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A novel application for multiple form protein separation by the conjugation of disc electrophoresis and isoelectric focusing: Re-evaluating cylindrical disc-PAGE tube system for isoelectric focusing and potential outcomes for future usage of this approach

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Abstract

Aim: There are various forms of isoelectric focusing. But involving a single glass column and stabilized by the presence of a sucrose gradient, they are preferably used and are the most common pre-application step in 2D electrophoresis. The cylindrical disc-PAGE tube system is possibly not evaluated for isoelectric purposes. The goal here was to try a modified application for the separation of isoform proteins via the disc gel system. And to establish isoform separation and evaluate the implications of this approach for future use.

Methods: Essentially, basic features of disc and isoelectric focusing were integrated. Although conventional disc electrophoresis requires three-layers of gel, but here only one form of gel layer was configured. As a second attempt, the homogenate and ampholite, were deliberately included in the unpolymerised gel medium as if they were gel preparation factors. Then, the separation of the isoform proteins, which were thought to be homogenously distributed in the presence of ampholite, was achieved.

Results: The observation of isoforms of proteins revealed the presence of a distinct pattern of protein bands in their respective isoionic areas. From an analytical perspective, this application exhibited very clear band patterns. The results obtained regarding the isoforms of hemoglobin proteins were looking as informative and descriptive.

Conclusions: The band pattern observed in the present approach clearly showed multiple isoform characters and simply demonstrated the differences of this novel application. It can be used to analysis and separate multiple of proteins in the first dimension of conventional two-dimensional electrophoresis.

Keywords: IEF, IPG-Dalt, ampholite, isoform proteins, hemoglobins multiplicity, disc-PAGE System

1. Introduction

A treatise shows that various isoelectric focusing models have been applied for protein separation. Discontinuous (briefly named as disc or disk) electrophoresis was generated in the early 1960s [1], and has been accepted as a common form of zone electrophoresis. And it was also considered as an attainable research tool after that date [2–3]. This technique is also carried out in polyacrylamide column gel. In acrylamide application, the gel acts like a molecular sieve, and the separation is based on either the charge or the size of the macromolecule. And these three components are essential for disc electrophoresis. In the conventional disc application, gels are commonly applied in cylindrical blocks and consist of polyacrylamide suspension between two buffer or electrolyte reservoirs. And in this case the sieving effect of the gel medium created by the gel medium is ignored. The polyacrylamide gel is divided into three parts, called the *sample*, *stacking*, and *separating* gels, as depicted in Fig. 1. In this case, the sample gel contains the mixture of proteins to be applied. And it is prepared at low acrylamide concentrations so that pore sizes are large and do not create background artifacts with protein migration. Typically, the upper reservoir is filled with an

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anionic buffer such as NaOH (10-20 mM) with a pH around 12, and the lower buffer is H_3PO_4 (10 mM) with a pH around 2.00. The use of a buffer gradient results in an increase in the pH value from the cathode to the anode.

On the other hand, isoelectric focusing (IEF) is a common form of electrophoretic method in which proteins are separated as a function of their own pIs, and it was developed at almost the same time as such disc electrophoresis in the 1960s by Svensson [4-5] and Westerberg [6]. Since proteins carry positive, negative, or zero net electrical charges, as shown in Fig. 2, they can be clearly separated in linear pH gradients, and this is the key to the success of IEF. When an electrical potential is applied, ampholite initially migrate to their isoionic points, establishing a pH gradient, and then electrophoresis moves charged proteins to their isoelectric positions in the gradient, creating a series of band models [7]. When a detailed treatise has been carried out, IEF can be traced back to the early work of Svenson [8-11], and then the extensions of his theory have been followed by Rilbe [12]. Rilbe's attempts have been questioned in a number of ways and no additional ideas have been presented for a long time. As a result, their basic concepts have remained the same up to the present day. On the other hand, an extension of IEF is immobilised pH gradients (IPGs) for isoelectric focusing, introduced by Bjellqvist [13]. Some other applications and t results followed this tradition, and then the methodology became a conventional common technique, included in some methodological textbooks [1-14].

After initial problems with the handling of IPGs, a basic protocol of two-dimensional electrophoresis (2-DE) with IPGs in the first dimension followed by horizontal or vertical SDS electrophoresis (IPG-Dalt) was established in 1988 [15-16]. Since then, a number of different approaches or modifications of these protocols have been developed and are still in use for a variety of purposes. However, the basic concept of 2D electrophoresis has not changed so much and remains unchanged and is still largely used in its original application format [16-17].

