 doi:10.20944/preprints202107.0159.v1

Article

On the Applicability of Electrophoresis for Protein Quantification

Karina Dome [1,*], Zoya Akimenko [1], Aleksey Bychkov [1,2], Yuri Kalambet [3] and Oleg Lomovsky [1]

[1] Institute of Solid State Chemistry and Mechanochemistry, Siberian Branch, Russian Academy of Sciences, Kutateladze Str. 18, Novosibirsk 630128, Russia; domekarina@ya.ru (K.D.), akzoia56@mail.ru (A.Z.), lomov@solid.nsc.ru (O.L.)
[2] Department of business, Novosibirsk State Technical University, K. Marks Ave. 20, Novosibirsk 630073, Russia; bychkov.a.l@gmail.com (A.B.)
[3] "Ampersend ltd.", Russia, Moskow, 2 Kurchiatov pl., 123182; kalambet@ampersand.ru (Y.K.)
* Correspondence: domekarina@ya.ru; Tel.: +7-960-790-87-72 (K.D.)

Abstract: Polyacrylamide gel electrophoresis (PAGE) is widely used for studying proteins and protein-containing objects. However, it is employed most frequently as a qualitative method rather than a quantitative one. In this paper, we show the feasibility of routine digital image acquisition and mathematical processing of electrophoregrams for protein quantification. Both the well-studied model protein molecules (bovine serum albumin) and more complex real-world protein-based products (casein-containing isolate for sports nutrition), which were subjected to mechanical activation in a planetary ball mill to obtain samples characterized by different protein denaturation degrees, were used as study objects. Protein quantification in the mechanically activated samples was carried out. The degree of destruction of individual protein was shown to be higher compared to that of protein-containing mixture after mechanical treatment for an identical amount of time. The methodological approach used in this study can serve as guidance for other researchers who would like to use electrophoresis for protein quantification both in individual form and in protein mixtures. The findings prove that photographic imaging of gels followed by mathematical data processing can be applied for analyzing the electrophoretic data.

Keywords: electrophoresis; protein; mechanical treatment; quantification.

1. Introduction

Protein chemistry methods are currently used to control, optimize, and elaborate novel technologies in molecular biology, pharmacology, bioengineering, and food technology [1–5]. Such efficient, fast, illustrative, and reproducible methods as HPLC, HPLC–MS, and PCR coupled with Sanger sequencing are used for protein quantification [6–9]. Despite the rapid progress in fast and efficient techniques employed for protein identification and quantification, simpler and more accessible analytical techniques (e.g., the conventional colorimetric measurements) also remain relevant [4, 10, 11]. Thus, these methods are used for Lowry protein assay in solutions in a reaction with the Folin reagent [11] or Bradford protein assay with Coomassie dye [12].

Protein-containing objects are usually analyzed by 1D and 2D polyacrylamide gel electrophoresis (PAGE), with sodium dodecyl sulfate (SDS) used as a detergent [10, 13–17]. Staining with dyes that bind irreversibly to protein molecules but do not form stable bonds with polyacrylamide gel is often employed for protein detection in the gel [18–20]. The intensity of stained bands in gel depends on the amount of the applied sample; i.e., it is assessed according to the laws of colorimetric measurements: staining intensity is directly proportional to protein content.

Electrophoregrams are illustrative and informative. However, this technique is most typically used as a qualitative method and quite rarely as a semi-quantitative test (only visual assessment of band staining intensity is performed). The colorimetric approach (usually the visual one) is also employed in individual cases typically related to molecular biology for measuring the resolution during protein separation in polyacrylamide gel, as well as for protein quantification. In quantification assay, electrophoretic separation is used together with enzyme-linked immunosorbent assay or western blotting [21, 22], which require respective immune sera against the target proteins.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 July 2021 doi:10.20944/preprints202107.0159.v1

In recent practical studies, there is demand for protein quantification in complex systems containing numerous impurities of protein and non-protein nature. Thus, we previously used polyacrylamide gel electrophoresis to determine the depth of hydrolysis of pea seed proteins [23]. The resulting hydrolysate enriched with free amino acids and peptides was used as a component of functional foods. The method combines the qualitative and quantitative assays of a protein mixture by polyacrylamide gel electrophoresis and simultaneous assessment of concentrations of the mixture components. It can also be used to develop special nutrition products containing pea seed proteins [24].

