Studies on Chromatographic Fractioning by Cations Exchangers of a Bovine Hemoglobin Hydrolysate

ALINA DIANA PANAINTE[1], IONELA DANIELA MORARIU[1]*, NELA BIBIRE[1], MADALINA VIERIU[1], GLADIOLA TANTARU[1], ALINA COSTINA LUCA[2]*, MIHAI APOSTU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Pharmacy, 16th Universitatii Str., 700115, Iasi, Romania
[2]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Medicine, 16th Universitatii Str., 700115, Iasi, Romania

A peptidic hydrolysate has been obtained through hydrolysis of bovine hemoglobin using pepsin. The fractioning of the hydrolysate was performed on a column packed with CM-Sepharose Fast Flow. The hydrolysate and each fraction was filtered and then injected into a HPLC system equipped with a Vydak C4 reverse phase column (0.46 x 25 cm), suitable for the chromatographic separation of large peptides with 20 to 30 amino acids. The detection was done using mass spectrometry, and the retention time, size and distribution of the peptides were determined.

Keywords: hemoglobin, cation exchangers, RP-HPLC

Enzymatic protein hydrolysis is used in the food industry to improve organoleptic and functional properties such as solubility, emulsification, gelling or for large-scale preparation of protein hydrolysates used in hypoallergenic diet of children and in nutritional therapy [1, 2]. Blood proteins are also used in the pharmaceutical industry. For example, hemoglobin is the main constituent of Proferrin® (Colorado Biolabs, Inc., CO, USA), a heme iron supplement used to address iron deficiency. Many beneficial health effects have been described for theose molecules, (e.g., antimicrobial, antioxidative, antithrombotic, anti-hypertensive and immunomodulatory) [3-5].

One of the most common ways to obtain biopeptides from protein sources is enzymatic hydrolysis. Active peptides obtained during enzymatic hydrolysis of food proteins are often intermediates and are generally found in a complex peptide population in which their concentration is very poor. Their preparation requires rather complicated fractioning and purification techniques. In order to simplify those problems, numerous researches have been carried out using biphasic organic solvent extraction, systems that can isolate these active peptides from protein hydrolysates. In 1985, Brantl et al [6] have isolated for the first time biologically active peptides based on *in vitro* enzymatic hydrolysis of bovine hemoglobin, a method that has been used extensively. Currently, 150 fragments of hemoglobin have been isolated, and the biological activity of 18 of them has already been determined [7,8]. Bovine hemoglobin was selected for use in bioactive hydrolysates with potential use as functional food ingredients for the prevention of various disorders such as hypertension, obesity and diabetes[9-12].

The objective of the study was to fractionate using ion exchange chromatography a bovine peptic hydrolysate hemoglobin in order to obtain fractions with a small number of peptides.

Experimental part

Material and method

Quantitative determination of bovine hemoglobin

24.16 grams of powdered hemoglobin (Sigma, ref. H2625) were weighed and dissolved in distilled water. The solution was centrifuged for 20 min at 4000 rpm to remove the insoluble fractions. The concentration of the supernatant was determined using the method described by Crosby et al., [7] based on the convertion of hemoglobin in the presence of Drabkin reagent, into a stable cyanomethemoglobine complex with a maximum of absorption at 546 nm. The procedure involved mixing 50 μL of supernatant with 12.5 mL of Drabkin reagent. After 15 minutes of rest at room temperature, the absorbances of the sample was measured at 546 nm against the Drabkin reagent. Knowing that the 14.7% standard hemoglobin solution had a 0.4 absorbance, the concentration of hemoglobin was calculated using the formula: C% = 14.7 × (absorbance/0.400).

Denaturation of hemoglobin using urea

A volume of stock solution corresponding to 5 g of hemoglobin was added to 80 g of urea dissolved in water. The mixture was incubated at 37°C for one hour, and then the *p*H was adjusted to 3 with a concentrated hydrochloric acid, and it was all filled up to 250 mL with distilled water.

