QUALITY EVALUATION OF FREEZE-DRIED AMMODYTES AMMODYTES VENOM BY CGE ON-A CHIP WITH LASER INDUCED FLUORESCENCE DETECTION

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Abstract: *Our study represents the first attempt to develop a protocol for evaluating the quality freeze-dried venom by using current proteomics analysis techniques*
The research carried out represents the initial step in the validation of the method for protein analysis by capillary electrophoresis gel with laser induced fluorescence detection , being our first attempt in determine the impairment of protein fractions during long storage of the freeze-dried venom .
Freeze-drying was used as a method of advanced dehydration Ammodytes ammodytes venom and the yield of these procedure went up to 25.92 %.
On a long-term storage at -20 ° C freeze-dried venom of the factions protein degradation occurs , the most affected being that with the molecular weight of approx . 28 and 37 kDa , respectively identified as Vammin and phospholipase A2 , as demonstrated on the one hand, by decreasing the % of these fractions and on the other hand, by increasing the % of protein fractions obtained by degradation.

Key words*: ammodytes ammodytes venom, freeze-dry, CGE-on a chip*

The use of use of Ammodytes ammodytes viper venom in therapeutics has become a very topical subject (7,8,12), which is also exploited by the vipers farmers market in our country.

Processing by lyophilization of Ammodytes ammodytes viper venom is a preparing method for the conservation of this biological material with high value on pharmaceutical market (10, 14,17,19)..

Due to the lack of a widely accepted protocol for assessing the quality of the venom, the study proposed in this paper represents the first attempt to develop a protocol for assessing the quality of freeze-dried venom by using current techniques of proteomics analysis (2,6,15,16).

The research carried out started with the validation of the method for analysis of proteins by capillary electrophoresis in gel with detection in laser-induced fluorescence (18,21), in an attempt to first determine the impairment of protein fractions according to the duration of the storage of freeze-dried venom.

1. MATERIALS AND METHODS

Sampling

Venom samples were harvested on the farm Viperin Invest SRL Ineu, Arad county, which has a nest of 450 Ammodytes vipers and Ligia Borşanu Individual with a workstation in Marga village, Caras Severin county, with 30 Ammodytes ammodytes vipers.

Fig.1. Venom sampling

Samples were collected in sterile urine containers harvested by rubber membrane (Fig.1). The harvest period was 2013-2015, collected venom being stored at -20C until processed further. The samples were rated from 1 to 10 and their collection period is shown in Table 1.

Table 1.

Sample harvesting periods

Sample no.	1	2	3	4	5	6	7	8	9	10
Year/month of harvest	2015/ 01	2015/ 05	2015 /05	2015/ 05	2014/ 02	2014/ 06	2014/ 10	2014/ 11	2013/ 03	2013/ 06

Freeze-drying venom

Freeze drying venom samples was performed at USAMVBT Interdisciplinary Research Platform in the Biochemistry Research Laboratory.

The method includes the following steps: weighing of the initial liquid venom, freezing the vials and lyophilized, freeze-drying, homogenization of the final product, the final weighing. The freeze drying machine used was IlShin Europe, made in the Netherlands, model: Kryptonstraat 11 6718 WR EDE, lyophilization conditions are the following: -54ºC temperature, pressure of 5 mTorr for lyophilization time 48 hours.

The validation method was performed by determining the solid substance according to the standardized method at 103ºC (3).

The 10 samples were freeze-dried at the time of harvesting and were stored in a freezer at -20 ° C in Eppendorf tubes sealed with paraffin film in order to prevent the wetting of the samples. The yield of freeze-drying was calculated as a percentage of dry matter obtained based compared to the initial amount taken into it.

Determination of protein content

Protein content analysis was performed with the Thermo Scientific ™ Coomassie (Bradford) (22) protein test kit, a colorimetric method based on the property of Coomassie blue dye to bind proteins in an acidic environment with an immediate change of the absorption maximum from 465nm to 595nm and a color change from brown to blue.

Analysis was performed at the Interdisciplinary Research Platform in Biochemistry Research Laboratory using UV-VIS spectrophotometer Specord 210, Analytic Jena and data processing was performed with RidaWin software.