In order to optimize the procedure for analyzing the drug aprotinin, the currently existing labor-intensive technique of electrophoretic analysis was replaced with HPLC analysis [25]. Meanwhile, as aprotinin derivatives have a protein nature, they can be analyzed by polyacrylamide gel electrophoresis. The target aprotinin and its impurities can be detected by gel electrophoresis as clearly as by chromatography [26]. This approach can also be proposed for monitoring product purification in various bioengineering processes (novel forms of food products [24] or novel sorbents for protein purification [27]) and in the development of pharmaceuticals [28].

Therefore, this approach can be employed for manufacturing pharmaceutically important products, such as bovine serum albumin. Simultaneous quantitative and qualitative monitoring of purification of the target product, albumin, will be useful in novel technologies [27].

The patent for an invention of a method for antibody isolation and purification can be mentioned as an example of using this technique for pharmaceutical products [28]. In this and similar studies, it is also convenient and efficient to perform manufacturing process monitoring and simultaneous quantitative assessment of concentrations of immunoglobulin components both during the purification stages and in the target products using PAGE.

Having analyzed our experience, it is fair to say that the applicability of protein quantification by electrophoresis is currently limited by the following factors. First, there are certain difficulties related to obtaining digital images of the gels. The currently available scanners and densitometers are not common equipment; their resolution is insufficient to work with a densitogram like with a chromatogram. Second, the existing software mostly specializes on electrophoresis of nucleic acids and therefore uses a different signal-to-noise ratio.

This study makes a methodological attempt to use electrophoresis for protein quantification. The specially designed test bench for digital imaging of gels and optimally selected software allows one to quickly and easily determine the molecular weight distribution of protein molecules in the samples and perform quantitative assay. This will enable quality control of protein products according to the quantitative contents of fractions of protein molecules and presence of impurities.

2. Materials and Methods

Materials. Bovine serum albumin (#SLBB7759V, Sigma Aldrich, USA) was used as the model study object. This protein was applied both in its non-modified form and after vigorous mechanical treatment.

A protein-containing product, Kultlab Isolate ISO 90% sports nutrition supplement (Kultlab, Russia) with 90% casein content, was used as an experimental study object.

Mechanical treatment. In order to obtain samples characterized by various degrees of protein molecule destruction, BSA and the Kultlab sports nutrition supplement predominantly containing casein were subjected to mechanical treatment on an AGO-2 laboratory planetary ball mill (acceleration of the grinding media, 200 m/s2). Treatment duration was varied between 5 and 30 min. Weight of the sample loaded into the reaction jars was 5 g per 200 g of the grinding media (steel balls 6 mm in diameter).

SDS-polyacrylamide gel electrophoresis was carried out using the Laemmli protocol [20]. Polyacrylamide concentration in the stacking and resolving gels was 5% and 13%, respectively. Gel was stained using Coomassie R-250 dye (ThermoFisherScientific, USA). Unstained protein MW marker (ThermoFisherScientific, USA) with protein molecular weight ranging between 14.4 and 116 kDa was used as a protein marker.

BSA solution in a lysing buffer (2 mg/mL) for being applied onto the gel lanes was prepared according to the Laemmli protocol [20]. The calibration BSA solutions were prepared by twofold serial dilution. BSA concentration in the calibration solutions ranged from 0.0125 to 0.2 mg/mL. For calibration, the solutions were applied in such a manner that BSA concentration on the polyacrylamide gel lanes was sequentially reduced twofold. The samples of protein mixtures (components of sports nutrition) after mechanochemical activation were prepared using the same procedure. Dilution of sports nutrition samples was selected so that the band intensity lay within the calibration plot.

Protein quantification. In order to save the electrophoresis results, we took photos of the gel with a camera (Olympus, Japan) with a 64 MP resolution. The photos were taken on a specially designed test bench with six light sources ensuring uniform illumination of the object.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 July 2021 doi:10.20944/preprints202107.0159.v1

