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Simultaneous determination of *Pseudomonas aeruginosa* elastase, human leukocyte elastase and cathepsin G activities by micellar electrokinetic chromatography

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Abstract

Micellar electrokinetic chromatography (MEKC) is a new method for analysing proteolytic activities simultaneously present in incubation mixtures. Here we demonstrate that MEKC differentiates between the enzymatic activities of *Pseudomonas aeruginosa* elastase (PsE) and human leukocyte elastase (HLE) or cathepsin G (Cat G) in assays using the chromogenic peptide substrates Suc–Ala–Ala–Ala–NA or Suc–Ala–Ala–Pro–Phe–NA, respectively (where Suc=succinyl and NA=4-nitroaniline/u-nitroanilide). When PsE and Cat G were incubated at equimolar ratio with Suc–Ala–Ala–Pro–Phe–NA, the PsE-specific cleavage products PheNA and Suc–Ala–Ala–Pro were detected whereas inhibition of the metalloproteinase PsE with EDTA resulted in detection of NA and Suc–Ala–Ala–Pro–Phe only. Similarly, when PsE and HLE were incubated at equimolar ratio with Suc–Ala–Ala–Ala–NA, the PsE-specific cleavage products Suc–Ala and Ala–Ala–NA were detected whereas at an PsE-HLE ratio 1:50, both the PsE-specific and the HLE-specific cleavage products NA and Suc–Ala–Ala–Ala were separated. MEKC also allowed determination of the kinetic constants for the interactions of PsE, Cat G and HLE with the substrates considered. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Micellar electrokinetic chromatography; *Pseudomonas aeruginosa*; Enzymes; Elastases; Cathepsins; Peptides

1. Introduction

Several eukaryotic and prokaryotic cells produce proteinases which cleave elastin, proteoglycans and collagen and thus may play a pathogenic role in clinical conditions such as inflammation and infection. For example, human polymorphonuclear leukocytes may secrete several serine proteinases including elastase (HLE, EC 3. 4. 21. 37) and cathepsin G (Cat G, EC 3. 4. 21. 20) [1,2]. The opportunistic bacterial pathogen *Pseudomonas aeruginosa* also produces three extracellular proteinases, elastase (PsE, EC 3. 4. 21. 14) [3], alkaline proteinase (EC 3. 4. 24. 4) [4] and LasA [5,6] which act on a variety of substrates including elastin. Assays to detect elastolytic activity include the determination of clearance zones in elastin plates and the quantification of labelled elastin hydrolysis by biological fluids [7,8], the use of suitable synthetic peptides [9–14] and elastin cleavage products [15]. These assays, however, do not generally differentiate between elastases from different origins; one way to circumvent this problem is the establishment of antigen-specific radioimmunoassays or enzyme-

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linked immunosorbent assays for the detection of the proteolytic enzyme in question [9,16–18]. However, the results of these assays do not allow us to answer the question whether a given concentration also reflects enzyme activity in biological fluids. Alternatively, specific proteinase inhibitors such as α1-proteinase inhibitor or phenylmethylsulfonyl fluoride (PMSF) will inhibit HLE and Cat G but will not impair the enzymatic activity of PsE [19,20]. On the other hand, ethylenediaminetetraacetic acid (EDTA) will inhibit PsE [19,20]. A typical clinical example where such a differentiation is necessary is cystic fibrosis (CF), a genetic disorder characterized by chronic respiratory infection [21]. The predominant species colonizing the CF respiratory tract is *P. aeruginosa* and PsE has been shown to play an important role in CF [22] together with the continuously recruited neutrophils releasing considerable amounts of lysosomal enzymes [23]. The objective of the present study was to investigate the role of capillary electrophoresis (CE) in allowing the simultaneous determination of different proteinase activities (PsE, HLE and Cat G).

In a previous investigation [24] we showed that micellar electrokinetic chromatography (MEKC), with its capability of separating the product(s) from the substrate of the enzymatic reaction, was indeed a very useful method for monitoring protease activity. Although successfully applied to the determination of the proteolytic activity of a few commercial proteases, the study was limited to elastases that specifically cleaved the substrates at the C-terminal nitroanilide linkage.

In the present report we extend our study to the identification of reaction products obtained from elastases with different specificities of bond cleavage simultaneously present in the assay mixture. The high resolution of the MEKC technique allowed the detection of all products of enzymatic reactions and the separation of fragments prior to quantification also allowed us to distinguish clearly between the different elastase activities. The methodology described appears to be more reproducible and more practical than other techniques as far as concerns ensuring accurate and representative measurements of each protease activity present in biological fluids containing different proteases. Moreover, since it should be equally useful for other enzymes not investigated here, it may lead to the development of a general approach that could provide the complete determination of proteinases present in lung secretions, such as sputum, or in bronchoalveolar lavage fluid of patients affected by lung disease.