The IEF system can be used for both preparative and analytical purposes. As a preparative target, IEF has been shown to have many favorable properties, such as providing high resolution separations of large amounts of material and good recoveries [18]. Initially, the application was experienced by means of a sucrose density gradient, which also prevents the convection that is used in electrophoresis [1-5]. The most commonly used configuration for analytical IEF is the horizontal polyacrylamide slab gel. However, high-grade agarose gels or high-molecular-weight proteins are also used to avoid the molecular sieving effect of polyacrylamide [1,4–6,19–23]. Similarly, in the column version, the column is stabilized by the presence of a sucrose density gradient to prevent diffusion. And the mixture of proteins to be separated could be able initially to be confined to a specific region of the column. Also uniformly distributed throughout the density gradient without a supporting matrix as shown in Fig. 2. In the other commonly used variant of the column, the protein sample is applied to the bottom of the wells by means of a syringe [1,4–6].

In this presentation, we are reevaluating the application of disc and iso-focus electrophoresis. And we are simply pointing out an approximation for separating isoform proteins. In our application, a known but not commonly used and also a different form of disc-PAGE vertical tube system was evaluated. And the isoelectric separation potential of this application was used to separate and compare different fish isohemoglobins, and also some other known proteins were depicted (see Fig. 3). In the general cylindrical vertical acrylamide IEF application, the homogenate protein to be resolved is usually applied onto polymerized gel (generally into the sample gel layer). However, in our modified application, the homogenate was applied to vertical and cylindrical Disc-PAGE glass tube devices, as if it were a chemical ingredient of the gel (before the gel or matrix in general was unpolymerised). In this context, the system could clearly distinguish between protein band profiles and demonstrate hemoglobin differences in a single analytical step. Even if the application system presented here is considered old-fashioned and difficult enough for a practical application, this approach has led to some remarkable and informative bands results. As a common outcome, some possible post-applications of this approach, e.g., after the gel has been dispelled from the cylindrical tubes, were also tested for native and denatured SDS-PAGE conditions.

2. Experimental

2.1. Reagents and chemicals

All the chemicals used in the experiment were of analytical reagent grade. Abbreviations for common chemicals are as follows: Potassium chloride (KCl), trichloroacetic acid (TCA), ammonium persulphate (APS), hemoglobin (Hb), human adult hemoglobin (HbA_2), myoglobin, protein molecular mass marker, tetramethylethylenediamine (TEMED). Acrylamide and bis-acrylamide, beta-mercaptoethanol, urea (sequencing grade), ampholite (3,5-10), natrium hydroxide,

phosphoric acid, Coomassie blue R (G), acetic acid, methanol, and absolute alcohol.

2.2. Apparatus

Cylindrical vertical disc-PAGE glass tube gel (5 mm internal diameter by 150 mm in length and with 2 ml internal size) electrophoresis apparatus, Heidelberg GmbH Instruments, Desaga, "Desaphor VC System for Circulator Gel Electrophoresis", Heidelberg (the very old system, and possibly one that cannot be produced commercially and currently). The name of this apparatus application is still cited in some old or recently published manuscripts [19–23]. Some laboratories still use it as an isoelectrophoretic system instead of the original vertical column focusing system [23].

2.3. Procedure

2.3.1. Gel preparation

The application was essentially based on the cited methodology [23], but the chemical composition consisted of a different approach of an adapted urea analytical protocol system and was briefly as follows: A column with a height of approximately 100 mm (ca. 1.5 ml by vol. per tube) was filled with an acrylamide gel with the following composition: 5.64 ml H_2O, 2 ml 30 % (w/v) acrylamide-1 % (w/v) bis-acrylamide, 48 µl ampholite solution pH 3.5-10, 6.0 gr new and freshly prepared urea (sequencing purity or grade), 250-300 µl protein solution (between 10-20 µg/ml in concentration [17,24]. Urea was first dissolved in warm water, and then the above-mentioned substances were added. In the last step, 25 µl of 10 % APS and 20 µl of TEMED were added.

