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Multiplexed on-column protein digestion and capillary electrophoresis for high-throughput comprehensive peptide mapping

Yan He, Wenwan Zhong, Edward S. Yeung*

Ames Laboratory, US Department of Energy and Department of Chemistry, Iowa State University, 332 Wilhelm Hall, Ames, IA 50011, USA

Abstract

A novel scheme based on multiplexed capillary electrophoresis (CE) has been developed for high-throughput, low-cost and comprehensive peptide mapping. Orthogonal peptide maps of the protein of interest were obtained by using multiple reaction conditions with three different enzymes (trypsin, pepsin, and chymotrypsin), and multiple separation conditions with six zone electrophoresis buffers and two micellar electrokinetic chromatography (MEKC) buffers. Fifteen nanoliters of two protein samples (β-lactoglobulin A and β-lactoglobulin B) were separately mixed on-column and digested independently at 37 °C for 10 min to produce peptides in a 20-capillary system. The resulting peptides were detected simultaneously at 214 nm by a photodiode array detector. The overall analysis time from reaction to detection was about 40 min.

Keywords: Protein digestion; Peptide mapping

1. Introduction

With the recent completion of the rough draft of the human genome, understanding the structure and function of the numerous proteins has challenged analytical chemists to develop new tools for high-throughput and low-cost proteome analysis. Although protein profiles based on molecular masses can be obtained from the matrix-assisted laser desorption ionization (MALDI) MS of biological tissues [1,2], further characterization is needed. The primary structure of protein is most commonly determined by Edman degradation [3] and subsequent direct reading of the ordered amino acids. With the advent of MS, direct sequencing based on reassembly of the fragment ions has been highly successful [4]. De novo sequencing of proteins is however not always necessary. With the availability of the genomic sequence, the coding regions of a given organism can be readily translated to the expected protein sequences. Identification of proteins by comparison with the known (expected) proteins is a much simpler problem. Since the action of proteolytic enzymes are well known [5,6], one can even predict the set of peptides that will be generated under ideal conditions. The smaller peptide fragments are readily analyzed by MS and the molecular masses can be used as unique identifiers of the starting proteins. Both MALDI [7–9] and electrospray [10–12] methods have been applied. Much of the success is due to the availability of peptide data bases and automatic searching algorithms [13–17].

While MS analysis of protein digests is clearly a rugged and definitive way to characterize proteins,

*Corresponding author. Tel.: +1-515-294-6342; fax: +1-515-294-0105.

E-mail address: yeung@ameslab.gov (E.S. Yeung).

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the sample throughput is still a limitation. Even though the MS scan itself is fast, samples have to be introduced into the MS sequentially. The commercial eight-channel liquid chromatography (LC) MS simply alternates among the channels to achieve pseudo-parallel operation and is not cost effective. On the other hand, capillary electrophoresis (CE) analysis can be performed in a highly parallel fashion. 96- or even 384-channel units are now commercially available. This provides good incentive for developing CE-based methods for protein characterization.

Peptide mapping is one of the most powerful and successful tools for protein identification and characterization. It has been widely used for the verification of the primary structure of proteins produced by recombinant DNA technology [18] and for the detection of post-translational modifications [19]. Peptide mapping usually consists of enzymatic [20,21] or chemical cleavage [22,23] of the protein into a number of smaller peptide fragments followed by separation and detection. Proteolysis can be performed in two ways: by homogeneous reaction or by solid-phase digestion. In the homogeneous environment, generally 1 part of enzyme digests 20 parts of protein at 37 °C for several hours [24]. Conventional homogeneous reaction is convenient and is performed in plastic tubes requiring tens of microliters sample volume and micromoles of highly purified protein. In the case of heterogeneous reaction, the protein passes through a bed of enzyme that has been immobilized on the solid support. Solid-phase microreactors have the advantages of rapid reaction rate, long enzyme lifetime, less enzyme auto-proteolysis and easy automation. Some problems encountered are poor reproducibility, incomplete digestion or overdigestion.

Frequently, the proteins of interest are isolated and purified in small amounts (e.g. sub-micrograms) from biological samples. There is thus a need to establish rapid and reliable techniques to obtain peptide maps from nanomoles and lower quantities of protein. There have been a number of publications which demonstrated proteolytic reactions in capillary tubes for both homogeneous and heterogeneous protein digestion. Heterogeneous proteolytic reactions have been carried out in relatively large capillaries (≈500 μm I.D.) in which enzymes were immobilized [25–27]. The consumption of proteins and enzymes in most solid-phase micro-reactors is still at the microliter scale. Recently, homogeneous protein digestion has also been investigated in 20 and even 1.5 μm I.D. capillaries, with sample volumes ranging from nanoliter to picoliter [28,29].

After protein digestion, separation of the resulting peptides is typically performed with methods such as high-performance liquid chromatography (HPLC) [30–35], slab gel electrophoresis [36], and thin-layer chromatography [37]. Among these techniques, HPLC is the most popular form. HPLC usually requires tens of microliters of sample volume and consumes large amounts of solvent. Moreover, complete separation of all peptide fragments is often difficult to achieve even under gradient elution, due to the complexity of proteolytic products. In recent years, CE has been increasingly employed in peptide analysis due to its high separation efficiency, minimal sample consumption, lack of organic waste and compatibility with biological materials [25–29]. Versatility results from the ease of changing the separation mode and affecting selectivity simply by altering the buffer composition. Despite the impressive separation power, the complete separation of all peptides in a digest by using a single buffer condition is unlikely to be achieved. This difficulty has been addressed by our recent work on the use of six different separation buffers in a 96-capillary array CE system [38]. However, even though submicroliters are injected into each capillary for CE, the protein digestion step was still performed at tens of microliters scale in plastic tubes. So, the potential of extremely low sample consumption by CE has not been exploited.