Analysis of enzymatic activity of pepsin

The enzymatic activity of pepsin was evaluated spectrophotometrically, using the following solutions:

- pepsin stock solution obtained by dissolving 14.5 mg of pig pepsin (Sigma ref. P6887) in 5.18 mL of 0.25 M sodium acetate buffer solution (*p*H = 3). It was diluted 1:200 with distilled water;
- 5% hemoglobin solution denatured using urea at *p*H=3.

Denatured 5% hemoglobin solution was mixed with diluted pepsin solution at 37°C. Then, the samples were prepared by mixing 5 mL hemoglobin solution with 1 mL diluted pepsin solution, and incubating the mixtures for 10 min at 37°C. The reaction was stopped by adding 10 mL of 5% (w/v) tricloracetic acid. A blank sample was prepared by mixing 10 mL of 5% (v/v) tricloracetic acid with 1 mL of diluted pepsin solution, and 5 mL of hemoglobin solution was added before incubation. The samples were left to rest at room temperature for 30 min. The solutions were then centrifuged at 4000 rpm for 15 min in order to remove any precipitate. The absorbance values of the samples were measured at 280 nm against the blank.

An enzymatic activity unit (E.A.U.) is defined as the amount of enzyme required to cause an increase in absorbance (ΔA) at 280nm of 0.001 per minute when the

*email: ionela.morariu@umfiasi.ro; acluca@yahoo.com

optical path is 1 cm long [13,14]. The formula used for calculating the enzymatic activity of pepsin solution was:

Enzimatic activity (E.A.U./mL = ΔA.1000.200/10.

Pepsic hydrolysis of hemoglobin solution

The following solution were used:

- pepsin stock solution was prepared by dissolving 100 mg pepsin in 20 mL distilled water;
- 14.36% hemoglobin solution was prepared firstly, and then through suitable dilutions 1% (w/v) concentration was achieved;
- 5M sodium hydroxide solution.

50 mL of 1% (w/v) hemoglobin solution (*p*H = 3.0 adjusted with 1M HCl) was mixed with 10 mL pepsin solution (3200 UA/mg) corresponding to a 2.5% (w/v) enzyme/substrate ratio. The hydrolysis reaction was stopped after 2.5 min, using the 5M NaOH to adjust the *p*H value to 9.5 that inactivated the pepsin. The hydrolysate was analyzed by reverse phase high performance liquid chromatography [15,16].

Fractioning of hemoglobin hydrolysate using ion exchange chromatography

A XK 26 column (19.5 × 2.5 cm) filled with a CM-Sepharose Fast Flow gel (Amersham Biosciences, U.S.) was used for the fractioning of the pepsic hemoglobin hydrolysate. The gel conditioning and washing solution was a 20mM ammonium buffer (*p*H = 6.5). The elution solution was a 1M ammonium buffer (*p*H = 6.5). All solutions and the gel had been previously degassed under vacuum and filtered through a 0.45μm Millipore filter. The elution rate was set to 1 mL/min. Elution for fractioning of the hydrolysate was done according to the data from table 1. Each resulting fraction was analyzed by reverse phase high performance chromatography.

Table 1

ELUTION PROGRAM

Volume (mL)	Gradient for elution
0-160	0%
160-560	30%
560-700	70%
700-860	100%

Fractioning the samples by high performance liquid chromatography

The HPLC system used included a Waters TM 600 pump controller and a Waters 996 diode bar detector. A Vydak C4 column (0.46 × 25 cm) was used for separation, because it was suitable for the separation of large peptides, with 20 to 30 amino acids. Schroder et al [17] highlighted its efficacy for the separation of globin chains in non-human hemoglobin.

The mobile phase consisted in an A eluent containing water and trifluoroacetic acid (TFA) in a 1000:1 (v/v) ratio, and a B eluent composed of acetonitrile, water, and TFA in a 600:400:1 (v/v) ratio. The solutions were degassed continuously, thanks to a membrane degasser. Prior to injection, the samples were filtered through a 0.2 μm cellulose acetate filters (AIT Chromato). Separation was carried out in an elution gradient at a flow rate of 1 mL/min. The gradient consisted of a linear increase of the eluent B ratio in the mobile phase from 0 to 60% for the first 30 min, and then from 67 to 87% during the next 35 min.

Fraction analysis by mass spectrometry

All spectrometric measurements were performed in positive ionization mode on a Quatro II triplequadripol equipped with an electrospray ion source.

