

A NOVEL, HIGHLY SENSITIVE AND RAPID ASSAY SYSTEM FOR PROTEOLYTIC ENZYMES

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1. Introduction

Existing methods using chromophoric peptide derivatives as substrates for measuring proteinases suffer from the disadvantages that some proteinases do not hydrolyse low- M_r substrates [1], that peptidases may interfere, and that due to the specificities of proteinases a large number of substrates must be purchased or prepared if a complex or unknown mixture is to be studied. Other methods using protein substrates and measuring the production of new groups (e.g., NH_2 groups) during hydrolysis generally suffer from the disadvantage that the parent protein contains many such groups, giving high background readings. To date, all methods using protein substrates are essentially passive in character since one released fragment usually represents only one molecule of product for any detection system. The novel aspect of our approach has been to bind substrate proteins to a 'reporter' enzyme so that fragments released by proteolysis bear an active label which can transform many molecules of its own substrate, thereby achieving a considerable amplification factor over the initial peptide bond cleavage. The method is of high sensitivity and of general applicability to the measurement of proteinases and proteinase inhibitors in purified fractions and in complex biological fluids.

The key to our procedure lies in the preparation of a suitable substrate protein-reporter enzyme complex and the approach we have followed consists of coupling the substrate protein to an insoluble support (to facilitate subsequent removal of unhydrolysed substrate) followed by coupling the reporter enzyme to the substrate protein. For the support we have used Sepharoses, purely on grounds of availability and convenience. Ideal substrates should have a random structure and be readily cleaved by proteolytic enzymes (e.g., heat-denatured proteins or proteins with little

well-defined structure such as the caseins). We have used purified individual α_{s1} - or β -caseins [2] or isoelectrically precipitated total casein (Hammarsten casein). The reporter enzyme should be an enzyme not present in the samples to be measured. We have used commercial preparations (Sigma Chemical Co.) of β -galactosidase (EC 3.2.1.23) and aryl sulphatase (EC 3.1.6.1) satisfactorily, but because it is available in high specific activity at low cost, we generally use β -glucosidase (EC 3.2.1.21). Coupling of substrate to support should be as 'leak-free' as possible. We have employed several procedures including the use of commercial supports (Pharmacia Fine Chemicals AB) such as epoxy-activated Sepharose 6B and activated-CH Sepharose 4B as well as periodate-activated Sepharose 4B [3], but most often the traditional CNBr-activation procedure [4]. Typical protein cross-linking agents are then used to couple reporter enzyme to the substrate protein (e.g., dimethyl suberimidate and *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester) but we routinely use glutaraldehyde. Glutaraldehyde reacts rapidly with NH_2 groups, so to preserve substrate activity towards proteinases with a trypsin-like specificity a proportion of these groups must be protected during the coupling stage, for example, by citraconylation [5].

2. Experimental

2.1. Preparation of the substrate complex

Sepharose 4B was activated by the cyanogen bromide procedure [4] and 100 g (moist wt) mixed with 100 ml 0.2 M NaHCO_3 (adjusted to pH 9.5 with 1 N NaOH) containing 2 g casein. After stirring gently at 4°C for 24 h, ethanolamine was added to 0.5 M to block residual active groups on the Sepharose and stirring continued at 4°C for 48 h. The mixture was then washed thoroughly [4] and the moist filter cake mixed with 250 ml 0.1 M sodium phosphate and the