Protein concentrations were estimated by reference to the absorbance obtained for a dilutions series of protein standard which are analyzed together with unknown samples. Since the Coomassie color response is nonlinear with the protein concentration increases, a standard curve is required to complete each test.

The calibration curve is a 4 parameters curve type, made by 8 points, albumin concentrations in standards being 2000, 1500, 1000, 750, 500, 250, 125, 25 µg/ml.

The amount of sample used or standard is 30ul over which is 1.5ml Coomassie reagent pured and shaken. Incubate for 10 min. at room temperature and read against distilled water in the spectrophotometer set at 595nm. At the same time a blank is carried out where the sample is replaced with deionized water.

Quality control of the results was carried out with certified reference material Sigma Aldrich code A1933.

The analysis of proteins by capillary gel electrophoresis with laser-induced fluorescence detection

Analysis of samples of freeze-dried venom by capillary gel electrophoresis with laser induced fluorescence detection (CGE) was conducted in the USAMVBT`s Horia Cernescu Research Laboratories Complex - CLC-HC, in the Molecular Biology Laboratory (A2). The equipment used was a Agilent 2100 Bioanalizor and for the analysis of protein fractions a Protein 80kit kit of the same manufacturer (21) was used according to the method described by Halassy and colab. (9).

Ladder (standard fractions) has the following molecular weight values: 1.60, 3.5, 6.50, 15.00, 28.00, 46.00, 63.00, 95.00 kDa.

The steps in the test procedure are described in the insert kit and are the following: preparing the mixture of gel-dye, prepaping the discoloration solution, preparation the distortion solution, sample and the ladder preparation, charging the chip with the mixture of gel-dye and solution fade, loading the chip with ladder and testing, migration, writing of the analysis report.

2. RESULTS AND DISCUSSION

Lyophilization

Lyophilisation yield expresses the percentage of dry matter relative to the initial quantity. The water content of the samples was ranged from 78.4 to 74.08% (Figure 2). The literature indicates a variation of the parameter range between 70-80% (3, 13).

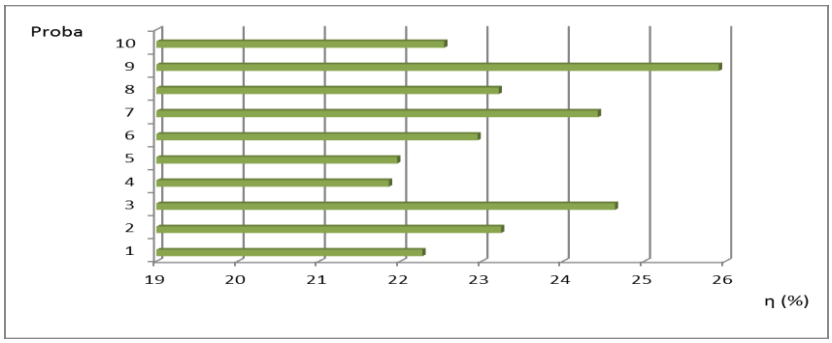

Fig. 2. Graphical representation of the variation in yield lyophilization

Analysis of protein content

The calibration curve obtained and its equation is shown in Figure 3, remarking the regression factor of the curve with a value of 0.995. Quality control of the results was ensured by using certified reference material Sigma-Aldrich, with a declared value of the protein content of over 98%. The value obtained for the reference material was 98.56%.

