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Short communication

Analysis of cathepsin D from breast tissues by capillary electrophoresis

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Abstract

A rapid and simple method for analyzing cathepsin D in breast tissue based on capillary zone electrophoresis (CZE) is described. After incubating the tissue extracts with hemoglobin as a substrate, a specific peptide is cleaved and separated by CZE in less than 5 min. This peptide is not produced by the action of pepsin or trypsin. It is inhibited by the addition of pepstatin, a specific inhibitor for cathepsin D. Human hemoglobin acted as a better substrate than bovine hemoglobin. The test compared well to a radioimmunoassay. We have shown that peptides can be stacked by the use of acetonitrile. The method demonstrates the advantages of CZE for assay of proteolytic enzymes in general.

Keywords: Cathepsins; Enzymes

1. Introduction

Cathepsin D is a proteolytic lysosomal enzyme with an optimum pH of 3.5. It is secreted from some tumor cells [1], aiding the cells in metastasis. Tissue enzyme levels have been found to be a good predictor of tumor malignancy in general [2,3] and of breast carcinoma in particular [4–7].

Initially the enzyme was assayed by its catalytic activity on several proteins including hemoglobin [8] and more recently by immunoassays [6]. Both of these methods are time consuming requiring about a day to perform, leading to a high cost.

CZE is a relatively new technique in the clinical laboratories. It has a good potential for analyzing wide variety of compounds. Because it can separate specific peptides and measure their concentration at 214 nm without staining or using synthetic substrates it is quite suited for the rapid measurement of proteolytic enzyme activities using natural substrates [9–11]. Small changes in absorbance can be measured by CZE rendering the technique very sensitive.

Here, we describe a rapid method for assaying cathepsin D based on CZE after incubating the enzyme with the substrate. The CZE step is rapid, less than 5 min; while the incubation step is about 20 min. This work illustrates the potential of CZE for analyzing other proteolytic enzymes.

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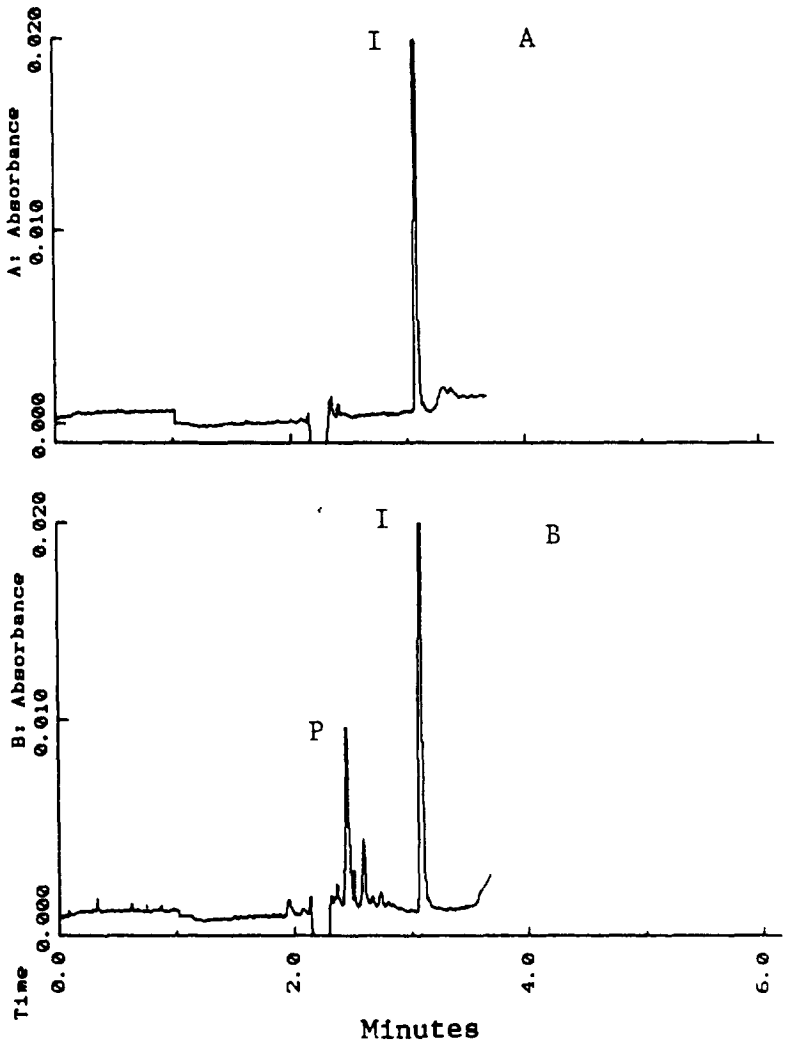