2. Experimental

2.1. Chemicals

Tris(hydroxymethyl)aminomethane (Tris) used as incubation buffer of enzymatic reactions, EDTA and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, MO, USA). Doubly distilled water utilized for preparation of CE buffers was obtained from a Millipore (Bedford, MA, USA) Milli-Q purification system. All other reagents, of analytical grade, were used without further purification.

2.2. Determination of elastolytic activity

Pseudomonas aeruginosa elastase (260 units mg^{-1} protein), human neutrophil cathepsin G (2800 units mg^{-1} protein) and human leukocyte elastase (865 units mg^{-1} protein) were obtained from Elastin (Owensville, MO, USA). The peptide-substrates used with these enzymes: Suc–Ala–Ala–Ala–NA and Suc–Ala–Ala–Pro–Phe–NA were purchased from Sigma and their solutions were freshly prepared at the moment of use (Suc=succinyl; NA=4-nitroaniline/u-nitroanilide).

The procedure followed for the CE assay was essentially the same as that previously described [24] except that the enzymatic reaction was started by simultaneous addition of different proteinases to a solution containing an appropriate amount of peptide substrate in 50 m*M* Tris–HCl buffer (pH 7.8). At various times 100 μl-aliquots were withdrawn and the reaction was stopped by addition of 10 μl of 0.45 *M* TCA. The mixture was then centrifuged at 5000 *g* for 10 min and supernatants were submitted to capillary electrophoretic analysis.

2.3. Capillary electrophoretic instrumentation and running conditions

The Biofocus 3000 system (Bio–Rad, Richmond,

CA, USA) equipped with a high-speed UV–Vis scanning detector was used. Separations were performed using fused-silica capillaries of 57 cm (50 cm effective length)×50 μm I. D. (Beckman, Palo Alto, CA, USA) and 35 m*M* sodium tetraborate buffer (pH 9.3) containing 65 m*M* sodium dodecyl sulfate (SDS) and 15% (v/v) methanol as run buffer.

Samples were injected hydrodynamically for 1 s at 0.034 MPa to give a volume of about 9 nl. UV absorbance was monitored at 200 nm. Temperature was maintained at 20±0.1°C by means of a cooling liquid circulating through the cartridge. A voltage of 25 kV was applied and samples migrated through positive to negative polarity.

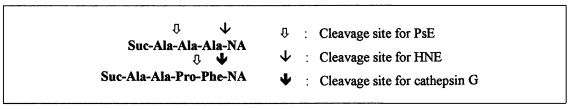
Between runs the capillary was rinsed with 0.5 *M* NaOH for 2 min followed by water for 3 min and electrophoresis buffer for 2 min.

2.4. Amino acid and mass spectrometry (MS) analyses

Fractions from 20 consecutive micropreparative MEKC runs were collected in microvials containing 30 μl of 50 m*M* Tris–HCl buffer (pH 7.8) and divided in two aliquots. Volumes of 10 μl were used for amino acid analyses, 20 μl for MS analyses. For amino acid analyses, samples were hydrolyzed in 6 *M* HCl vapor at 106°C for 24 h and analyzed using the AminoQuant amino acid analyzer based on the HP 1090 HPLC system (Hewlett–Packard, Palo Alto, CA, USA) with fully automated pre-column derivatization using both *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) reaction chemistries. UV absorbance was measured at 262 and 338 nm. The analyses of fragments were performed on a Finnigan MAT (Finnigan, San Jose, CA, USA) LCQ ion trap MS with an electrospray ionization (ESI) source.

3. Results and discussion

Due to its high power of resolution MEKC is capable of separately analyzing different proteolytic cleavage products and, for this reason, it could be used as a new method for identifying and quantifying proteolytic activities present simultaneously in incubation mixtures and possibly in biological fluids. Here we demonstrate that MEKC differentiates between the enzymatic activities of PsE and HLE or Cat G in assays using the synthetic chromogenic peptide substrates Suc–Ala–Ala–Ala–NA or Suc–Ala–Ala–Pro–Phe–NA whose cleavage sites are indicated in the scheme below reported.



As a tentative representative model of 'in vitro' lung secretion PsE and Cat G were incubated with the peptide substrate Suc–Ala–Ala–Pro–Phe–NA: the assay was carried out at 37°C by incubation of saturating concentrations of the substrate previously indicated (its final concentration in the assay being 15 m*M*) in 400 μl of 50 m*M* Tris–HCl buffer (pH 7.8) and then starting the enzymatic reaction by the simultaneous addition of PsE and Cat G. At selected times aliquots (100 μl) were withdrawn, the reaction was stopped by addition of 10 μl of 0.45 *M* TCA and the supernatant obtained after centrifugation for 10 min at 5000 *g* was loaded onto the capillary. Unless otherwise stated, all electrophoretic separations were performed in 35 m*M* sodium tetraborate buffer (pH 9.3) containing 65 m*M* SDS and 15% (v/v) methanol.

