Biotechnology Techniques **13:** 621–623, 1999.

Determination of proteolytic activity with magnetic dye-stained gelatine

Mirka Šafaříková[1,*] & Ivo Šafařík[1,2]
[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*
[*]*Author for correspondence (Fax: +420 38 45719; E-mail: mirkasaf@uek.cas.cz)*

Received 4 June 1999; Revisions requested 15 June 1999; Revisions received 16 July 1999; Accepted 16 July 1999

Key words: gelatine, insoluble chromolytic substrate, magnetic separation, proteolytic activity

Abstract

A procedure for the determination of proteolytic activity with dyed magnetic gelatine as an insoluble chromolytic substrate is described. The magnetic nature of the substrate enables magnetic separation of unhydrolysed substrate from the hydrolysed dyed peptide fragments. Such type of substrates could enable the development of new automated protease assays based on the principle of Flow Injection Analysis (FIA).

Introduction

Many methods have been developed for the quantitative measurement of proteolytic activity. Protein-based substrates are often used for this purpose because of their sensitivity to the majority of proteases. These substrates are especially useful when activity of endoproteases with unknown specificity is to be determined such as in the case of newly discovered and isolated proteases of microbial origin. Both soluble and insoluble protein-based substrates can be used in the assays. Proteins are usually labelled with an appropriate marker, such as a dye (Šafařík 1988), fluorescent label (De Lumen & Tappel 1970) or an appropriate radioactive label (Gisslow & McBride 1975). The simplest assays use chromolytic substrates with spectrophotometric detection.

When soluble chromolytic proteins are used as substrates, unhydrolysed and slightly hydrolysed protein molecules have to be removed from the assay mixture after finishing the enzyme reaction. Usually precipitation with trichloroacetic acid is used, followed by the filtration or centrifugation step. Then the clear filtrate or supernatant is measured spectrophotometrically.

Insoluble chromolytic proteinaceous substrates are also often used for the spectrophotometric determination of proteolytic activity in animal, plant and microbial samples. Naturally occurring insoluble proteins such as fibrin, elastin, keratin or collagen (Wunderwald 1984) or soluble proteins rendered insoluble by cross-linking with a suitable bifunctional reagent (Šafařík 1989) or by entrapment into an appropriate polymer matrix (Šafařík 1988) may be used for the assay after labelling with a suitable dye or pigment. At the end of the reaction no precipitation step is necessary, the insoluble substrate is filtered or centrifuged out and the solution containing the released labelled peptides or pigment is used for the measurement (Šafařík 1989).

Using both types of substrates, the automation of the assay procedure is difficult. Therefore a new type of insoluble chromolytic protein-based substrates containing ferrimagnetic iron(II,III) oxide (magnetite) within the substrate particles has been developed. The insoluble magnetic substrate is incubated with the protease and cleavage of the substrate releases dye-stained peptides. The presence of magnetic moiety in the insoluble substrate enable to remove the unhydrolysed substrate particles from the reaction mixture using an appropriate magnetic separator, thus leaving

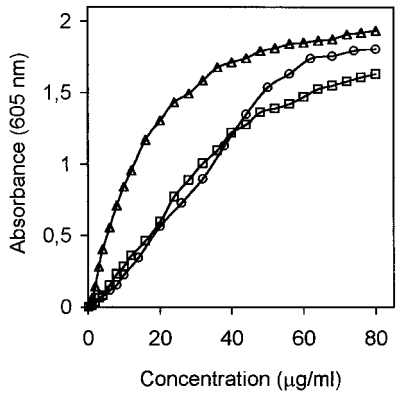

Fig. 1. Hydrolysis of magnetic insoluble chromolytic substrate as a function of proteases concentration. △ trypsin; □ alkaline protease; ○ Pronase P.

the dye-labelled peptides in the solution. The amount of hydrolysed peptides in the supernatant fluid is determined spectrophotometrically. This new magnetic substrate can be prepared easily and can be used for the determination of proteolytic activity of various proteases. Such a type of procedure might be particularly important when multiple assays should be performed.

Materials and methods

Materials

Gelatine and common chemicals were obtained from Lachema, Czech Republic. Glutaraldehyde was from Serva, and magnetite powder [iron(II,III) oxide, $< 5\ \mu$m] was from Aldrich, USA. Reactive textile dye (Ostazin Blue S-R) was from Spolek pro chemickou a hutní výrobu, Czech Republic. Pronase P (a protease from *Streptomyces griseus*) was from Serva, Germany, while trypsin was from Zdravotnické zásobování, Czech Republic. A crude preparation of alkaline bacterial protease was obtained from a local biodetergent producer. Test tube magnetic separators MPC-1 and MPC-6 were from Dynal, Norway. Flat magnetic separator (trough shape, dimensions 320 × 205 × 70 mm, active magnetic area 270 × 200 mm comprising 7 magnet poles of alternating polarity) was described recently (Šafaříková *et al.* 1996).