Fig. 1. Catalytic activity of purified cathepsin D: A=0 min and B=20 min. (P=peptide, I=iothalamic acid).

2. Experimental

2.1. Reagents

Cathepsin buffer: formic acid 0.3 mol/l, pH 3.4 was prepared in NaCl (12.5 g/l). Triton X-100; 0.5 ml/l was added also to the buffer.

Hemoglobin solution: lyophilized human hemoglobin 24 g/l (Sigma; St. Louis, MO, USA) was dissolved in water.

Substrate: equal volumes of hemoglobin solution and cathepsin buffer were mixed and kept on ice.

Deproteinization reagent: iothalamic acid, 70 mg (Malinckrodt; St. Louis, MO, USA) was dissolved in 1 l of acetonitrile and used as an internal standard.

2.2. Tissue extracts

Tissue extracts preparation have been described early [8]. Protein analysis was performed by the Bradford method [12].

2.3. Method

The substrate 150 μl in a 1.5-ml centrifuge tube was brought to 37°C. Tissue homogenate (calibrator,

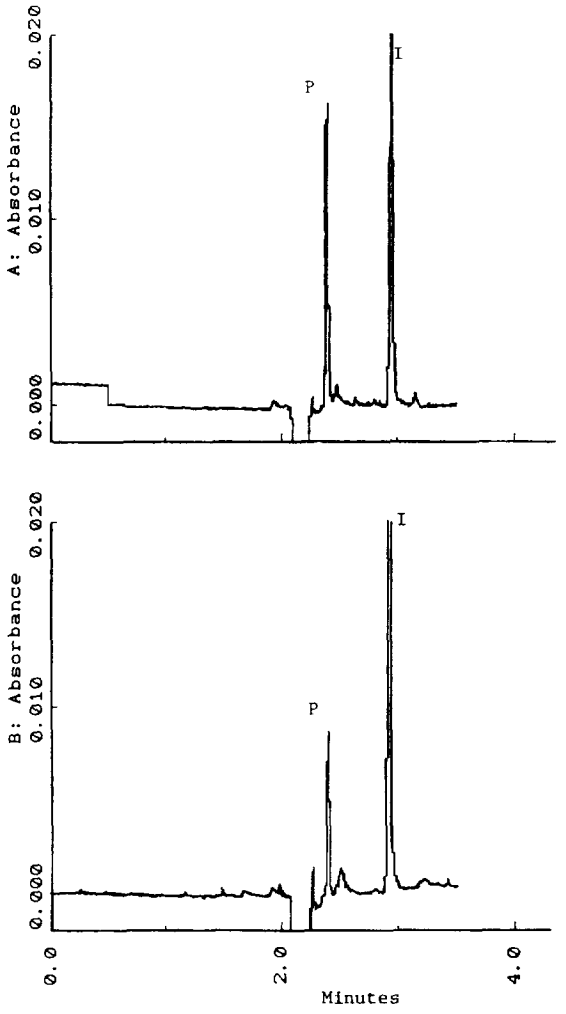

Fig. 2. Effect of hemoglobin source on human breast tissue cathepsin D activity: (top) human hemoglobin and (bottom) bovine hemoglobin.

control or patient), 50 μl is mixed with the substrate and incubated for 20 min at 37°C. At the end of the incubation period, 500 μl of the deproteinizing reagent was added. The tube contents were mixed and centrifuged for 30 s. The supernatant was injected into the capillary within 1 h.