2.3.2. Gel application and insertion of glass tubes

After gentle mixing, the solution was immediately injected with a syringe into the combined applicator (each side consisting of 12 combination cylindrical tubes) according to *the composite or unified container principle*. The gel was allowed to polymerize for 10 minutes. Then glass tubes immediately were dispelled from the applicator and inserted into the running chamber. The catholyte (10-20 mM NaOH, ~pH:12.00) into the upper and anolyte (10 mM H_3PO_4, ~pH:02.00) into the lower chambers were added as a buffer system [25]. Unlike the Robertson et al.'s [17] application, the inner chamber was heated to about 37 °C by a circulatory bath to prevent re-crystallization of urea. The circuit has been given as recommended elsewhere [17].

2.3.3. Removing, staining, and storing of gel tubes

Gels were removed from glass tubes as described by Parrish and Marchalonis [23], incubated in KCl and TCA as described by Bolag et al., [24], and then stained with Coomassie Blue R (G)-250 gel stain and destained as described by Robertson et al. [17]. The gel background artefact was eliminated by incubating the gels in a serial descending ethanol gradient solution and then storing them in a 3 % TCA solution (which can be stored for any length of time and does not lose its visual color over the years). If the gel is intended to be used for preparative purposes, the dispelled but unfixed gel pieces that have been separated or combined can be subjected, even under freezing conditions, to other gel systems, such as a 2D system, or to any other possible desired post-application. This is one of the most impressive results of this application, as the protein preservation in the gel is one of the most effective approaches, especially for post-electrophoretic applications.

2.4. A Trial for determination of immobilized pH gradient

In contrast to Robertson et al. [17], for an attempt, a hypothetical and practical immobilized pH gradient was calculated in order to determine the immobilized pH gradient. This was done by removing the ampholyte from the gel. The gels were then stained with the dye to reveal all protein band profiles. And an empirical calculation model was experienced. The upper anolyte pH was recorded as approximately 12.00 (due to 10/20 mM NaOH), and the lower catholyte pH was recorded as 02.00 (due to 10 mM (H_3PO_4) (ca. 11.50 cm of gel length). Also, assuming that each range corresponds to a pH scale, the intervals to be taken into account between the first, second, etc., visible protein bands were considered to be at 1 cm (10 mm). Then, 11.50 units of the pH range were taken into account and the position of the protein bands corresponding to the sequentially numbered pH points was recorded [26-28] (but data not detailed here) (See Fig. 5a-j).

Secondly, as recommended elsewhere [24], the gels dispensed from 12 cylindrical tubes containing only ampholite (the gel not containing applied or running protein) were arranged side by side and each cylindrical gel was divided into slices at 1 cm interval (depending on the approach mentioned or assumed above). Each corresponding putative intervals disc-shaped slice was joined in the same tube and gently crushed by pressing or grinding in the same tubes. Then a brief centrifugation and the pH of the upper supernatant phase solution of each slice were recorded. The likely observed or calculated pH gradient values were compared with the expected pH ratio using this modelling (see Fig. 5f).

3. Results and discussion

3.1. A comprehensive results and discussion

Isoelectric focusing in a cylindrical gel tube system on the acrylamide gel has been experienced earlier [3–12].

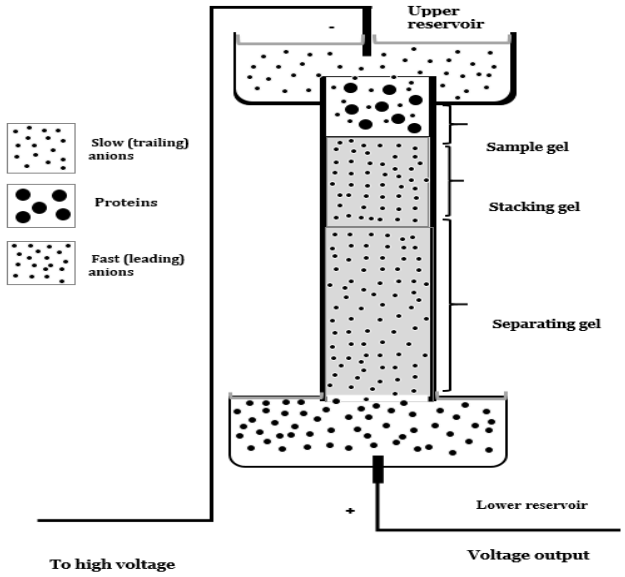

Figure 1. Demonstration of the main components of a discontinuous electrophoresis gel and cylindrical blocks of polyacrylamide, suspended in between two reservoirs of electrolyte. As can be seen in this demonstration, the block is divided into three sections as *sample*, *stacking* and *separating* gels, and the upper reservoir and sample/stacking gel have the same pH. However, the separating gel differs from the other two regions in that it has a higher pH and greater gel concertation. As shown in the figure, the protein sample applied to the layer of sample gel and low concentration of gel has no effect on migration. In this way, the protein separation starts from the sample phase layer, continues to migrate through the stacking and separating gel layers and is finally concentrated in the separating gel (data taken from cited textbooks [3] and adapted as described above).

