1 or MPC-6; Dynal, Norway) the free solution was removed. Then 2 ml Tris/HCl buffer (pH 7.6) and 0.2 ml of BAPNA solution (26 mM in dimethyl sulfoxide) were added and the mixture was incubated at 30 °C for 10 min. The reaction was stopped by the addition of 0.3 ml 30% (v/v) acetic acid. After magnetic capture the absorbance of the solution was measured at 405 nm. One BAPNA unit (1 U) of trypsin activity was defined as the increase of the absorbance of 0.001 per min at 405 nm under standard conditions.

Determination of trypsin inhibition

The following procedure was used to detect and quantify the presence of trypsin inhibitors in water solutions. Washed suspension of the magnetic particles with immobilized trypsin (0.2 ml, corresponding to the sedimented volume 0.1 ml) was mixed with 1 ml of tested solution. Incubation proceeded under occasional mixing at room temperature for 1 h. Then magnetic particles were separated from the solution using a test tube magnetic separator and washed several times with distilled water or saline (detection of organic inhibitors) or with 0.9% $NaNO_3$ solution (detection of heavy metal ions).

Decrease of trypsin activity after incubation of trypsin inhibitor containing sample with immobilized trypsin was determined using the standard procedure described above. Percentage of inhibition was calculated using the standard formula:

$$\% \text{ of inhibition} = (A_N - A_I) \cdot 100/A_N \qquad (1)$$

where A_N and A_I are the activities of non-inhibited and inhibited trypsin samples, respectively.

Results and discussion

To verify the applicability of the proposed assay system, ferrofluid-modified bovine trypsin was used. This proteolytic enzyme is inhibited by selected heavy metal ions (Ag^+, Pb^{2+}) and organic compounds (e.g., 4-aminobenzamidine, bacitracin, safranin and thionin).

The native magnetic nanoparticles used for the immobilization exhibited lognormal particle size distribution with the following parameters: mean diameter $D_{TEM} = 9.6$ nm and standard deviation $\sigma_{TEM} = 0.22$. Immobilization of trypsin on these particles was performed using CDI as a coupling agent. Carbodiimide coupling was done in the one-step process when all the components were present in the reaction mixture at the same time (Ezpeleta *et al.* 1996). Under optimum conditions, 72% of the added trypsin was immobilized. After trypsin immobilization larger magnetic complexes were formed; the diameter of majority of particles ranged between 10 and 60 μm.

Inhibition of immobilized trypsin was monitored using the batch procedure. Figure 1 shows the dependence of percentage of trypsin inhibition on the concentration of the inhibiting silver and lead ions. As can be seen, almost complete trypsin inhibition was achieved with Ag^+ ions at 0.1 M while Pb^{2+} at 0.1 M caused 86% inhibition. On the other hand, almost no trypsin inhibition was observed for the concentrations of both ions below 10^{-6} M. The dependence exhibits a course, which is typical for similar type of assays.

Also selected organic compounds can inhibit the activity of immobilized trypsin (see Figure 2). These

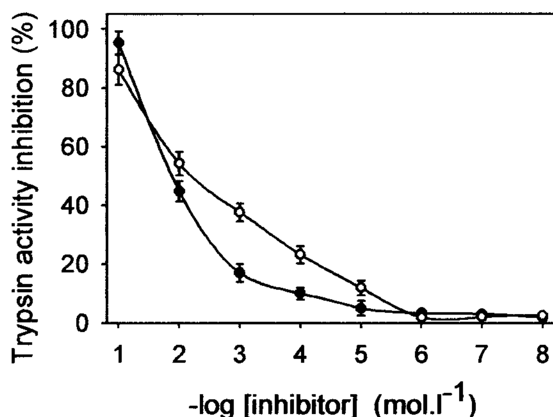

Fig. 1. Dependence of the inhibition of the trypsin activity on the concentration of Ag^{+} (•) and Pb^{2+} (○). The results represent the mean ± SD from three independent experiments. Magnetic particles with immobilized trypsin (0.2 ml, corresponding to the sedimented volume 0.1 ml) were mixed with 1 ml of solution of tested inhibitor. After 1 h incubation magnetic particles were separated from the solution and washed with 0.9% $NaNO_3$ solution. Decrease of trypsin activity was determined spectrophotometrically using BAPNA as a substrate and the method of constant time. Percentage of inhibition was calculated using the formula: % of inhibition $= (A_N - A_I)\cdot 100/A_N$ (A_N and A_I – activities of non-inhibited and inhibited trypsin samples, respectively). The activity of immobilized trypsin during the assay at 0% inhibition was 45 U.