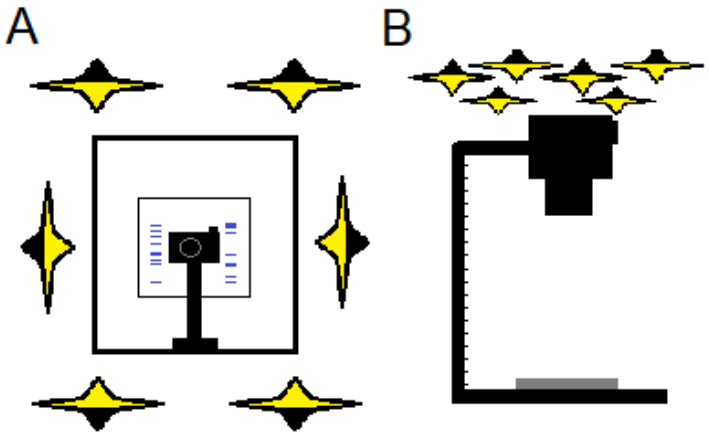

Figure 1. A schematic diagram of the photographic test bench: (A) top view; (B) side view.

Taking photos of the gel under these conditions allows one to obtain a densitogram with a resolution being manifold higher than the resolution attainable using scanners for gels. Below, we will demonstrate that densitograms can be handled in the same manner as chromatograms.

The grey-tone photo images of polyacrylamide gels with stained protein bands were used for protein quantification. Mathematical data processing was performed using the MultiChrom Planar software in order to obtain a dependence between protein concentration and band color intensity/peak area [29]. The results of quantitative measurements were processed and saved using the MultiChrom Planar software.

3. Results and Discussion

As already mentioned, the electrophoresis results are most often assessed visually, and it is a qualitative assessment. In this study, the results were analyzed using the MultiChrom Planar software consisting of two programs: the Planar software for image conversion to densitograms and the MultiChrom-Planar software performing quantitative processing of densitograms (Fig. 2) [29].

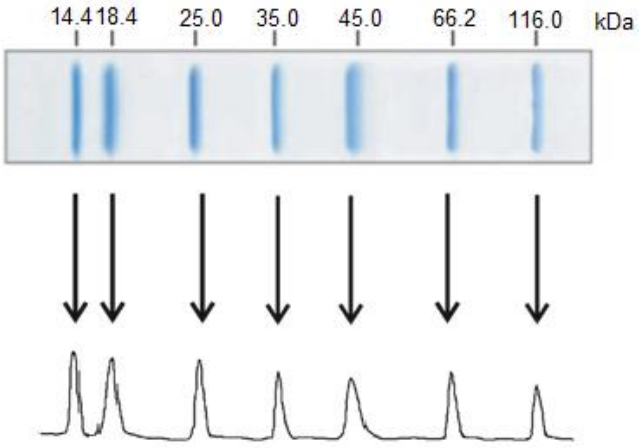

Figure 2. Converting the electrophoretic profile of a protein marker into a densitogram using the MultiChrom Planar software.

The area of the resulting chromatographic peaks in the densitogram depends on band color intensity, which corresponds to protein content. The calculations were performed for each peak according to the standard operating procedure of the MultiChrom Planar software.

Figure 3 shows an example of the electrophoregram of calibration BSA samples prepared by serial dilution. One can see that band staining intensity in the solutions applied onto the gel varies in accordance with protein content (Fig. 3).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 July 2021 doi:10.20944/preprints202107.0159.v1