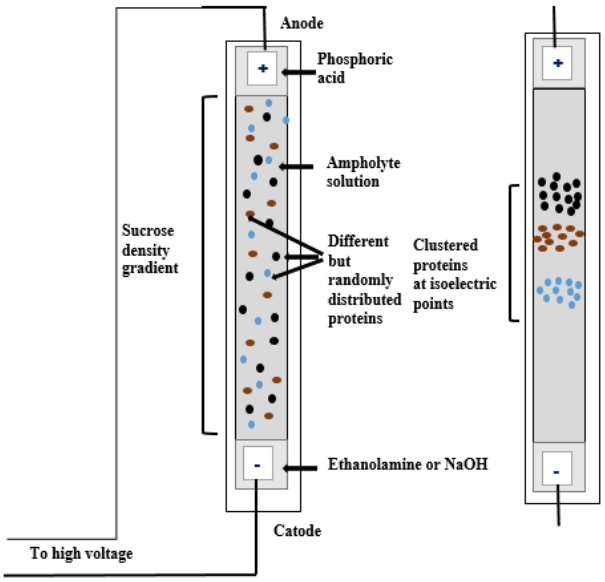

Figure 2. Visualization of sucrose density gel application before and after gel application. In this common application model, the mixture of protein to be separated or condensed can be initially confined to a specific area of the column present or uniformly distributed throughout the sucrose density gradient. In this application, the protein is applied to a single column and then allowed to separate or condense within the sucrose density without the molecular sieving effect of the support material. As can be seen from this scheme, since sucrose was used in this common model and in any attempt to obtain protein from sucrose or attempt to observe protein bands in the sucrose gel, it will not be so easy to obtain or transfer to the next step gel application (data taken from cited textbooks [3] and adapted as described above).

But in this application the protein sample to be run in the electric field was applied to the polymerised gel after being layered on top, under a protective stacking gel layer. The carrier ampholyte in the gel medium or as a layer is preferentially run. Whereas in ours every and all constituents of the gel medium (including ampholite and protein to be separated) were dispersed throughout the gel and run simultaneously. In the essential components of disc electrophoresis, the polyacrylamide gel is usually divided into three segments, known as *sample*, *stacking*, and *separation* gels. But in this presented application there was only one form of gel, consisting of separation gel (see Fig. 1 and Fig. 2).

As shown earlier, in the conventional application, the sample gel is used for protein penetration and is prepared with low polyacrylamide to keep the pore size large and not affect the migration rate. The stacking gel is identical to the sample gel but initially contains no protein. Under these circumstances, the upper reservoir, the sample, and the stacking gel are at the same pH range (Fig. 1 and Fig. 2). However, the separation gel differs from the above conditions in terms of pH and gel concentration, as it consists of a higher pH and a higher acrylamide concentration. In our application, we have only made one form of gel system, known as a separating gel, as in the conventional system, and the application consisted of only these (Fig. 3 and Fig. 4). Therefore, our application was only constructed from this single form of gel when compared to the traditional one. And since the sample and stacking gel layer were neglected, it corresponds only to the separation gel.

As is expected, since the upper pH directly contacts the applied material, it makes the upper pH site of the initial conditions. In this way, the most notable configuration of gel content in the present applications is the samples to be applied and ampholite to migrate protein bands at the same time and place when the current was applied in the fields. As the gel acts as a molecular sieve and the separation is based on both the charge and the size of the proteins in application conditions. We tried to keep the gel content as low as possible to avoid the anti-connective effect of a higher gel sieving effect and necessarily preferred as much as gel concentrations in some applicable limits.

This electrophoretic application has the potential to be a new form of application, and it was shown to be separate from different fish hemoglobin multiplicity even in a single step. (see Fig. 5a-e). All species tested, including different populations of the same species, showed different band models and were distinguished by hemoglobin multiplicity. (Fig. 5a-e), [14-17].