After incubating the two proteinases at equimolar ratio (the concentration of both enzymes in the assay being 75 n*M*) with the substrate for 5 min, the electropherogram yielded the pattern of peaks shown in Fig. 1A, lower trace. As shown, apart from the signals indicated by arrows whose presence was associated with the use of Tris (these peaks were found to be practically identical in height in all experiments performed throughout the study presented here), the electropherogram contained three main peaks (namely peaks 2, 4 and 5) whose migration times were 16.12±0.15 min, 34.40±0.2 min and 41.20±0.16 min, respectively, and two other very small peaks: peak 1, migration time 15.20±0.1 min and peak 3, migration time 22.80±0.15 min. As incubation progressed (the incubation time was prolonged to 30 min) the ratios of the different products formed changed; peaks 1 and 3 increased (as did peaks 2 and 4) while peak 5 disappeared completely (Fig. 1A, upper trace). In

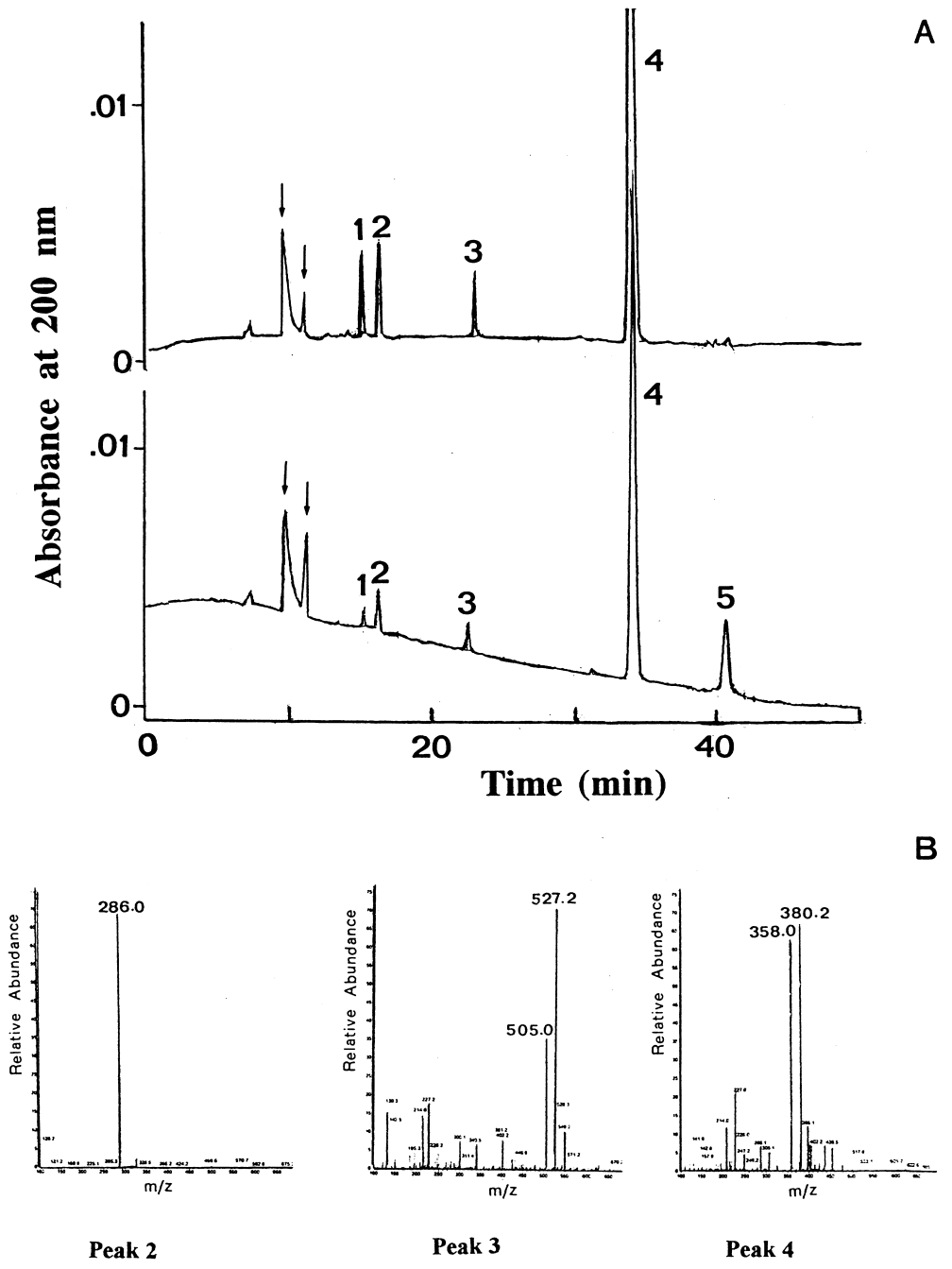


Fig. 1. (A) Micellar electrokinetic chromatography (MEKC) profiles showing the proteolytic cleavage of peptide Suc–Ala–Ala–Pro–Phe–NA after incubation for 5 min (lower trace) and 30 min (upper trace) with *P. aeruginosa* elastase (PsE) and human leukocyte cathepsin G (Cat G) at equimolar molar ratio (75 n*M*). Arrows indicate the peaks of Tris; peak 1: NA; peak 5: intact substrate. For identification of peaks 2, 3 and 4 see below. Runs were carried out at 25 kV using 35 m*M* sodium tetraborate buffer (pH 9.3) containing 65 m*M* SDS and 15% (v/v) methanol as electrolyte. Analytes were monitored at 200 nm; temperature: 20°C ±0.1°C. Positive to negative polarity. (B) Identification of peaks 2, 3 and 4 (left to right, respectively) by ESI–MS; peak 2: Phe-NA (286.0 $[M+H]^+$); peak 3: Suc–Ala–Ala–Pro–Phe (505.0 $[M+H]^+$ and 527.2 $[M+Na]^+$); peak 4: Suc–Ala–Ala–Pro (358.0 $[M+H]^+$ and 380.2 $[M+Na]^+$).

order to confirm the assumption that both proteolytic activities could be responsible for the observed pattern we proceeded to the identification and exact characterization of all fragments produced.

Peaks 1 and 5 were identified as *p*-nitroaniline (NA) and the intact peptide substrate by the migration time of the authentic compounds (15.25±0.1 and 40.90±0.18 min, respectively) and by co-injection of different amounts of standards with samples examined. Identification of peaks 2, 3 and 4 was achieved by collecting sufficient material to perform MS and amino acid analyses: to this purpose 20 consecutive micropreparative runs were performed on the mixture incubated 30 min and each sample was collected and analyzed as indicated in the experimental section. The mass spectra of these peaks, obtained by ESI–MS gave a signal at m/z 286.0 for peak 2, at m/z 505.0 and 527.2 for peak 3 and at m/z 358.0 and 380.2 for peak 4 (Fig. 1B, left to right). A rigorous assignment of chemical structures that could match the molecular masses found was achieved determining the amino acid composition of these samples. As shown in Table 1 peak 2 could be identified as Phe-NA (M_r 285), peak 3 as the peptide Suc–Ala–Ala–Pro–Phe (M_r 504.0) and peak 4 as the peptide Suc–Ala–Ala–Pro (M_r 357.0).