2.4. Instrument

A Model 2000 capillary electrophoresis instrument (Beckman Instruments; Fullerton, CA, USA) was set

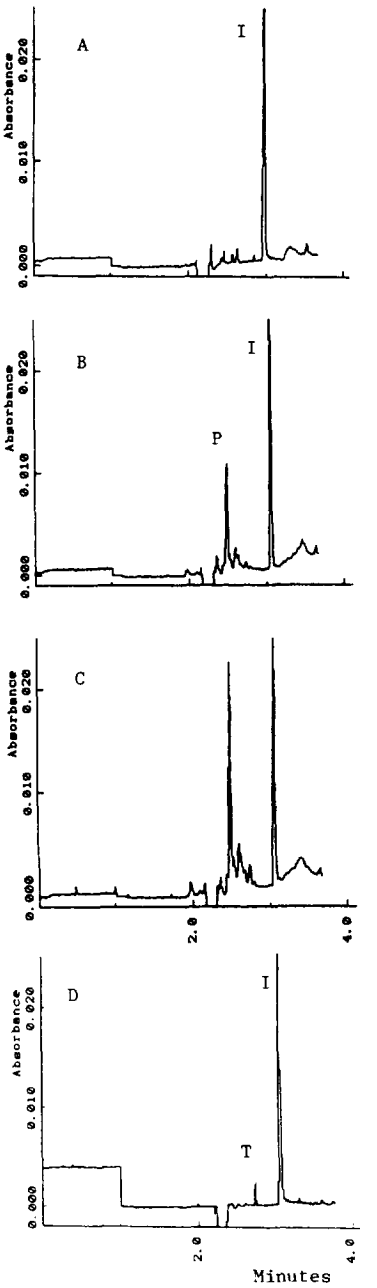

Fig. 3. Enzymatic activity of breast tumor homogenate activity (106 pmol/mg protein) at different periods of incubation: A=0 min; B=10 min; C=20 min and D=at 20 min in presence of pepstatin. (P=split peptide, I=Iothalamic acid; and T=pepstatin).