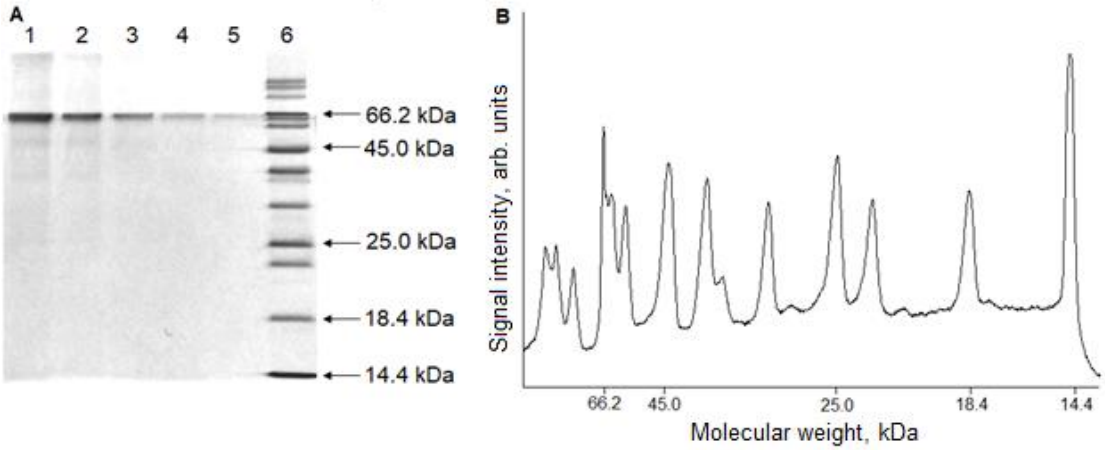

Figure 3. (***A***) An example of the electrophoregram of calibration BSA solutions with concentrations 0.2, 0.1, 0.05, 0.025 and 0.0125 mg/mL, respectively (lanes 1–5) and the reference sample with known molecular weights (lane 6). Volume of the applied sample = 10 μL. (***B***) A densitogram of the reference sample.

The electrophoregram was converted to densitograms using the MultiChrom Planar software (Fig. 3B). As a result of data processing, the area of analytical peaks depending on band color intensity in the gel was measured for the BSA samples with different protein concentrations (lanes 1–5). Table 1 summarizes the results of intensity measurements (peak area in arb. units).

Table 1. The resulting data for plotting the calibration plot.

Lane number	Protein content in the sample, μg	Peak area, arb. units
1	2.0	1288±49
2	1.0	934±35
3	0.5	763±29
4	0.25	525±20
5	0.125	348±13

The resulting data were used to build a calibration plot "protein concentration vs. peak area" for BSA (the calibration protein) (Fig. 4). A quadratic calibration dependence was obtained:

$$Q = 0.1 * 10^{-5} * S^2 + 2.6 * 10^{-4} * S, \quad (1)$$

where Q is the protein content (μg) and S is the area of the chromatographic peak on the densitogram. This dependence is standard for planar chromatography or gel electrophoresis [30].

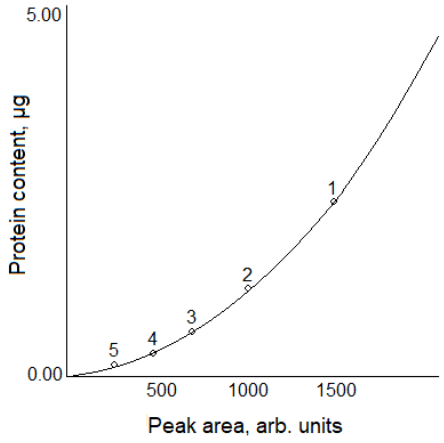

Figure 4. The calibration plot for BSA quantification.

The relative deviation was 3.8%. The molecular weight of the analyzed bovine serum albumin (68.53 kDa) was determined using the known molecular weights of protein marker. The results correlate with the UniProt database values [31, 32].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 July 2021 doi:10.20944/preprints202107.0159.v1

For further studies, the calibration and test samples were applied on the same gel. In this case, all the calculations conducted for the same gel prevent the problems related to the possible non-uniformity of background staining and differences in gel concentration.

In order to obtain BSA samples characterized by different degradation degrees, they were subjected to mechanical treatment for different time. Sample concentration for electrophoresis analysis was selected so as the intensity of the stained bands lay within the calibration curve plotted for native BSA (shown in the same gel on lanes 6–10) (Fig. 5).

