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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 333–339
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Analysis and determination of biological activity of short-chain peptides from porcine brain hydrolysate

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Received 17 May 2004; received in revised form 23 October 2004; accepted 25 October 2004
Available online 13 December 2004

Abstract

Gel permeation chromatography fractions of short-chain peptides from a hydrolysate product, which in turn was from the purified porcine brain through enzyme hydrolysis, were tested for their biological activities. The results showed that the fractions A4 and A5 had significant biological activities. The two fractions were analyzed with analytical techniques such as high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), isoelectric focusing (IEF), discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Preliminary results showed that the main components of these two fractions were short-chain acidic peptides with a relative molecular mass (M_r) of less than 2400.
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Keywords: Porcine brain protein hydrolysate; Short-chain peptides; HPLC; CZE; IEF; SDS-PAGE

1. Introduction

The hydrolysate products of animal brain are used as a medicine for some cerebral functional diseases. In deed, one such product, Cerebrolysin (Ebewe Pharmaceutical, Austria), has been in clinical application for more than 40 years. Its physiology activities have been confirmed by researchers all over the world [1–6]. It is used for the treatment of neural system diseases, especially Alzheimer's disease (AD) [7–9]. It prevents the age-dependent dementia characterized by progressive loss of cognitive capability, and by pathological changes in the brain [10]. Cerebrolysin is also reportedly used for the treatment of cerebral concussion, brain injury, brain arteriosclerosis, brain anoxemia of the new-born and a number of other diseases [11–13].

As the producer reported, cerebrolysin is produced from purified brain proteins by enzymatic breakdown. It is known to be a mixture of 75% free amino acids and 25% short-chain peptides, probably only a part of the peptides are physiologically active [6,14]. Although this preparation has been in clinic use for quite a long time, most studies were on its pharmacological activities, far less were on the study of its analysis. In this work we studied the hydrolysate of degreased fresh porcine brain with several analytical techniques, to analyze its active components as well as to find the appropriate means of the quality control of the product.

2. Materials and methods

2.1. Materials

Fresh porcine brains were purchased from the market, Kunming mice (KM), used for the test of the biological activity of the hydrolysate, were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

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doi:10.1016/j.jpba.2004.10.031

2.2. Biological products, reagents and standards

Isoelectric protein standards with isoelectric point (pI) values from 3 to 10, PhastGel IEF [pI 3–9, 43 mm × 50 mm × 0.45 mm ($W \times L \times T$)] and Sephadex G-15 were from Amersham Bioscience (Uppsala, Sweden). The acidic proteinase (25,000 u/g) was obtained from Northern enzymatic products Co. (Zhaodong, China), bovine insulin from Sigma (St. Louis, MO, USA), albumin from North China Pharmaceutical Co. (Shijiazhuang, China), also was the physiological saline used in the work. The scopolamine hydrobromide injection (0.3 mg/ml) was from Hefeng Pharmaceutical Ltd. (Shanghai, China). Acetonitrile (Chromatographic grade) was from Fisher (Fair Lawn, NJ, USA). Tris [Tris(hydroxymethyl)aminomethane], acrylamide, bisacrylamide and tricine (*N*-[Tris(hydroxymethyl)methyl] glycine) were all from Sigma (St. Louis, MO, USA).

2.3. Instruments

Following instruments were used in this work: Waters 650E Protein Purification System (Milford, MA, USA), Virtis AD2.0ES cryodesiccator (NY, USA), JXDT-1 step-down apparatus (Jixing Experimental Instruments Co. Ltd., Shijiazhuang, China), Waters 2487 HPLC (Milford, MA, USA) with a millennium^{32} workstation using a C_{18} column (200 mm × 4.6 mm, 5 μm, Agilent, Palo Alto, CA, USA), Beckman P/ACE 5000 Electrophoresis apparatus (Fullerton, CA, USA) equipped with fused silica capillary column (50 μm i.d., Yongnian Optic Fiber Co. Ltd., Hebei, China), PhastSystem Automatic horizontal IEF (Amersham Bioscience, Uppsala, Sweden), Mini Protean II vertical electrophoresis apparatus (Bio-Rad, Hercules, CA, USA).