The definite unambiguous identification of all fragments obtained from cleavage of substrate provided a strong body of evidence that MEKC was indeed capable of detecting two different proteolytic activities simultaneously involved in the assay and of discriminating between them. These data were confirmed by further experiments in which PsE was individually pre-incubated either with the metallo-proteinase inhibitor EDTA or with the substrate prior to the addition of the second proteinase.

Thus, PsE was pre-incubated 1 h at 25°C with 20 m*M* EDTA in 50 m*M* Tris–HCl buffer (pH 7.8) and the reaction was then started by addition of substrate. Aliquots of 100 μl were withdrawn after 5 min and 30-min incubation and submitted to electrophoretic separation: as expected the enzymatic activity of the metallo elastase was totally inhibited and, in both cases, the electropherograms exhibited only the peak of the intact substrate (peak 5 in the inset of Fig. 2). The addition of Cat G (in equimolar ratio with PsE) to the above mixture yielded (after a 30-min incubation time) the pattern shown in Fig. 2, lower trace, in which only the Cat G-specific cleavage products (peaks 1 and 3) were identified (peak 5 being the residual intact substrate). Similarly, pre-incubation for 15 min of the substrate with PsE before addition of Cat G resulted (after a 30-min incubation time) in the production of PsE-specific peaks 2 and 4, peak 5 being totally hydrolyzed under these experimental conditions (Fig. 2, upper trace). The 'complementarity' of peaks shown in the electropherograms of Fig. 2 clearly indicated that MEKC was a method very sensitive to all changes induced in the assay mixture. The experiments described above also evidenced the well-known competition of these two enzymes for the substrate Suc–Ala–Ala–Pro–Phe–NA. In fact, in agreement with earlier findings [11] our data showed that the addition of this substrate to PsE before Cat G completely abolished the latter's proteinase activity on account of very fast degradation of the substrate by PsE. In contrast, the simultaneous addition of the two enzymes to the substrate allowed Cat G to compete favorably with PsE and Cat G activity increased substantially with an increase of Cat G:PsE molar ratio. The rate of formation of NA was used for kinetic determinations of Cat G activity in samples where PsE activity was also present. After preparing a calibration curve for NA (not shown), which allowed the measurement of the amount of NA released by the enzymatic hydrolysis of the substrate, concentration-dependent experiments were carried out incubating PsE and Cat G at the molar ratios of 1:10, 1:20 and 1:50. The electropherograms reported in Fig. 3, panel A show that the height of peaks 1 and 3, produced by cleavage of the substrate at the C-terminal nitroanilide linkage, increased