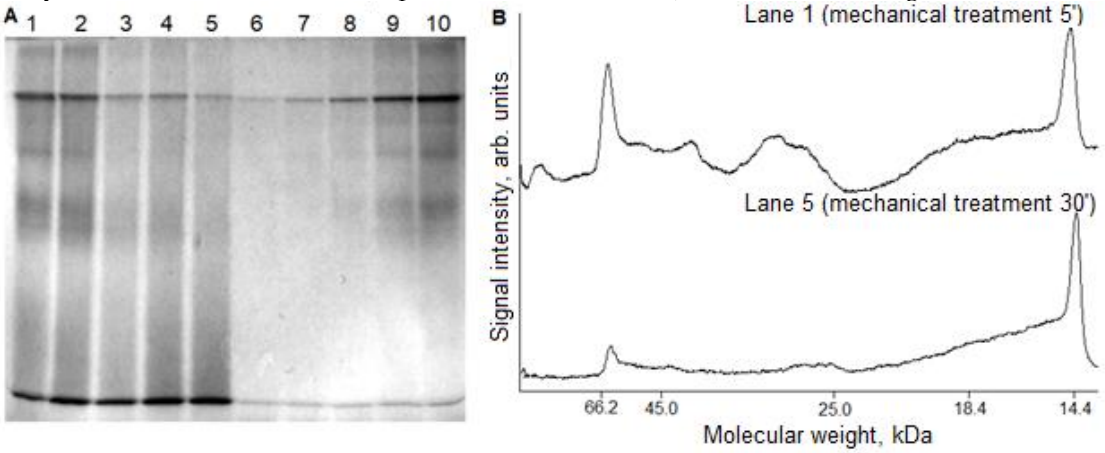

Figure 5. (***A***) Electrophoregram of the BSA degradation products after mechanical treatment for 5, 10, 15, 20, and 30 min (lanes 1–5) and the calibration BSA samples with concentration ranging from 0.0125 to 0.2 mg/mL (lanes 6–10); volume of the applied sample = 10 μL. (***B***) Densitograms of the BSA degradation products after mechanical treatment for 5 and 30 min (lanes 1 and 5).

The calibration plot was used as the standard of quantitative measurements to calculate the amount of BSA remaining in the sample after mechanical treatment (Fig. 6). Figure 6 shows the data on the degree of degradation (α) of protein molecules calculated using the formula:

$$\alpha = \frac{\Delta S}{S_0} = \frac{S_0 - S_t}{S_0} * 100\%, \quad (2)$$

where ΔS is the change in the area of the peak corresponding to the native protein molecule after mechanical treatment (treatment duration t); S_t is the area of the peak corresponding to the native protein molecule after mechanical treatment (treatment duration t); and S_0 is the area of the peak corresponding to the native protein molecule before mechanical treatment.

Protein molecules in BSA subjected to 30-min mechanical treatment were degraded by 92 ± 3%.

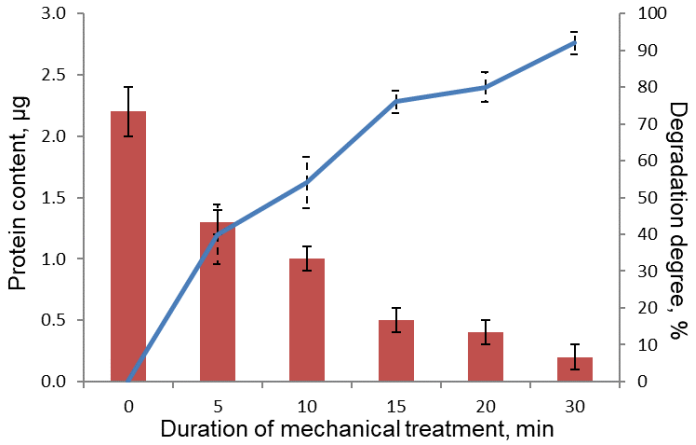

Figure 6. Protein content in the samples of mechanically treated BSA.

The experiment involving mechanical treatment of milk protein isolate (a sports nutrition mix) and quantitative calculation of casein degradation products during this treatment was conducted in a similar way.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 July 2021 doi:10.20944/preprints202107.0159.v1

Figure 7 shows the electrophoregram of the samples of milk protein isolate before and after mechanical treatment for 5, 10, 15, 20, and 30 min. One can see that destruction of casein protein molecules also takes place during mechanochemical treatment (Fig. 7).