Results and discussions

The results obtained for the quantitative determination of bovine hemoglobin are presented in table 2. The calculated mean concentration of stock hemoglobin solution was 14.36% (w/v).

Table 2

HEMOGLOBIN DETERMINATION

Sample	Absorbance (λ = 546nm)	Hemoglobin % (w/v)
1	0.391	14.36
2	0.391	14.36
3	0.390	14.35

Table 3 shows the absorbance values that were measured for the blank and for the samples during the analysis of enzymatic activity of pepsin.

Table 3

ANALYSIS OF ENZYMATIC ACTIVITY OF PEPSIN

No	Absorbance (λ = 280 nm)
blank	0.561
sample 1	0.953
sample 2	0.959

ΔA_{280nm} = 0.953 + 0.959 – 0.561 = 0.4

Specific activity = [(0.4 × 1000 × 200)/10] × (20/50) = 3200 E.A.U./mg pepsin

Pepsin activity in solution = (0.4 × 1000 × 200)/10 = 8000 E.A.U./mL

The fractioning of the hydrolysate was performed for 2.5 min on a column packed with 80 mL of CM-Sepharose Fast Flow gel mounted on a Pharmacia LKB apparatus. The elution program used allowed the fractioning of the hydrolysate into 9 fractions (fig. 1).

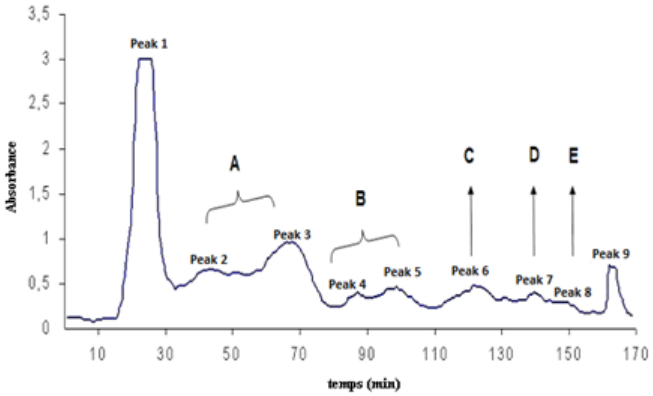


Fig. 1. Chromatographic profile of hemoglobin hydrolysate

The hydrolysate and each fraction recovered on CM-Sepharose Fast Flow was filtered and then injected (50μL) in the RP-HPLC system. Figure 2 shows the chromatograms obtained.

The first peak contains the complex peptide that could not be fractioned on the ion exchange support and went out in the dead volume of the column. Then, there were various peaks marked 2 to 9 containing various peptide fractions. The chromatographic profiles of those peaks showed a reduction in the number of peptides. In addition there were similarities between some of the fractions which allowed grouping the peaks from 2 to 9 into five characteristic fractions marked A to E and which are shown in the figure 1. Those five fractions were analyzed by LC-MS to determine the value of the molecular mass of each peptide. A coupling between a HPLC apparatus fitted with a C4 reverse phase column and an electrospray ionization mass spectrometer (EIMS) was used for the analysis of