In this paper, we report the integration of on-column protein digestion and separation of the resulting peptides in a 20-capillary array system. The technique potentially provides a powerful solution to high-throughput, low-cost and comprehensive peptide mapping. The utility of this technique was demonstrated in the analysis of two closely related proteins, β-lactoglobulin A and β-lactoglobulin B at the nanoliter scale. Comprehensive peptide mapping was attained by using not only different separation conditions but also varied reaction conditions. The technique can be easily expanded to a 96-capillary array to provide one-step characterization of up to nine protein samples in one analysis.

2. Experimental

2.1. Reagents and chemicals

β-Lactoglobulin A and B (BLGA and BLGB), TPCK-treated trypsin, chymotrypsin, pepsin, tricine (*N*-tris[hydroxymethyl]methylglycine), ammonium acetate and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Formic acid, phosphoric acid, sodium acetate and Tween 20 were from Aldrich (Milwaukee, WI, USA). Sodium hydroxide, sodium tetraborate, trisma-base (tris[hydroxymethyl] aminomethane) and sodium phosphate-monobasic were from Fisher Scientific (Fairlawn, NJ, USA). Water used to prepare buffer and reaction solutions was purified by a Milli-Q purification system (Millipore, Worcester, MA, USA).

2.2. Single-capillary electrophoresis system

All separations for capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) were first optimized on an ISCO (Lincoln, NE, USA) model 3140 Electropherograph System before multidimensional multiplexed CE runs. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 45 cm effective length and 69 cm total length (75 μm I.D. and 360 μm O.D.) were used. Six different buffer systems for CZE separations and two different buffer systems for MEKC separations were investigated. Their compositions were as follows: Z1, 40 m*M* phosphate (pH 3.3 with HCl); Z2, 0.12 *M* sodium acetate (pH 4.0); Z3, 70 m*M* sodium acetate (pH 5.0); Z4, 0.05 *M* trisma base–0.2 *M* tricine (pH 7.0); Z5, 0.1 *M* trisma base–0.1 *M* tricine (pH 8.1); Z6, 20 m*M* sodium borate (pH 9.3); M1, 1% Tween 20, 40 m*M* phosphate (pH 3.3); M2, 10 m*M* SDS, 0.1 *M* trisma base–0.1 *M* tricine (pH 8.1). The solutions of enzymes, proteins and buffers were injected with hydrodynamic flow. The applied electric field was 180 V/cm, and electrophoresis was performed at room temperature. The detection wavelength was set at 214 nm for monitoring peptide fragments. After each run, the capillary was rinsed with 0.1 *M* NaOH for 10 min, water for 5 min, and running buffer for 10 min.

2.3. The 20-capillary array electrophoresis system

The experimental CE setup for multidimensional 20-capillary array reaction and electrophoresis is similar to the 96-capillary array system described in Ref. [39]. Fused-silica capillaries, 75 μm I.D. and 360 μm I.D. with 45 cm effective length and 69 cm total length, were packed side by side at the detection window and clamped between two flat surfaces of a plastic mount. Capillaries A1, A2, C2 and D3 were filled with buffer Z1, capillaries B1 and D2 with buffer Z2, capillaries C1 and E2 with buffer Z3, capillaries D1 and F2 with buffer Z4, capillaries E1, B2, G2 and E3 with buffer Z5, capillaries F1 and H2 with buffer Z6, capillaries G1 and A3 with buffer M1, and capillaries H1 and B3 with buffer M2. An eight-position valve was used to distribute nitrogen gas from a gas cylinder to eight buffer vials to simultaneously fill the 20 capillaries with eight buffers. At the injection (high voltage) end, the 20-capillary array was spread and mounted along the first three rows on a copper plate with spacings that fit into a 96-well microtiter plate for sample introduction. Gold-coated pins (MillMax) were mounted on the copper plate near each capillary tip to serve as individual electrodes, with the capillary tips slightly extended (~0.5 mm) beyond the electrodes to guarantee contact with small-volume samples. A high-voltage power supply (Glassman High Voltage, Whitehorse Station, NJ, USA) was used to drive the electrophoresis.

A 213.9-nm zinc lamp (model ZN-2138, Cole-Parmer) was used as the light source for UV absorption detection. The light source, interference filter, capillary array holder, and photodiode array (PDA) detector were all contained in a light-tight metal box attached to an optical table. The transmitted light from the capillary array passed through an interference filter (Oriel) and a quartz lens (Nikon; focal length=105 mm; f/4.5). An inverted image of the capillary array at a nominal magnification factor of 1.2 was created by the quartz lens on the face of the PDA. The PDA (Hamamatsu model S5964, Hamamatsu, Japan) incorporated a linear image sensor chip (1024 diodes, 25 μm in width, 2.5 mm in height), a driver/amplifier circuit, and a temperature controller. The built-in driver/amplifier circuit was interfaced to an IBM-compatible com-

puter via a National Instrument PCI E Series multifunction 16-bit data acquisition board. All codes used to operate the PDA and to acquire data were written in-house using Labview 5.0 software (National Instruments, Austin, TX, USA). The raw data sets were converted into single-diode electropherograms by another in-house Labview program. Data treatment and analysis were performed using Microsoft Excel 97 and Grams/32 5.05 (Galactic Industries).

2.4. Integration of multiplexed on-column digestion and separation

The two reaction buffers were prepared as follows: RB1, tris 10 m*M*, sodium acetate, 50 m*M*, pH 8.1, and RB2, 5% (v/v) formic acid, 0.02% (v/v) Tween 20, pH 1.9. Samples were prepared as 2 mg/ml BLGA in RB1 (GA1), 2 mg/ml BLGA in RB2 (GA2), 2 mg/ml BLGB in RB1 (GB1), and 2 mg/ml BLGB in RB2 (GB2). Enzymes were prepared as 0.2 mg/ml trypsin in RB1 (t), 0.2 mg/ml chymotrypsin in RB1 (c-t), and 0.2 mg/ml pepsin in RB2 (p).

Five standard 96-well microtiter plates were used to hold the sets of separation buffers, reaction buffers, protein samples and proteolytic enzymes. Their configurations are displayed in Fig. 1. First, 180 μl of each buffer were pipetted into the vials on two separation buffer (SB1 and SB2) plates. SB2 was built in-house with a brass sheet glued to the base. SB2 was in contact with water heated to 40 °C.