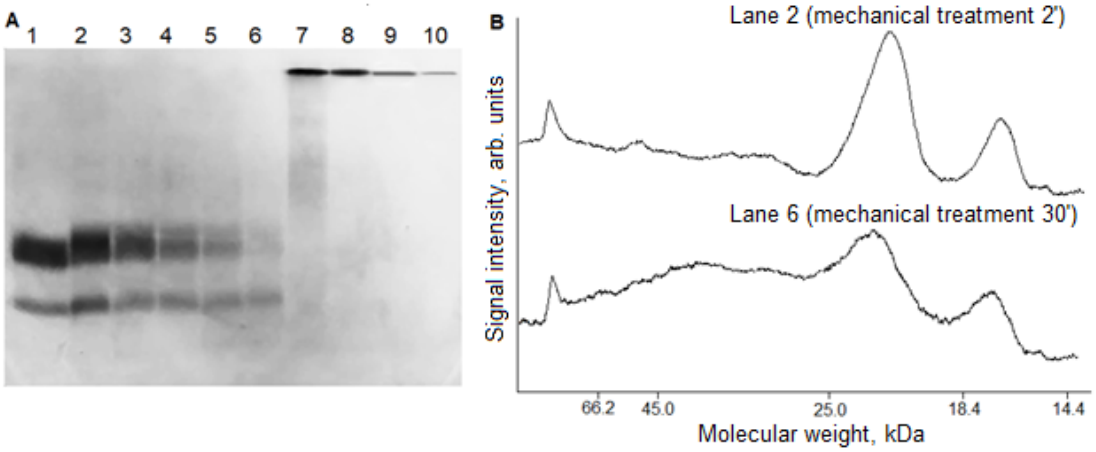

Figure 7. (*A*) Electrophoregram of the initial sample of milk protein isolate (lane 1) and the samples of milk protein after mechanical treatment for 30, 20, 15, 10, and 5 min, respectively (lanes 2–6), as well as the calibration BSA samples with concentration ranging from 0.125 to 2.0 mg/mL (lanes 7–10). (*B*) Densitograms of BSA degradation products after mechanical treatment for 5 and 30 min (lanes 2 and 6).

The BSA calibration plot was used to obtain a dependence that allowed one to calculate casein content in the samples (in μg) before and after mechanical treatment for 5, 10, 15, 20, and 30 min. The results are shown in Fig. 8.

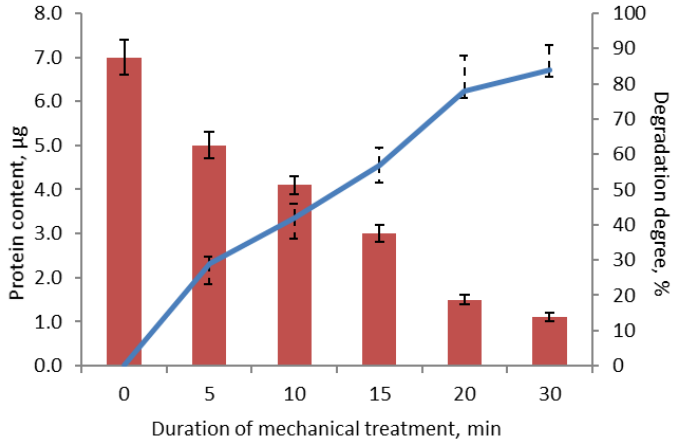

Figure 8. Protein content in milk protein isolate before and after mechanical treatment for 5, 10, 15, 20, and 30 min.

It was demonstrated that the degree of degradation of protein molecules within the sports nutrition product after mechanical treatment for 30 min was 85 ± 2%, being comparable to the data for individual protein (BSA).

Hence, it has been shown that polyacrylamide gel electrophoresis coupled with simultaneous recording photographic images of the gels and mathematical data processing using the MultiChrom Planar software allows one to measure protein content in the test sample directly in polyacrylamide gel (identically to the known colorimetric methods for protein quantification). This technique has made it possible to estimate the degree of protein degradation for the model BSA protein and casein (a component of sports nutrition product). The procedure allows protein quantification for various applied problems such as performing control over protein impurities or regulating the content of target protein substances with simultaneous control over the molecular weight distribution.

4. Conclusions

Photographic imaging of gels followed by mathematical data processing using the MultiChrom Planar software was used for quantifying band intensities of proteins in polyacrylamide gel. The findings have proved that this algorithm can be applied to process the electrophoretic data. The relative inaccuracy of the method was estimated using calibration solutions. To make protein quantification more accurate, it was suggested to use calibration solutions to-

gether with test samples so that all the variable factors during the analysis and recording the results could be taken into account.

Protein-containing substances, BSA (as a model protein) and casein within sports nutrition product, were subjected to mechanical treatment. The proposed method was used to obtain the data on dependence between the degradation degree of protein molecules and duration of mechanical treatment. Mechanical treatment of BSA for 30 min caused degradation of protein molecules by 92 ± 3%, while protein molecules within sports nutrition product were degraded by 85 ± 2%. The degree of destruction of individual protein was higher compared to that of protein-containing mixture after mechanical treatment for an identical amount of time.

The methodological approach used in this study can serve as guidance for other researchers who would like to use electrophoresis for protein quantification both in individual form and in protein mixtures.

Author Contributions: Conceptualization, A.B.; funding acquisition, A.B.; investigation, K.D. and Z.A.; methodology, Z.A., A.B. and Y.K.; software, Y.K.; resources, A.B. and O.L.; writing—original draft preparation, K.D. and Z.A.; writing—review and editing, A.B., Y.K. and O.L.; visualization, K.D.

