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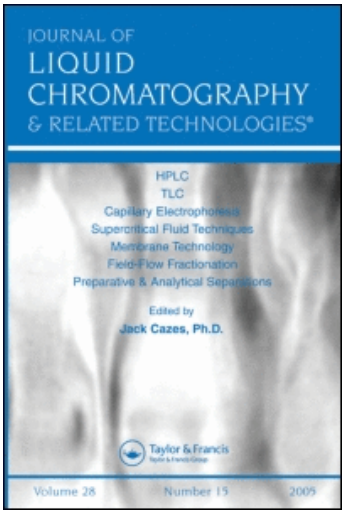

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An HPSEC Method for Determining the Cleavage Position of a Protein in Enzymatic Hydrolysis

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An HPSEC Method for Determining the Cleavage Position of a Protein in Enzymatic Hydrolysis

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ABSTRACT

A method for determining the cleavage position of a protein in enzymatic hydrolysis using high performance size-exclusion chromatography (HPSEC) was established, in terms of an example of bovine serum albumin (BSA) in tryptic hydrolysis. The molecular relative mass distribution of the hydrolysates is characterized by the chromatogram. Comparing the molecular relative mass of theoretical peptides based on the primary structure of BSA with that from experiments, the amino acid sequences of theoretical peptides matching to the peaks in chromatogram are obtained. Thus, the possible cleavage position of BSA in tryptic hydrolysis can be deduced by analyzing the appearing frequency of some amino acid serial.

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Key Words: Bovine serum albumin; Trypsin; Cleavage position; High performance size-exclusion chromatography.

INTRODUCTION

Enzymatic hydrolysis of proteins is a common procedure to improve the functional and nutritional properties.[1–3] Compared with the original protein, physical and chemical performance of the peptides is evidently changed because the molecular relative mass and the structure of peptides are quite different from the protein. The bioactive mechanism and structure of some peptides have been identified. For example, insulin-stimulating peptide, which can enhance the action of insulin in vitro on fatty acid synthesis, has been obtained from a tryptic digest of bovine serum albumin (BSA).[4,5] The specific information of the hydrolysates of protein can be characterized by electrophoresis or HPLC.[6–9] It is a very effective way to determine the functional peptides by analyzing the cleavage positions of protein hydrolyzed.

This work makes an attempt to establish a method of analyzing the cleavage position of protein in enzymatic hydrolysis utilizing high performance size-exclusive chromatography (HPSEC). Taking BSA as example, it is possible to deduce its cleavage position in tryptic hydrolysis since the complete amino acid sequence of BSA has been identified and trypsin is a kind of specific incision enzyme. The cleavage position of BSA in tryptic hydrolysis could be deduced in terms of comparing the molecular relative masses of the hydrolysates corresponding to the peaks in the chromatogram to those of theoretical peptides.

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EXPERIMENTAL

Materials

Bovine serum albumin as a substrate is of chromatographic purity, which was obtained from Tianjin Institute of Blood Research (Tianjin, China). Trypsin was purchased from Sigma Corp. (MI, USA). Five standard substances for determining the calibration curve of the molecular relative mass of the HPSEC column: BSA (67 kDa), ovalbumin (43 kDa), bovine hemoglobin (64 kDa), cytochrome C (12 kDa), and vitamin B12 (1355) were also purchased from Sigma Corp. All other chemicals of analytical grade were commercially available.

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Hydrolysis of Bovine Serum Albumin

Bovine serum albumin (1 g dissolved in 250 mL water) was hydrolyzed with trypsin (enzyme/substrate: 1/100) in a 5 L batch stirred tank reactor. The reaction was carried out at $40 \pm 0.1^{\circ}$C. The pH was kept at 8.0 ± 0.1 by adding 0.1 M NaOH solution with a pH-stat apparatus (Shanghai Sanyuan Scientific Instrument Co., China). Samples of hydrolysates were taken at each 5 min interval, and the reaction terminated by heating it in boiling water for 10 min.

Equipment and Chromatographic Conditions

A HPSEC (WDL-95, Elite Scientific Instrument Co., China) equipped with a Protein Pak 125 column (8 mm × 300 mm i.d., Waters Co., USA) was used in this study. All samples were filtrated by 0.2 μm membranes prior to injection. The injection volume was 20 μL. The elution was 0.1 M phosphate buffer, pH 7.0 at a flow rate of 0.5 mL/min. The temperature of the column is kept at $25 \pm 0.1^{\circ}$C. Elution was monitored by a UV detector at 215 nm. The molecular relative mass of each hydrolysate corresponding to a peak in the chromatogram was determined with the retention time.