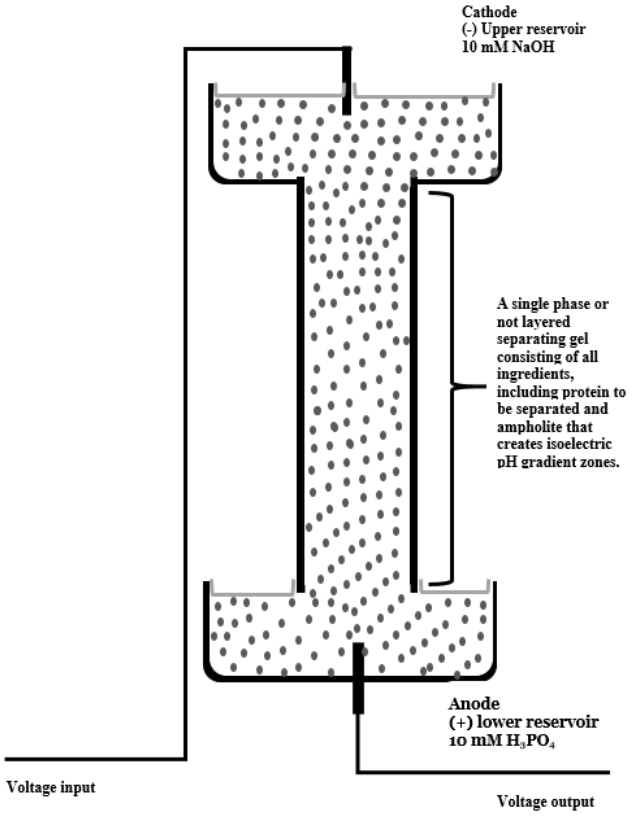

Figure 3. A column appearance of compound column model experienced in this application. Different from common models shown in Fig. 1, column is consisted of one form gel and layer, and polyacrylamide gel used instead of sucrose as seen in Fig. 2. In this way, the present application model consists of conjugation of general form of disc and isoelectric focusing model. A single gel layer instead of the multi-layer model of disc application and a single form in lower concentration of acrylamide instead of sucrose density gradient were applied. As can be seen in the post-application attempt, the very sharp and clear protein band pattern was obtained by this modified application (see Fig. 5). Full details are given in the method. In this way, the new application provides a new approximation for this purpose.

Among the numerous applications of IEF reviewed by several authors, no similar attempts have been made [17,23–26]. Vertical cylindrical PAGE tube systems have been tried for the general form of electrophoresis, but not for a form like IEF [7,14, 23–26]. The bands' pattern of the proteins in the gel, which spans the range of pH 3.50-10.50 (**Fig. 5f**), was also found informative with regard to ecological conditions of the fish tested (**Fig. 5a-e**) [27–28].

The present application also distinguishes itself from the conventional sucrose density gradient method [2,3], which is the other similar method to our experiment. In a conventional system, a uniform column and sucrose density are preferred, and as is known, protein samples can initially be confined to a specific region of the column. Whereas in ours, multiple-column model and lower density polyacrylamide are structured. By using a very low concentration of acrylamide, which provides a lower support matrix, high resolution and ultimately clear band patterns were achieved in the gel. In this way, a large number of columns can be obtained with a single sample application trial in one sample attempt.

In order to test the effectiveness of this application, some of the other pure and well-known protein samples were also applied at various stages. For this experiment, pure forms of human HbA_2 and myoglobin, laboratory-purified human hemoglobin, and standard molecular weight markers for proteins were also assayed. The results are shown in the respective figure (Fig. 5g-i). Human hemoglobin obtained in the lab is separated in a similar manner and exhibits very clear band patterns (Fig. 5g). Commercially pure HbA_2 and myoglobin were deliberately mixed as shown (Fig. 5h). A marked separation was also shown by the protein molecular mass marker (12-212 kDa) in a similar way. (Fig. 5i). These proteins were clearly separated from each other. And the proteins with similar properties, charge, and shape, which were expected to cluster in the same line, formed similar clusters as predicted (Fig. 5g-i).