SB1 and SB2 Plate

Z1	Z2	Z3	Z4	Z5	Z6	M1	M2
Z1	Z5	Z1	Z2	Z3	Z4	Z5	Z6
M1	M2		Z1	Z5			

RB Plate

RB1	RB1	RB1	RB1	RB1	RB1	RB1	RB1
RB2	RB1	RB1	RB1	RB1	RB1	RB1	RB1
RB1	RB1		RB2	RB1			

E Plate

t	t	t	t	t	t	t	t
p	c-t	t	t	t	t	t	t
t	t		p	c-t			

P Plate

GA1	GA1	GA1	GA1	GA1	GA1	GA1	GA1
GA2	GA1	GB1	GB1	GB1	GB1	GB1	GB1
GB1	GB1		GB2	GB1			

Fig. 1. Configurations of solutions in three rows of each of five 96-well microtiter plates. Only 20 of the 24 capillaries were utilized.

Each vial contains 180 μl of reaction buffer (RB1 and RB2) on the reaction buffer (RB) plate, or 25 μl of each protein (BLGA and BLGB) on the protein (P) plate, or 25 μl of each enzyme (trypsin, chymotrypsin and pepsin) on the enzyme (E) plate.

All solutions were injected into the capillaries with hydrodynamic flow. The process started with injection of 15 nl enzymes from vials on the E plate, followed by injection of 15 nl protein samples from vials on the P plate and 20 nl of separation buffers from vials on the SB1 plate. Finally, all capillary tips were immersed in the solutions on the SB2 plate. It should be noted that the capillary tips should also be dipped briefly in the reaction buffers on the RB plate prior to injection of enzyme, and between injections of enzyme and protein. This is to guarantee the optimal reaction conditions around the boundaries between the enzyme and protein zones. In addition, the SB2 plate was placed on a thermally insulated surface to reduce heat dissipation. After 10 min of on-column protein digestion, the temperature of solution in the vials on the SB2 plate dropped from 40 to 37 °C. After the reaction, SB2 was replaced by SB1, 14 kV were applied at the two ends of the capillaries, and electrophoresis was performed at ambient temperature. Between runs, the capillary array was rinsed with 0.1 *M* NaOH for 10 min, deionized water for 5 min, and then the separation buffers for 10 min. The pressure from the gas cylinder is set at 10 p.s.i.

3. Results and discussion

3.1. Separation of various off-column protein digests with complementary buffers

In our previous work [38] on comprehensive peptide mapping, model proteins were digested with only one enzyme (trypsin), and the resulting peptides were separated with six CZE buffers and two MEKC buffers. In this work, model proteins were digested with three enzymes (trypsin, chymotrypsin and pepsin), and the digests were separated with eight CZE buffers and two MEKC buffers in order to achieve even more comprehensive peptide maps.

Trypsin, chymotrypsin and pepsin are three principal digestive proteinases [24,40]. Trypsin is remark-

ably similar in chemical composition and in structure to the chymotrypsin. Both enzymes are most active in basic solutions with optimal pH in the range of 7.5–8.5; they appear to have similar mechanisms of action, with residues of histidine and serine found in the active sites of both. However, trypsin and chymotrypsin are different in their cleavage specificity. Each is active only against the peptide bonds in protein molecules that have carboxyl groups related to certain amino acids. For trypsin these amino acids are highly basic and hydrophilic (Arg and Lys). For chymotrypsin, they are aromatic (Phe, Tyr, Trp) or large hydrophobic (Leu and Met) amino acids. Similar to chymotrypsin, pepsin is most efficient in cleaving bonds involving aromatic amino acids (Phe, Trp, and Tyr). The chief difference is that pepsin cleaves preferentially C-terminal to these aromatic amino acids while chymotrypsin severs effectively the N-terminal. Additionally, pepsin works well in acidic solutions with optimal pH in the range of 2–4. Similar to micelles, relatively hydrophobic amino acids (Phe and Leu) are more likely located inside the globular protein while highly hydrophilic amino acids (e.g. Arg and Lys) tend to be spread on the surface. So, digestion of proteins with these three different enzymes is expected to produce a diverse view on the primary structure of protein, and can even reveal subtle differences in protein conformation.

Fig. 2 shows the separation of protein digests from trypsin, chymotrypsin and pepsin, respectively. Comparison of Fig. 2A and B confirms that trypsin and chymotrypsin have similar digestion efficiency, but different specificity reflected by the different features in the peptide maps. The difference between trypsin and pepsin is substantial as shown by the peptide maps in Fig. 2C and D. For accurate comparison, separation of protein digests from those two enzymes are carried out in identical acidic buffers. The huge first peak in Fig. 2C is from the intact protein,