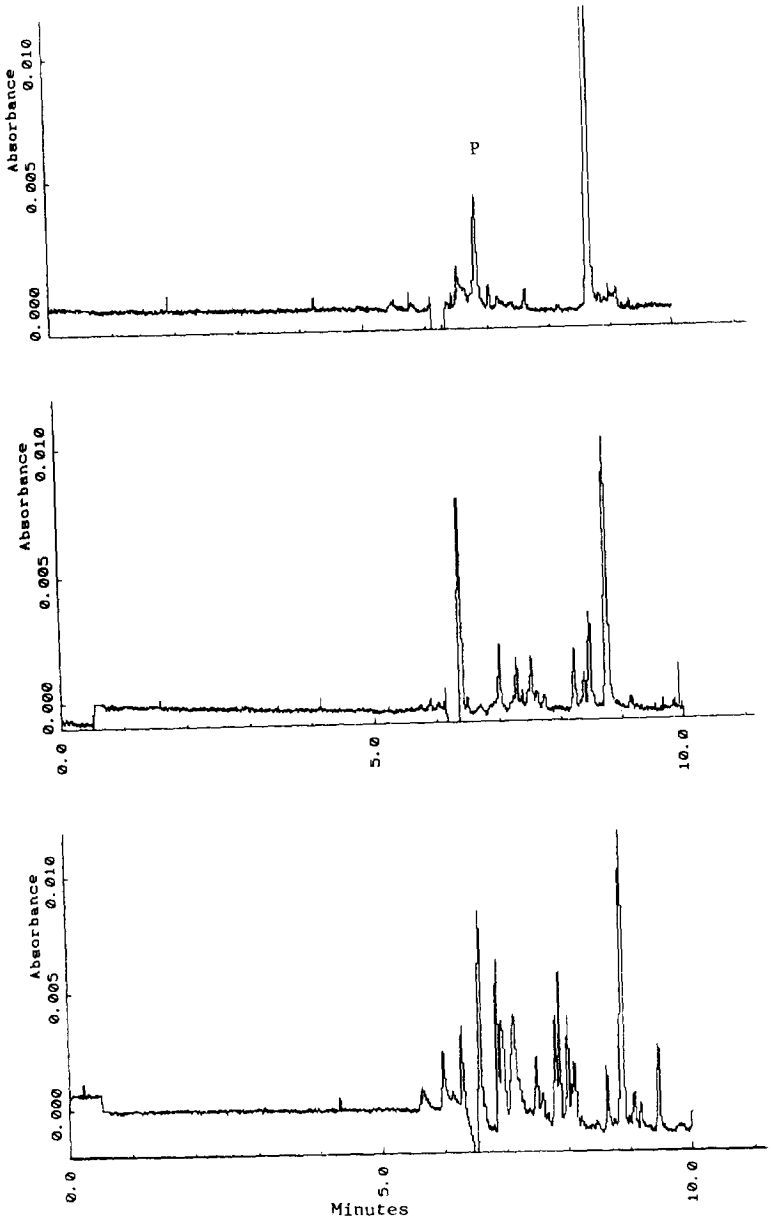

Fig. 4. Reaction on human hemoglobin: (top) cathepsin D of human breast tissue; (middle) trypsin (1000 U of bovine pancreas in Tris buffer 50 mmol/l, pH 8.1) and (bottom) pepsin (60 U of porcine stomach in 200 mmol/l HCl). Samples incubated for 20 min. The separation was performed on 50 μm (I.D.)×60 cm capillary at 200 V/cm.

at 360 V/cm, 24°C and 214 nm. The capillary was 42 cm (35.5 to the flow cell)×50 μm I.D.. The electrophoresis buffer was boric acid 175 mmol/l adjusted to pH 8.4 with 4 mol/l NH_4OH. Samples were introduced by pressure injection for 10 s (2.5% of the capillary to the detector). The capillary was

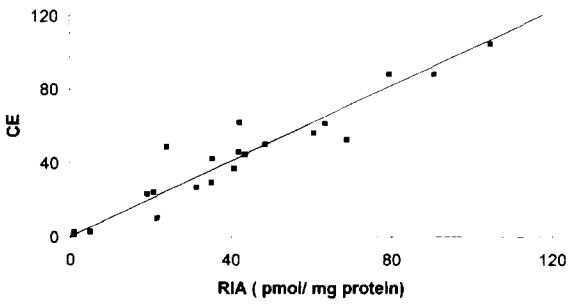

Fig. 5. Correlation of the RIA and the CE (r=0.949; n=21).

washed for 1 min with 0.2 mol/l NaOH and filled with electrophoresis buffer for 1.0 min after each run.

3. Results and discussion

Initially we optimized the assay for both the enzymatic activity as well as the CZE conditions. Enzymatic activity was measured here by using the traditional substrate, hemoglobin, in a formate buffer at pH 3.4. Purified cathepsin D from beef spleen cleaved a major peptide with a migration time of about 2 min which can be monitored at 214 nm, Fig. 1. The migration time for the internal standard was about 3 min. Human hemoglobin, as a substrate, gave about twice the activity compared to bovine hemoglobin, Fig. 2. This was the case for the cathepsin D from human breast as well as that from beef spleen.

Fig. 3 illustrates the activity at different periods of time for a sample from breast carcinoma. The peptide peak height increased with time. Pepstatin (0.2 mmol/l, Sigma), which is a specific inhibitor peptide for the cathepsin D activity, completely inhibited the production of the fragment. Pepstatin migrated as peak between the internal standard and the split peptide, Fig. 3. Pepsin and trypsin produced several major and minor peptides which are different from the one produced by cathepsin D, Fig. 4. This suggests a better specificity of protein cleavage by cathepsin D compared to these other two enzymes. A great degree of specificity of cathepsin D arises from the unique optimum pH which is different from other proteolytic enzymes, in addition to the single specific peptide. Based on sample dilution, the test was linear between 5–160 pmol/mg protein. Increased risk of tumor malignancy occurs at values >30 pmol/mg protein [13]. Other investigators have used different values also. The within-run R.S.D. was 9.1% (mean 25 pmol/mg, n=10). Fig. 5 illustrates a good correlation between the CE and the RIA method (r=0.949, n=21).