3. Methods

3.1. Preparation of short-chain peptides

Fresh porcine brains, after washing and removing blood vessels and meninges, were chopped into small pieces, extracted thoroughly with acetone and ether to obtain degreased porcine brain protein powder. The powder was mixed with water into slurry, acidic proteinase was subsequently added into the slurry, its pH value adjusted to 2.00. Hydrolysis of the porcine brain protein was carried out for 36 h at 50 °C to obtain the hydrolysate. The product was subsequently separated on a Sephadex G-15 column eluted with 0.9% NaCl (w/v). Fractions were collected according to the elution of chromatographic bands shown by a UV detector (Fig. 1). The collected fractions were subsequently desalted and cryodesiccated.

3.2. HPLC analysis

Solutions of the raw hydrolysate as well as peptide factions were analyzed with HPLC at 210 nm, using a C_{18} column (200 mm × 4.6 mm, 5 μm), the mobile phase (flow rate 1.0 ml/min) was acetonitrile (AC)/0.1% (v/v) trifluoroacetic acid water solution, the gradient program was as follows: the AC concentration was kept at 5% for 2 min, subsequently raised linearly to 10% in 8 min, then linearly to 90% in 25 min, and kept at that for 5 min. All the experiments were carried out at room temperature. The concentrations of all the sample solutions were at 1 mg/ml.

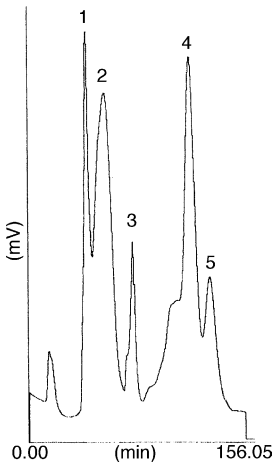

Fig. 1. Elution curve of the peptide fractions on Sephadex G-15 A: fraction A4, B: fraction A5, C: raw hydrolysate. Conditions: column: 100 cm × 2.6 cm i.d., eluent: 0.9% (w/v) NaCl at 3.4 ml/min.

3.3. CZE analysis

Analysis of peptides using CZE technique has been reported by many authors, excellent separations were reported [15–17]. In our analysis of the hydrolysate a 27 cm × 50 μm i.d. fused silica capillary (effective length 20 cm) was used, 0.1 M NaH_2PO_4 (adjusted to pH 2.0 with 0.3 M phosphoric acid solution) was used as the buffer. A 1 kV × 2 s was applied for electrokinetic injection, the electrophoretic voltage was 20 kV and the current was about 120 μA. The analysis was carried out at a temperature of 30 °C, detection was at 214 nm. Concentrations of the samples were all 0.5 mg/ml.

3.4. Isoelectric focusing analysis

The experiments were carried out according to the PhastSystem manual. The electrophoresis time is about 30 min with the voltage 2 kV and the current 25 mA. Isoelectric protein standards (pI 3–10) and Albumin (pI 4.7–5.2) were used as markers. After the completion of the separations, the gel was fixed with a mixture of 6.25% (v/v) glutaraldehyde, 12.5% (v/v) glacial acetic acid, 50% (v/v) ethyl alcohol solutions. Silver staining was used for the dyeing of the gel [18].

3.5. SDS-PAGE separation

Many authors, e.g., Schägger [19], reported the separation of small-molecule peptides with SDS-PAGE technique.

