A simplified linked enzyme assay procedure (LEAP) for measuring proteinase activity

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1. INTRODUCTION

We have recently reported the development of a novel and sensitive method for the general detection of proteinases and proteinase inhibitors [1,2]. The approach adopted was to couple a 'reporter' enzyme to a substrate protein which in turn was immobilised on a particulate support, usually Sepharose. Thus, during an incubation with proteinase-containing solution peptides bearing an active enzyme label are released into the supernatant phase and at the end of the required incubation unhydrolysed complex can be removed easily by filtration or centrifugation. In a second incubation step the labelled peptides transform many hundreds of molecules of the reporter enzyme substrate, resulting in a substantial amplification of the initial peptide bond hydrolysis by the proteinase, and hence a great increase in terms of the sensitivity of proteinase detection.

Since the particulate support, the substrate protein, the reporter enzyme and the coupling methods can all be varied considerably to suit individual requirements and convenience, a wide range of variations on the basic philosophy can be envisaged and to date we have explored only a relatively small number. All methods however rely upon the release by proteinases from a substrate complex of peptides bearing an active enzyme label which is subsequently determined giving an indirect assessment of the proteinase. We therefore refer to such methods as Linked Enzyme Assay Procedures (LEAP) for proteinase measurement.

Our previous LEAP showed very high sensitivity for detection of proteinases but preparation of the substrate complexes was quite lengthy and the resulting complexes were not ideal because there was some non-specific leakage of label off the complex, even during storage at 4°C, giving higher background readings than desirable. This leakage could be reduced to manageable proportions by thorough pre-washing but can probably never be eliminated with Sepharose-linked complexes because of the low but still finite solubility of Sepharose. For these reasons we have sought to simplify and improve the preparation of LEAP complex substrates. We now wish to report the preparation and use of LEAP for proteinase measurement using an insoluble protein as combined support and substrate in a simplified procedure.

2. EXPERIMENTAL

2.1. *Preparation of citraconylated hide powder*

Hide powder (2 g) was suspended in water (50 ml) and the pH adjusted to 8.2. Citraconic anhydride (0.8 ml) was added at room temperature with rapid stirring in portions of 100 μl over a period of 4 h, maintaining pH close to 8.2 throughout by addition of about 5 N NaOH. After a further 2 h stirring, the mixture was filtered under gentle vacuum through a scintered glass funnel, washed with at least 2 l of 0.1 M sodium phosphate buffer (pH 8.0), dried in a freeze dryer and stored at 4°C.

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2.2. *Preparation of hide powder–glucosidase complex*

Before using the citraconylated hide powder to make a complex it is necessary to unmask some of the amino groups. A series of experiments showed that the complex most sensitive for proteinase measurement resulted from using about 10% unmasked hide powder. Hide powder (2 g) was suspended in 50 ml 0.5 M sodium phosphate (pH 4.2) and incubated for 18 min at 40°C. The pH was then readjusted to 7.0 and the mixture allowed to cool to room temperature (18–20°C). Solid (400 mg) β-glucosidase (EC 3.2.1.21) was added and dissolved by gentle stirring. The stirring rate was increased and 5% aqueous glutaraldehyde (4 ml) added. After a further 15 min, sodium borohydride (2–3 mg) was added to destroy excess glutaraldehyde and reduce aldehydic groupings. The complex was then thoroughly washed on a scintered glass funnel with at least 2 l of 0.1 M sodium phosphate buffer (pH 5.0) to remove loosely associated β-glucosidase and the filter cake maintained at pH 5.0 for 6 h (or overnight at 4°C). Finally the complex was freeze-dried and stored at 4°C.