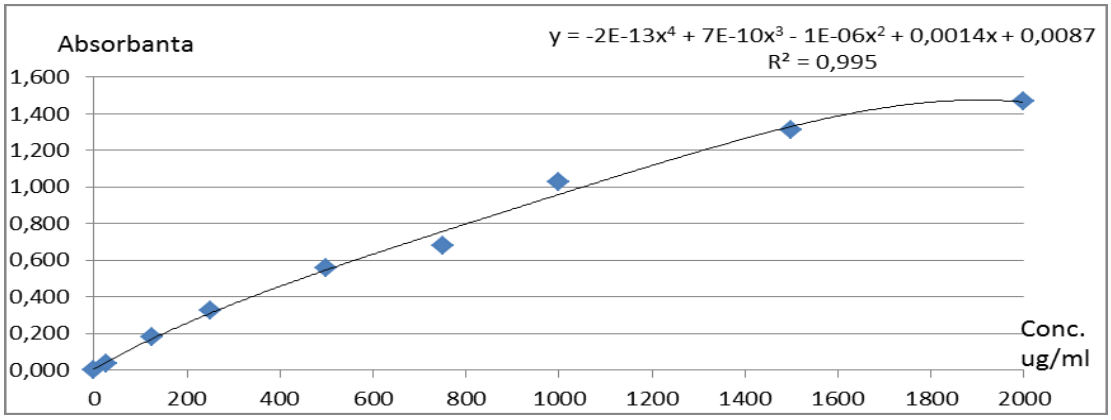

Fig 3. The calibration curve type 4 parameters - Blue Commasie method

The protein content of the analyzed samples ranged from 88.85 to 92.40 g%. Existing data in the literature on the analysis of the venom protein content of Ammodytes ammodytes indicate varying intervals depending on the method used. Thus, using the biuret method values in the range of 80-85% (3) wre obtained, while the values obtained using the Lowry method were over 95% (9).

By comparing the methods Comassie and Lowry, the values obtained were higher in the second case, this being attributed to false positives reaction of the phenolic reagent with low molecular weight phenolic compounds type from venom (11) .

The graphical representation of the variation of the protein content is given in Figure 4.

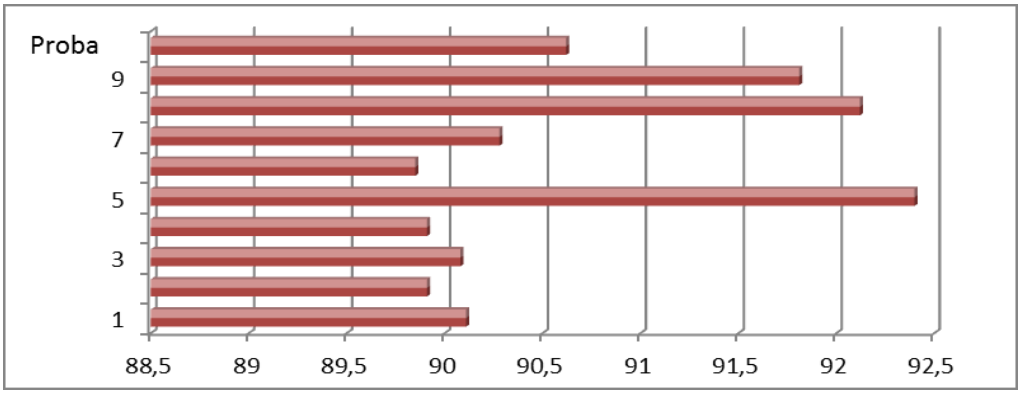

Fig. 4. Variation in protein content

2. 3. The analysis of proteins by capillary gel electrophoresis with laser-induced fluorescence detection

The gel obtained after migration is shown in Figure 5. The first position shows the ladder's migration and the next 10 correspond to the samples.

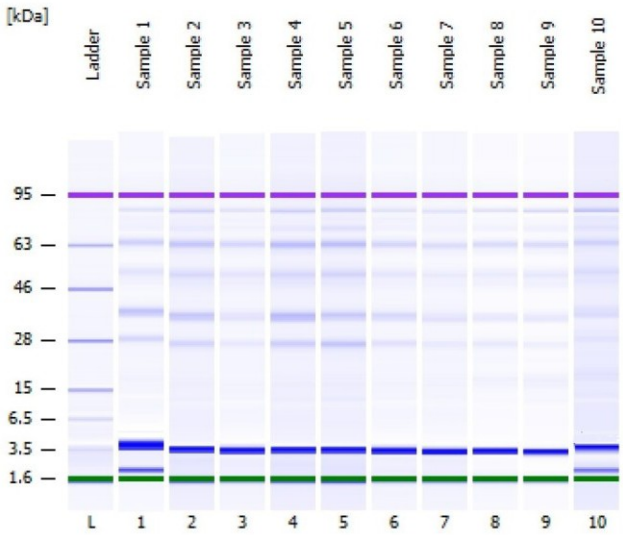

Fig5. Appearance of the gel after protein fractions migration

Percentage distribution analysis of the protein fractions identified 5 fractions of interest namely 28kDa, 37kDa, 53kDa, 64kDa and 85kDa, the difference being represented by other factions in a proportion not exceeding 3% for each of the values obtained are shown in Table 2. The marker is found at the lowest and the highest 1,6kDa to 95kDa. Peaks system are contained in the range of 2.2 and 3.5 kDa.