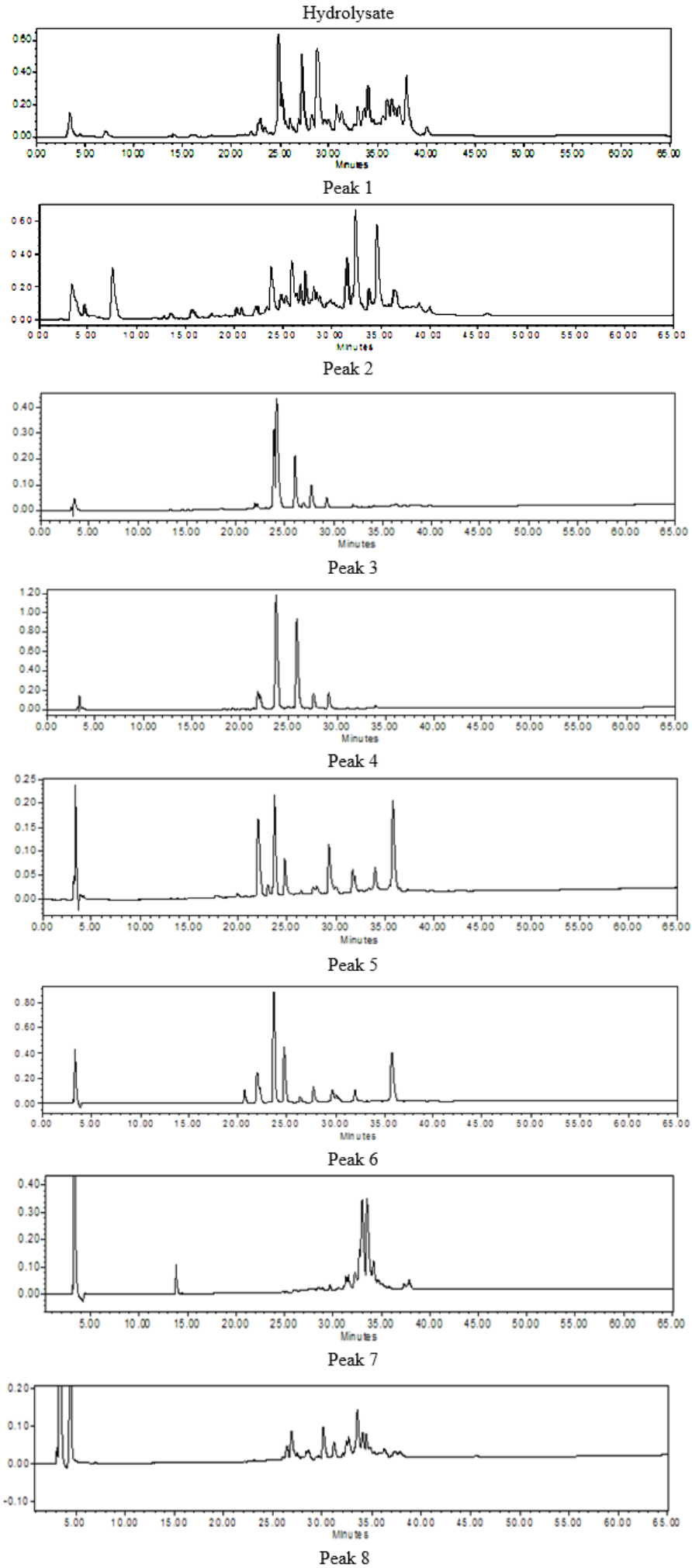
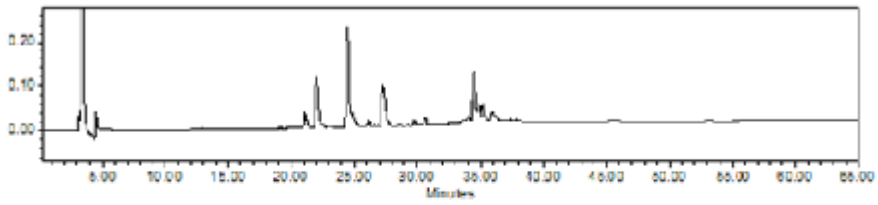


Fig. 2. The chromatograms obtained for the hydrolysate and for each fraction



Peak 9

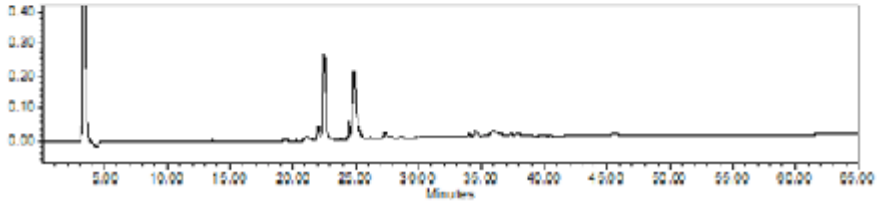


Fig. 2. Continuated

A-E fractions obtained through ion-exchange chromato-graphy (fig. 3).