This application can also be used for preparative purposes. As an example of this application, stained and unstained gels are first aligned, and the areas corresponding to the stained area on the unstained line are cut with a scalpel. Each section was then transferred directly to the next gel (sometimes with a combination of different gel fragments) or to tubes containing native or denaturing loading buffer and gently minced. After a brief centrifugation, the upper phase was applied to a native or SDS gel system (Fig. 5j). It can also be used to recover the pure form of protein bands from the gels [27,28].

When the gel was transferred to native or denatured gel systems, single-band patterns and four monomeric subunits were obtained in SDS gel media, as shown in Fig. 4i. These results were considered to indicate that the hemoglobin subunits remained in tetrameric form and did not cause any perturbation in chemical composition during polymerization [27,28]. To avoid protein carbamylation, new and freshly prepared urea (sequencing grade) was especially preferred.

3.2. Evaluation of immobilized pH gradient profiles.

In order to determine the immobilized pH gradients (IPGs), a theoretical and practical pH determination was carried out as described in the method. Depending on the pH of the polyanionic ampholyte in the gel, the location of the proteins on the focused gel was found to be predominantly in the range of pH 3.50-10.50. The expected IPGs and the obtained results were found to be in agreement with the experimental results. From an analytical point of view, this result was found to be consistent with the results of the general distribution of

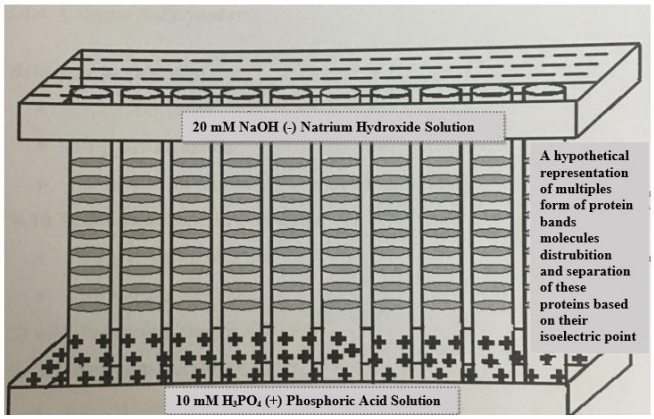

Figure 4. Pictorial representation of the general form of a multiple tube or channel model vertical PAGE disc gel electrophoresis system (see references [1,2,5,6]). The model consists of two parallel tube systems placed side by side and the entire column is filled by an applicator with an available concentration of acrylamide at the same time as the protein sample to be separated and ampholyte solutions, which will have an isoelectric focusing effect. An upper and a lower buffer, each with its own higher pH point, for the maintenance of higher electrical currents.

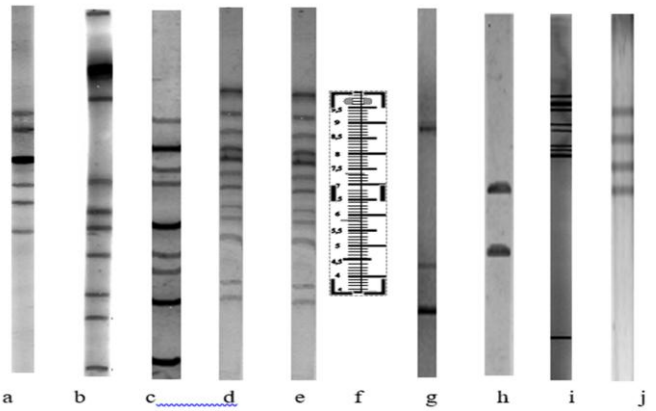

Figure 5. Some results of this new modified approach and obtained isoform protein profiles of multiple fish Hb, which were adapted to different ecological conditions. A representation for multiple form hemoglobin appearances and other known proteins, and determined IPGs in the cylindrical tube gel: (**a)** represent cold acclimated salmonid fish hemoglobin; (**b-e)** represent various carp species which are adapted to chemically and environmentally different conditions; (**f**) theoretical pH scale depending on observed results for immobilize pHs determination; (g) shows human Hb; (**h**) mixture of commercial HbA_2 and myoglobin; (**i**) represents known protein molecular mass marker (12- 212 kDa); (**j**) is representing a line in the SDS-PAGE electrophoresis application as an example of post-IEF application (more detail *see* [27,28].

multiple forms of hemoglobin as observed in fish and other lower vertebrate organisms [19,20].