Funding: The research was funded within the state assignment to ISSCM SB RAS (project No. FWUS-2021-0005).

Determination of energy consumption was carried out with the support of the Russian Science Foundation (project No. 19-73-10074).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nice, E.C. The separation sciences, the front end to proteomics: An historical perspective. *Biomedical Chromatography* **2021**, *35*, # e4995.
2. Tyanova, S.; Albrechtsen, R.; Kronqvist, P.; Cox, J.; Mann, M.; Geiger, T. Proteomic maps of breast cancer subtypes. *Nat Comms* **2016**, *7*, # 10259.
3. Stadlmann, J.; Taubenschmid, J.; Wenzel, D.; Gattinger, A.; Dürnberger, G.; Dusberger, F.; Elling, U.; Mach, L.; Mechtler, K.; Penninger, M. Comparative glycoproteomics of stem cells identifies new players in ricin toxicity. *Nature* **2017**, *549*, pp. 538-542.
4. Garrido, B.C.; Souza, G.H.M.F., Lourenco, D.C.; Fasciotti, M. Proteomics in quality control: Whey protein-based supplements. *Journal of Proteomics*, **2016**, *147*, pp. 48-55.
5. Nwachukwu, I.D.; Aluko, R.E. A systematic evaluation of various methods for quantifying food protein hydrolysate peptides. *Food chemistry*, **2019**, *270*, pp. 25-31.
6. Gagnon, P.; Goricar, B.; Mencin, N.; Zvanut, T.; Peljhan, S.; Leskovec, M.; Strancar A. Multiple-monitor HPLC assays for rapid process development, in-process monitoring, and validation of AAV production and purification. *Pharmaceutics*, **2021**, *13*, # 113.
7. Liu, H.; Chen, F.; Wang, B.; Wang, H.; Jin, S.; Yang, Z.; Chen, Y.; Quan, Y.; Xiang, X. A Sensitive HPLC-MS/MS method for the quantification of selegiline in Beagle dog plasma: application to a pharmacokinetic study. *Current Pharmaceutical Analysis*, **2021**, *17*, pp. 140-148.
8. Lagoutte-Renosi, J.; Royer, B.; Rabani, V.; Davani, S. Method for the determination of plasma ticagrelor and its active metabolite useful for research and clinical practice validation of an HPLC–MS/MS. *Molecules*, **2021**, *26*, # 278.
9. Faller, A.C.; Arunachalam, T.; Shanmughanandhan, D.; Kesanakurti, P.; Shehata, H.R.; Ragupathy, S.; Newmaster, S.G. Investigating appropriate molecular and chemical methods for ingredient identity testing of plant-based protein powder dietary supplements. *Scientific reports*, **2019**, *9*, # 12130.
10. De Silva Neto, G.F.; de Andrade Rodrigues, M.L.; Fonseca, A. A new quantitative gel electrophoresis method with image-based detection for the determination of food dyes and metallic ions. *Talanta*, **2021**, *221*, # 121602.
11. Peterson, G.L. Determination of total protein. *Methods in Enzymol*, **1983**, *91*, pp. 95-119.
12. Serackis, A.; Matuzevicius, D.; Navakauskas, D.; Sabanovic, E.; Katkevicius, A.; Plonis, D. A robust identification of the protein standard bands in two-dimensional electrophoresis gel image. *Electrical, Control and Communication Engineering*, **2017**, *13*, pp. 63-65.
13. Kurien, B.T.; Scofield, R. H. *Electrophoretic separation of proteins: methods and protocols*; Humana Press: New York, USA, 2019; 523 p.
14. Tanford, C.; Reynolds J.A. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta*, **1976**, *457*, pp. 33- 70.
15. Macheleidt, J.; Kniemeyer, O. Serological Proteome Analysis for the Characterization of Secreted Fungal Protein Antigens. In: *Host-fungal interactions. Methods in molecular biology*; Bignell, E, Eds.; Humana: New York, USA, 2021; Volume 2260, pp. 15-26.
16. Dietrich, M.A., Judycka, S.; Słowińska, M.; Kodzik, N.; Ciereszko, A. Short-term storage-induced changes in the proteome of carp (Cyprinus carpio L.) spermatozoa. *Aquaculture*, **2021**, *530*, # 735784.
17. Tans, R.; van Rijswijck, D.M.H.; Davidson, A.; Hannam R.; Ricketts, B.; Tack, C.J.; Weccels, H.J.C.T.; Gloerich, J.; van Gool, A.J. Affimers as an alternative to antibodies for protein biomarker enrichment. *Protein Expression and Purification*, **2020**, *174*, # 105677.