pH adjusted to 8.2. Stirring vigorously, 8 portions of citraconic anhydride (0.25 ml each) were added over 4 h and the pH maintained close to 8.2 (e.g., 7.6–8.6). About 30 min after the last addition the mixture was filtered and washed successively with 250 ml H₂O, 250 ml 0.1 M sodium phosphate buffer (pH 7.0) and 1 litre of 0.2 M NaHCO₃. The product was suspended in 250 ml 1.0 M sodium phosphate (pH 5.0) and the pH readjusted to 5.0. After 30 min at 18–20°C ~15% of the NH₂ groups have become deblocked and the pH was readjusted to 7.0. The mixture was filtered, washed successively with H₂O, and 0.1 M sodium phosphate (pH 7.0) and resuspended in 100 ml 0.1 M sodium phosphate buffer (pH 7.0) warmed to 37°C and 0.7 ml 6% glutaraldehyde solution added with stirring. After 5 min, 5.0 ml 0.1 M sodium phosphate buffer (pH 7.0) containing 0.4 g β -glucosidase was added and the mixture stirred thoroughly. After a further 30 min at 37°C the mixture was filtered, washed briefly with phosphate buffer, resuspended in 200 ml 1 M Tris–HCl buffer (pH 7.0) and solid NaBH₄ (0.5 g) added in small portions with stirring over a period of a few minutes. The mixture was kept at 18–20°C overnight, filtered and then suspended in 250 ml 0.1 M sodium phosphate buffer (pH 5.0). After a minimum of 4 h at 37°C to remove remaining citraconyl blocking groups, filter and wash thoroughly with at least 3 litres of 1 M NaCl, followed by 5 litres of 3 M urea containing 0.5 M NaCl and 0.2 M NaCO₃ and then 5 litres of H₂O. Finally, remaining loosely-adsorbed glucosidase, or enzyme that will readily 'leak' off the complex, was removed by incubating the filter cake resuspended in 1 litre of 0.1 M sodium phosphate (pH 7.0) overnight at 37°C, filtering and washing with 1 litre of H₂O.

This procedure seems long but is not very intensive and the final substrate complex is stable for at least 3 months as a moist filter cake stored at 4°C, so relatively large batches can be prepared at a time. Due to slow 'leakage' of material linked by the CNBr procedure to Sepharose [3], the background reading of glucosidase in subsequent proteinase assays gradually increases with storage of the complex. Therefore, if a sample of substrate complex more than 2–3 weeks-old is to be employed, it is advisable to repeat the above final overnight washing step (2–4 h incubation is usually sufficient, if more convenient) shortly before use.

2.2. Proteinase assays with the complex

Proteinase assay with the complex is simple. Vol-

umes, weights and incubation times can be varied to suit the requirements of sensitivity and speed, but as a general compromise giving a reasonably rapid assay, combined with good sensitivity, we used the following:

Weighed portions (0.2 g) of complex were mixed with 1.0 ml test solution, standard proteinase solution or buffer (blanks) and incubated for 1–3 h at 37°C, preferably with shaking. An appropriate proteinase inhibitor or buffer solution (2.0 ml) was added and the mixtures filtered. If proteinase inhibitor is not used, samples must be diluted, cooled and filtered as rapidly as possible to minimise continued digestion of the substrate complex. Portions (1.0 ml) of filtrate were then assayed for β -glucosidase by mixing with 1.0 ml *p*-nitrophenyl- β -D-glucopyranoside (2.0 mg/ml) in 1.0 M sodium phosphate buffer (pH 5.0) incubating at 37°C for 1 h, adding 1.0 M NaOH (1.0 ml) and reading optical densities at 410 nm. Levels of proteinase in the samples are obtained by reference to readings given by standard solutions containing known amounts of proteinase.

3. Results and discussion

3.1. Proteinase assays

Results we have obtained with a number of different proteinases are shown in fig.1 and as can be seen the lowest practical limit for detection of trypsin and chymotrypsin was $\sim 3 \times 10^{-10}$ g/ml and for plasmin and bromelain $\sim 3 \times 10^{-8}$ g/ml, although even lower levels still fell on the linear plot. The upper limit was at least 30-fold higher than the minimum practical values. Sensitivity would be enhanced by more sensitive assays for the reporter enzyme (e.g., fluorimetric procedures) and we chose nitrophenol-based assays only on grounds of convenience and simplicity. In addition, the enzymes shown in fig.1 were all routine commercial products (Sigma Chemical Co.) and were unlikely all to be 100% pure proteinase (e.g., the bromelain was only a practical grade), so the minimum detection limits of our method are almost certainly much lower than indicated above.

Our data showed that the method is of general applicability and can be used to detect and measure proteinases belonging to different classes. We have used it to detect proteinases in column fractions obtained during the gel filtration of sodium caseinate from normal bovine milk and in fractions from the ion-exchange chromatography (DEAE-cellulose) of high cell count milk from cows with mastitis (fig.2).