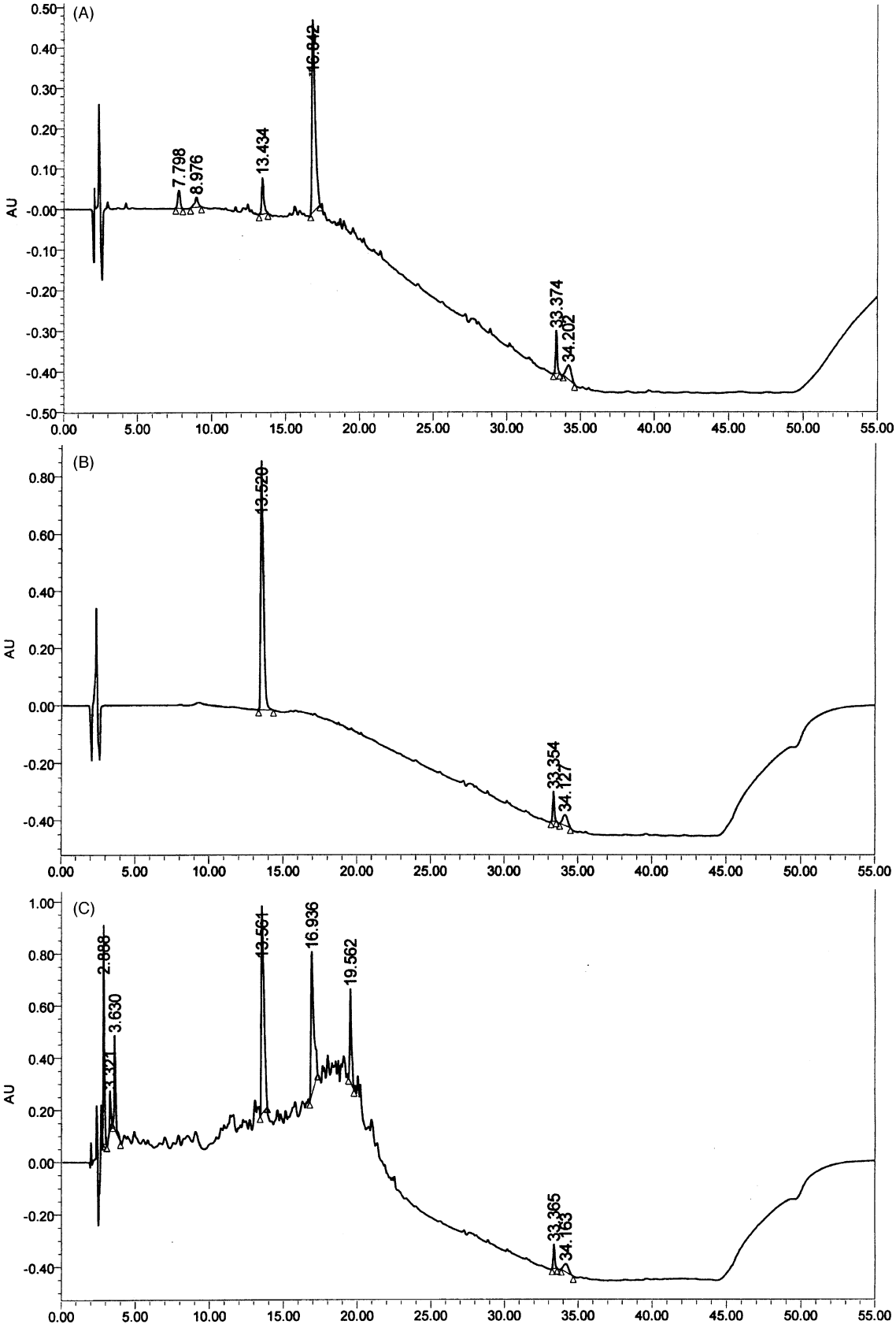

Fig. 2. Chromatogram of fraction A4, A5 and the raw hydrolysate by HPLC A: fraction A4, B: fraction A5, C: raw hydrolysate. For chromatographic conditions, see text.

