Front. Chem. China (2006) 2: 199–202
DOI 10.1007/s 11458-006-0016-y

RESEARCH ARTICLE

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Quantitative analysis of complex casein hydrolysates based on chromatography and membrane

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Abstract The enzymatic hydrolysates of casein are so complex that there is no effective method to do quantitative analysis. The common techniques, such as high performance chromatography and SDS-PAGE, can only carry out qualitative analysis. On the basis of membrane separation and high performance size exclusion chromatography (HPSEC), standard peptides with different molecular mass range were prepared, and the linear relationships between mass concentration of the standard peptides and the ultraviolet absorption of corresponding peak areas were established. Consequently, mass concentration of the different hydrolysates at different reaction times could be accurately calculated. The combination of chromatography and membrane separation is of great importance to the quantitative analysis of the complex hydrolysates, which can also be applied to the other macromolecular systems, such as carbohydrates.

Keywords casein, enzymatic hydrolysis, high performance size exclusion chromatography (HPSEC), complex system, membrane separation, quantitative analysis

1 Introduction

Through multi-enzymatic hydrolysis of casein, more than one hundred active peptides can be obtained by further separation and purification of hydrolysates. Due to their complex components and various sequences, these active peptides have many physiological functions, such as immunological competence, free radical scavenger, mineral carrier, bacteriostatic agent, anticancer, decompression, nervinae and anaesthesia. Therefore, enzymatic hydrolysis has become one of the most attractive research methods to prepare active peptides [1, 2]. However, the hydrolysates are so complex that it is difficult to carry out quantitative analysis via traditional gel electrophoresis or high performance liquid chromatography (HPLC), which is beneficial to efficiently prepare peptides.

Based on the difference of molecular mass, high performance size exclusion chromatography (HPSEC) has become an effective tool to analyze enzymatic hydrolysates. In our previous work, the reaction process of casein tryptic hydrolysis system has been characterized, and its reaction mechanism was also deduced from the chromatograms [3]. In this paper, membrane separation [4] and chromatography technology are combined to divide enzymatic hydrolysates into several lumping components according to their molecular mass. Each lump with a narrow molecular mass range is approximately regarded as a pseudo peptide. Then the relationship between mass concentration and absorption peak area is established, and the distribution of multiple hydrolysates concentration at different reaction time can be calculated. The above method is helpful to the establishment of a kinetic model for enzymatic reaction and to purification of target peptides. It can also be applied to some other macromolecular systems, such as carbohydrates.

2 Experimental

2.1 Materials

Casein was provided by Tianjin Hematology Institute, Chinese Academy of Medical Sciences. Trypsin and molecular standard samples, including bovine serum albumin (BSA) (Molecular mass: 66,409), ovalbumin (43,000), bovine hemoglobin (31,000), cytochrome C (12,327), and vitamin B12 (1,355), were purchased from Sigma. All the other chemicals were of analytical grade.

Translated from *Chinese Journal of Chromatography*, 2005, 23(2) (in Chinese)

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2.2 Apparatus

ZD-2 pH-stat (Shanghai Precision & Scientific Instrument Co., Ltd.); HPSEC system with Echrom 98 chromatography work station, a UV-200 II ultraviolet detector and a P200 II high-pressure and constant-flow pump (Elite Scientific Instrument, Dalian, China); TSK- G3000PW, 7.5 mm ID×30.0 cm L (TOSOH Co.); SMB-20 ultrafiltration membrane with molecular mass cut off 10,000 and 100,000, effective membrane area 0.6 m^2 and retention rate over 90% (Shanghai Atomic Energy Research Institute); Allegra 21R Centrifuge (BECKMAN Co.); FREEZONE 4.5 freezing drier (LABCONCO Co.)

2.3 Experimental methods

2.3.1 HPSEC operating conditions

The mobile phase was 0.1 mol/L phosphate solution adjusted to pH 7.0 with flow rate of 0.5 mL/min. Injection volume was 20 µL. Detection was performed by UV-light absorption at 214 nm, and the column temperature was maintained at 25 ºC.

2.3.2 Standard curve of molecular mass

Five standard samples were analyzed respectively by HPSEC, and the linear relationship between molecular mass (M_r) and retention time (t_R) was determined by regression analysis as: $\lg M_r=-0.1899t_R+6.9879$, $R^2=0.9906$.