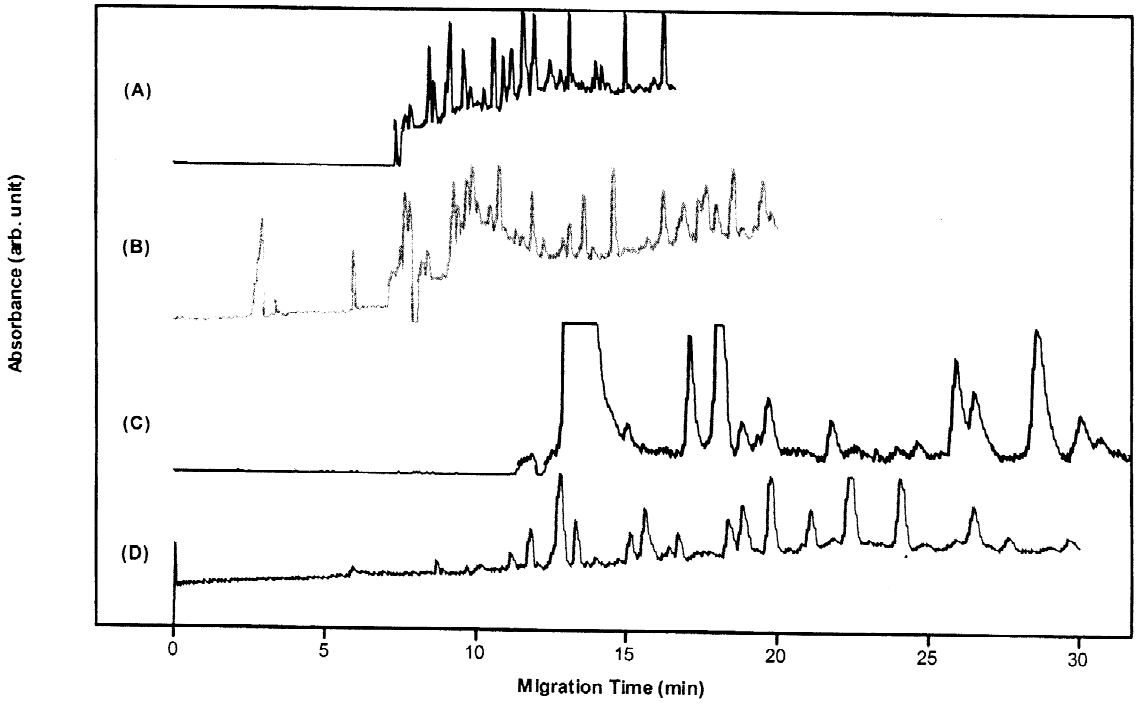

Fig. 2. Electropherograms showing the peptide maps of 1 mg/ml BLGA digested by (A) 0.1 mg/ml trypsin, (B) 0.1 mg/ml chymotrypsin, (C) 0.1 mg/ml pepsin, and (D) 0.1 mg/ml trypsin. Separation buffers: (A) and (B), 0.1 *M* Tris–0.1 *M* Tricine, pH 8.1; (C) and (D), 40 m*M* phosphate, pH 3.3. Applied electric field: +175 V/cm. Column: bare fused-silica capillary (75 μm I.D. and 360 μm O.D.) with effective/total length of 45/69 cm.

BLGA. As can be seen, the digestion efficiency of BLGA by pepsin is much lower than that by trypsin. Moreover, the peak patterns created by the two enzymes are very different.

With regard to the separation of protein digests, CZE buffers with six different pH values ranging from 3.3 to 9.3, and MEKC buffers with either ionic (SDS) or neutral (Tween 20) surfactants were used to provide detailed information on the composition of these complex mixtures. Since peptides are amphoteric, they are ideally suited for characterization by electrophoretic separations. Best selectivity is generally obtained at one or two pH units above or below the pI of the peptide, which varies from pI=3 to pI=10. Slight changes of pH in this range will cause big variations in migration behavior of most peptides. Hence, method development usually entails the use of many pH conditions to produce comprehensive peptide maps that are complementary to each other. On the other hand, the use of extreme pH values ($<$3 and $>$10) for peptide mapping is not desirable, as it can decrease the resolution of many peptides by equalizing their charges and thereby minimizing mobility differences. The separation mechanism of peptides in MEKC buffers is based on their differences in not only the charge-to-mass ratio but also hydrophobicity. Fig. 3 shows the separation of tryptic digests of BLGA by CZE at six pH conditions and MEKC with either SDS or Tween 20 as micelles. As can be seen, the number of peaks, the migration times and the shape of the peaks are all different under the variety of separation conditions.

3.2. On-column mixing of enzyme and proteins

For heterogeneous protein digestion, the enzyme is immobilized onto the capillary wall or the packed bed. Proteins are digested as they pass through the capillary [25–27]. For homogeneous protein digestion, the mixing of enzymes and proteins in the capillary presents some technical challenges. In previous work [28,29], the enzyme and protein are electrokinetically mixed by the use of the difference in electrophoretic mobilities. The method requires accurate determination of the mobilities of enzymes and proteins. In addition, the electroosmotic flow and

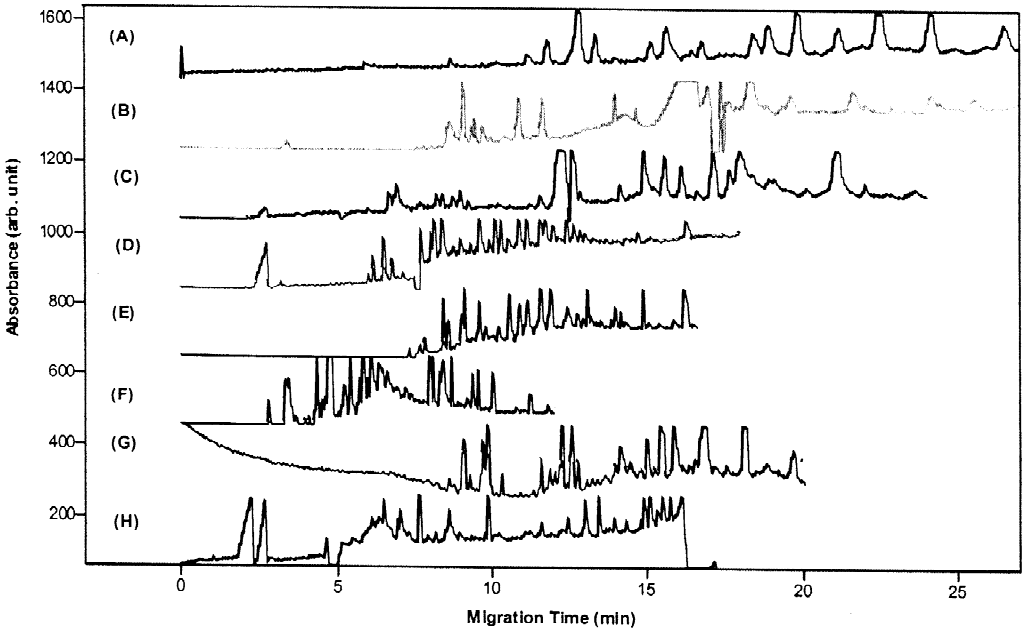

Fig. 3. Electropherograms showing peptide maps of BLGA obtained at six CZE, and two MEKC conditions. Separation buffer: (A) Z1, (B) Z2, (C) Z3, (D) Z4, (E) Z5, (F) Z6, (G) M1, and (H) M2. Composition of the buffers are described in the experimental section. Other conditions are the same as those in Fig. 2.

electrophoretic mobilities are strongly affected by the buffer composition. So, such an approach cannot be used for simultaneous on-column mixing of different enzymes and proteins in a multiplexed capillary system where different capillaries are filled with different buffers.