Table 1
Amino acid composition of fragments obtained after a 30-min digestion of peptide Suc–Ala–Ala–Pro–Phe–NA by *P. aeruginosa* elastase and cathepsin G and separated by CE

Peak	Amino acid[a]		
	Ala	Pro	Phe
2	–	–	8.21 (1)
3	16.21 (2)	8.00 (1)	8.15 (1)
4	16.12 (2)	8.10 (1)	–

[a]The values reported (expressed as pmoles) are the mean of two independent determinations. The values reported between brackets are moles per mole of fragment in integer numbers.

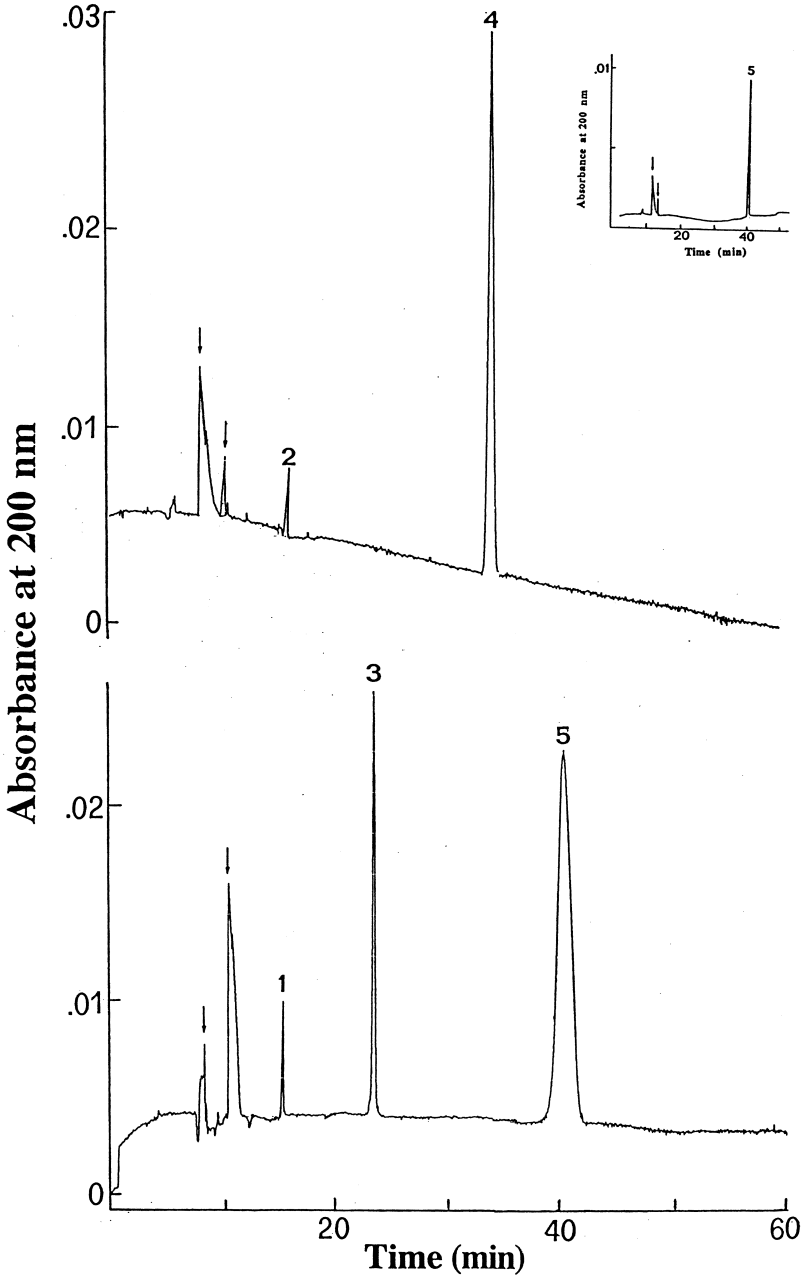


Fig. 2. MEKC profiles showing the proteolytic cleavage of peptide Suc–Ala–Ala–Pro–Phe–NA after incubation with a mixture of Cat G and PsE inhibited by 20 m*M* EDTA (lower trace) and with PsE followed by addition of Cat G (upper trace). Inset: electropherogram obtained upon incubation of Suc–Ala–Ala–Pro–Phe–NA with PsE inhibited by 20 m*M* EDTA. Identification of peaks and all other experimental conditions as in Fig. 1.