Consulting [19], the preparation of separating and stacking gels suitable for the separation of the hydrolysate peptides were tested. The best gels were prepared using the following proportions of the materials: 16.5% T and 6% C to prepare the uniform separating gel (86 mm × 46 mm × 1.0 mm, $W \times L \times T$), and 5% T and 3.3% C for the stacking gel (86 mm × 22 mm × 1.0 mm, $W \times L \times T$). The samples and bovine insulin were boiled for 3–5 min at 100 °C in a mixture of 4% (w/v) SDS, 5% (w/v) glycerol, 10% (v/v) mercaptoethanol, 125 mM Tris and 0.05% (w/v) bromphenol blue, adjusted with HCl to pH 6.8. Bovine insulin was used as the molecular mass marker (the relative molecular mass of its chain A is 2400 and chain B 3300). The electrophorestic plate was cooled by circulative water. The electrophoretic runs started at 12 mA to introduce the whole sample band into the separating gel, then the current was adjusted to 20 mA for separation. The total electrophoresis time was about 5 h. Fixing and staining procedures were the same as in the IEF experiments shown above.

4. Results and discussion

4.1. The yields of purification of the porcine brain protein and the hydrolysate fractions

From 1800 g fresh porcine brain, 67 g of the degreased protein powder was obtained (yield = 3.7%). From the degreased protein, the hydrolysate yield was 31.2%. Five main fractions were collected after the gel permeation chromatographic separation of the hydrolysate (see Fig. 1), the total yield of the separation was 94.5%. It was found that although it seemed straightforward to elute the peptide fractions with deionized water, however in this way only four fractions could be collected and the yield was down to 79.4%. It can be postulated that when pure water was used for elution, some peptide fractions would be adsorbed on Sephadex gel more strongly by non-specific adsorption.

4.2. Determination of physiological activity

The physiological activity of the five fractions were tested using a model of step-down type passive avoidance [20]. Briefly, groups of mice were firstly injected i.p. with biological saline (normal control and model control groups) or the solutions of one of the purified peptide fraction (test groups). One injection per day was kept for 7 days, after the final injection, scopolamine hydrobromide injection was administered i.p. to the mice to cause memory obstruction, apart from those of the normal control group, which was injected with biological saline. To test the biological activities of the fractions, all the mice were firstly trained in the step-down apparatus. When they were placed on the platform of the apparatus, they would normally jump immediately down to the metal grid below, where they would suffer electric shocks (alternating current at 36 V). After reiterative trainings, the mice would learn to stay as long as possible to avoid shock. Twenty-four hours later, the step-down latencies (the sum of time intervals between the mice in a group being put on the platform to their jumping-down to the grid) and the numbers of errors (the numbers of their jumping-down) were experimented and recorded as memorial indices. When a mouse remained on the platform for more than 5 min, its latency was taken as 300 s. A cross-design of the experiment was followed so that the mice were trained and tested by turns.

By using the Student's *t*-test to test the significance between the step-down latencies and the numbers of errors of the animals in the individual groups in the step-down type passive avoidance test, the results (See Table 1) showed that, of the five groups tested with corresponding fractions, A3, A4 and A5 groups showed significantly extension of latencies in comparison with the model control group, whereas

