Chemiluminescence Determination of Proteases by Flow Injection Using Immobilized Isoluminol

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A flow-injection system for protease determination is described. A tripeptide–isoluminol derivative was immobilized on an affinity support, and incorporated into the flow system. Injected enzyme sample solutions (0.025 cm³) catalysed the hydrolysis of some of the derivatives at pH 8.47, and the released isoluminol was detected by the chemiluminescence produced by its cobalt-catalysed oxidation with hydrogen peroxide. Linear calibration results were obtained for α-chymotrypsin (from 5×10^{-4} to 0.41 mg dm^{-3}), trypsin (from 4×10^{-2} to 4 mg dm^{-3}) and a commercial protease (from 2×10^{-3} to 4 mg dm^{-3}). The limits of detection were 2.7×10^{-4}, 4×10^{-2} and 2×10^{-3} mg dm^{-3}, respectively.

Keywords: *Chemiluminescence; flow injection; isoluminol; immobilized reagent; protease*

Introduction

Proteolytic enzymes are prominent in the control and regulation of certain physiological processes. They are also finding increasing application in industrial manufacture such as detergent products. Progress in both areas has necessitated the development of specific, sensitive and rapid analyses for monitoring low levels of these enzymes. A highly sensitive technique is required to overcome possible interferences caused by the matrix of complex systems such as commercial detergent products, which contain a variety of anionic, cationic and non-ionic surfactants, builders and other functional additives. Such matrix effects can be overcome by dilution when necessary.

Various methods have been developed to determine proteolytic activity, with many advances being made since the classical assays of Anson[1] and Kunitz.[2] Synthetic protease substrates containing chromogenic and fluorogenic leaving groups have been developed, including β-naphthylamide,[3] 7-amino-4-methylcoumarin,[4–6] 4-nitroanilides,[7–9] peptide thioesters[10–13] and 4-aminophthalhydrazide (isoluminol).[14,15] Several methods of labelling native proteins with fluorescent leaving groups such as fluorescein[16,17] and 2-methoxy-2,4-diphenylfuran-3-one[18] have also been described.

Despite their sensitivity, these procedures are often inconvenient and require large amounts of substrate. Long periods of incubation are always necessary and reaction products often require separation from unreacted starting material before analysis. The sensitivity possible from chemiluminescent reactions is far superior to chromogenic assays. Flow injection (FI) provides a system with rapid throughput and ease of handling, and is well suited to chemiluminescent measurements.[19] This paper describes a procedure for protease determination based on chemiluminescence monitoring in an FI system. It is based on a novel assay for proteases which uses an immobilized tripeptide–isoluminol substrate and enzyme-catalysed hydrolysis.[14,15] In the procedure the enzyme passes through a mini-column of substrate, and the released isoluminol is determined by chemiluminescence caused by oxidation on merging with a stream of H_2O_2 in the presence of a cobalt ion catalyst.

Experimental

Reagents

The concentrations of trypsin (Fluka), α-chymotrypsin (Sigma), and a commercial protease (Procter and Gamble) were determined by FI. The synthetic substrate alanine–alanine–phenylalanine–isoluminol·HBr (AAPIL·HBr) was synthesized, purified and kindly donated by Sigma. Affi-gel 10 was purchased from Bio-Rad Laboratories. Ultra-pure deionized water (Elgastat) was used for preparing the solutions. All reagents were of analytical-reagent grade.

FI Manifold

Optimum conditions are given throughout. The flow system used is shown in Fig. 1. A peristaltic pump (Minipuls 3, Gilson) was used to deliver each solution at a flow rate of 0.9 cm³ min^{-1}. Poly(tetrafluoroethylene) tubing (0.5 mm i.d.) was used for the flow system. The cobalt and enzyme carrier streams were combined at a Perspex Y-piece. After passing through 50 mm of tubing the solution mixed with the peroxide solution at a Y-piece 30 mm before entering the detector coil.

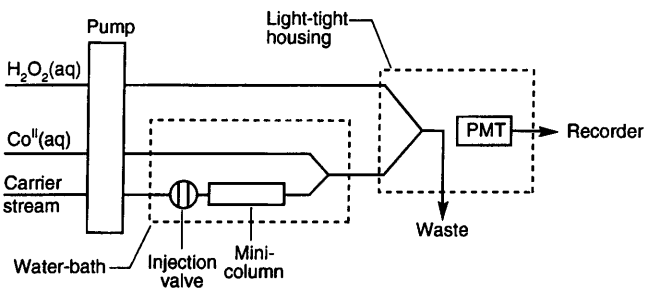

Fig. 1 FI manifold for protease determination (PMT = photomultiplier tube).