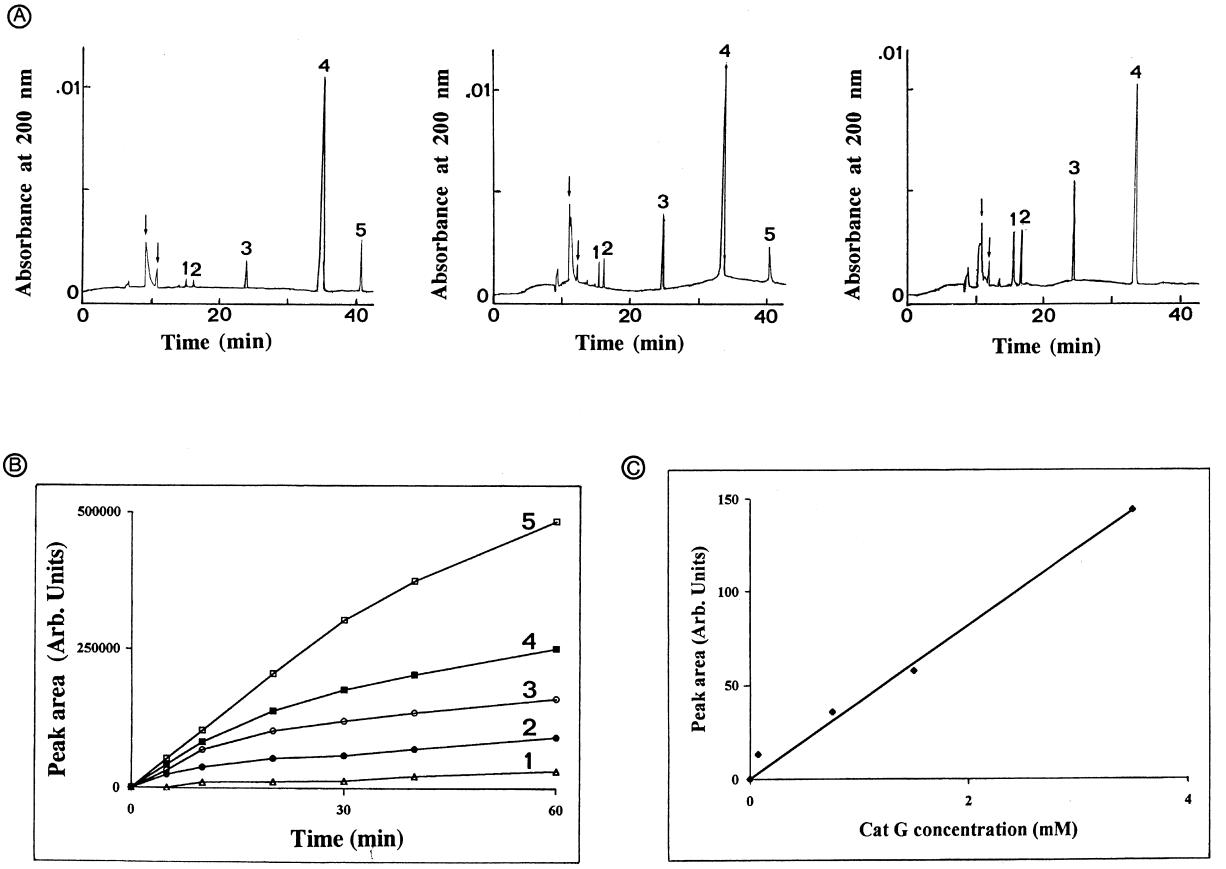


Fig. 3. (A) MEKC profiles obtained incubating the substrate Suc–Ala–Ala–Pro–Phe–NA with a mixture of PsE and Cat G at the molar ratios 1:10, 1:20 and 1:50 (left to right, respectively). Identification of peaks and all other experimental conditions as in Fig. 1. (B) Kinetic profiles showing the formation of NA at PsE:Cat G molar ratios 1:1 (curve 1), 1:10 (curve 2), 1:20 (curve 3), 1:50 (curve 4), 1:1 but with PsE inhibited by 20 m*M* EDTA (curve 5). (C) Formation of NA upon incubation of 15 m*M* substrate with the above reported Cat G concentrations.

proportionally with the amount of Cat G added (from PsE:Cat G molar ratio 1:10 to 1:50, left to right, respectively) and that, for each molar ratio PsE:Cat G chosen, the amount of NA released increased with incubation time (curves 1 to 4 of Fig. 3, panel B) the greatest amount obviously being released when PsE was totally inhibited by EDTA (curve 5 of the same figure). Moreover, as expected, at a fixed concentration of substrate (15 m*M*), the rate of substrate hydrolysis by Cat G (calculated from the peak areas of NA produced that were converted to molar quantities by reference to a calibration curve, not shown, obtained from injection of variable, known amounts of standard NA) was a linear function of the amount of Cat G included in the assay (Fig. 3, panel C). The finding that the proteolytic coefficient k_{cat}/K_m for PsE is about 30-fold higher than that for HLE (Table 2) clearly rationalizes all the above reported observations.