18. Lu, Y.; Fu, T. Performance of commercial colorimetric assays for quantitation of total soluble protein in thermally treated milk samples. *Food analytical methods*, **2020**, *13*, pp. 1337-1345.
19. Laemmly, U.K. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature*, **1970**, *227*, pp. 680-685.
20. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, **1976**, *72*, pp. 248-254.
21. Wang, P.; Yang, Y.; Hong, T.; Zhu, G. Proximity ligation assay: an ultrasensitive method for protein quantification and its applications in pathogen detection. *Appl Microbiol Biotechnol*, **2021**, *105*, pp. 923-935.
22. Posch, A.; Kollmann, F.; Berkelman, T.; Dreskin, E. Sample preparation of secreted mammalian host cell proteins and their characterization by two-dimensional electrophoresis and western blotting. In: *Proteomic profiling. methods in molecular biology*; Posch, A., Eds.; Humana: New York, USA, 2021; Volume 2261, pp. 507-524.
23. Gavrilova, K.V.; Bychkov, A.L.; Bychkova, E.S.; Akimenko, Z.A.; Chernonosov, A.A.; Kalambet, Ju.A.; Lomovskii, O.I. Mechanically activated hydrolysis of protein plant materials for the production of food components. *Food and Raw Materials*, **2019**, *7*, pp. 255-263.
24. Pogue, G.P.; Vojdani, F.; Palmer, K.E.; Whate, E.; Haydon, H.; Bratcher, B. Production of pharmaceutical grade recombinant native aprotinin and non-oxidized aprotinin variants under greenhouse and field conditions. In: *Commercial plant-produced recombinant protein products*. Howard, J.; Hood, E., Eds.; Springer: Berlin, Germany, 2014; Volume 68, pp. 65-80.
25. Shkuratova, N.A.; Neprienko, E.N.; Sharova, O.V.; Makarova, E.A.; Doroshenko, A.S. Comparative assessment of aprotinin quantification using different methods. *Siberian Journal of Clinical and Experimental Medicine*, **2009**, *2*, pp. 25–29.
26. Boursier, B.; Delebarre, M.; Lis, J.; Marquilly, Ph. RF Patent No. 2008108500/13, September 10, 2009. *Textured pea proteins* / RF Patent No. 2398444, July 25, 2006. Bulletin No. 25.
27. Zemskova, L.A.; Sheveleva, I.V.; Sukhoverov, S.V.; Voit, F.V.; Sergienko, V.I.; Avramenko, V.A. RF Patent No. 2005106613/13, August 20, 2006. The method for isolation and purification of bovine serum albumin / RF Patent 2289588. 2005. Bulletin No. 23.
28. Hickman, R.K.; Huang, Q.; Weed, C.L.; Ennis, S.T.; Perilli-Palmer, B.; Wan, M. RF Patent No. 2011120191/10, November 27, 2012. Isolation and purification of antibodies using protein A affinity chromatography / RF Patent No. 2520838. October 20, 2009. Bulletin No. 33
29. Mikhailova, K.; Krasilov V.; Malahova, I.; Kozmin, Y.; Kalambet, Y. Data processing in planar chromatography and gel electrophoresis using "Chrom&Spec" software. *Analytics*, **2014**, *19*, pp. 56-61.
30. Moldoveanu, S.C.; David, V. *Selection of the HPLC Method in Chemical Analysis*, 1st ed.; Elsevier: Amsterdam, Netherlands, 2016; 598 p.
31. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res. Oxford University Press*, **2019**, *47*, pp. D506–D515.
32. Bairoch, A.; Apweiler, R.; Wu, C.H.; Barker, W.C.; Boeckmann, B.; Ferro, S.; Gasteiger, E.; Huang, H.; Lopez, R.; Magrane, M.; Martin, M.J.; Natale, D.; O'Donovan, C.C.; Redashi, N.; Yeh, L.L.; Boutet, E. The Universal Protein Resource (UniProt). *Nucleic Acids Res*, **2005**, *33*, pp. D154-D159.