The method, as described, can measure values as low as 10 pmol/mg protein. However, it can be modified for much greater sensitivity e.g., by longer incubation, wider capillary diameter or by increasing the sample size. In CZE, the sample loading usually is kept at less than 1% of the capillary volume [14]. We have demonstrated earlier, that in the presence of acetonitrile and sodium chloride the sample size can be greatly increased [14–17]. In this method we incorporated sodium chloride in the formate buffer in order to induce sample stacking with the acetonitrile. Increasing the sample size to a loading of 12.5% of the capillary volume increases the sensitivity about six-fold, Fig. 6. This will allow values of about 2 pmol/mg tissue to be measured. This work illustrates, also, that small peptides can be concentrated on the capillary (stacked) by the use of acetonitrile [16,17]. Our previous data showed that the predicative value of cathepsin D by immunoassay, or by measuring its catalytic activity, is comparable [8].

We used peak height to calculate the results. In order to convert peak height to enzymatic concentration (/activity) we used a secondary calibrator, i.e. a sample of known enzymatic concentration analyzed by the RIA method as a standard. The peak height/area of the unknown samples are compared to that for the known sample, similar to that commonly used on the automated chemistry analyzers in the clinical laboratories [18]. Table 1 illustrates that this method can also be extended to other tissues.

The two main advantages of this method are relative speed and low cost. Both RIA and the traditional enzymatic assay require more than a day to complete the reaction and the cost especially for RIA, is very high. Here the cost, of the reagent is negligible. Although we used 50 μl of the homogenate for convenience, the test can be scaled down at least ten-fold. Since this method uses a wavelength

