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Isolation and removal of proteolytic enzymes with magnetic cross-linked erythrocytes

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Abstract

New magnetic adsorbents for batch isolation and removal of various proteolytic enzymes were prepared by glutaraldehyde cross-linking of bovine, porcine and human erythrocytes in the presence of fine magnetic particles. Trypsin, chymotrypsin, alkaline bacterial protease and proteases present in various commercial enzyme preparations were efficiently adsorbed on these adsorbents; on the contrary, proteins without proteolytic activity were not adsorbed. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Proteolytic enzymes represent a very important group of enzymes, both from the point of view of the basic research and potential applications. Many procedures have been developed for the isolation of proteases, many of them being based on the principles of affinity chromatography. Affinity chromatography and other affinity-based procedures are of special interest because they allow the desired proteases to be isolated in a single step. In these methods various affinity ligands, such as protease inhibitors, specific peptides and amino acids, substrates, substrate analogues, antibodies, etc., have been immobilised on chromatography car-

riers [1]. The most general approach is based on the application of protease substrates, i.e., proteins. Soluble proteins such as haemoglobin, casein and gelatine have been used as affinity ligands immobilised on an appropriate carrier, while some insoluble (or insolubilized) proteins such as collagen, keratin or thermally modified casein have been directly used as affinity adsorbents [2–5].

Not always proteolytic enzymes are the target compounds to be isolated. Proteases, present in crude extracts or culture fluids, can also cause great problems in the course of the isolation and purification of other enzymes or proteins of interest. The unwanted proteolysis can result in the loss of enzyme activity and in the fragmentation of the target protein molecule.

For maximum protection of a target protein during its purification and storage, a mixture of protease inhibitors is usually added to the solutions. Due to the fact that proteases differ widely in their mechanism, no one protease inhibitor can

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effectively eliminate all proteases from a biological extract. Usually a mixture of protease inhibitors is used, containing e.g. leupeptin, phenylmethanesulphonyl fluoride (PMSF), pepstatin and disodium EDTA [6].

Another possibility to solve this problem is to remove contaminating proteases from the samples. Usually affinity adsorbents based on immobilised inhibitors (e.g. aprotinin or α_2 -macroglobulin) are used for this purpose [6,7]; many of them are commercially available, but they are usually rather expensive.

To simplify the isolation of the target proteolytic enzymes or the removal of contaminating proteases magnetic affinity adsorbents would be useful. New specific magnetic adsorbents, based on modified bovine, porcine and human erythrocytes, have been developed and are described in this paper. These adsorbents enable the isolation or removal of selected proteases present in the sample while retaining the non-protease enzymes or non-enzyme proteins of interest non-influenced.

2. Methods

2.1. Materials

Bovine, porcine and human erythrocytes were prepared from corresponding citrated blood by centrifugation and repeated washing with isotonic phosphate buffer (5 mM sodium phosphate buffer, containing 150 mM of sodium chloride, pH 7.4). Trypsin and chymotrypsin were from Léčiva, Czech Republic. Pronase P (protease from *Streptomyces griseus*) and glutaraldehyde (25% solution, w/v) were from Serva, Germany. Alkaline bacterial protease from *Bacillus* sp. was obtained from Liko, Bratislava, Slovak Republic. Neutral protease was from Amano, Japan. Technical enzyme preparations Brewers protease and Filtrase BR (containing proteases, amylases and hemicellulases) were from Gist-Brocades, The Netherlands. Acetylcholinesterase from bovine erythrocytes was from Boehringer, Germany. Cellulase from *Penicillium funiculosum*, alpha-amylase from *Bacillus licheniformis*, acetylthiocholine iodide, DTNB (5,5-dithio-bis-2-nitrobenzoic acid; Ellman's reagent)

and azocasein were from Sigma, USA. Black gelatine, black starch and black CM-cellulose (insoluble chromolytic substrates for the determination of proteolytic, alpha-amylase and cellulase activities, resp.) were prepared according to the described procedures [8–10]. Magnetite (iron(II,III) oxide, declared particle size $< 5 \mu\text{m}$) was from Aldrich, USA. Azoalbumin (bovine) and bacto-peptone were from Calbiochem and Difco, USA, respectively. Common chemicals were from Lachema, Czech Republic. Magnetic separators MPC-1 and MPC-6 were from Dynal, Norway.