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RESULTS AND DISCUSSION

Standard Curve for Molecular Relative Mass

The relationship between molecular relative mass (MW) and retention time (R_t) was determined by five standard substances as:

$$R_t = -8.135 \log(MW) + 53.927, \quad r^2 = 0.9992 \tag{1}$$

Chromatographic Analysis of Bovine Serum Albumin Tryptic Hydrolysis Reaction Products

Figure 1 (a) is the chromatogram of BSA before hydrolysis. It can be seen that the substrate is at high pure level. The main peak is at 14.65 min in the chromatogram. Figure 1 (b), (c), (d), and (e) are the chromatograms of hydrolysates of BSA at 2.5 min, 20 min, 60 min, and 90 min, respectively. There are nine absorption peaks at 15.40, 19.60, 20.95, 22.01, 24.00, 26.05, 27.40, 27.55, and 31.70 min. The molecular relative masses corresponding to

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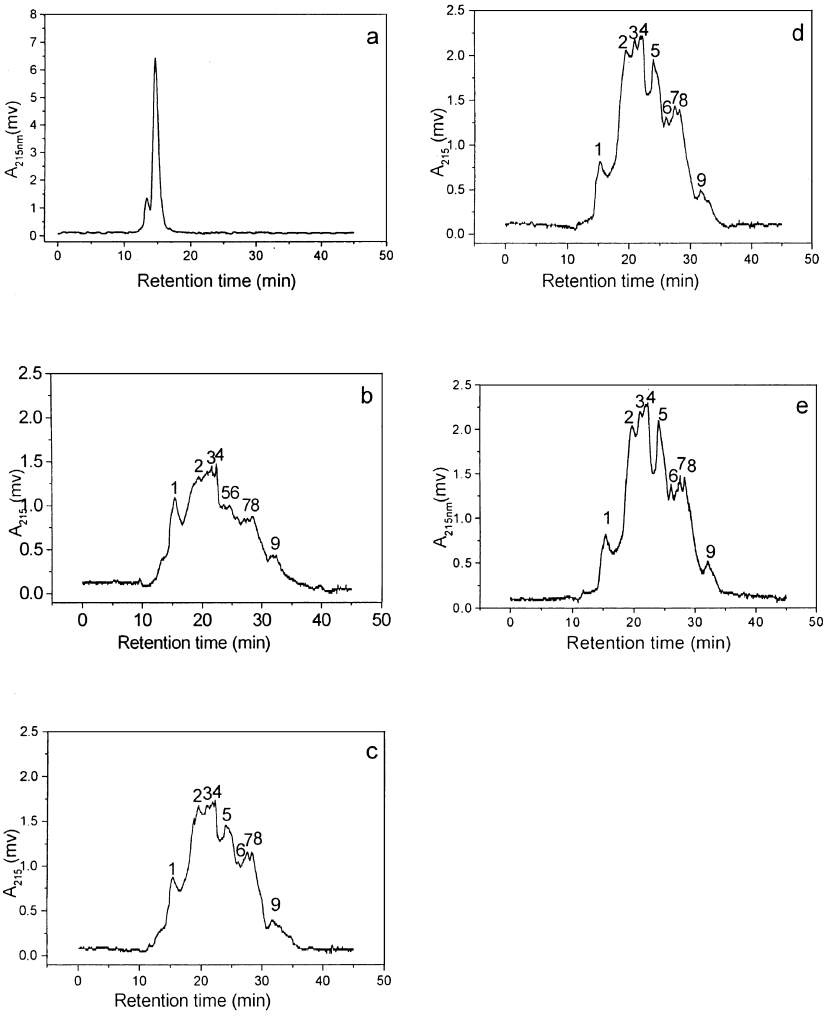

Figure 1. High performance size-exclusive chromatography of tryptic hydrolysates of bovine serum albumin: (a) BSA before hydrolysis; (b) hydrolysates after 2.5 min; (c) hydrolysates after 20 min; (d) hydrolysates after 60 min; (e) hydrolysates after 90 min.

the peaks determined by Eq. (1) are 54,400, 16,600, 11,300, 8,380, 4,770, 2,670, 1,820, 1,750, and 540. Compared with the chromatogram of pure BSA, there is no absorption peak at 14.65 min in the chromatogram of the hydrolysates, which showed that BSA did not exist any more. It can be concluded

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from Fig. 1 (b), that BSA can be hydrolyzed easily by trypsin and can decompose to polypeptides, peptides, and oligopeptides in a short time. In Fig. 1 (b), the absorption intensity of peak 1 is high, which means the polypeptides corresponding to peak 1 can be generated easily, after a short time hydrolysis. The percentage of polypeptides corresponding to peak 1 is about 57% at 2.5 min.