2.3. *Measurement of protease activity*

Portions (20 mg) of dry complex were mixed with proteinase solution (2 ml) and incubated at 37°C in a shaking water bath. After suitable time intervals, portions (20 μl) of an appropriate proteinase inhibitor solution were added. Soybean trypsin inhibitor Type 1-S (Sigma) was used in measurements with trypsin and 2-nitro-4-carboxyphenyl-*N,N*-diphenyl carbamate (NCDC) was used in α-chymotrypsin (EC 3.4.21.1) experiments. The suspensions were filtered through Whatman no.4 filter papers as earlier experiments, using Millipore discs, resulted in poor replication, perhaps due to the β-glucosidase binding to the filter matrix. Portions of filtrate (0.5 ml) were incubated at 37°C with 0.5-ml portions of *p*-nitrophenyl-β-D-glucopyranoside (NPGP) (3 mg/ml) in 0.5 M sodium phosphate buffer (pH 5.0). After 15 min 0.3 M NaOH (2.0 ml) was added and optical densities read at 410 nm.

3. RESULTS AND DISCUSSION

The results reported here refer to hide powder linked to β-glucosidase with 5% aqueous glutaraldehyde. The decision to use glutaraldehyde was made after extensive studies using other cross-linking reagents such as dimethyl suberimidate and *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester, which were unsuitable either because insufficient reporter enzyme could be linked to the substrate protein or because cross-linking was so extensive that the complex was resistant to hydrolysis by proteinases. The optimum level of 5% aqueous glutaraldehyde used for cross-linking was determined in the following way. Portions (250 mg) of citraconylated hide powder, unblocked to the extent of about 10% by treatment at pH 4.2 for 20 min, were suspended in 25 ml of 0.1 M Na phosphate (pH 7.0) and mixed with 25 mg solid β-glucosidase. Varying amounts of 5% aqueous glutaraldehyde solution were added and preparation of batches of substrate complex continued in the usual way. Measurement of the total amount of β-glucosidase bound to hide powder (table 1) showed the optimum level to be 2 μl glutaraldehyde/mg hide powder. Further measurements also showed (table 1) that this level was the optimum in terms of the proportion of immobilised β-glucosidase that was released from the complex during subsequent digestions with trypsin.

If unmodified hide powder was used, large amounts of β-glucosidase were bound during

Table 1

The influence of varying the level of glutaraldehyde used for cross-linking on the properties of the substrate complex used for proteinase assays

Vol. of 5% aqueous glutaraldehyde added (μl)	Relative amounts of β-glucosidase unmobilised (arbitrary units)	Activity released by trypsin (25 μg/ml) in standard assay (A_{410})
60	0.186	0.322
125	0.539	1.077
250	1.060	1.763
500	1.551	3.049
1000	1.350	3.016