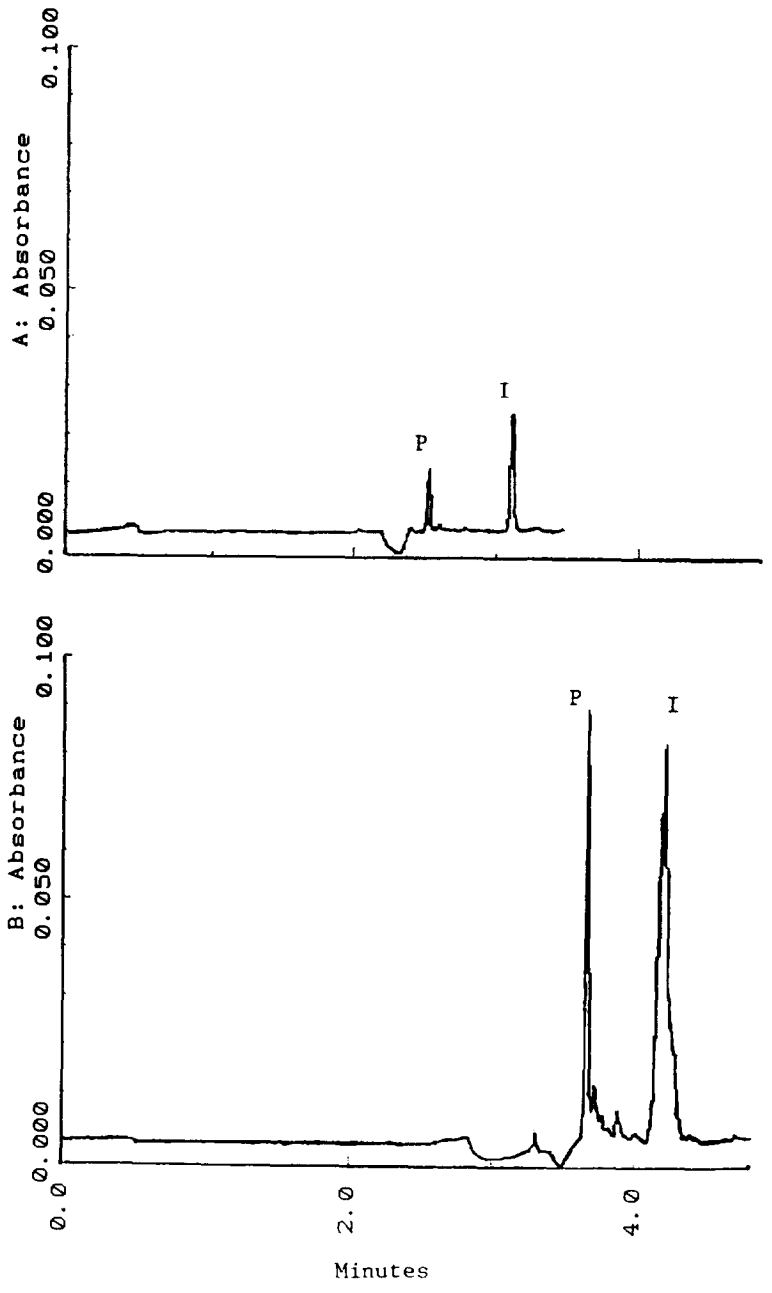

Fig. 6. Effect of the sample volume on the sensitivity of cathepsin D. (Top) sample injected 10 s (2.5% of the capillary at 360 V/cm and (bottom) the same sample injected for 50 s at 240 V/cm (12.5% of the capillary).

at 214 nm, a great sensitivity is obtained compared to 280 nm which is used traditionally [8]. Because a specific peptide is monitored, other proteolytic enzymes which might be present in the tissues, such as pepsin or trypsin, do not interfere in this assay.

Proteolytic enzymes are quite common in the body

Table 1
Cathepsin D activity from different tissues of the nude mouse

Tissue	Activity (pmol/mg protein)
Lung	28
Heart	29
Intestine	70
Uterus	83
Liver	74
Spleen	183
Tumor 1	58
Tumor 2	161

e.g., renin, angiotensin converting enzyme, trypsin, collagenase, pepsin and many of the clotting factors belong to this group. This work illustrates how CZE can offer a simple method for analysis of this group of enzymes using natural substrates.

References

[1] F. Capony, M. Morrisset, A.J. Barrett, J.P. Capony, P. Broquet, F. Vignon, M. Chambon, P. Louisot and H. Rochefort, J. Cell. Biol., 104 (1987) 253.
[2] G. Leto, N. Gebbia, L. Rausa and F.M. Tumminello, Anticancer Res., 12 (1992) 235.
[3] T. Nazeer, J.H. Malfetano, T.G. Rosano and J.S. Ross, Am. J. Clin. Pathol., 97 (1992) 764.
[4] G. Leto, N. Gebbia, L. Rausa and F.M. Tumminello, Cancer Cells, 2 (1990) 383.
[5] H. Rochefort, Sem. Cancer Biol., 1 (1990) 153.
[6] H. Rochefort, Eur. J. Cancer, 28A (1992) 1780.
[7] M.J. Duffy, D. Reilly, J.P. Brouillet, E.W. McDermott, C. Faul, N. O'Higgins, J.J. Fennelly, T. Maudelonde and H. Rochefort, Clin. Chem., 38 (1992) 2114.
[8] T.E. Kute, Z.M. Shao, N.K. Sugg, R.T. Long, G.B. Russell and L.D. Case, Cancer Res., 52 (1992) 5198.
[9] Z.K. Shihabi, Ann. Clin. Lab. Sci., 22 (1992) 398.
[10] A. Vinther, K. Adelhorst and O. Kirk, Electrophoresis, 14 (1993) 486.
[11] F. Mulholland, S. Movahedi, G.R. Hague and T. Kasumi, J. Chromatogr., 636 (1993) 63.
[12] M.M. Bradford, Anal. Biochem., 72 (1976) 248.
[13] S. Romain, X. Muracciole, I. Varette, C. Bressac, H. Brandone and P.M. Martin, Bull. Cancer, 77 (1990) 439.
[14] Z.K. Shihabi and L.L. Garcia, in J. Landers (Editor), Handbook of Electrophoresis, CRC Press, Boca Raton, FL, 1994, pp. 537–548.
[15] Z.K. Shihabi, J. Chromatogr. A, 680 (1993) 471.
[16] Z.K. Shihabi, J. Cap. Elec., 2 (1995) 267.
[17] Z.K. Shihabi, J. Chromatogr. A, (1996) in press.
[18] D.W. Moss, A.R. Henderson and J.F. Kachmar, in N.W. Tietz (Editor), Textbook of Clinical Chemistry, Sanders, Philadelphia, 1986, pp. 619–763.