2.2. Preparation of magnetic erythrocytes

Washed erythrocytes (50 ml, sedimented volume) and magnetite (5 g) were stirred in a 600 ml beaker using a mechanical stirrer (400 rpm) and 5 ml of 25% glutaraldehyde were quickly added. After 20 s the mixture was diluted with 100 ml of water and after another 30 s 300 ml of water was added. The suspension was then heated at 100°C under stirring for 30 min. During this period magnetic particles were formed. After cooling the magnetic particles were washed with water. The suspension was then homogenised by mixing to obtain fine magnetic particles which were then washed with water. To the washed suspension 5 ml of ethanolamine were added and the suspension was stirred at ambient temperature for 24 h. To increase the adsorption capacity of the adsorbent chloroform treatment was performed. The water-washed adsorbent was gradually transferred into methanol, acetone and chloroform. After 20 min chloroform treatment and one chloroform washing the adsorbent was transferred into acetone, methanol and water and thoroughly washed with water. The magnetic erythrocytes were stored in 0.02% sodium azide solution.

2.3. Isolation and removal of proteolytic enzymes

An appropriate amount of magnetic erythrocytes (usually 0.3 ml, sedimented volume) was mixed with 3 ml of the protease-containing solution. The magnetic erythrocytes were removed after 30 min of adsorption using a magnetic separator. The adsorbent was washed several times with water and the

adsorbed protease was eluted with 3 ml of glycine-HCl buffer, pH 2.2.

2.4. Fast protein liquid chromatography

The fast protein liquid chromatography was performed on a FPLC system (Pharmacia, Sweden) equipped with a MONO S HR 5/5 column, using 0.05 M acetate buffer, pH 4.6, as a mobile phase A, and the same buffer containing sodium sulphate (1 mol dm^{-3}) as a mobile phase B. The flow rate was 1 ml min^{-1} . The distribution of proteins in the effluent was monitored at 280 nm using a HP 1040 A diode-array detector (Hewlett-Packard, USA).

2.5. Analytical procedures

Determination of activities of proteases, alpha-amylases and cellulases was performed using black gelatine, black starch and black CM-cellulose as substrates, as described previously [8–10]. Acetylcholinesterase activity was determined with acetylthiocholine iodide as a substrate according to the procedure described previously [11]. Concentrations of azocasein and azoalbumin were determined spectrophotometrically at 400 nm. Lysozyme concentration was determined with cation exchange chromatography. Size distribution of the particles was determined using the particle size analyser Cilas 920 (France).

3. Results

Modification (cross-linking) of erythrocytes with glutaraldehyde in the presence of fine magnetic particles led to the formation of insoluble granular magnetic particles. The particles of modified erythrocytes were sufficiently rigid to enable batch magnetic separations. Magnetic particles prepared from all three types of erythrocytes had similar properties. To prepare the magnetic adsorbent with higher adsorption capacity the original particles were homogenised in a mixer and treated with chloroform. More than 90% of the final particles had the diameter smaller than $180 \mu\text{m}$ and 80% of the particles had the diameters in the range 20 and $180 \mu\text{m}$.

Various proteolytic enzymes (e.g. trypsin, chymotrypsin, alkaline bacterial protease from *Bacillus* sp., proteases present in various commercial enzyme preparations) were selectively adsorbed on this new adsorbent. There was not a significant difference in proteases binding on adsorbents prepared from various erythrocytes. Treatment of magnetic erythrocytes with chloroform increased the adsorption capacity of all types of modified erythrocytes. For example, magnetic porcine erythrocytes without chloroform treatment could adsorb approximately $400 \mu\text{g}$ of trypsin/ml of adsorbent, while after chloroform treatment the adsorption capacity was 10–15% higher. Chloroform treatment probably removed membrane structures from the modified erythrocytes and released more cross-linked haemoglobin, which is responsible for the specific binding of proteolytic enzymes. Proteases adsorbed to modified erythrocytes to various degree, depending on their type (see Table 1).