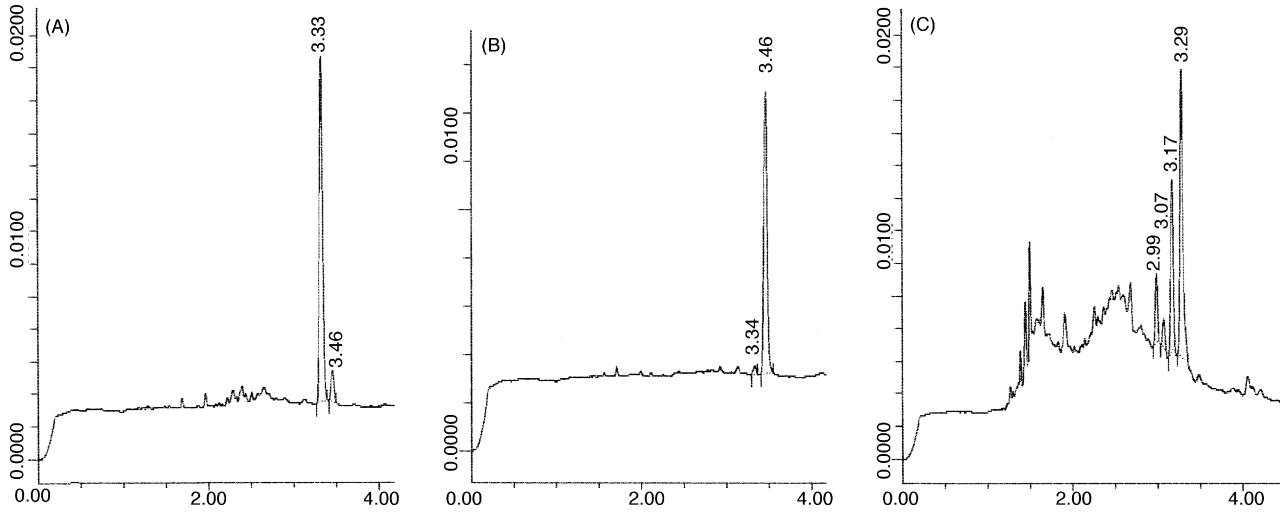

Fig. 3. Electrophoregram of fractions A4 and A5 by CZE. For electrophoretic conditions, see text.

Table 1
Statistical test results (P) of biological activities of the hydrolysate fractions of porcine brain protein using the Student's t-test

Parameter	Control	Fraction				
		A1	A2	A3	A4	A5
Latency	Normal	3.7*	0.4**	20.3	11.0	33.9
	Model	5.3	15.0	0.7**	0.8**	0.2**
Number of errors	Normal	6.0	3.9*	4.6*	33.4	41.5
	Model	9.8	8.0	50.0	0.7**	0.3**

* $1\% < P < 5\%$.
** $0.1\% < P < 1\%$.

their latency differences with normal control group were not significant. For groups A1 and A2, contrary results were obtained.

Statistical tests on the numbers of errors obtained from the groups of mice showed that results from these mice made significantly fewer mistakes in comparison with the model control group, the numbers, however, were not differ significantly with those obtained from the normal control group. Groups A1, A2 and A3 showed contrary result (apart from group A1, which did not show significant difference).

These results suggested that, taking both the step-down latency and the numbers of errors of the animals into

