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Peptide mapping of bovine and chicken cytochrome *c* by capillary isoelectric focusing with universal concentration gradient imaging

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Abstract

Capillary isoelectric focusing with universal concentration gradient imaging detection was used to separate and detect tryptic peptides from bovine and chicken cytochrome *c*. For a desalted sample of peptide angiotensin 2, the isoelectric point (p*I*) measured by the instrument agreed well with the p*I* calculated from amino acid p*K* values. For the cytochrome digests, correlations between measured and calculated p*I* values were imprecise because peak positions shifted slightly from test to test. This problem is thought to be caused by the inefficient desalting process used on the samples, leaving salt residues which caused distortion in the pH gradient during the focusing process. However, this system differentiated between the two cytochrome *c*'s. The concentration gradient imaging detected peptides which contain no tyrosine and no tryptophan amino acids, which a UV absorption detector operating at 280 nm could not. The separation and detection steps took only 5–7 min because no mobilization was necessary after the focusing process.

1. Introduction

Peptide mapping involves the fragmentation of a protein through selective cleavages of peptide bonds by proteolytic enzymes or by chemical treatments, followed by separation and detection of these smaller peptide fragments [1,2]. The methods of peptide mapping are important for the structural identification and characterization of proteins.

Presently, the most common method for separating and identifying peptide fragments is reversed-phase high-performance liquid chromatography (RP-HPLC) because of its speed, sensitivity, and the easy recovery of the peptides for further analysis [3–12]. This method uses the hydrophobic nature of the peptides to separate them. The complex variety of peptides, however, often requires more than one separation technique to isolate them.

Capillary zone electrophoresis (CZE) is an important complementary method to chromatography in peptide mapping [13–19]. Because it depends on the charge to size ratio of the peptides, CZE can resolve peptides that coelute in RP-HPLC. Furthermore, the technique of capillary electrophoresis can provide analytical information from very small amounts of sample with high speed [20].

Capillary isoelectric focusing (cIEF), developed in 1985 [21], has been successfully applied to the analysis of protein samples [22–25].

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Recently, cIEF was used by Mazzeo *et al.* [26] to map peptides. This method is also orthogonal to RP-HPLC because it depends on the isoelectric point (p*I*) property of the peptide. However, conventional cIEF has drawbacks in peptide mapping. First, conventional cIEF detectors require the fluid to be drawn out of the capillary which distorts the linear pH gradient and thus deteriorates the precision of p*I* measurements [27]. Second, the carrier ampholytes used to create a pH gradient interfere with UV detection [22], so that the detector must be operated at wavelength 280 nm which only detects peptides containing tryptophan and tyrosine amino acid residues.

To address these limitations, a detector that measures concentration gradients was applied to peptide mapping with cIEF. This universal detection system monitors the full length of the capillary, is real-time, and is independent of light wavelength [28]. Therefore, it eliminates the need for mobilization and no background signal is generated by the wide zones of carrier ampholytes [29]. This paper describes a preliminary experiment of peptide mapping by cIEF with concentration gradient detection.

2. Experimental

2.1. Reagents

All chemicals were reagent grade, and solutions were prepared using distilled water. 10 m*M* H_3PO_4 and 20 m*M* NaOH were used as anolyte and catholyte, respectively [25]. Ammonium hydrogencarbonate was purchased from BDH (Poole, UK) for protein buffer. Bovine cytochrome *c*, chicken cytochrome *c*, angiotensin 2, and carrier ampholytes (Pharmalyte pH 3–10) were purchased from Sigma (St. Louis, MO, USA). N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin was purchased from Worthington Biochemicals (Freehold, NJ, USA). Before use the lyophilized protein samples were dissolved into 0.5 ml of water to a concentration of 2.0 mg/ml and were desalted using dialysis membranes (MWCO: 500) obtained from Spectrum Medical Industries (Houston, TX, USA). Samples were then mixed with the carrier ampholytes to a final concentration of 2% ampholytes [27].