In our studies, we found a simple and versatile method for mixing proteins and enzymes in capillaries. The method relies on the parabolic flow profile characteristic of hydrodynamically injecting short plugs of enzyme, protein and separation buffer sequentially. Mixing is achieved by both axial and radial dispersion of the solutes in the capillary. The mixing process is similar to what happens in a flow injection analysis system where the solute peaks are skewed with a sharp rising edge and slow falling edge [41]. For the enzymes used in our experiments (especially trypsin and chymotrypsin with p*I* around 9.7), peak tailing should be particularly serious due to their adsorption on the capillary wall (at pH below their p*I*). Here, the plugs of enzyme and protein are both 3 mm long with corresponding volumes of about 15 nl each. It was observed that movement of enzyme and protein by 6–9 mm along the capillary allowed adequate interfacial mixing. It was not determined as to what fraction of the enzymes and proteins was completely mixed at their interface. Nevertheless, the high degree of similarity between Fig. 4a and A in peak patterns and in signal intensities suggests that ≈1 mm plug (≈5 nl) around the boundary was sufficiently well mixed. The similarity between the peptide maps from off-column digestion and from on-column mixing and digestion in the other traces in Fig. 4 also indicates adequate interfacial mixing of chymotrypsin with protein in basic buffer and pepsin with protein in acidic buffer, respectively.

3.3. Limited on-column digestion of proteins

In the on-capillary protein digestion for peptide mapping, a stop-flow incubation period within the

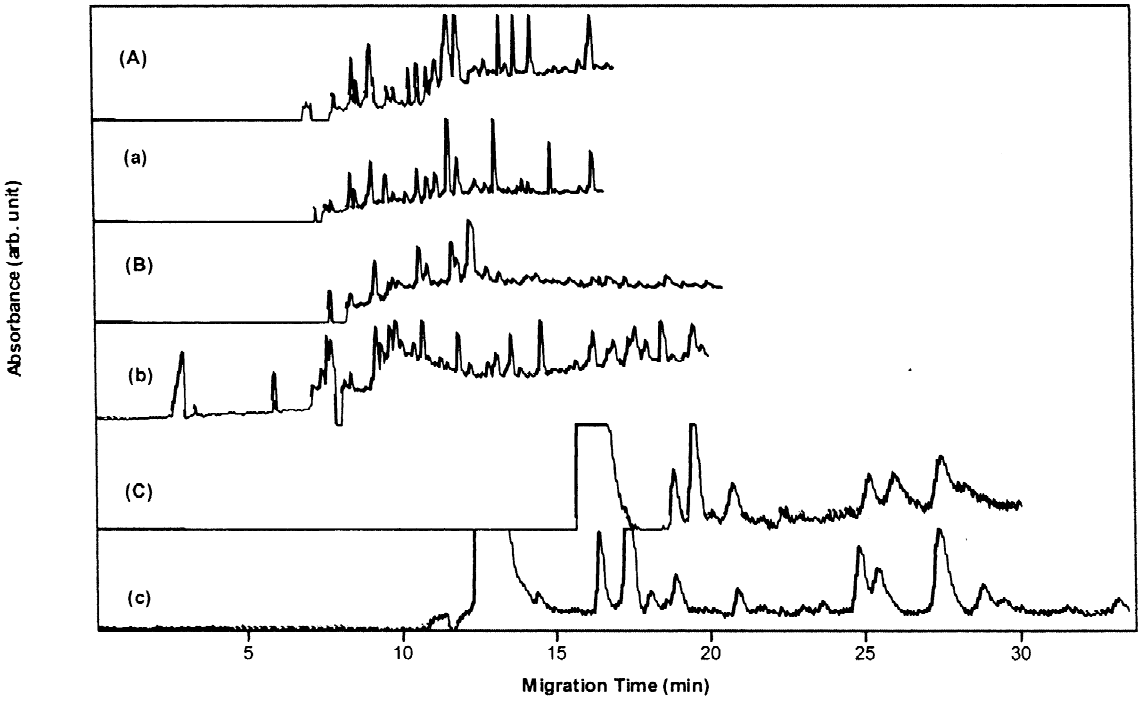

Fig. 4. Comparison of on-column (A, B, C) and off-column (a, b, c) digestion of BLGA with different enzymes. (A) and (a) trypsin; (B) and (b) chymotrypsin; and (C) and (c) pepsin. Injection: (A)–(C) 15 nl enzyme, 15 nl BLGA and 20 nl separation buffer; (a)–(c) 5 nl reaction products from microvials. Reaction: 37 °C for 10 min. Separation buffer: (A), (a), (B) and (b), Z5, pH 8.1; (C) and (c), Z1, pH 3.3.

capillary is required to allow products to accumulate to enhance detection. Complete protein digestion often requires several to over 20 h. However, the resolving power of the CE separation suffers greatly due to diffusional broadening of the product zone after lengthy incubation periods. Diffusion also leads to increasing dilution of the products with incubation time. Another critical consideration is the suitability of the separation buffer for the enzyme-mediated chemistry. The success of on-column peptide mapping is compromised when the pH and composition of the optimal reaction buffer differs greatly from those of the optimal separation buffer. The optimal reaction pH for trypsin and chymotrypsin is in the range of 7.5–8.5, while the optimal reaction pH value for pepsin is from 2 to 4 [42]. In addition, enzymes can be inhibited by many substances, such as high levels of coomassie blue, ampholines, and some surfactants. Considering all these factors, it is difficult to achieve simultaneously complete on-column protein digestion and efficient separation of the resulting peptides in a given buffer condition. Fortunately, these problems can to some extent be resolved by performing limited on-column protein digestion.