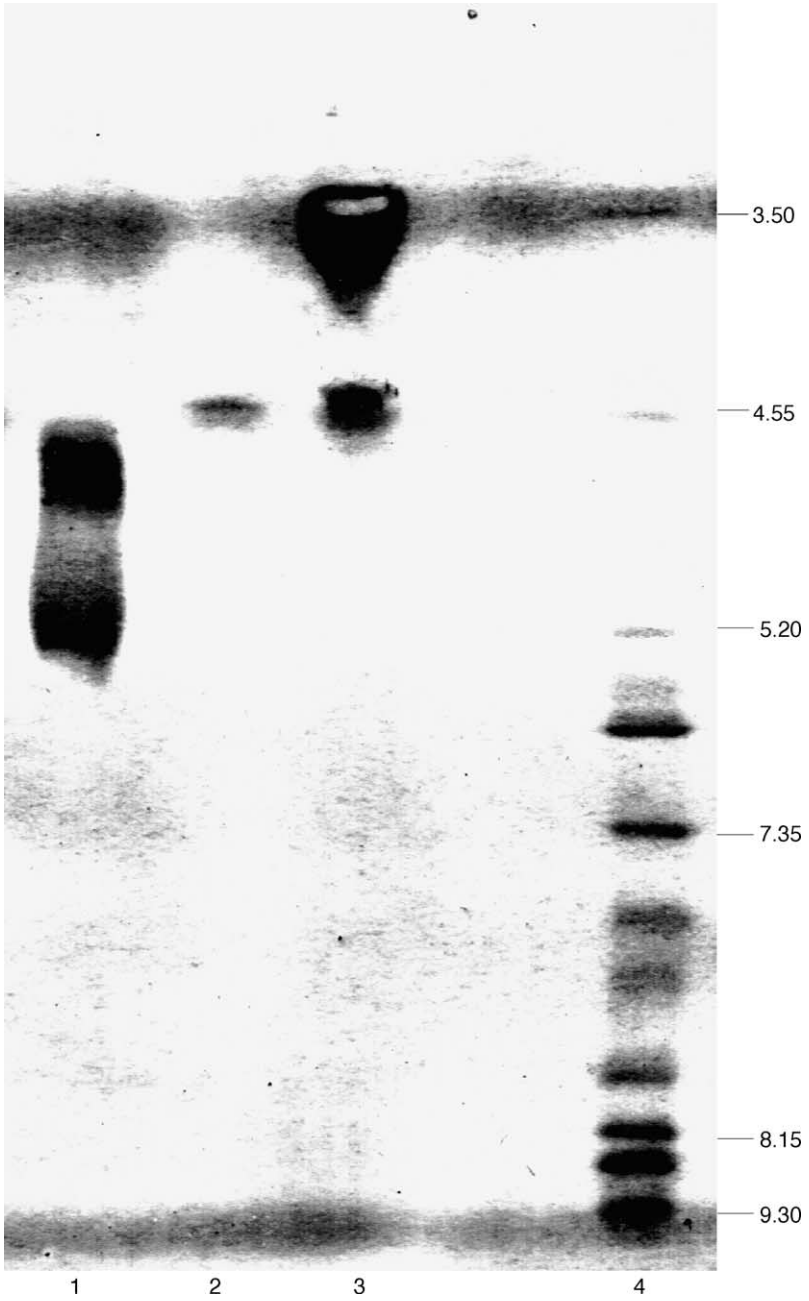

Fig. 4. IEF analysis of the peptide fractions. Sample identities: (1) albumin (pI 4.7–5.2); (2) fraction A5; (3) fraction A4; (4) protein pI standards.

consideration, fractions A4 and A5 were able to significantly improve the capability to learn and memorize of the mice having acquired memory obstruction by scopolamine hydrobromide injection. Other fractions were significantly less effective, therefore only these two fractions were analyzed later on.

4.3. Results from HPLC analysis

Fig. 2 shows chromatograms of biologically active fractions A4, A5 and the raw hydrolysate by HPLC. It can be seen from the figures that from fraction A4, six main peaks could be separated, among which the most abundant one (retention time 16.8 min) accounted for 65% of the total peak area; while from fraction A5, three peaks were separated in which one with a retention time of 13.5 min accounted for 85% of the total area. A5 appeared to bear more main component (supposedly the effective one) than A4. As can be expected, the hydrolysate chromatogram was more complicated.

4.4. Results from CZE analysis

Fig. 3 shows the electrophoregrams of A4, A5 and the raw hydrolysate samples by CZE analysis, it can be seen that each of the fractions showed a main peak. In accordance with the HPLC analytical results, A5 was more homogeneous than A4, as can be seen from the fewer impurity peaks it contained. Also, the electrophoregram from the analysis of the raw hydrolysate was more complicated.

In a study on the separation conditions, it was found that when the pH was increased, the resolution between A4 and A5 turned worse. When pH > 3.0, the main component in A4 and that in A5 became non-separable. Accordingly, a pH range of the buffer solution between 2.0 and 2.5 was chosen.

At temperatures of 20, 25 and 30 °C, the resolution between the main components in A4 and A5 were 1.60, 2.25 and 2.52, respectively. This suggested that high separation temperature favored better separation, and a temperature of 30 °C was adopted for the separation. Considering that with a longer column, the separation time is proportionally elongated, a shorter column is chosen in case sufficient resolution could be attained.