Since PsE is reported to interfere also with the assay of human leukocyte elastase (HLE) in lung secretions [11], we explored the possibility of evaluating the activity of HLE in combination with PsE applying the CE approach. Once again the rationale behind this assay was the use of CE to attempt the separation of the reaction products of two proteinases with different intrinsic catalytic activity, i.e. the identification and quantification of proteolytic

Table 2
Kinetic parameters obtained by MEKC from incubation of *P. aeruginosa* elastase, cathepsin G and human leukocyte elastase with the substrates indicated

Enzyme	Substrate	K_m (m*M*)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
PsE	Suc–Ala–Ala–Ala–NA	0.67	3.16	4600
	Suc–Ala–Ala–Pro–Phe–NA	0.99	45	46000
Cat G	Suc–Ala–Ala–Pro–Phe–NA	2.9	4.2	1400
HLE	Suc–Ala–Ala–Ala–NA	4.5	1.5	330

activities simultaneously present in the incubation mixture. The synthetic peptide used for this assay was Suc–Ala–Ala–Ala–NA, a substrate of both PsE and HLE that is cleaved at the Ala–Ala bond by the former enzyme and at the C-terminal nitroanilide group by the latter. The peptide substrate, at a concentration of 15 m*M*, was incubated with the two proteinases at different molar ratios, the concentration of the two enzymes being 75 n*M* at a molar ratio PsE:HLE 1:1. The other molar ratios investigated were the same as those previously indicated and the procedure followed was identical as that described above. Thus, after a 5-min incubation of the two proteinases (at a equimolar molar ratio) with the substrate, the typical electrophoretic pattern obtained was that exemplified in Fig. 4 (lower trace). The pattern showed only two peaks (namely peak 1 and peak 3, retention times 14.35 ± 0.13 min and 29.50 ± 0.18 min, respectively) identified as the intact substrate (peak 1) and as the fragment Ala–Ala–NA (peak 3) which is one of the products of PsE digestion. Due to its low UV absorbance we were unable to detect the other small fragment (Suc–Ala) produced by the cleavage of substrate. The above pattern did not change even after 30-min or 60-min incubation and since the k_{cat}/K_m value of PsE was calculated to be about 14 times greater than that of HLE for the used substrate (Table 2) no HLE-specific cleavage products were detectable. Interestingly these results were consistent with those previously reported by Pedrines and Bieth [25] using α1-proteinase inhibitor (α1-PI) instead of chromogenic peptide substrates. In fact, it is well-known that α1-PI is the major naturally occurring inhibitor of HLE but it acts as a substrate for PsE, which splits its Pro357–Met358 peptide bond [26]. Pedrines and Bieth demonstrated that, as a function of the k_{cat}/K_m value of the PsE-catalyzed α1-PI cleavage and of the K_{ass} value for the HLE-α1-PI association (9×10^4 M^{-1} s^{-1} *vs* 1.7×10^7 M^{-1} s^{-1}), at equimolar concentrations of PsE and HLE, α1-PI almost fully forms complexes with HLE and therefore is not available as PsE substrate, whereas in the presence of an excess of PsE most α1-PI is cleaved. Thus, the HLE-specific cleavage products were detected either at PsE:HLE molar ratios of 1:10, 1:20 and particularly 1:50 or at PsE:HLE molar ratios 1:5 and even 1:1 after inhibiting PsE activity by addition of 20 m*M* EDTA as previously indicated. The electropherogram of Fig. 4, upper trace, shows infact the formation (after 30-min incubation at a molar ratio 1:50) of two new peaks in addition to peaks 1 and 3: peak 2, migration time 15.20 ± 0.12 min, was identified as NA, and peak 4, migration time 38.50 ± 0.15 min represented the substrate shortened by NA. As expected, peaks 2 and 4 were the only products present in the electrophoretic pattern (after 30-min incubation at a molar ratio 1:1) when PsE activity was inhibited by EDTA (inset of Fig. 4).

Thus, under the above described experimental conditions, the proteolytic activity of HLE could be accurately detected despite the presence of PsE. This provided a further interesting example that the ability of CE technique to achieve the separation of all products released from substrates (not necessarily the synthetic p-nitroanilide peptides) by digestion with proteinases does, indeed, represent a very useful tool for studying the hydrolytic activity of different proteinases simultaneously present in the reaction mixture.