Limited protein digestion produces useful information on the structure of proteins by revealing subtle variations of protein conformation, especially in the disordered region [43,44]. Another benefit of limited protein digestion is that auto-proteolysis of the enzyme is greatly reduced, thereby giving relatively clean background and more reproducible peak patterns in peptide maps. Limited protein digestion also saves time. The peptide map of BLGB from limited off-column protein digestion by trypsin is shown in Fig. 5. The reaction was cut off at 10 min. Nevertheless, at least 25 peptide fragments were produced while the peak area of the intact protein was reduced to 25%.

Since the time required for on-column limited protein digestion is short, dilution of reactants and peak broadening by diffusion become negligible. In addition, the influences of pH and composition of the separation buffer on the reaction are also greatly

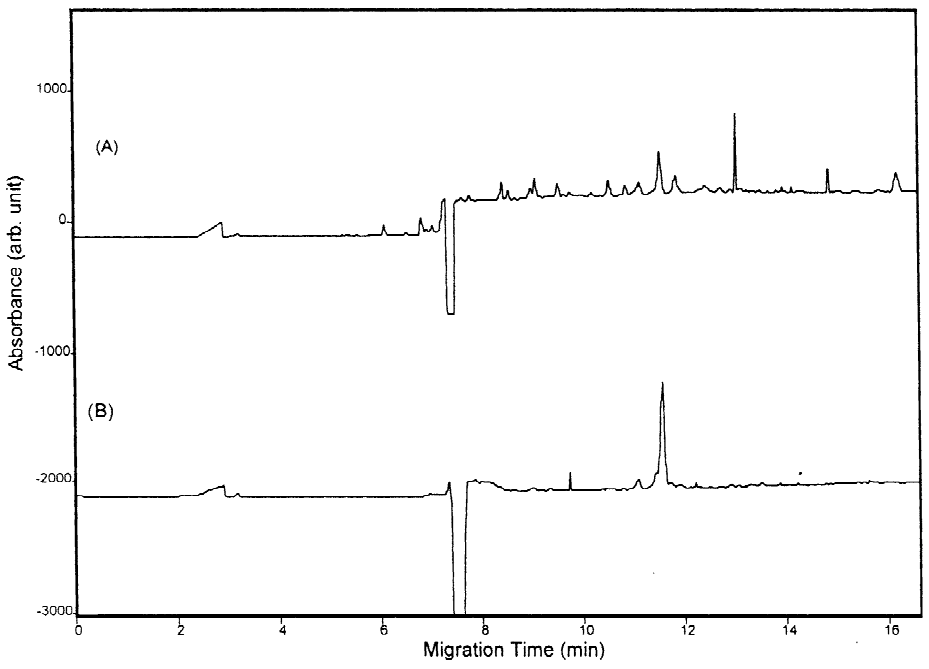

Fig. 5. Comparison of limited digestion of BLGA with control. (A) 1 mg/ml BLGA digested by 0.1 mg/ml trypsin at 37 °C for 10 min, and (B) 1 mg/ml of intact BLGA. Separation buffer: 0.1 *M* Tris–0.1 *M* tricine, pH 8.1. Other conditions are the same as those in Fig. 2.

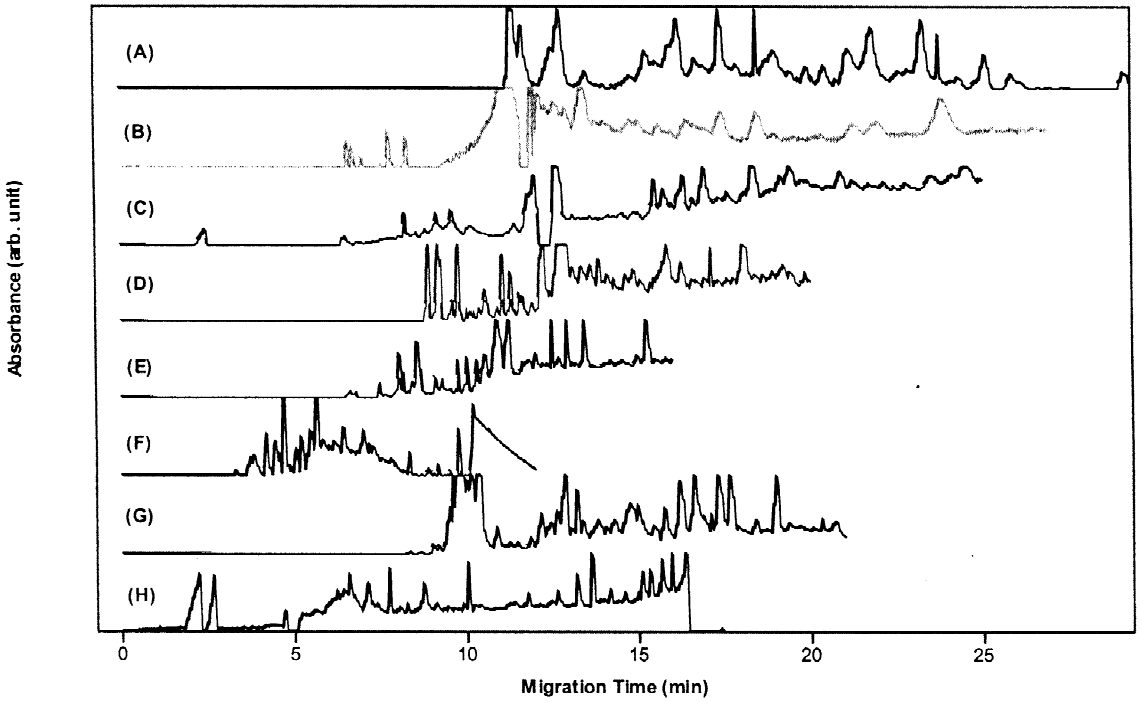

Fig. 6. Electropherograms comparing the peptide maps from limited off-column digestion of BLGA by trypsin under different separation conditions. Other conditions are the same as those in Fig. 3.