2.2. Tryptic digestion

Bovine and chicken cytochrome *c* were dissolved in 100 m*M* ammonium hydrogencarbonate buffer, pH 8.0, to obtain two 0.5-ml solutions at concentrations of 2.0 mg/ml. Trypsin was dissolved in the same buffer at a concentration of 0.2 mg/ml. A 0.5-ml volume of trypsin solution was added to 0.5 ml of the cytochrome *c* suspensions and incubated for 24 h at 37°C. After digestion, trypsin was deactivated by heating at 100°C for 5 min. The samples were then lyophilized. This digestion followed the procedure reported in refs. 26 and 30. During the experiment, the samples were kept on ice to minimize peptide denaturation.

2.3. Instrumental procedures

This procedure is the same as reported in ref. 31. A 4 cm × 100 μm I.D. square glass capillary (Vitro Dynamics, Rockaway, NJ, USA), coated with non-cross-linked acrylamide to eliminate electroosmosis [24], was used for separation. A light beam from a He–Ne laser (Uniphase, San Jose, CA, USA) was expanded and focused by a cylindrical lens through the capillary, focused again through an optical stop, and then intercepted by a 1024-pixel charge-coupled device (CCD) sensor (Type S3903-1024Q, Hamamatsu, Hamamatsu City, Japan). The distance between the capillary and the CCD was optimized previously to 5 mm [31]. This configuration monitored 25 mm of the capillary [31]. The position of capillary cartridge could be moved so that different parts of the capillary could be monitored. The whole system was mounted on a vibration isolation table.

The signals from the CCD were collected by an IBM DACA board, in a PC-AT personal computer, using ASYST software (Asyst Software Technology, Rochester, NY, USA). For each electropherogram recorded, the signal from

each pixel in the CCD was measured ten times in 1 s, and the ten measurements were averaged to reduce random noise. The background signals, recorded before the separation voltage was turned on, were also subtracted. Thus, the measured data gives a profile of light intensity changes created by the refractive index gradient of concentration changes inside the capillary due to focused peptide zones.

2.4. Isoelectric focusing process

The samples were pipetted into the cathode reservoir and introduced into the capillary by pressure with a syringe. A plug of a 1% agarose gel (prepared in the anolyte, 10 m*M* H_3PO_4) was placed in the reservoir of the anodic end of the capillary to avoid hydrodynamic flow inside the capillary. A 3.5-kV voltage was applied to the two ends of the capillary. The current which passed through the capillary dropped from 30 to about 3 μA in 2 min before stabilizing. All experiments were done in triplicate to ensure reproducibility.

2.5. Safety considerations

A Plexiglass box should be used to isolate the anodic end of the capillary because of the high d.c. voltage applied to this end.

3. Results and discussion

Estimates of p*I* values of the tryptic peptides from both bovine and chicken cytochromes were calculated using equation [32]:

$$\text{net charge} = 0 = \sum \frac{n_i}{\frac{K_i}{[H^+]} + 1} - \sum \frac{n_j}{\frac{[H^+]}{K_j} + 1} \quad (1)$$

where the peptide has n_i of weakly basic groups and n_j weakly acidic groups. The dissociation constants of groups, K, are assumed equal to that of the free amino acids. Common basic groups include the α-amino group, the imidazole group of histidine, the ϵ-amino group of lysine and the guanidinium group of arginine. Common acidic groups include the α-carboxyl group, the β-carboxyl of aspartic acid, the γ-carboxyl of glutamic acid, the phenolic group of tyrosine and the thiol of cysteine.

Peptide peak p*I* values obtained in the experiments were estimated by the peak position, taking into account the adjustable position of the capillary. Inside the capillary, a linear pH gradient was created. The cathodic endpoint was assumed to be pH 3.0 and the anodic endpoint was assumed to be pH 10. The p*I* value measured by this method is accurate to within 0.06 pH units [31].

To validate cIEF with concentration gradient imaging detection for peptide mapping, a desalted sample of angiotensin 2 was run (Fig. 1). Angiotensin 2 was estimated by Eq. 1 to have a p*I* value of 7.5 and the observed peak was estimated from its position in the capillary to have a p*I* value of 7.8. The estimates are relatively close (0.2 pH unit difference).