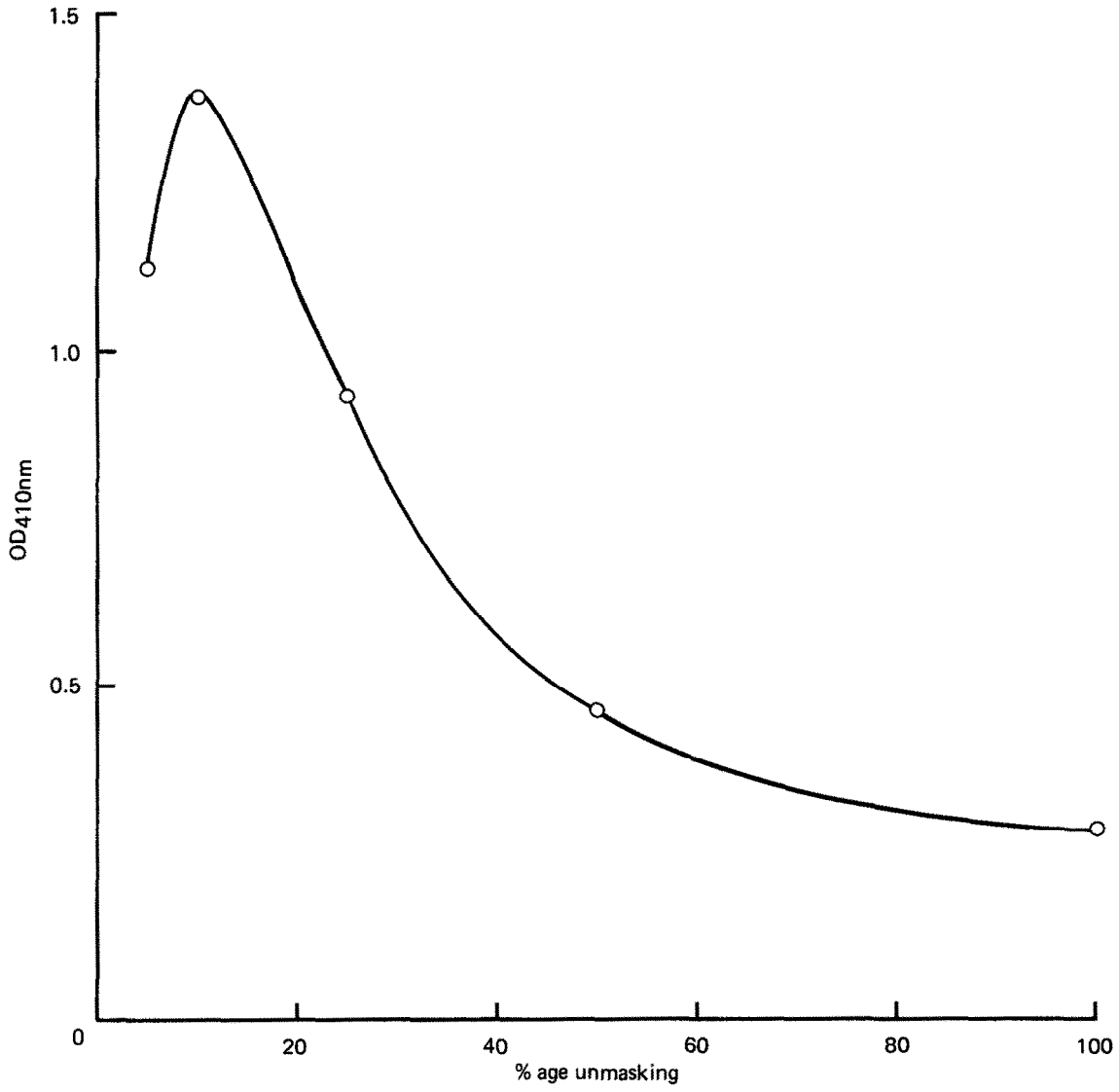

Fig.1. The relation of percentage unmasking of citraconylated hide powder in the complex to release of β-glucosidase. Portions (20 mg) of the complexes in 0.1 M Na phosphate buffer (pH 7) (2 ml) were incubated with trypsin (25 μg/ml) for 30 min at 37°C. Aliquots (0.5 ml) were removed from the filtrate and assayed in the usual way (see text).

glutaraldehyde treatment but little was subsequently released by proteinases. It was found to be necessary to protect a large proportion (~90%) of the available NH_2 groups on the hide powder by citraconylation [3] in order to prevent excessive cross-linking within the matrix by the glutaraldehyde treatment step. This also preserved a number of available sites for cleavage by trypsin-like enzymes which cleave polypeptide chains adjacent to Lys and Arg residues [4,5]. Fig.1 shows how the amount of trypsin-released β-glucosidase depended on the percentage unmasking of the citraconylated hide powder used in the complex, and it can be seen that around 10% unmasking allowed the greatest release of β-glucosidase.

The hide powder–β-glucosidase complex was very stable for at least 6 months when stored dry at 4°C. There was negligible leakage of β-