The concentration of peptides corresponding to these peaks changes with time. Table 1 is the experimental results of molecular relative mass distribution at different times. The proportion of the polypeptides corresponding to peak 1 declined, while those of the other peptides increased. The decreasing of the concentration means that polypeptides are hydrolyzing to peptides and oligopeptides continuously. The mass concentrations of the other peptides increased because their generation rate is faster than the decomposition rate. It indicates that these kinds of peptides can be produced easily and decomposed with difficulty. However, the changing rate of the concentration of peptides declined with time, which can be seen clearly especially from 60 min to 90 min. The reason is that the number of reactable amino acid residue in peptides decreased and the trypsin denatured with time.

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Deduction of Cleavage Position of Bovine Serum Albumin

Trypsin is a kind of high specificity protease that only attacks the long alkaline amino acid residues such as arginine and lysine, and cuts off the protein chain at the carbonyl end. Bovine serum albumin is composed of 583 amino acid residues and contains 83 arginine and lysine, as showed in

Table 1. Molecular weight distribution at different reaction time.

Peaks	Corresponding molecular weight	Percentage of peptides at 2.5 min	Percentage of peptides at 20 min	Percentage of peptides at 60 min	Percentage of peptides at 90 min
Peak 1	54,400	57.14	42.41	31.35	26.50
Peak 2	16,600	18.26	23.62	26.20	27.15
Peak 3	11,300	5.99	8.55	11.02	12.41
Peak 4	8,380	3.88	5.25	7.14	8.26
Peak 5	4,770	4.66	7.61	10.16	11.18
Peak 6	2,670	1.49	2.04	2.31	2.45
Peak 7	1,820	2.50	3.50	4.03	4.19
Peak 8	1,750	4.57	5.08	5.57	5.77
Peak 9	540	1.49	1.94	2.21	2.30