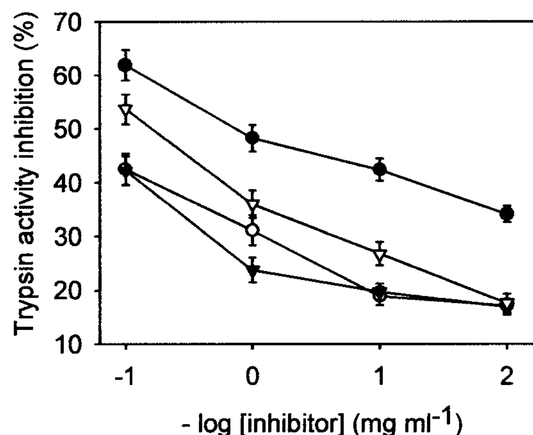

Fig. 2. Dependence of the inhibition of the trypsin activity on the concentration of different inhibitors. Thionin (•), 4-aminobenzamidin (▽), safranin (○), bacitracin (▼). The results represent the mean ± SD from three independent experiments. Magnetic particles with immobilized trypsin (0.2 ml, corresponding to the sedimented volume 0.1 ml) were mixed with 1 ml of solution of tested inhibitor. After 1 h incubation magnetic particles were separated from the solution and washed with distilled water or saline. Decrease of trypsin activity was determined spectrophotometrically using BAPNA as a substrate and the method of constant time. Percentage of inhibition was calculated using the formula: % of inhibition $= (A_N - A_I)\cdot 100/A_N$ (A_N and A_I – activities of non-inhibited and inhibited trypsin samples, respectively). The activity of immobilized trypsin during the assay at 0% inhibition was 35 U.

compounds comprehend both the well-known trypsin inhibitors and the dyes found to inhibit trypsin activity in the preliminary experiments. Detection of inhibition activity of colored compounds could be performed using photometric procedure thanks to trypsin immobilized on magnetic carrier. The possibility of simple washing of the immobilized trypsin due to the magnetic nature of the carrier is very important.

As can be seen from the results, appropriate enzymes immobilized on magnetic carriers could be used for monitoring of selected xenobiotics acting as enzyme inhibitors. Immobilization on magnetic carriers is advantageous due to the fact that the reaction can be performed also in the presence of suspended solid and colored compounds, which could otherwise cause problems in standard photometric assays. This type of assays based on the application of specific enzymes immobilized on magnetic carriers could be also used (due to its simplicity) for field measurements. On the other hand, automated assay systems employing magnetic separation process could be developed. Selection of enzymes used for this type of assay will depend on the type of the analyzed compounds and on the sensitivity of the enzyme against these inhibitors. It should be also pointed out that suitable immobilized enzymes could be also used for the detection of target ions, which are acting as enzyme activators.

Acknowledgements

This research is part of the Research Intention of ILE, No. AV0Z6087904. The experimental work was supported by the NATO Science Programme (Collaborative Linkage Grant No. LST.CLG.977500), Ministry of Education of the Czech Republic (grant project No. OC 523.80) and Slovak Academy of Sciences (Project No. 7020).

References

Ezpeleta I, Irache JM, Stainmesse S, Chabenat C, Gueguen J, Orecchioni AM (1996) Preparation of lectin-vicilin nanoparticle conjugates using the carbodiimide coupling technique. *Int. J. Pharm.* **142**: 227–233.

Han SB, Zhu M, Yuan ZB, Li X (2001) A methylene blue-mediated enzyme electrode for the determination of trace mercury(II), mercury(I), methylmercury, and mercury-glutathione complex. *Biosens. Bioelectron.* **16**: 9–16.

Huber GK (1986) Organophosphorus and carbamate residues in water. Colorimetric method. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis*, Vol 12. Weinheim: VCH, pp. 412–417.

Kindervater R, Künnecke W, Schmid RD (1990) Exchangeable immobilized enzyme reactor for enzyme inhibition tests in flow-injection analysis using a magnetic device. Determination of pesticides in drinking water. *Anal. Chim. Acta* **234**: 113–117.

Koneracká M, Kopčanský P, Antalík M, Timko M, Ramchand CN, Lobo D, Mehta RV, Upadhyay RV (1999) Immobilization of proteins and enzymes to fine magnetic particles. *J. Magn. Magn. Mater*. **201**: 427–430.

Krawczyk TKV, Moszczynska T, Trojanowicz M (2000) Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. *Biosens. Bioelectron.* **15**: 681–691.

Preininger C (1999) The enzymatic determination of mercury and copper using acid urease. The effects of buffers. *Mikrochim. Acta* **130**: 209–214.

Šafařík I, Šafaříková M (1997) Overview of magnetic separations used in biochemical and biotechnological applications. In: Häfeli U, Schütt W, Teller J, Zborowski M, eds. *Scientific and Clinical Applications of Magnetic Carriers.* New York and London: Plenum Press, pp. 323–340.