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Capillary electrophoresis in the assay of the hydrolysis of glycine-containing peptides by a protease from *Pseudomonas aeruginosa*

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Abstract

A rapid and simple capillary zone electrophoresis (CZE) method for measuring the activity of a minor protease from *Pseudomonas aeruginosa* is described. When glycine-containing oligopeptides were used as substrates, it was possible to separate and quantify substrate and products. Moreover oligopeptide hydrolysates were analysed by fast atom bombardment mass spectrometry providing the sites of splitting of the substrates. By comparison with CZE calibration curves constructed with reference peptides, the initial rates of hydrolysis were calculated. The method, validated for pentaglycine hydrolysis, was also used for the analysis of reaction mixtures and for monitoring the enzymic hydrolysis of various peptides in order to investigate enzyme specificity. © 1997 Elsevier Science B.V.

Keywords: *Pseudomonas aeruginosa*; Peptides; Proteases; Peptides; Proteases

1. Introduction

Pseudomonas aeruginosa secretes two major proteases (an alkaline protease and an elastase) together with various other enzymes among which are minor proteases with staphylolytic activity [1,2]. These minor enzymes have been demonstrated to enhance the elastolytic activities of different proteases, including human leukocyte elastase [3,4]. In severe infections, this activity may amplify the destruction of lung components, as is the case for pulmonary complications associated with cystic fibrosis [5]. Spectrophotometry is not very useful for activity measurements because of the low absorbances of the substrates and of the very low hydrolysis rates. Peptides which have previously been used as substrates are glycine oligomers, and qualitative identification of the hydrolysis products was carried out by thin-layer chromatography [1]. Separation of mono- to pentaglycine oligomers may be performed with ion-pair HPLC in the presence of octanesulfonic acid [6]. By this means it was demonstrated that pentaglycine (Gly_5) was hydrolyzed into di- and triglycine (Gly_2 and Gly_3) for example. Due to its rapid analysis times (5–20 min) and small sample volumes [7] capillary zone electrophoresis (CZE) is often used for the separation of small peptides, and has been applied to the measurement of enzymatic activities [8,9]. Here, we describe a rapid CZE method for assaying *Pseudomonas aeruginosa* proteases, after incubation of the enzyme with the pentapeptides, pentaglycine being considered as a model. This work demonstrates the usefulness of CZE for product separation and rate measurements.

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2. Experimental

2.1. Reagents

Di-, tri- and pentaglycine were obtained from Sigma (L'Isle-d'Abeau, France). Glycine-containing peptides were prepared by the Tea-bag Fmoc procedure, according to the Houghten method [10]. Peptides were dissolved in PBC buffer (25 m*M* $Na_3PO_4 \cdot 12H_2O$, 50 m*M* boric acid, 12.5 m*M* citric acid, pH 9.6) (final concentration: 1 mg ml^{-1})

2.2. Protease purification

Protease was purified according to the procedure described by Peters and Galloway [3], using the crude supernatant of PA 220 strain after a 16-h culture on liquid L-broth medium.

2.3. Apparatus

A Quanta 4000 automated capillary electrophoresis system (Waters) was used for all CZE experiments. The samples were introduced into a 40 cm× 50 μm I.D. coated capillary cartridge by an electrophoretic injection.

Fast atom bombardment (FAB) spectra were recorded with a VG ZAB2-SEQ mass spectrometer in the Centre d'Analyse du CNRS-Solaize. Samples were deposited onto a probe tip containing the matrix (glycerol or thioglycerol).

2.4. Methods

The injection and the analysis were performed under a 20 kV constant voltage with a current limit of 30 μA. The compounds were detected at 185 nm. The running buffer was 40 m*M* citrate (pH 2.7).

Aliquots of 300 μl of 1 mg ml^{-1} oligopeptide in PBC buffer (pH 9.6) were mixed with 10 μl of purified protease and hydrolysis was performed inside the apparatus at 30°C. Separation of the products was performed in 12 min, followed by a 2-min wash with citrate buffer. As the injection time was 1 min, four analyses could be carried out every hour. The extent of hydrolysis of glycine-containing pentapeptides was compared with that of pentaglycine under the same experimental conditions.

3. Results and discussion

We first optimized the experimental conditions for CZE in order to achieve a rapid and unambiguous separation of glycine oligomers (Fig. 1). Identification of di- and triglycine (Gly_2 and Gly_3) peaks was made by comparison with pure commercial peptides. Our device is able to deliver variable amounts of the mixture to be analyzed, by varying the injection time. We first tested the linearity of peak area with injection time, i.e. with the amount of peptide introduced into the capillary cartridge. A linear relationship could be demonstrated for injection times ranging from 12–100 s (correlation coefficients=0.99). Consequently it was possible to use variable injection times depending on the initial concentrations of stock solutions, in order to measure area with optimal precision.