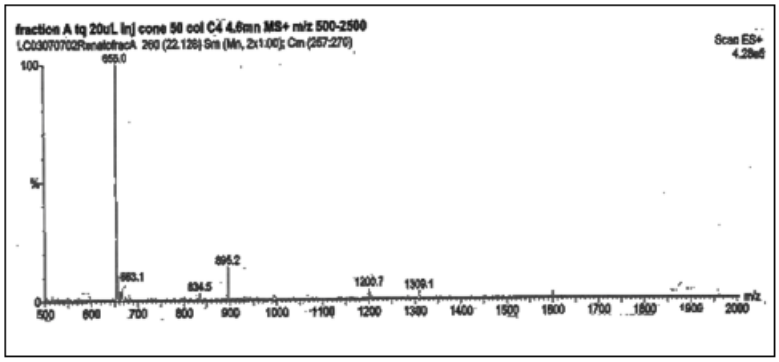


Fig. 3. MS profile of fraction A

Fraction	Retention time (min)	Molecular mass (Da)	Peptide sequence
A	16.09	2656	α (1-27)
	16.09	2727	α (1-28)
	18.05	2840	α (1-29)
	18.05	1812	β (14-30)
	20.43	3256	α (1-32)
	26.63	1813	β (14-30)
	22.13	1308	β (31-40)
	23.91	4795	β (40-83) or (41-84)
	28.84	3750	-
	30.88	3787	α (107-141)
B	14.48	2236	α (1-23)
	16.01	1748	α (87-98)
	17.79	2747	-
	17.79	2840	α (1-29)
	18.89	3165	-
	20.17	3256	α (1-32)
	20.17	1842	-
	26.38	1813	β(14-30)
	22.13	3333	β(31-40)
	22.13	1308	α (33-45) or (34-45)
	23.91	1585	-
	23.91	3402	β (40-83) or (41-84)
	25.44	1995	-
	26.38	2193	β (126-145)
	28.50	3851	-
	28.50	3537	-
	29.78	3472	-
	30.54	3787	α (107-141)
C	19.07 & 20.94	4001	-
	20.94	3740	-
	22.72	2640	
	24.34	3994	-
	25.44	4601	-
	26.89	4846	-
	26.89	5094	-
	26.89	5277	-
	28.59	5421	α (33-83)
	28.59	7002	α (33-97) or (34-98)
	28.59	7151	-
	29.44	4030	-
	31.73	3964	-

Table 4
IDENTIFICATION OF PEPTIDES

D	20.68	4001	-
	20.68	3740	-
	22.55	2640	-
	23.66	4678	-
	26.63	5094	-
	26.63	5277	-
	26.63	4942	-
	28.00	5421	α (33-83)
	28.00	7002	α (339 7) or (34 98)
	28.00	7151	-
E	14.73	1431	α (87-98)
	16.43	1733	β (130-145)
	18.98	3760	-
	20.17	1813	β (14-30)
	20.17	3553	β (114-145)
	21.36	3564	-
	21.36	3681	-
	22.63	4647	-

Table 4
CONTINUATED

If such a peptide is fractionated several times during its passage through the quadrupoles of the mass spectrometer, we noticed the occurrence of several successive peaks that allowed us to identify it [18,19].

For each fraction, table 4 shows: retention time in minutes; molecular weight in Dalton, and the sequences of the α or β chains of the peptides from the bovine hemoglobin eluted in the HPLC column and identified by mass spectrometry.

The results showed the existence of peptides with the same sequences in fractions A and B (e.g. α (1-29), α (1-32), β (14-30), β(31-40), β) or in fractions C and D (e.g.,α (33-83), α (33-97), α (34-98) [13]. Fraction E contained peptides found in no other fraction. At the same time, many values of the molecular mass remained to be determined.

Conclusions

In the present work a peptidic hydrolysate has been obtained by hydrolysis of bovine hemoglobin using pepsin. The bovine hemoglobin used in this study is a model substrate because it combines exceptional features such as its structure and its perfectly known sequences and the possibility of being obtained in pure state [14, 15]. The 2.5 minute hydrolysate showed a peptide population between 35 and 38 which was composed of large peptides. The MS analysis of the isolated protein revealed a series of multiple charged species of bovine α- and β-globin chains [16, 17]. The majority of the sequences were identified.

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