reduced, since hydrogen ions and other components of the separation buffer cannot effectively enter the central reaction region by diffusion. We can compare the CE separation of on-column (Fig. 6) vs. off-column (Fig. 3) tryptic digests of BLGA with six different CZE buffers and two MEKC buffers. The peptide map in Fig. 6E was obtained by performing on-column limited protein digestion with the separation capillary filled with buffer Z5, whose pH (8.1) is within the range of optimal pH (7.5–8.5) for trypsin. Under this condition, the peptide maps are very similar for on-column (Fig. 6E) and off-column (Fig. 3E) reactions in the number of peaks and their relative migration times. Pairs of similar peptide maps were also obtained between off-column and on-column digestions when the capillary was filled with buffers which are, to varying extents, not compatible with enzymatic digestion in terms of pH and/or buffer composition, although the similarity is somewhat less as compared to that between Figs. 6E and 3E.

It is interesting to find that on-column protein digestion was sometimes affected more by the composition than the pH of the separation buffer (Fig. 7). As can be seen, peptide maps from on-column reaction were very different from those from off-column reaction when Ches buffer was used (Fig. 7a and A), but were similar to those from off-column reaction when borate buffer was deployed (Fig. 7b and B). As enzymes can be inhibited by many different substances, it is difficult to predict which separation buffer compositions are compatible with on-column enzymatic digestion. In practice, each combination must be studied to identify the best separation buffer.

3.4. Multiplexed on-column reaction and separation in a 20-capillary array

Multiplexed on-column reaction and separation in a capillary array allows high throughput generation and characterization of protein digests from multiple

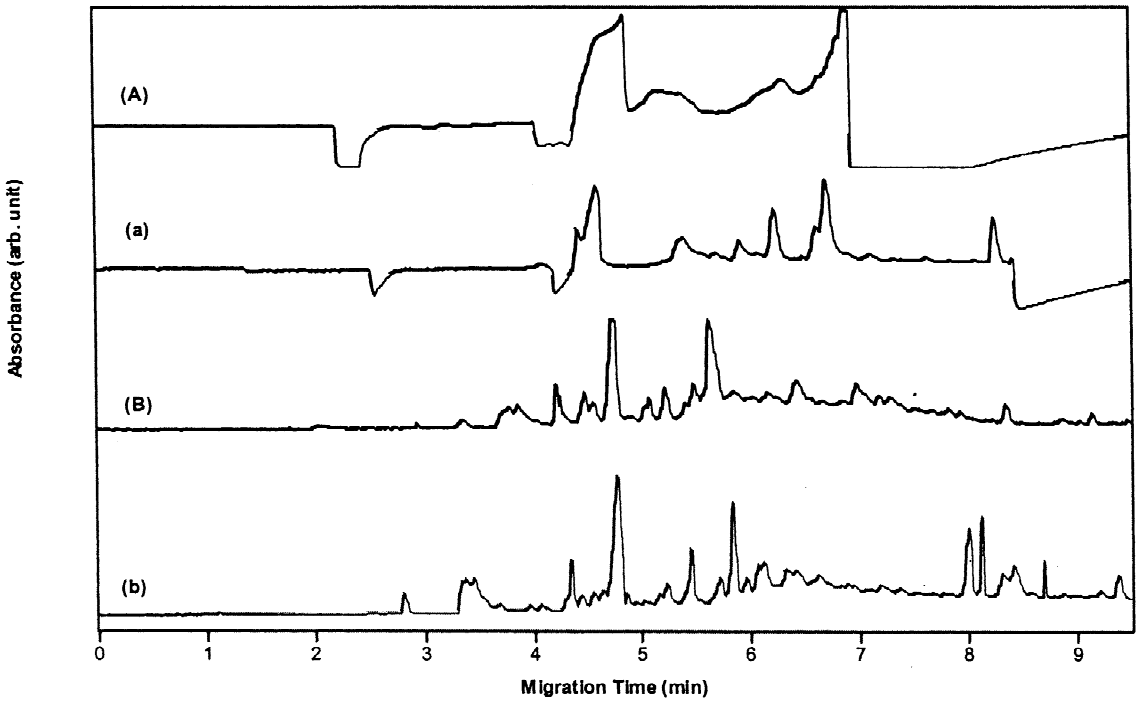

Fig. 7. Influence of separation buffer on on-column protein digestion. Separation buffers: (A) and (a) 0.1 *M* Ches–0.1 *M* NaOH, pH 9.3; (B) and (b) 20 m*M* borate, pH 9.3. Protein: BLGA. Enzyme: trypsin. Other conditions are the same as those in Fig. 2.

nanoliter samples. Comprehensive peptide mapping of two proteins, BLGA and BLGB was simultaneously tested in a 20-capillary array. BLGA and BLGB are two variants of protein BLG which has 162 residues [38]. BLGA and BLGB differ at two sites: aspartic acid (D) 64 in BLGA is changed to glycine (G) in BLGB and valine (V) 118 in BLGA is changed to alanine (A) in BLGB. With the use of 20-capillary system, 10 different peptide maps were simultaneously generated for each protein variant by using eight separation conditions (six CZE and two MEKC buffers) and three enzymes (trypsin, chymotrypsin and pepsin). In each case, the peptide maps of the two proteins generated under the same reaction and separation conditions are distinct. More importantly, while the small difference between BLGA and BLGB can barely be recognized under certain conditions, they can be easily recognized under other conditions (data not shown). Therefore, comprehensive peptide mapping under the present complementary conditions provides not only more information on a given protein, but also allows reliable discrimination among very similar protein variants.