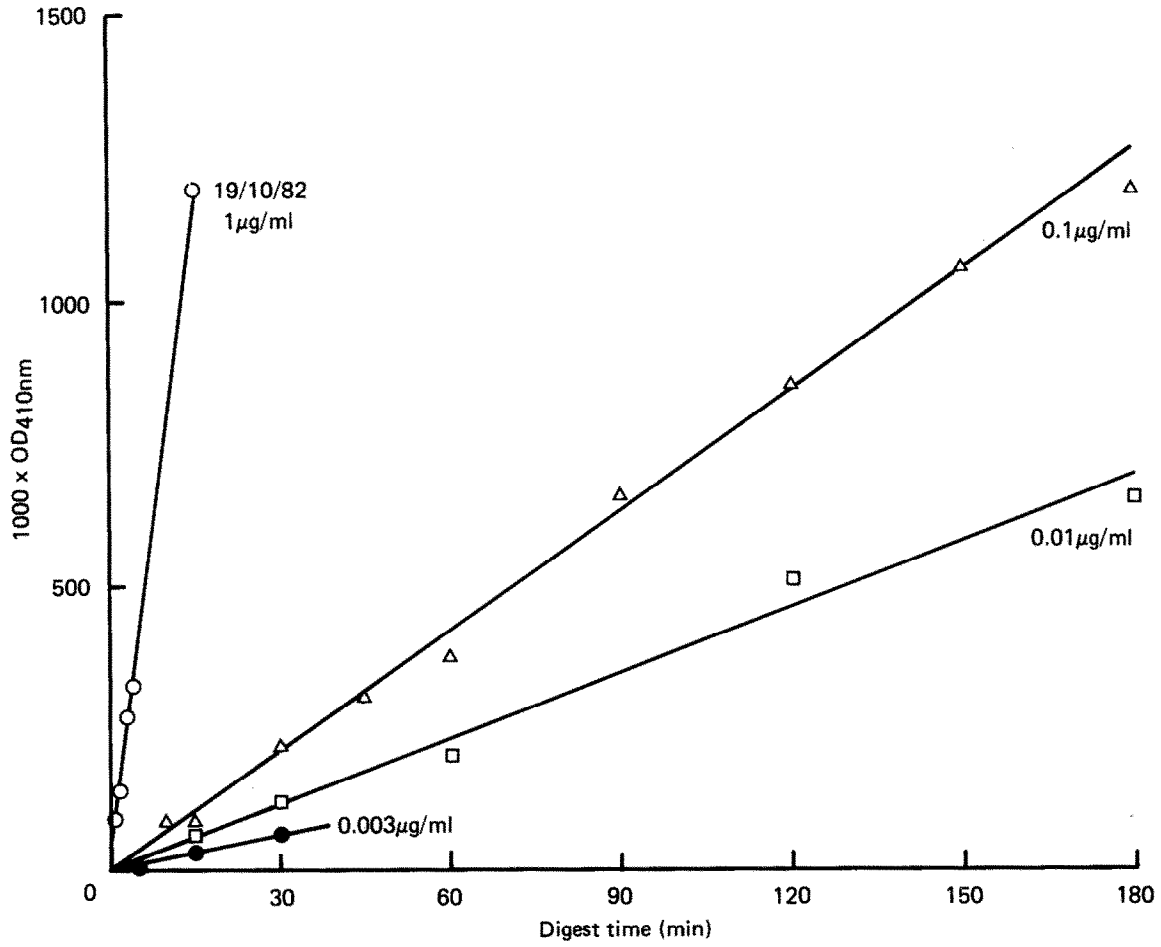

Fig.2. The relation of β-glucosidase released from the complex to varying levels of trypsin. Portions (20 mg) of the complex in 0.1 M Na phosphate buffer (pH 7, 2.0 ml) were incubated with trypsin for 3 h at 37°C. Digestion was stopped by addition of SBTI at various times. β-Glucosidase released was measured in aliquots (0.5 ml) of the filtrate using the standard assay. (○---○) Trypsin at 1 μg/ml; (△---△) 0.1 μg/ml; (□---□) 0.01 μg/ml; (●---●) 0.003 μg/ml.

glucosidase off the complex which had been a problem with Sepharose-linked substrate complexes [2]. The amount of β-glucosidase released from the complex by a well-defined quantity of trypsin remained constant throughout this storage period when tested under similar conditions.

It is likely that other insoluble proteins would make good complexes and in earlier tests fibrin was used, but hide powder was found to be more sensitive in detecting proteinases.