Differences in the elution of adsorbed proteases were observed. Various non-specific elution systems (high ionic strength, low pH, combination of both principles) were tested. The best elution of trypsin and chymotrypsin was achieved with glycine-HCl buffer, pH 2.2. However, other adsorbed proteases could not be easily eluted neither with this elution buffer nor other elution systems tested (see Table 1).

Specific adsorption of trypsin on magnetic erythrocytes was also tested in the presence of high concentrations of non-enzyme proteins. Trypsin solution was mixed with azocasein or azoalbumin which served as ballast proteins. Trypsin adsorption on magnetic erythrocytes was not substantially influenced by the presence of these proteins. The results are shown in Table 2.

Specific magnetic adsorbents enable efficient adsorption of the target analytes also from larger volumes of the samples [12]. Fig. 1 shows the adsorption of the constant amount of trypsin activity from volumes of solutions ranging between 3 and 100 ml, and the proteolytic activity eluted from the adsorbent. It can be clearly seen that the efficacy of the trypsin adsorption was relatively high also in the case when low protease concentrations are present in large sample volumes. Removal

Table 1

Adsorption of various proteolytic enzymes (from 3 ml solution) on porcine magnetic erythrocytes treated with chloroform (0.3 ml, sedimented volume) and their subsequent elution with 3 ml glycine-HCl buffer, pH 2.2. The values are given in percent of the initial proteolytic activity

Protease	Concentration (µg/ml)	Proteolytic activity adsorbed (%)	Proteolytic activity desorbed with buffer pH 2.2 (%)
Trypsin	50	90	80
Chymotrypsin	1500	94	80
Alkaline protease	100	75	0
Pronase P	100	25	5
Neutral protease	750	30	0
Brewers protease	500	70	0
Filtrase BR	500	60	0

Table 2

Adsorption of trypsin activity (from 3 ml solution containing 50 µg/ml of trypsin) on porcine magnetic erythrocytes treated with chloroform (0.3 ml, sedimented volume) in the presence of 5 mg contaminating proteins and subsequent elution of trypsin activity with 3 ml glycine-HCl buffer, pH 2.2. The values are given in percent of the initial proteolytic activity

Sample	Adsorption of trypsin activity (%)	Elution of trypsin activity (%)
Trypsin	90	80
Trypsin + azocasein	70	55
Trypsin + azoalbumin	70	55

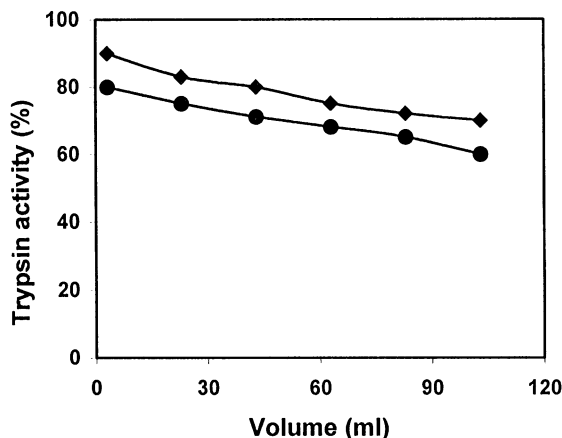


Fig. 1. Adsorbed (◆) and eluted (●) trypsin proteolytic activity after adsorption of the constant amount of trypsin (150 µg) from various volumes of solutions and subsequent elution of trypsin activity with low pH buffer. Chloroform-treated magnetic porcine erythrocytes (0.3 ml, sedimented volume) were used as the specific adsorbent. The values are given in percents of the initial proteolytic activity.

of contaminating proteases can also be improved using higher amounts of magnetic erythrocytes.