4. Conclusions

Comprehensive peptide mapping provides a powerful approach for detailed characterization of the target protein and reliable differentiation among similar protein variants. The utility of a multiplexed capillary system for high-throughput, low-cost and comprehensive peptide mapping has been demonstrated in the analysis of two protein variants, BLGA and BLGB. By integrating protein digestion and separation in capillaries, the consumption of rare proteins and expensive enzymes was greatly reduced. With the use of different reaction and separation conditions, a multiplexed capillary system provides a quick, economical and almost universal solution for characterizing proteins. This method should also be useful for the verification of the structure of proteins

produced by recombinant DNA technology and in the detection of post-translational modifications.

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References

[1] P. Chaurand, M. Stoeckli, R.M. Caprioli, Anal. Chem. 71 (1999) 5263.
[2] V. Ryzhov, C. Fenselau, Anal. Chem. 73 (2001) 746.
[3] P. Edman, Mol. Biol. Biochem. Biophys. 8 (1970) 211.
[4] K. Biemann, Anal. Chem. 58 (1986) 1288A.
[5] K.A. Cobb, M.V. Novotny, Anal. Chem. 64 (1992) 879.
[6] D.H. Patterson, G.E. Tarr, F.E. Regnier, S.A. Martin, Anal. Chem. 67 (1995) 3971.
[7] T.M. Billeci, J.T. Stults, Anal. Chem. 65 (1993) 1709.
[8] A. Tsarbopoulos, M. Karas, K. Strupat, B. Pramanik, N.T. Nagabhushan, L.F. Hillenkamp, Anal. Chem. 66 (1994) 2062.
[9] T.J. Griffin, S.P. Gygi, B. Rist, R. Aebersold, A. Loboda, A. Jilkine, W. Ens, K.G. Standing, Anal. Chem. 73 (2001) 978.
[10] M. Wilm, G. Neubauer, M. Mann, Anal. Chem. 68 (1996) 527.
[11] V. Ling, A.W. Guzzetta, E. Canova-Davis, J.T. Stults, W.S. Hancock, T.R. Covey, B.I. Shushan, Anal. Chem. 63 (1991) 2909.
[12] D. Figeys, I. van Oostveen, A. Ducret, R. Aebersold, Anal. Chem. 68 (1996) 1822.
[13] J.R. Yates III, J.K. Eng, A.L. McCormack, Anal. Chem. 67 (1995) 3202.
[14] A.L. McCormack, D.M. Schieltz, B. Goode, S. Yang, G. Barnes, D. Drubin, J.R. Yates III, Anal. Chem. 69 (1997) 767.
[15] C.L. Gatlin, J.K. Eng, S.T. Cross, J.C. Detter, J.R. Yates III, Anal. Chem. 72 (2000) 757.
[16] S.E. Martin, J. Shabanowitz, D.F. Hunt, J.A. Marto, Anal. Chem. 72 (2000) 4266.
[17] D.R. Goodlett, J.E. Bruce, G.A. Anderson, B. Rist, L. Pasa-Tolic, O. Fiehn, R.D. Smith, R. Aebersold, Anal. Chem. 72 (2000) 1112.
[18] T. Nadler, C. Blackburn, J. Mark, N. Gordon, F.E. Regnier, G. Vella, J. Chromatogr. A 743 (1996) 91.
[19] E.S. Sorensen, L.K. Rasmussen, P. Hojrup, T.E. Petersen, M.Z. Atassi, E. Appella (Eds.), Methods in Protein Structure Analysis, Plenum Press, New York, 1995, p. 217.
[20] G.E. Tar, J.W. Crab, Anal. Biochem. 131 (1983) 99.
[21] M.J. Geisow, G.H. Beaven, Biochem. J. 161 (1977) 619.
[22] E. Gross, Methods Enzymol. 11 (1967) 238.
[23] Y. Degani, A. Patchornik, Biochemistry 13 (1974) 1.
[24] P. Matsudaira, A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press, San Diego, 1993, p. 55.
[25] L. Licklider, W.G. Kuhr, Anal. Chem. 66 (1994) 4400.
[26] K.A. Cobb, M. Novotny, Anal. Chem. 61 (1989) 2226.
[27] E. Bonneil, M. Mercier, K.C. Waldron, Anal. Chim. Acta 404 (2000) 29.
[28] H.-T. Chang, E.S. Yeung, Anal. Chem. 65 (1993) 2947.
[29] J.B. Shear, E. Okerberg, Anal. Chem. 73 (2001) 1610.
[30] W.S. Hancock, C.A. Bishop, R.L. Prestidge, M.T.W. Hearn, Anal. Biochem. 89 (1978) 203.
[31] M.W. Dong, A.D. Tran, J. Chromatogr. 499 (1990) 125.
[32] M. Cor, R. Shapira, K.D. Wilkinson, Anal. Biochem. 154 (1986) 345.
[33] C.S. Fullmer, R.H. Wasserman, J. Biol. Chem. 254 (1979) 7208.
[34] L. Leadbeater, F.B. Ward, J. Chromatogr. 397 (1987) 435.
[35] W.H. Vensel, V.S. Fujita, G.E. Tarr, E. Margoliash, H. Kayser, J. Chromatogr. 266 (1983) 491.
[36] D.W. Cleveland, S.G. Fisher, M.W. Kirschner, U.K. Laemmli, J. Biol. Chem. 252 (1977) 1102.
[37] R.E. Stephens, Anal. Biochem. 84 (1978) 116.
[38] S.H. Kang, X. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3014.
[39] X. Gong, E.S. Yeung, Anal. Chem. 71 (1999) 4989.
[40] W. Konigsberg, J. Goldstein, R.J. Hill, J. Biol. Chem. 238 (1963) 2028.
[41] B. Karlber, G.E. Pacey, Flow Injection Analysis, Elsevier, New York, 1989, p. 25.
[42] P. Matsudaira, A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press, San Diego, 1993, p. 57.
[43] M. Herold, D.N. Heiger, R. Grimm, Am. Lab. 1 (1993) 20J.
[44] R.J. Beynon, J.S. Bond (Eds.), Proteolytic Enzyme: A Practical Approach, IRL Press, Oxford, 1989, p. 163.