4. Conclusions

The CE procedure presented in this paper offers a reliable and versatile assay for easy determination of activity of different proteinases simultaneously present in the reaction mixture. As a model of ‘in vitro’

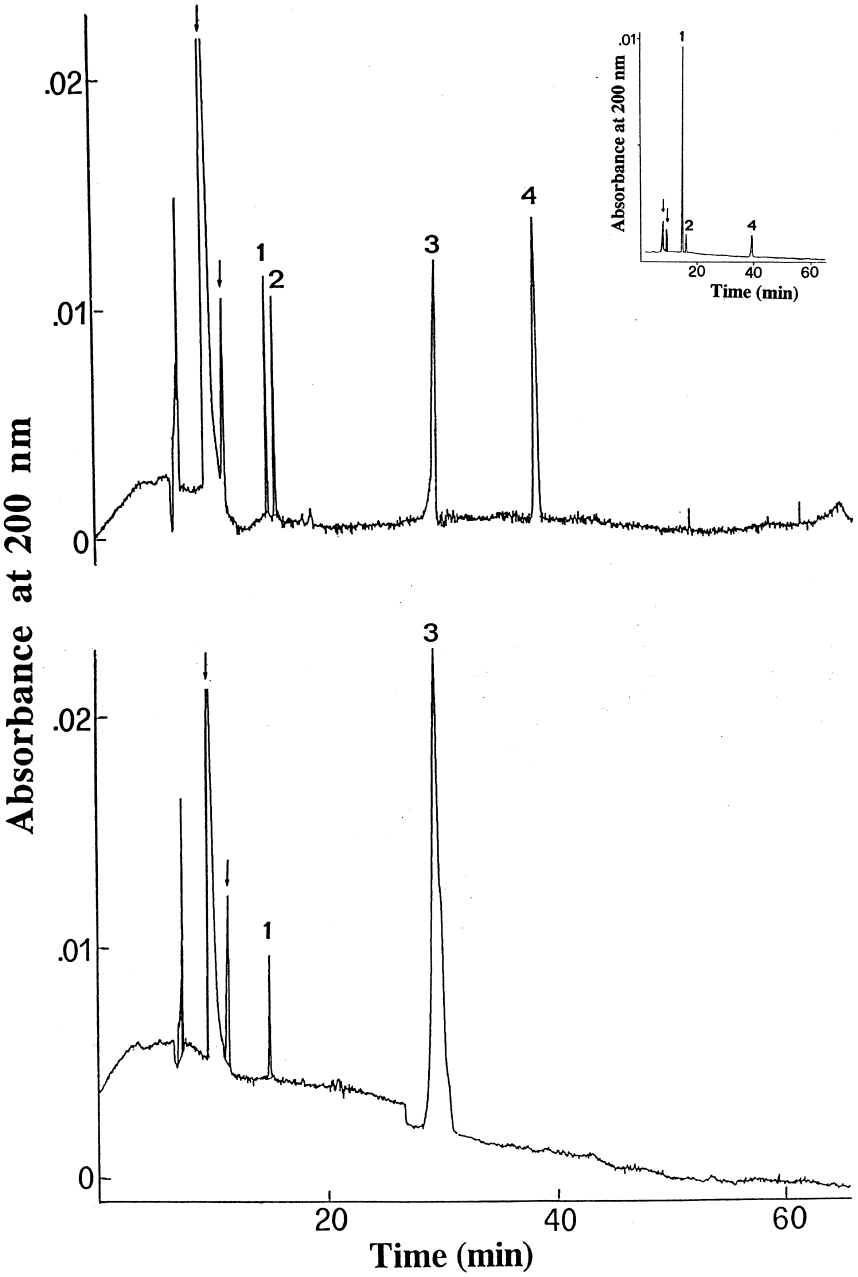


Fig. 4. MEKC profiles showing the proteolytic cleavage of peptide Suc–Ala–Ala–Ala–NA after 5-min incubation with PsE and human leukocyte elastase (HLE) at an equimolar ratio (lower trace) and after 30-min incubation at a PsE:HLE molar ratio 1:50 (upper trace). Inset: electropherogram obtained after 30-min incubation of the peptide above mentioned with PsE and HLE at a molar ratio 1:1 but with PsE inhibited by 20 m*M* EDTA. Identification of peaks. Peak 1: intact substrate; peak 2: NA; Peak 3: Ala–Ala–NA; peak 4: Suc–Ala–Ala–Ala.

lung secretions we have studied the behaviour of two pairs of proteolytic enzymes against their synthetic peptide substrates. The results we obtained from both the *Pseudomonas aeruginosa* elastase/cathepsin G pair and the *Pseudomonas aeruginosa* elastase/human leukocyte elastase pair suggest that the electrophoretic assay is very sensitive to the composition of the mixture examined and is also able to detect individually all the products released upon enzymatic digestion. The versatility of this method could probably allow simultaneous detection of a larger number of proteinases than described here, the only limitation being the capacity of the technique to separate all fragments obtained with a good resolution.

In this respect CE might be a good tool for precise evaluation of proteinase activities in lung secretions from patients affected by conditions, such as cystic fibrosis or pulmonary emphysema, characterized by an excess of mixed proteinases, both of host and non-host origin.

In addition, it might provide evidence for potential therapeutic effects in clinical trials dealing with specific proteinase inhibitors.

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