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Sample solutions (0.025 cm^3) were injected *via* a Rheodyne injection valve.

The detector was a modification of a design previously decribed.[20,21] The flow coil was made of glass tubing (2 mm i.d.), spiralled to a diameter of 25 mm and backed by a polished stainless-steel mirror for good light reflection. A photomultiplier tube (PMT) (9924B, Thorn EMI) was placed directly in front of the coil, encased in a light-tight housing. The PMT was powered by a stabilized EHT power supply (−940 V). The response of the PMT was recorded on a flat-bed chart recorder (Kipp and Zonen).

Immobilization Procedure

AAPIL·HBr was coupled to Affi-gel 10 following the manufacturer's instructions.[22] AAPIL·HBr (0.0288 g, 0.0542 mmol) was dissolved in KH_2PO_4 (0.1 mol dm^{-3}, 3 cm^3) kept at 4 °C. Affi-gel 10 suspension (3 cm^3) was filtered through a sintered glass filter and washed with cold KH_2PO_4 (0.1 mol dm^{-3}). The moist gel was added to the AAPIL·HBr solution and gently agitated at 4 °C. After 4 h the gel was filtered off, added to aqueous ethanolamine (0.1 mol dm^{-3}, 3 cm^3) at 4 °C, and gently agitated for a further 30 min. The resulting gel was washed with KH_2PO_4 (0.1 mol dm^{-3}). It was stored at 0 °C in NaN_3 solution (0.2%, 3 cm^3) until required.

When required for use, Affi-gel 10–AAPIL suspension (0.5 cm^3) was washed with $NaHCO_3$ (0.1 mol dm^{-3}) and filtered off. The moist gel was slurried in $NaHCO_3$ (0.1 mol dm^{-3}, 2 cm^3) and packed into a glass column (2.5 × 0.3 cm i.d.). A small plug of glass wool was inserted at each end of the column, and silicone rubber tubing (0.8 mm i.d.) was used to connect the column to the FI manifold. The column was washed with $NaHCO_3$ (0.1 mol dm^{-3}) for 6 h (0.9 cm^3 min^{-1}). If stored at 4 °C, the packed column is stable for over 4 weeks.

Enzyme Assays

Stock enzyme solutions were prepared by dissolving a weighed amount of enzyme, typically 40 mg dm^{-3}, in a sodium tetraborate buffer solution (0.08 mol dm^{-3} $Na_2B_4O_7·10H_2O$–0.25 mol dm^{-3} KCl), pH 9.41. These solutions were stable for approximately 1 week. Standard solutions were prepared daily by appropriate dilution of a stock solution with carbonate–chloride buffer (0.1 mol dm^{-3} $NaHCO_3$–1 mol dm^{-3} KCl), pH 8.47, and thermostated at 30 ± 0.1 °C in a Grant circulating water-bath.

For chemiluminescence measurement, each enzyme solution was injected into a carbonate–chloride buffer (0.1 mol dm^{-3} $NaHCO_3$–1 mol dm^{-3} KCl); after passing through the column, the solution was mixed with a Co^{II} solution (1 μg cm^{-3} in 0.1 mol dm^{-3} $NaHCO_3$). Both streams were thermostated at 30 °C. Hydrogen peroxide (1×10^{-5} mol dm^{-3} in 0.1 mol dm^{-3} $NaHCO_3$), kept in ice–water to prevent decomposition, was mixed with the combined enzyme and cobalt solution before entering the detection coil. The chemiluminescence intensity was recorded using a chart recorder and the peak height measured.

Enzyme Assays in a Detergent Matrix

A solution of a commercial powder detergent (10 g) was prepared in water (1 dm^3). The solution was boiled for 1 h to denature the enzymes present. The cooled solution was filtered and 40 cm^3 were diluted to 1 dm^3 using the carbonate–chloride buffer (0.1 mol dm^{-3} $NaHCO_3$–1 mol dm^{-3} KCl) described. This solution was used to prepare a series of standards of a commercial protease for comparison with the chemiluminescence intensity of solutions of the same commercial protease prepared in the carbonate–chloride buffer without any powder detergent. The chemiluminescence intensity was recorded as above. Results are presented in Table 1.