For a given injection time (60 s), linear relationships of areas with oligopeptide concentrations could be derived from the experimental data using Gly_2, Gly_3 and Gly_5 solutions, either in the 0–2.5 m*M* or in the 0–0.5 m*M* range (correlation coefficients= 0.99). Moreover, their slopes were approximately in the ratio 1:2:4 for Gly_2, Gly_3 and Gly_5, due to the major absorbance band at 185 nm of the peptide bonds. A similar relationship was obtained with a 5-fold lower injection time (12 s), the slopes then being 5-fold lower.

Reproducibility of the measurements was evaluated from the slopes of six different linear plots of area vs. concentration, for all three glycine oligopeptides. The calculated slopes in arbitrary units were: (3.64±0.06), (6.85±0.14) and (16.3±0.4), for Gly_2 Gly_3 and Gly_5, respectively, and the percentage R.S.D. values were 1.6%, 2% and 2.4%, respectively. This precision confirmed that CZE could be used for precise and reliable measurements of rates of enzyme-catalyzed hydrolysis.

We performed the hydrolysis of pentaglycine by a solution of the purified protease from *Pseudomonas aeruginosa*. The areas were derived from electropherograms obtained from semi-automatic cycles including alternate runs and wash cycles. Samples of the reaction mixture could then be separated and measured every 15–20 min. By comparison of the area with the above calibration curves we could calculate the initial rates of Gly_2 and Gly_3 formation.

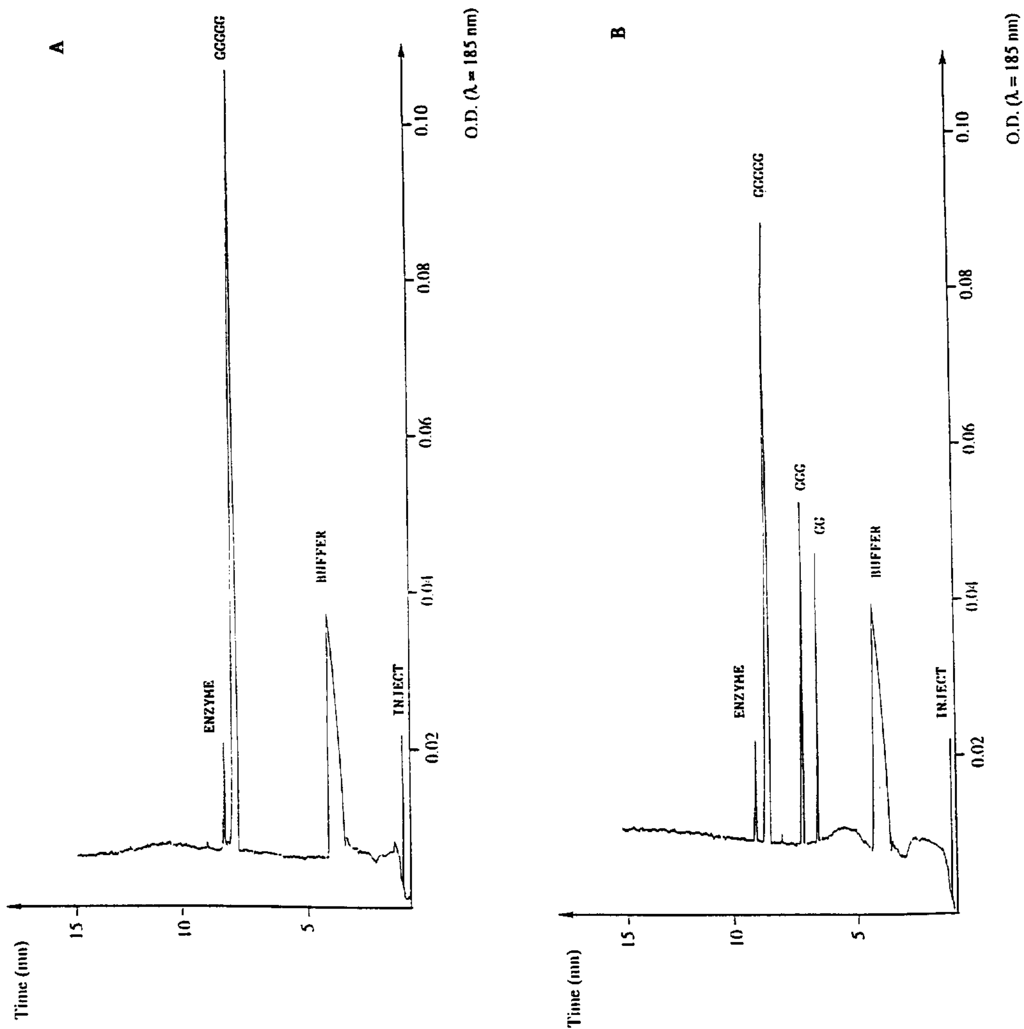

Fig. 1. Electropherograms showing the increase in amounts of Gly_2 and Gly_3 paralleled by the decrease in Gly_5 in relation to the progress of the enzyme-catalyzed hydrolysis. (A) just after addition of the enzyme and (B) 12 h after addition. The separation conditions are described in Section 2.

The values were: (0.80 ± 0.03) μM min^{-1} and (0.86 ± 0.04) μM min^{-1}, respectively and the correlation coefficients were 0.99.