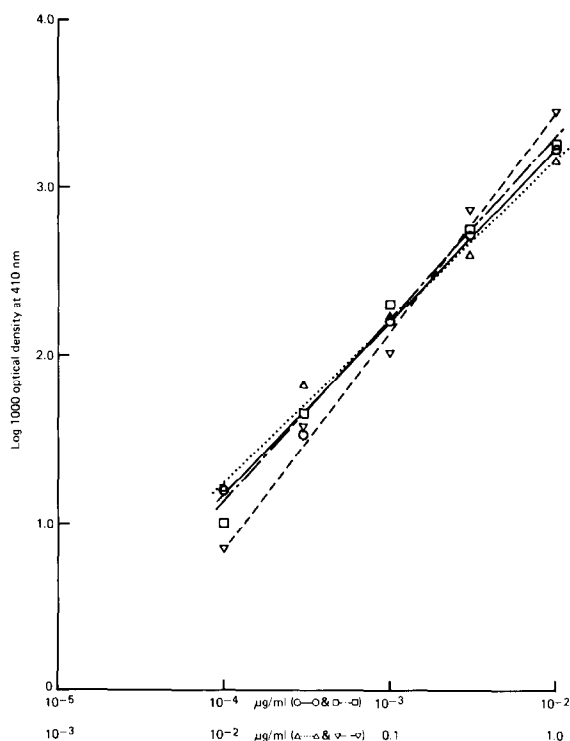


Fig. 1. The responses given by standard solutions of various proteolytic enzymes in the assay system. All data points are the average of triplicate analyses: (□—□) trypsin; (□---□) α-chymotrypsin; (Δ...Δ) porcine plasmin; (v---v) bromelain.

In the former a small peak due to plasmin was identified, but in the latter the 6 peaks of proteinase activity remain to be characterised further.

3.2. Measurement of proteinase inhibitors

We have also applied the method to the detection of proteinase inhibitors in gel filtration fractions. This was done by mixing 0.1 g portions of substrate complex with 0.5 ml of each fraction and 0.5 ml trypsin (0.1 µg/ml) in sodium phosphate buffer (pH 7.0). After 2 h at 37°C, 1.0 ml soya bean trypsin inhibitor solution (0.2 mg/ml) in H₂O was added and mixtures filtered and assayed for proteinase (glucosidase activity) as above. The proteinase inhibitors were readily measured in fractions obtained from gel filtration

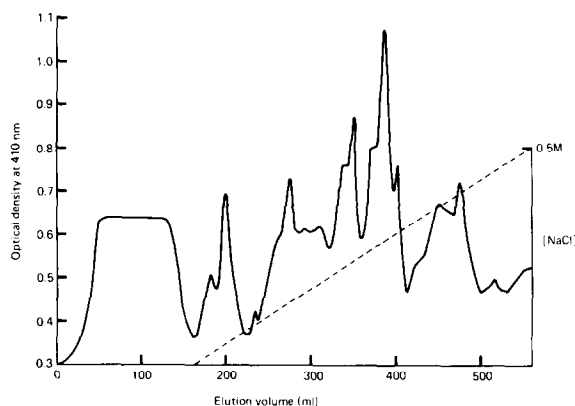


Fig. 2. Ion-exchange chromatography of proteinases in bovine milk (somatic cell count = 2.2×10^7 /ml) from an animal with clinical mastitis caused by infection with *Streptococcus agalactiae*. Milk (40 ml) was mixed with 40 ml 0.1 M Tris-HCl buffer (pH 7.0) and applied to a column (255 × 20 mm) of DEAE-cellulose pre-equilibrated in 0.05 M Tris-HCl buffer (pH 7.0). The column was then eluted with 80 ml of the same buffer followed by 400 ml of a 0.0–0.5 M NaCl gradient in 0.05 M Tris-HCl (pH 7.0). Fractions of 5 ml were collected throughout and 0.5 ml portions mixed with 0.1 g substrate complex for proteinase assay (see text).

experiments using columns (870 × 16 mm) of Sephadex G-150 to which samples of only 0.1 ml bovine blood serum or 2.0 ml bovine whey from normal milk had been applied.

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