A series of solutions were prepared using two commercial lipase enzymes (up to 10 mg dm^{-3}) in the same way as described above. No chemiluminescence was observed from these solutions when injected into the FI system.

Results and Discussion

Chemiluminescence, the phenomenon of light emission from a chemical reaction, has been exploited in numerous analytical applications, particualrly in conjunction with FI and liquid chromatography.[19,23] The absence of an irradiation source eliminates source light scatter and source noise, giving chemiluminescence detection inherently lower detection limits than other optical techniques. The H_2O_2 oxidation of luminol (3-aminophthalhydrazide)[24] is one of the most well known chemiluminescent reactions, and its use has been described in a wide range of analytical procedures.[25]

The protease assay described here depends on the ability of the enzyme to catalyse the hydrolysis of the amide bond formed between the terminal carboxyl group of phenylalanine and the amino group of isoluminol, or any other peptide link in the immobilized tripeptide–isoluminol substrate. On hydrolysis, the liberated products are eluted form the column and the isoluminol concentration, which is proportional to the enzyme activity, can be determined by its chemiluminescence, in the flow system.

Isoluminol has only about 10% of the chemiluminescent quantum efficiency of luminol.[26] However, on substitution at the amino group, a several-fold increase in chemiluminescence intensity is observed for isoluminol, whereas similar substitution on luminol leads to a decrease in chemiluminescence yield,[26] probably owing to steric hindrance. The protease is not limited to cleaving the isoluminol–peptide bond, but may attack any one of the amide bonds of the synthetic substrate. The eluted products will, therefore, be a mixture of substituted and free isoluminol.

A major advantage of using an immobilized, labelled substrate is that it can be used in a flow system. This allows ready separation of the soluble reaction products from the substrate, which remains on the column, and hence there is no interference of the substrate in the subsequent chemiluminescence measurements. Affi-gel 10, an affinity support, contains 15 μmol of *N*-hydroxysuccinimide ester groups per cm^3, which are capable of combining with the terminal amino group of the tripeptide–isoluminol substrate.[22] A slight excess of substrate was used during synthesis to ensure maximum loading and hence maximum continuing sensitivity during the column lifetime. The immobilized substrate was packed into columns that were placed in the system as shown in Fig. 1, so that the injected protease passed through the column, releasing the immobilized isoluminol by hydrolysis.

Table 1 Comparison of chemiluminescence intensity of commercial protease solutions prepared in a carbonate–chloride buffer with and without powder detergent*

Protease concentration/ mg dm^{-3}	Buffer only	Buffer + detergent
4	27.0	27.0
6	43.5	41.0
10	74.0	71.0

* Average of three replicate injections. Units are arbitrary.

Optimization of Conditions

In order to achieve the desired sensitivity for the chemiluminescence from isoluminol oxidation, four buffer solutions were examined for subsequent use in the protease assay. Low detection limits for isoluminol were not achieved in sodium tetraborate or phosphate solutions, but in Na_2CO_3 (0.1 mol dm^{-3}), pH 11.40, using the hematin-catalysed peroxide system, and in $NaHCO_3$ (0.1 mol dm^{-3}), pH 8.47, using a cobalt-catalysed peroxide system, detection limits of 1×10^{-10} mol dm^{-3} were achieved in both instances.

Problems were foreseen with the use of Na_2CO_3 buffer. The high pH would not be suitable for greatest enzyme activity and would result in base hydrolysis of the amide bonds in the immobilized substrate, thus increasing the background signal and noise. Therefore, protease asays were carried out in a carbonate buffer at pH 8.47. In order to prevent ionic interactions between the agarose support and enzyme, a high ionic strength was maintained using KCl.

After passing through the column, the enzyme-containing solution mixes with a second stream containing Co^{II}, which plays an essential catalytic role in the oxidation of isoluminol. Cobalt is thought to promote superoxide formation, although the mechanism by which the latter is generated from H_2O_2 has yet to be determined.[27] The efficiency of Co^{II} in such a reaction depends on its chemical environment. In the presence of strongly chelating ligands (citrate or ethylenediaminetetraacetic acid for example), the metal ion enhanced chemiluminescence of isoluminol is suppressed because the cobalt is masked. Amino acids also favourably bind with Co^{II}, reducing the free concentration of cobalt by complexation. The extent of complexation depends on the time for which the amino acids and cobalt ions are in contact. This reaction is slow and under the above conditions, when the eluted amino acid–isoluminol mixture and Co^{II} are in contact for only a short period before mixing with H_2O_2, cobalt can act as a satisfactory catalyst. Detectors for amino acids based on the suppression of chemiluminescence in the metal ion catalysed luminol–peroxide system have been described.[27–29]

Introducing Co^{II} after the enzyme has acted on the substrate has the added advantage that this avoids the possibility of metal ion inhibition of the proteases.