It was not possible to deduce the rate of Gly_5 hydrolysis, due to the small changes in the peptide areas, leading to a poor correlation of area with time. Thus for better precision it is necessary to measure the initial reaction rates for product formation rather than substrate hydrolysis. Nevertheless, the method is rapid and reproducible, and requires only small amounts of the reaction mixture. Furthermore, automatic injection allows measurement of the progress of the enzyme hydrolysis, from which initial rates may be derived.

Unfortunately, there are no Gly_5 sequences in the primary structure of human elastin, which could have explained the low but significant elastolytic activity of these minor proteases [11,12]. In order to locate possible sites of hydrolysis in human elastin, we have synthesized sixty pentapeptides containing at least three glycine residues in variable positions, all belonging to the primary structure of elastin. We

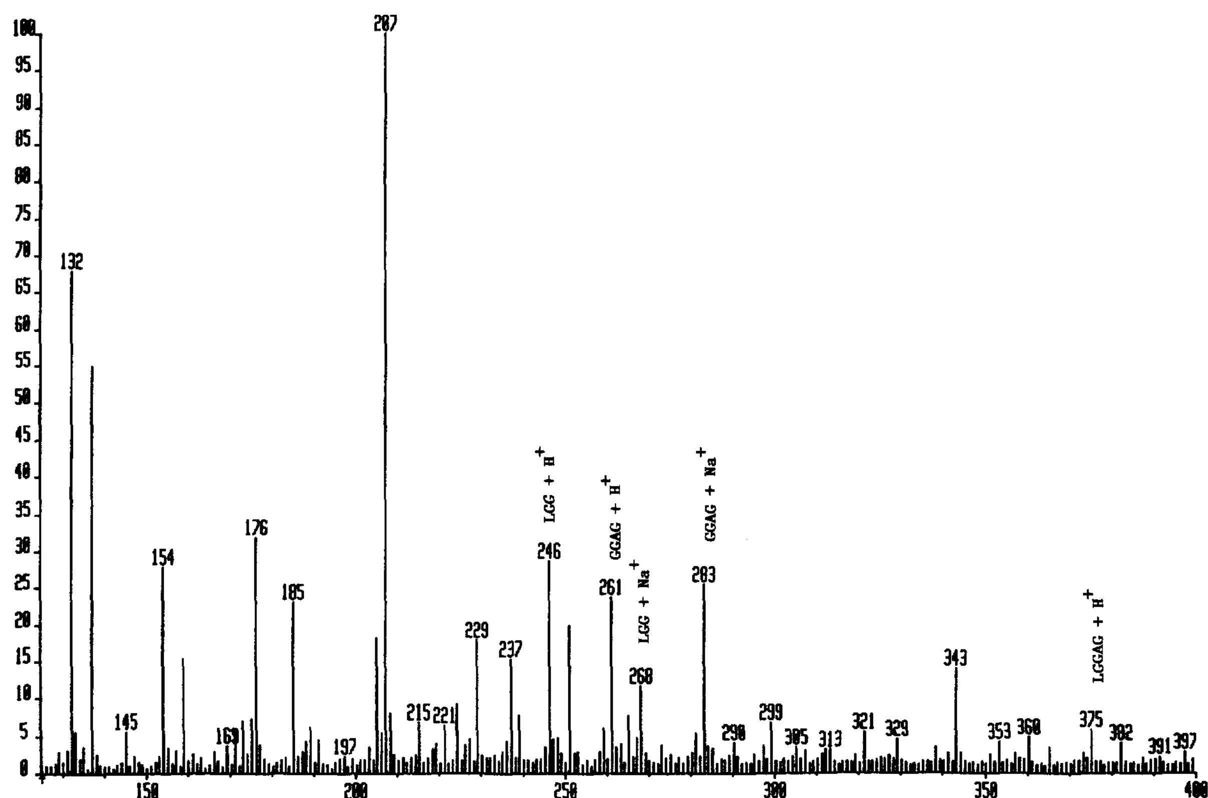

Fig. 2. FAB MS spectrum of the reaction mixture obtained after a 12-h hydrolysis of Leu–Gly–Gly–Ala–Gly (LGGAG) by the protease from *Pseudomonas*. The sites of hydrolysis are deduced from the observed masses (L+GGAG and LGG+AG, respectively).

have already obtained evidence for the splitting of some glycine-containing peptides, and characterized the position of hydrolysis using FAB-MS of the products, and we are currently measuring the hydrolysis rates and the kinetic parameters for the reactions. Fig. 2 demonstrates the results obtained for the enzymatic hydrolysis of Leu–Gly–Gly–Ala–Gly. Analysis of $[M+H]^+$ values clearly indicated that the peptide may be split either into Leu+Gly–Gly–Ala–Gly, or into Leu–Gly–Gly+Ala–Gly, confirming the number of peaks observed in the electropherograms. Use of both FAB-MS and CZE allows a direct and rapid identification of the reaction components and of the initial rates. Analysis of the hydrolysis of the sixty different peptides derived from the elastin sequence is currently in progress, using the same methodology.

Acknowledgments

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