Table2

Protein fractions percentage distribution

Sample	Faction 28kDa	Faction 37 kDa	Faction 53 kDa	Faction 64 kDa	Faction 85 kDa	Other fractions
1	19,7	34,1	10,6	18,2	5,5	11,9
2	17,7	26,7	14,2	20,1	7,1	14,2
3	14	23,1	16,1	24,6	8,5	13,7
4	17,9	20,1	17	21	6,6	17,4
5	24,7	22,2	17,4	21,9	8,2	5,6
6	17,9	28,7	12,6	21,1	7,1	12,6
7	15	27,6	10,4	21,9	9,2	15,9
8	17,1	15,5	12,3	21,8	8,5	24,8
9	8,9	16,2	14,1	17,7	9,2	33,9
10	5,7	16,4	12,7	16,4	14,4	34,4

It is noted that the fraction of approx. 28kDa is deeply affected by storage period, the percentage of registered samples collected in 2013 were up 19% lower than the maximum value obtained from samples collected in 2015. Yamazaki and colab. have identified this fraction as V ammin (endotelio-vascular venom growth factor like) (20).

37kDa fraction corresponding to phospholipase A 2 (5, 9) also shows a decreasing trend, yielding 17.7% lower values than the maximum recorded in 2015 for freeze-dried samples.

The fraction of approximately 53kDa identified in the literature as belonging to the ammotidase (1, 2, 9), does not show any significant variation in the percentage of long-term storage. The same trend was registered and the fraction of approximately 64kDa.

In a comparative analysis of protein fractions stored from 2013 samples (Samples 9 and 10) compared to those in 2015 (samples 1-5) there is a considerable increase in the proportion of other cuts which may be an indicator of quality degradation of the venom during long storage, by proteolysis (Figure 6).

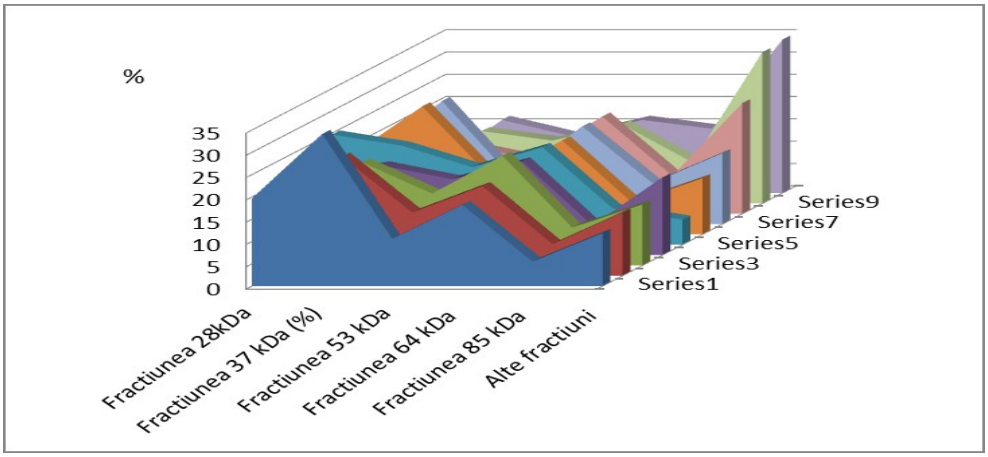

Fig 6. Comparative presentation of all factions in samples 1-10

3. CONCLUSIONS

3. 1. Freeze-drying used as a method of advanced dehydration of the Ammodytes ammodytes venom yielded a maximum of 25.92%.

3.2. The protein content of the freeze-dried venom varied in the 88.85 to 92.40 g% range.

3.3 In a long-term storage of freeze-dried venom at -20ºC, a degradation of the protein fraction appears, the most affected being the molecular weight of approx. 28 and 37 kDa, respectively identified as Vammin and phospholipase A2. After 3 years storage in this conditions, the degradation was demonstrated on the one hand by the % decrease of these fractions and on the other hand, by increasing the% of the protein fraction obtained by degradation.

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