The system was thermostated at 30 °C, which is not the optimum temperature for the protease reactions studied.[14] Above this temperature substrate columns were slowly denatured, as indicated by an increasing back-pressure in the flow system. At the temperature chosen, the column was used successfully for over 4 weeks.

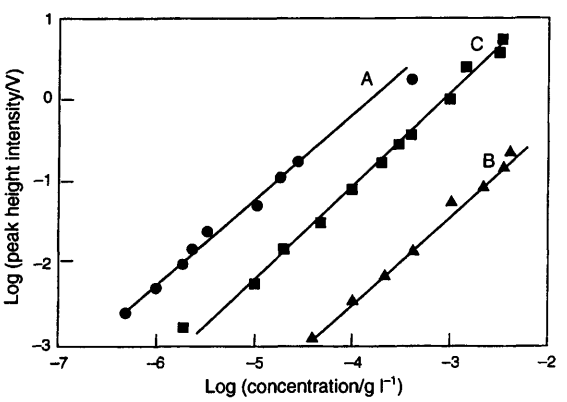

Fig. 2 Calibration graphs for A, α-chymotrypsin; B, trypsin; and C, commercial protease.

Analytical Performance

Under the conditions established, the calibration results for the proteases studied are shown in Fig. 2. The log–log calibration plots were linear at least over the range from 5×10^{-4} to 0.41 mg dm^{-3} for α-chymotrypsin (α-CT), from 4×10^{-2} to 4 mg dm^{-3} for trypsin and from 2×10^{-3} to 4 mg dm^{-3} for the commercial protease with regression coefficients of 1.03(0.03), 1.12(0.01) and 0.89(0.02), respectively. Errors are quoted as standard errors of the coefficients, which show continuing good reproducibility.

The limiting factor for sensitivity is the background noise produced by isoluminol bleed from the column. This major source of background noise, caused by non-enzymic hydrolysis of the peptide bonds, degrades the precison and detection limits. The limit of detection was governed by the peak-to-peak noise on the background signal, which was primarily due to cyclic variations in the flow rate through the detector caused by the peristaltic pump. A practical measure of the limit of detection, defined as twice the peak-to-peak noise, was 2.7×10^{-4} mg dm^{-3}, although the minimum detectable levels for trypsin and the commercial protease were 4×10^{-2} and 2×10^{-3} mg dm^{-3}, respectively. The higher detection limits for trypsin and the commercial protease are due to their lower selectivity towards the tripeptide used. Typically, the mid-range precision for eight replicate injections of α-CT (0.1 mg dm^{-3}) was 2.7% relative standard deviation.

Owing to the nature of the column substrate and protease interactions, the chemiluminescence response from injecting a sample of protease tailed before returning to the baseline. Increasing the ionic strength of the enzyme carrier stream reduced the magnitude of tailing, as did increasing the flow rate, but the latter was at the expense of the signal intensity. At the ionic strength used, typical sample rates of injection were 7.5 h^{-1}.

The useful lifetime of the immobilized substrate column was estimated by continuously pumping an α-CT solution (0.1 mg dm^{-3}) through the column. After 100 cm^3 (equivalent to 4000 injections), no deterioration in column performance was observed.

A comparison of the chemiluminescence signal obtained for the enzymes studied shows that the sensitivity of the assay is far superior for α-CT and the commercial protease than that for trypsin using AAPIL as the immobilized substrate, in agreement with Branchini *et al.*[14]

Initial work has been carried out to determine the suitability of the technique for determining protease activity in commercial powder detergents. The results from Table 1 indicate that with appropriate dilution any matrix effects can be eliminated so that there is no significant effect on the chemiluminescence signal and analysis of the protease. The specificity of the method towards determination of the proteases was confirmed when no chemiluminescence signal was observed for the two commercial lipases studied. Further work to investigate substrates specific for trypsin and other proteases and lipases of commercial importance is underway.

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