Specific adsorbents should exhibit low non-specific adsorption of various accompanying compounds, especially proteins. All three adsorbents were tested with various non-proteolytic enzymes and proteins without enzyme activity. In all cases the non-specific adsorption was very low, in most cases below 2% of the protein amount present. The results are shown in Table 3.

The course of trypsin purification from a model mixture containing vast amount of ballast proteins and peptides (bacto-peptone) is shown in Fig. 2. Cation exchange chromatography of the original protein mixture, protein mixture after adsorbent treatment and trypsin eluted with low pH buffer is shown. The ballast proteins were efficiently removed during the isolation procedure and that trypsin was substantially purified. Only low amounts of contaminating proteins or peptides were found in the purified trypsin preparation. The

Table 3

Non-specific adsorption of non-proteolytic enzymes and proteins without enzyme activity (1 mg/ml, total volume 3 ml) on chloroform-treated magnetic porcine erythrocytes (0.3 ml, sedimented volume). The values are given in percent of the initial enzyme activity or protein amount

Tested protein	Non-specific adsorption (%)	Analytical procedure
Alpha-amylase (Filtrase BR)	< 1	Activity assay
Cellulase	< 2	Activity assay
Acetylcholinesterase	< 1	Activity assay
Lysozyme	< 2	Chromatography
Azocasein	< 1	Photometry
Azoalbumine	< 1	Photometry

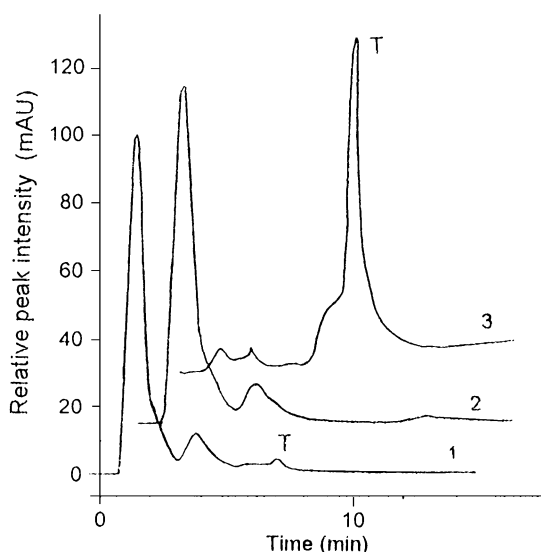


Fig. 2. Normalized cation exchange chromatograms of the original model mixture containing trypsin and bacto-peptone (curve 1), the same mixture after the treatment with porcine magnetic erythrocytes (curve 2) and trypsin eluted with glycine-HCl buffer, pH 2.2 (curve 3). T = trypsin.

chromatography also confirms that magnetic erythrocytes can be used for the specific removal of contaminating proteases from the samples, not influencing other proteins or peptides.

4. Discussion

Erythrocytes can be simply converted into magnetic particles. Modified erythrocytes can be successfully used during the separation process both for the isolation of selected target proteases and

removal of contaminating proteolytic enzymes from various samples. The specificity of the adsorbent is probably due to the haemoglobin present in the adsorbent. It was shown recently that immobilized haemoglobin can be used for efficient separation of wheat proteases [2]. In erythrocytes haemoglobin is the dominating protein and its cross-linking and subsequent particle formation may serve as an alternative procedure to prepare a specific protease adsorbent, in comparison to the covalent immobilization of haemoglobin to the inert carrier.

Magnetic erythrocytes represent an affinity adsorbent, where the adsorption of the proteolytic enzymes is caused by the interaction of the insolubilized substrate (haemoglobin) and the enzyme. Non-target proteins are only little adsorbed to magnetic erythrocytes. This is an important fact, which enables the application of this inexpensive magnetic adsorbent in the course of removal of contaminating proteolytic activity, without influencing the target proteins. Also lysozyme, which is a basic protein with the isoelectric point similar to that of trypsin is not adsorbed at all to the adsorbent on the contrary to trypsin.

Magnetic erythrocytes thus represent an inexpensive, easy-to-prepare affinity adsorbent for protease isolation and removal, which could be useful in many biochemical applications.

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