The proteolytic trypsin cleaves C-terminal side of lysine and arginine residues on a protein [33]. For bovine and chicken cytochrome *c*, some of their peptide fragments from the tryptic digest are listed in Table 1. Not all fragments are listed because (1) the concentration gradient detection is based on refractive index gradient, so the small peptides can only be detected with high sensitivity due to their small refractive indices,

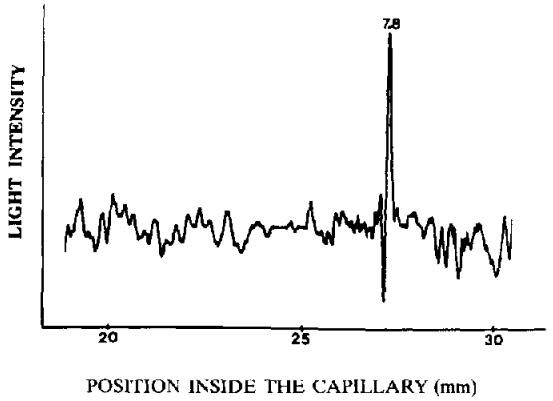

Fig. 1. Electropherogram of angiotensin 2. Peak is labelled with p*I* value estimated from its position inside the capillary.

Table 1
Peptide fragments from tryptic digests expected to be detected

Expected peptide fragment	pI value calculated from Eq. 1
Bovine cytochrome c	
(1) CAQCHTVEK	7.0
(2) EDLIAYLKK	6.8
(3) KATNE	6.6
(4) TGQAPGFSYTDANK	6.5
(5) TGQAPGFSY	5.6
(6) GITWGEETLMEYLENPKK	4.1
(7) EDLIAYLK	4.0
Chicken cytochrome c	
(1) CSQCHTTVEK	7.0
(2) DATSK	6.9
(3) KTGQAEGFSYTDANK	6.7
(4) VDLIAYLK	6.7
(5) GITWGDETLMEYLENPKK	4.3
(6) TGQAEGFSYTDANK	4.0

and (2) the two ends of the capillary are covered with glue so that this method is limited to p*I* values from 4.0 to 9.0.

Figs. 2 and 3 show the electropherograms for bovine cytochrome *c* peptides and for chicken cytochrome *c* peptides, respectively. The p*I* associated with each peak is estimated from its position inside the capillary. The position of the capillary was adjusted to cover all peaks. Both patterns were reproducible, but their position

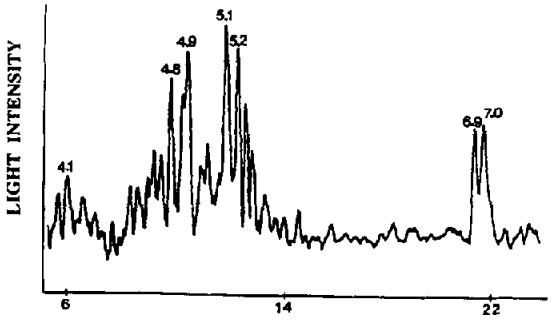

Fig. 2. Electropherogram of bovine cytochrome *c* tryptic digest. Peaks are labelled with p*I* values estimated from their positions inside the capillary.

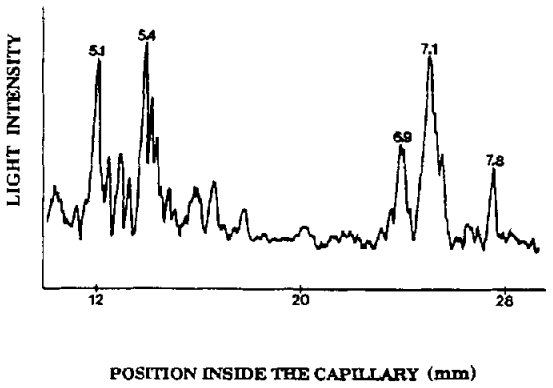

Fig. 3. Electropherogram of chicken cytochrome *c* tryptic digest. Peaks are labelled with p*I* values estimated from their positions inside the capillary.

shifted slightly from test to test. The peaks assigned with p*I* values on the figures are those which appear in all tests. The p*I* values are averaged ones of three tests. The measured p*I* values may not agree well to the calculated ones because of this shifting, and also the assumptions of Eq. 1 [26].