2.3.3 Preparation of standard polypeptides with different molecular mass range

The reaction of casein tryptic hydrolysis was stopped at 90 min by heating. Then, the hydrolysates were centrifuged at 9,000 rpm for 45 min. Thus an insoluble residue and a supernatant can be obtained. The supernatant was further filtrated by an ultrafiltration membrane with molecular mass cut off 100,000, and the retentate was kept. The permeate was further filtrated by an ultrafiltration membrane with molecular mass cut off 10,000, and the retentate and permeate were both kept. Three kinds of standard polypeptides with different molecular mass range were obtained by freezing drier.

2.3.4 Establishment of relationship between the concentration of standard polypeptides and corresponding chromatography area

The solutions of standard polypeptide with different concentrations were made and analyzed by HPSEC. Then, the linear relationship between chromatography areas and mass concentrations were obtained by regression analysis.

2.3.5 Preparation and pretreatment of enzymatic hydrolysates

Casein was dissolved in deionized water for 12 h, and then adjusted to pH 8.0 and heated up to 40 ºC. Trypsin was added at 1/50 (w/w) of casein concentration. Hydrolysis was carried out at constant temperature with pH maintained at 8.0. The reaction was stopped at different times by keeping the samples in boiling water for 10 min. All hydrolysate solutions were filtered through a 0.45 µL millipore film before HPSEC analysis.

3 Results and discussions

An unknown concentration of a single substance can be calculated according to the standard curve, which expresses the linear-regression relationship of mass concentration and corresponding chromatographic absorption area. However, protein enzymatic hydrolysates are so complex that the absorption coefficients of each component are distinctly different. Therefore, product concentrations cannot be calculated by the above method. In the present work, a lumping idea is adopted, which is commonly used in the chemical engineering field [5], i.e., different polypeptide components with relatively narrow molecular mass range should be firstly prepared, and then the quantitative analysis of complex hydrolysates could be accomplished according to the linear-regression relationship between mass concentration and absorption area of each lump.

3.1 Preparation of polypeptides with different molecular mass range

The hydrolysates of casein–trypsin system are mainly distributed in seven peaks, which belongs to three districts (Fig. 1a), and the height of each peak are changing with the reaction time [6]. Several polypeptide components with relatively narrow molecular mass range can be prepared by membrane separation. The chromatograms of each lumping components are shown in Fig. 1b–d, and the molecular mass values of the largest peptides are marked in each chromatograms.

3.2 The relationship between mass concentrations and peak areas for each lump

From Fig. 1d, it can be seen that the molecular mass of the permeate obtained by membrane with 10,000 molecular mass cut off is below 11,300. The linear equation of the peak area and mass concentration for this component is determined as follows:

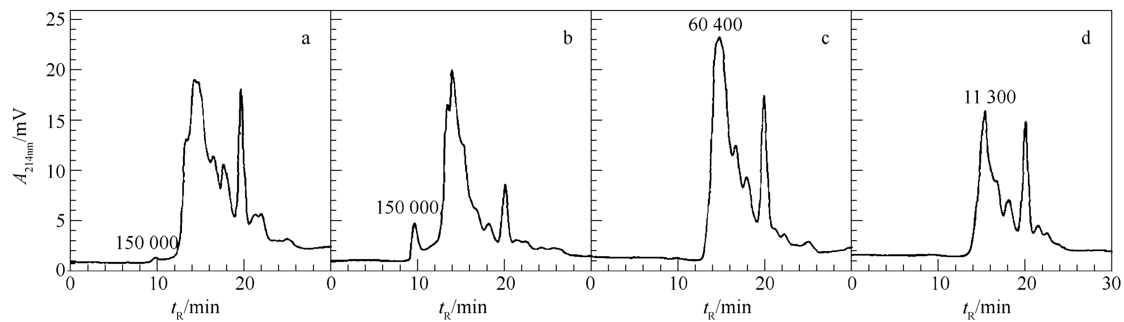

Fig.1 Chromatograms of hydrolysates and peptides with different molecular mass ranges prepared by membrane separation
(**a**) hydrolysates of casein by trypsin for 90 min; (**b**) retentate of membrane with 100,000 molecular mass cut off; (**c**) permeate of membrane with 100,000 molecular mass cut off; (**d**) permeate of membrane with 10,000 molecular mass cut off

$$S = 7.2695m + 1.8629 \quad (1)$$

in which S means absorption peak area, and m depicts mass concentration.