Preparation of magnetic substrate

The dyed magnetic gelatine was prepared in the following way. Gelatine (10 g) was suspended in 100 ml cold water for 30 min and then heated at 80 °C in a water bath. After the gelatine had dissolved, the solution was cooled to 37 °C and 4 g finely divided magnetite was added with thorough stirring. Then 10 ml of 10% (w/v) glutaraldehyde was added and the mixture was stirred intensively. The cross-linking was allowed to proceed for 24 h at ambient temperature. The cross-linked gelatine was then cut into small pieces and homogenised in a standard kitchen mixer to obtain fine particles. After washing the magnetic gelatine particles with water the dyeing procedure was performed as follows. The volume of the suspension was adjusted to 600 ml and 40 g NaCl and 10 g Ostazin Blue S-R were added. The suspension was stirred for 20 min at 40 °C and then 10 ml of 1.5 M NaOH was added. The mixture was stirred at the same temperature for 3 h and then left at ambient temperature overnight without stirring. The dyed magnetic gelatin particles were washed with water and methanol (with the aid of a flat magnetic separator) until the washings were colorless.

Assay of proteolytic activity

For the experiments, the buffered suspension of the substrate was usually used. The protease assay was performed in test tubes. The reaction mixture contained 2 ml of an appropriate buffer and 1 ml of the substrate suspension in the same buffer. Then 1 ml of protease solution was added to the substrate suspension (under mixing) and test tubes were incubated at an appropriate temperature (depending on the protease assayed) without mixing. In the standard procedure the reaction was stopped after 30 min by the insertion of the test tube into a magnetic separator for about 1 min and pipetting the supernatant into another test tube. The absorbance of the supernatant was measured at 605 nm against the substrate blank (1 ml of buffer was added to the substrate suspension instead of protease solution).

Results and discussion

Cross-linked, dyed gelatine was hydrolysed by a variety of microbial, animal and plant proteases (Šafařík 1989). Incorporation of fine magnetite particles into dyed gelatine gel has no influence on the ability of proteases to hydrolyse this magnetic substrate. It was shown in preliminary experiments that this substrate was hydrolysed with trypsin, chymotrypsin, papain, alkaline and neutral bacterial proteases and Pronase P.

Blue gelatine fragments are released during the substrate hydrolysis into the surrounding medium which begins to colour.

Bacterial alkaline protease, trypsin and Pronase P were used in the experiments to show the applicability of the magnetic substrate. The reaction mixture contained an appropriate buffer (0.1 M Tris/HCl buffer, pH 8.8 for trypsin and alkaline bacterial protease, and 0.1 M phosphate buffer, pH 7.5 for Pronase P). The settled volume of the magnetic chromolytic substrate (sedimentation at 1 g for 24 h) in 1 ml of the substrate suspension was 250 μl. The reaction was carried out at 37 °C without shaking. Hydrolysis of magnetic insoluble substrate as a function of protease concentration is shown in Figure 1. The dependence of absorbance on the concentration of protease was linear up to the absorbance value equal to one.

The absorbances of blanks, caused by the spontaneous leakage of dyed gelatine fragments into buffer during the assays, were low. Under the standard conditions the absorbances of supernatants did not exceed the value 0.08.

The described substrate can be prepared easily and can be used for the assay of a variety of proteases. Probably other magnetic protein-based insoluble substrates could be prepared. If higher specificity of the substrate is necessary, magnetic substrate with immobilised radiolabelled peptides can also be used, as described recently (Wu & Abeles 1995). Such type of substrates could enable the development of new proteases assays based on the principle of Flow Injection Analysis (FIA). The magnetic approach described in this paper represents an alternative way of integration of substrate hydrolysis and separation of hydrolysis products; another possibility is the application of aqueous two-phase systems for activity determination of enzymes hydrolysing macromolecular substrates (Mattiasson 1980).

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic, grant No. 203/98/1145.

References

De Lumen BO, Tappel AL (1970) Fluorescein-hemoglobin as a substrate for cathepsin D and other proteases. *Anal. Biochem.* **36**: 22–29.

Gisslow MT, McBride BC (1975) A rapid sensitive collagenase assay. *Anal. Biochem.* **68**: 70–78.

Mattiasson B (1980) A simple method involving aqueous two-phase separation systems for activity determination of enzymes hydrolyzing macromolecular substrates. *Anal. Lett.* **13**: 851–860.

Šafařík I (1988) An insoluble chromolytic substrate for the determination of proteolytic activity. *J. Biochem. Biophys. Meth.* **17**: 193–198.

Šafařík I (1989) Spectrophotometric determination of proteolytic activity in coloured solutions. *J. Biochem. Biophys. Meth.* **19**: 201–206.

Šafaříková M, Nymburská K, Blažek Z, Šafařík I (1996) Rapid removal of magnetic particles from large volumes of suspensions. *Biotechnol. Tech.* **10**: 391–394.

Wu Y, Abeles RH (1995) Protease assay based on magnetic beads. *Anal. Biochem.* **229**: 143–144.

Wunderwald P (1984) Proteinases (Proteins as substrates). In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis*, Vol. 5. Weinheim: Verlag Chemie, pp. 258–270.