Fig.2 shows the relationship of absorbance to different levels of trypsin. It can be seen that linear plots were obtained for trypsin levels from 1×10^{-8} g/ml to 1×10^{-6} g/ml and levels down to 3×10^{-9} g/ml were detected in 30 min. It should be noted that the stated weights of complex and times for proteinase digest and β-glucosidase assay could be varied to suit the levels of proteinase being measured.

Fig.3 shows the correlation of absorbance and increasing amounts of trypsin and chymotrypsin using a 3 h incubation with the proteinase. Although this clearly shows that the hide powder–glucosidase complex was a better substrate for trypsin, chymotrypsin was easily detected down to 1×10^{-9} g/ml.

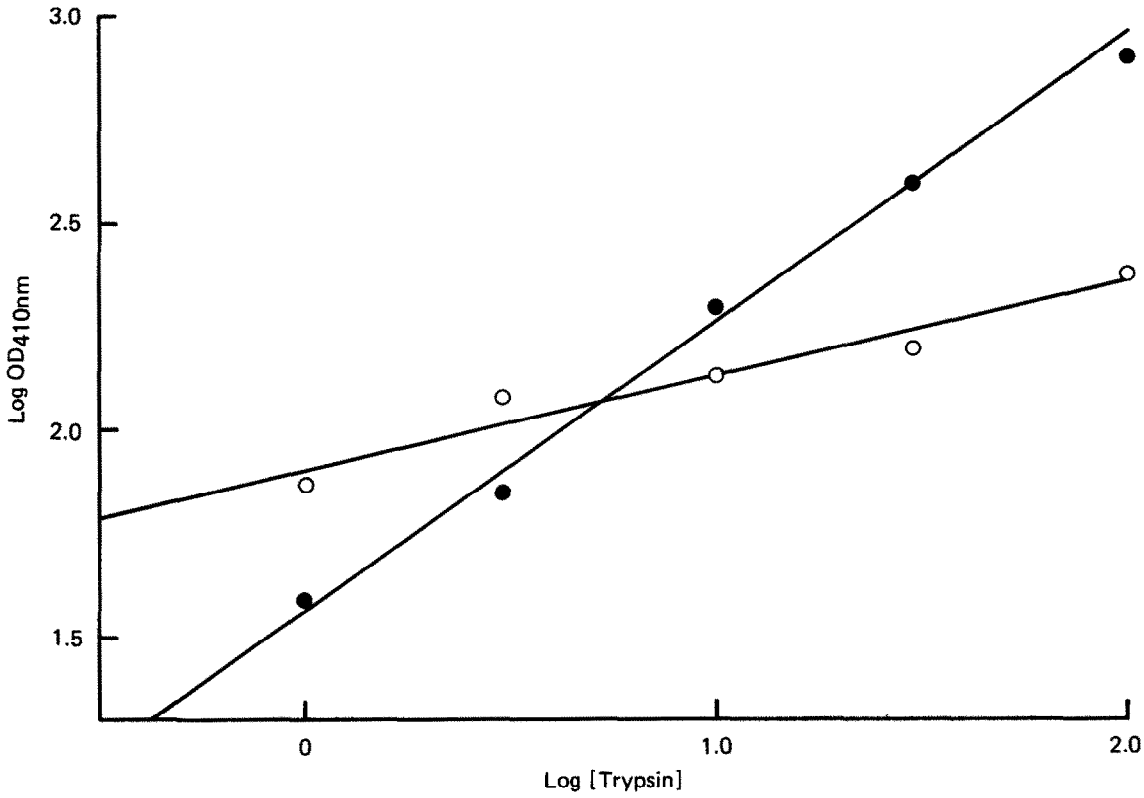

Fig.3. The relation between β-glucosidase released from the complex to increasing levels of trypsin and chymotrypsin. Portions (20 mg) of the complex in 0.1 M Na phosphate buffer (pH 7, 2.0 ml) were incubated with the proteinase for 3 h at 37°C. Aliquots (0.5 ml) of filtrate were assayed for β-glucosidase using the standard procedure. (●---●) Trypsin; (○---○) chymotrypsin.

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