The molecular mass of the permeate obtained by membrane with 100,000 molecular mass cut off is below 60,400, which is shown in Fig. 1c. Since S and m of the lumping component with molecular mass below 11,300 can be integrated and further calculated according to Eq. 1, the relationship between S and m of the lumping component from 11,300 to 60,400 can be deduced as follows:

$$S = 5.8566m - 2.5013 \quad (2)$$

By the same means, the expression between S and m of the lumping component from 60,400to 150,000 can also be obtained via twice iteration according to Fig. 1b as follows:

$$S = 12.654m + 4.8038 \quad (3)$$

2.3 Quantitative analysis of enzymatic hydrolysates at different reaction times

Based on the values of membrane molecular mass cut off, the tryptic hydrolysates of casein are divided into three lumps, i.e., 60,400~150,000 (A), 11,300~60,400 (B), and <11,300 (C). According to Eqs. 1–3, mass concentration of each lumping components with different molecular mass can be calculated at different reaction times. Then, the results are verified by adding them up on the basis of mass conservation, which are listed in Table 1. Furthermore, the distribution curves of mass concentration for each lump changing with reaction time are also shown in Fig. 2.

Table 1 Mass concentration of the hydrolysates at different reaction time g/L

Reaction time/min	Insoluble substance[1] D	Soluble substances[2]			Total mass concentration[3]
		A	B	C	
2.5	2.89	2.28	2.01	2.89	10.07
5	2.41	2.15	2.24	3.28	10.08
10	1.68	1.98	2.24	3.28	10.08
20	0.96	1.77	2.59	4.94	10.24
40	0.78	1.61	2.52	5.15	10.06
60	0.69	1.47	2.55	5.19	9.90
90	0.71	1.29	2.66	5.25	9.91

[1] Not hydrolysates, obtained by high-speed centrifugation; [2] A: M_r 60,400~150,000; B: M_r 11,300~60,400; C: M_r<11 300; [3] initial concentration of casein substrate was 10 g/L

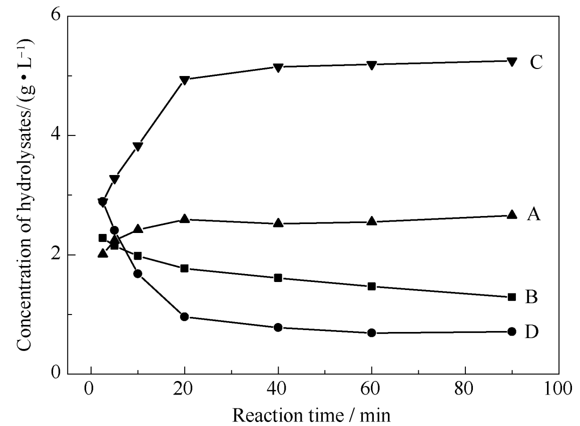

Fig. 2 Curves of mass concentration of hydrolysates *vs* reaction time. For A, B, C, see Table 1.

As shown in Fig. 2, the mass concentration of lump C, including mostly active peptides, evidently increased with reaction time at the beginning of the reaction. That means the longer the reaction time is, the more the bioactive peptides can be obtained. However, taking all the aspects into account, especially the process cost, the optimized reaction time may be 20 minutes for industrial production, because the concentration of lump C is nearly constant in the middle and last period of the reaction.

4 Conclusions

Through the combination of membrane separation and chromatography analysis, mass concentration of enzymatic hydrolysates with a certain molecular mass range can be quantitatively calculated. It is also an effective analysis method for some other complex macromolecular systems, such as carbohydrates. However, there are still some aspects

to be improved. For example, the division of districts can be more flexible and the calculation of concentration can be more precise by changing membrane separation into preparative chromatography. With the development of biochemical separation and the improvement of analysis method, quantitative analysis of complex polypeptide mixture derived from enzymatic hydrolysis of protein will be more precise and simple.

Acknowledgement The authors wish to thank the financial support from the Natural Science Foundation of China (No. 20306023).

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