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                10                  20                  30                  40
DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHV
                50                  60                  70                  80
KLVNELTEFARTCVADESHAGCEKSLHTLFGDELCKVASL
                90                  100                 110                 120
RETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPN
                130                 140                 150                 160
TLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKY
                170                 180                 190                 200
NGVFQECCQAEDKGACLLPKIETMREKVLTSSARQRLRCA
                210                 220                 230                 240
SIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKV
                250                 260                 270                 280
HKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDK
                290                 300                 310                 320
PLLEKSHCIAEVEKDAIPEDLPPLTADFAEDKDVCKNYQE
                330                 340                 350                 360
AKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECC
                370                 380                 390                 400
AKDDPHACYTSVFDKLKHLVDEPQNLIKQNCDQFEKLGEY
                410                 420                 430                 440
GFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKP
                450                 460                 470                 480
ESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESL
                490                 500                 510                 520
VNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEK
                530                 540                 550                 560
QIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAA
                570                 580
DDKEACFAVEGPKLVVSTQTALA
```

Figure 2. Amino acid sequences of bovine serum albumin.

Fig. 2.[10] So, the possible peptides can be obtained by cutting off the peptide chain theoretically at the position of arginine and lysine on the basis of the primary structure of BSA. If cleavage can take place at each arginine or lysine, the number of all the possible peptides is 3570. The molecular relative mass of each thoretical peptide can be calculated. In fact, the number of the peptides in hydrolysates is larger than that showed in HPSEC. Seventy-five peaks were observed in a reversed phase HPLC chromatogram of peptides of BSA tryptic hydrolysis for a long time, and sixty-six peptides were identified.[10] But many

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of them are similar in molecular relative mass. For instance, the molecular relative mass of position 564–573 is 1108.2 and that of position 148–156 is 1177.3. In other words, an absorption peak in HPSEC can correspond to many peptides whose molecular relative masses are similar. As a result, compared with the number of peaks in reversed phase HPLC, the number for hydrolysates decreased in HPSEC.

Comparing the molecular relative masses of the peptides from chromatograms with those of the theoretical peptides, some theoretical peptides can match the molecular relative masses corresponding to the peaks. For example, the peptide corresponding to the peak at 15.40 min, whose observed molecular relative mass is about 54,400, can be matched to the amino acid sequence at 1–474, 13–483, 65–537, 82–556, 99–573, and 107–583. The theoretical peptides, whose molecular relative mass can correspond to the peaks in the chromatograms, are shown in Table 2.

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Table 2. Molecular weight of peaks and corresponding theoretical peptides.

Peaks	Retention time (min)	*MW*	Corresponding theoretical peptides
1	15.40	54,400	1–474, 13–483, 65–537, 82–556, 99–573, 107–583
2	19.60	16,600	1–144, 42–185, 115–256, 132–275, 137–280, 218–362, 233–377, 243–388, 286–431, 376–520, 378–523, 436–583
3	20.95	11,300	1–98, 65–159, 94–187, 99–194, 115–208, 137–232, 145–242, 181–280, 186–285, 212–312, 281–377, 313–409, 317–412, 440–537, 484–583
4	22.01	8,380	42–116, 115–185, 145–217, 199–273, 212–285, 262–335, 317–388, 337–409, 397–471, 410–483, 432–504, 472–544, 485–556
5	24.00	4,770	13–51, 65–106, 145–185, 233–275, 240–280, 281–322, 397–439
6	26.05	2,670	52–76, 186–208, 195–217, 257–280, 436–458, 534–556
7	27.40	1,820	99–114, 117–132, 428–444, 459–474, 484–499, 505–520
8	27.55	1,750	117–131, 132–144, 218–232, 323–336, 363–377, 557–573
9	31.70	540	77–81, 133–136, 195–198, 257–261

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It can be found that the frequency of some cleavage position appearing in Table 2 is high. For instance, the amino acid serial 280 appears six times, and the serials 144,185 appear five times. This kind of cleavage position and corresponding peptides are shown in Table 3.

Although the theoretical peptides in Table 2 can match the absorption peaks, it cannot come to a conclusion that the terminal groups of these peptides are the cleavage positions. But, the appearing of amino acid serials time after time in Table 3 shows that the probability of cleaving of BSA chain at these positions is higher than other positions. Kazuo et al.[10] investigated the tryptic hydrolysates of BSA after hydrolyzing 8 h with the combination of frit-fast atom bombardment, mass spectrometry/liquid chromatography. Some peptides were reported as follows: 77–93, 82–98, 94–106, 126–130, 128–132, 128–136,

Table 3. Amino acid sequence of cleavage position and corresponding peptides.

Amino acid serial number	Amino acid residue	Amino acid sequences of theoretical peptides
12	Lysine	13–51, 13–483
64	Lysine	65–106, 65–159, 65–537
76	Lysine	52–76, 77–81
81	Arginine	77–81, 82–556
98	Arginine	1–98, 99–114, 99–194, 99–573
106	Lysine	65–106, 107–583
114	Lysine	99–114, 115–185, 115–208, 115–256
131	Lysine	117–131, 132–144
136	Lysine	133–136, 137–232, 137–280
144	Arginine	1–144, 132–144, 145–185, 145–217, 145–242
185	Arginine	42–185, 115–185, 145–185, 186–208, 186–285
232	Lysine	137–232, 218–232, 233–275, 233–377
242	Lysine	145–242, 243–388
280	Lysine	137–280, 181–280, 240–280, 257–280, 281–322, 281–377
312	Lysine	212–312, 313–409
377	Arginine	233–377, 281–377, 363–377
397	Arginine	397–471, 397–439
439	Lysine	397–439, 440–537
474	Lysine	1–474, 459–474
483	Arginine	13–483, 410–483, 484–499, 484–583
537	Lysine	65–537, 440–537

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115–144, 145–185, 148–156, 153–166, 157–159, 195–198, 300–320, 317–322, 397–413, 410–412, 564–573. The chain length of these peptides is small because of the long time hydrolysis, which is different from our work. However, some cleavage positions are the same: 77, 82, 98, 106, 115, 132, 136, 144, 185, and 397. In addition, insulin-stimulating peptides, in which the amino acid sequence is 115–185,[4] can be found in Table 2 and corresponds to peak 5. It is also shown in Table 3 that the appearing frequency of lysine and arginine is 14 and 7, respectively; and the ratio of lysine to arginine approximately equals the ratio of the two kinds of amino acid in the protein of BSA, which is 59 to 24. This means that the capacity with which lysine and arginine bind with trypsin and the probability that cleavage takes place at the position of the two amino acid residues is similar. In summary, the cleavage position of BSA can be deduced roughly by analyzing the hydrolysates with HPSEC.

CONCLUSION

The molecular relative mass of hydrolysates can be characterized quantitatively with HPSEC. A novel method of deducing the cleavage position of BSA in tryptic hydrolysis reaction is proposed. By comparing the molecular relative mass of theoretical peptides of BSA with that of the HPSEC absorption peaks, the cleavage position of BSA can be roughly deduced.

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