A blank enzyme digestion sample was also run. No peaks were observed in the pH region from 4.0 to 7.2. This result means that all peaks in Figs. 2 and 3 are due to the peptides digested from cytochrome *c*.

For bovine cytochrome *c*, two peaks were expected at around pH 4.0, one at around pH 5.5 and four between pH 6.5 and 7.0. However, at least six peaks were observed between pH 4.0 and 5.5 and only two were observed in pH region 6.0 to 7.0, as estimated by positions. In the latter region, some of the peptides may be cofocused because of close p*I* values, and peptide 3 (KATNE) may be too small to be detected by the instrument.

For chicken cytochrome *c*, two peaks were expected around pH 4.0 and three or four were expected between pH 6.0 and 7.0. However, two large peaks among some small peaks were observed in the pH region 5.0–5.5 and three were observed in pH region 6.5–8.0. Peptide 2 (DATSK) might not be detected because of its small size, and the broadness of peak at pH 7.1 could indicate that some peptides cofocused. The

peak at 7.8 was not expected but it appeared in all peptide maps of chicken cytochrome *c*.

The discrepancies observed are thought to be caused by inefficient removal of salts from the peptide samples prior to the focusing process. This cause was deduced from the correspondence between desalting procedure and peak shifts. The cIEF with concentration gradient imaging detection system showed, in our previous research, good accuracy for measurement of p*I* values of proteins desalted essentially [31]. Also, in this experiment, the commercially desalted sample of angiotensin 2 did not have the problem. Salts would cause the focused peptides to confine to the central of the capillary [25] which would explain why the peaks appeared in unexpected positions.

Despite the discrepancies, this system differentiated between the two species of cytochrome *c* tested. These results also show that cIEF with concentration imaging detection can detect peptides that do not have tyrosine or tryptophan residues, such as peptide 1 of both bovine cytochrome *c* and chicken cytochrome *c*. These peptides, having no tyrosine or tryptophan, would not be detected by an absorption detector using 280 nm UV light. Compared to the electroosmotic flow (EOF)-driven cIEF method [26] by which no neutral peptides were detected for both bovine and chicken cytochrome *c*'s, cIEF with concentration imaging detection detected several peptides in pH region 6.0–8.0. Since the position on the capillary corresponding to pH 6–8 is in the centre in which the distortion of pH gradient due to salt is minimum [25], this results should be more reliable than EOF-driven cIEF method in which both cathodic mobilization and anodic mobilization had to be applied for detecting neutral peptides [26]. The observation of these neutral peptides may be attributed to the on-line detection without the mobilization process of the latter.

Some technical improvements can be made to this method. Desalting was attempted for different lengths of time, and peak shifting during the focusing process only became reasonably stabilized when the samples were dialyzed against distilled water for 12 h. The ammonium hydrogencarbonate salt was still not sufficiently removed however, which interfered with the pH gradient and distorted the banding pattern. To overcome this problem, a microscale cartridge containing reversed-phase membrane (available in Bio-Rad Labs., Richmond, CA, USA) can be used for high-accuracy desalting of the samples. This desalting method would also reduce the dialysis time and minimize the denaturation of the peptides from long exposure to room temperature [34]. Also, the whole range of peptides can be monitored at one time with the use of a larger CCD or the use of ampholytes with larger pH range.

The preliminary results indicate that cIEF with concentration gradient imaging can be applied to peptide mapping. It was possible to differentiate between the two cytochrome *c*'s. Once optimized, cIEF with concentration gradient detection could complement other techniques in peptide mapping. Its advantages are detection of peptides without tyrosine and tryptophan, no mobilization and faster run time (5–7 min) than conventional cIEF (15–30 min).

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