There are far more peaks in the electrophoregram of raw hydrolysate in Fig. 3 than in the chromatogram of the raw hydrolysate in Fig. 2 This shows that CZE is a more powerful separation technique than HPLC, besides, a sample can be analyzed within only 5 min. Being highly efficient, rapid and economic, CZE is apparently a good alternative technique for monitoring the protein hydrolysis process and the like.

4.5. Results from IEF analysis

A result from IEF analysis (Fig. 4) showed that the fraction A4 gave rise to a cluster of bands between pH 3.5 and 4.55, further demonstrating that A4 was composed of a group of acidic short-chain peptides, whereas fraction A5 showed a single band at pH 4.55, meaning that it was a relatively homogeneous fraction with an isoelectric point of 4.55. The IEF separation result coincided with those from HPLC and CZE experiments.

4.6. Results from SDS-PAGE analysis

To test the SDS-PAGE conditions for the analysis of hydrolysate as well as its fractions, separating gels of 15% T 3.3% C, 18% T 3.3% C and 20% T 3.3% C were tested. The results showed that increasing the concentration of the separating gel did not significantly improve the separation of the bands.

The SDS-PAGE technique, which is usually used to analyze high molecular proteins, probably is not a good choice for the analysis of these short-chain peptides: considering that in the preparation of the fractions, A5 was the last to elute,

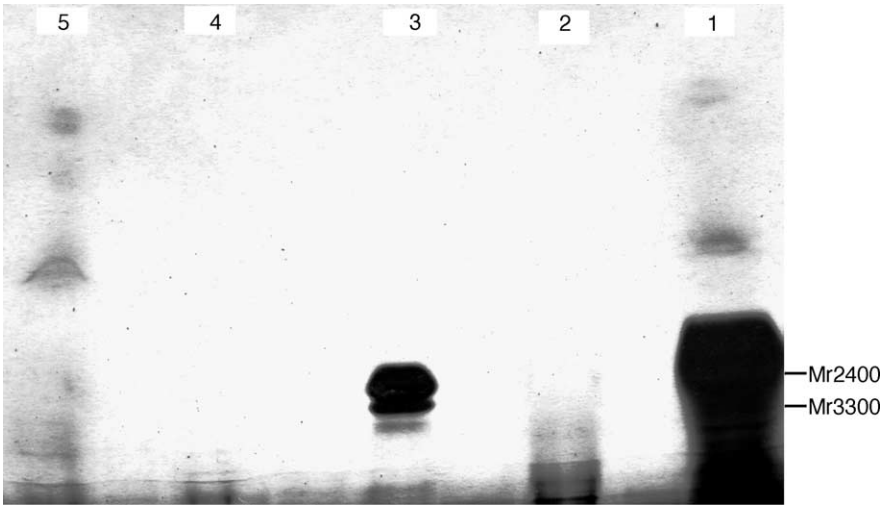

Fig. 5. SDS-PAGE analysis of the peptide fractions (separation gel: 16% T, 6% C). Sample identities: (1) raw hydrolysate; (2) fraction A5 (high concentration); (3) bovine insulin (M_r = 3300, 2400); (4) fraction A5; (5) fraction A4.

it should be composed of too small peptides to be properly focused at the interface between the gels and subsequently be sieved, although by using 16% T 6% C separating gel, better separation of A4 was achieved (Fig. 5). It showed 3 dispersed, relatively faint bands. The position of these bands suggested that their relative molecular masses were probably around or lower than 1000. Under the same conditions, A5 did not show clear bands.

5. Conclusion

Porcine brain hydrolysate product as well as the short-chain peptide fractions produced by the present authors were analyzed using traditional biochemical analytical techniques as well as some modern instrumental analytical methods. The discoveries should be able to play an active role in the study on the structures of short-chain peptides, as well as the quality control of the clinically applied peptide medicine.

Acknowledgements

The authors are heartily grateful for the great and selfless support from the Northern China Pharmaceutics (Group) Co., Northern enzymatic products Co. Zhaodong, China, they also appreciate very much C.Q. Niu, A.H. Dong and A.Y. Hao for their ceaseless support to this work.

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