3.3. The challenges faced

The application of the present modified approach involves some handling problems, as observed or experienced in the original application. In fact, when the basic protocol of two-dimensional electrophoresis (2-DE) was established in 1988 with IPGs for the first dimension, followed by horizontal/vertical SDS electrophoresis, some or similar handling problems were also reported [15,16]. The main obstacle in the study is that when the gel is removed from a tiny glass tube, very intensive and careful attempts are necessary. An easy way to overcome from this obstacle is to use the correct thickness of gel tube for pre– and post-gel applications. The sample detection appeared to be as laborious and often difficult as with the conventional isoelctrophoresis techniques.

It has been reported that any attempt to distinguish or resolve a protein mixture into a number of separate zones does not ensure that all the different proteins present in the original extract have been separated. Because for two or more variable proteins that are similar in size or structure, they can also have the same net charge in a given circumstance and exhibit the same electrophoretic motilities [19,20].

All these tests have been revealed by this new version of the application; it is not easy to ignore the novelty of this new approach [27,28]. However, since we have applied only very limited hemoglobin purification protocols as depicted in the above method, the results seen under these circumstances are worth considering.

3.4. Conclusions remarks

Different ways and some superiorities of the current modification can briefly be described as below: The appearance of protein bands is quite clear and very informative for discriminating between protein band patterns as observed in our fish hemoglobin trials. Since the homogenate is directly included to the gel before polymerization and the protein specimens are dispersed uniformly throughout every gel tube, this striking band profile is given. Conventional focusing is mostly applied after protein purification, but in our trials we did not use a protein purification protocol or detailed separation method; instead, we did only a simple hemoglobin homogenate extraction. This band discrimination is looking very informative for the phylogenetic description of fish, as it is expecting 2D electrophoresis. On the other hand, the gels containing protein profiles can be assessed for *in situ* activity determination when applied for isozyme or activity staining, especially enzymes, like dehydrogenase. Proteins profile can be stored in every column for long-term storage. Ultimately, the application can be applied for a very small amount of sample detection, and since homogenate (purified or crude form) is dispersed to every column matrix homogenously, each sample column can be transferred for post-application purposes or recovering sample from separately or after being matched or ordered side by side, the corresponding section can then be joined and used for different purposes.

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Declarations and funding:

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Data availability:

All data from this trial are presented in the manuscript. It may be used if cited.

Ethical responsibilities of authors:

The first author read, understood, and has complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors.

Conflict of interest:

The authors declare that they have no competing financial interests in related to this article. The authors alone are solely responsible for the content and writing with regarding of the paper.

References

[1] L. Ornstein, B, Davis, Disc or Discontinuous Electrophoresis, Editors: P.M. Sheeler, D. E. Bianchi, Cell and Molecular Biology (3. Edition), 1987, USA, Wiley.

[2] L. Ornstein, Disc Electrophoresis-I, Background and Theory, 121:2, 1964, 321-349.

[3] P.M. Sheeler, D.E. Bianchi, Isoelectric Focusing, Cell and Molecular Biology, (3. edition) 1987, USA, Wiley.

[4] H. Svensson, Isoelectric Focusing, Editors, P.M. Sheeler, D.E. Bianchi, Cell and Molecular Biology, (3. edition), 1987, USA, Wiley.

[5] H. Svensson and O. Westerberg, Isoelectric Focusing, Editors, P.M. Sheeler, D. E. Bianchi, Cell and Molecular Biology, (3. Edition), 1987, USA, Wiley.

[6] O. Westerberg, Chapter XI Isoelectric Focusing and Separation of Proteins, Editors, J.R. Norris, D. W. Ribbons, Methods in Microbiology, 1971, USA, Academics Press.

[7] A.T. Andrews, Theory, Techniques and Biochemical and Clinical Applications: Electrophoresis, 1986, England, Oxford University Press.

[8] H. Svenson, Gradients I, The Differantial Equation of Solute Concentrations at a Steady State ant its Solution for Simple Cases, Acta Chem Scand 15, 1961, 325-341.

[9] H. Svenson, Acta Chem Scand, Isoelectric Fraction, Analysis and Characterisation of Ampholytes in Natural pH Gradients II Buffering Capacity and Conductance of Isoionic Ampholytes, 16, 1962, 456-466.

[10] H. Svenson, Isoelectric fractionation, analysis and characterization of Ampholytes in natural pH gradients III Description of apparatus for electrolysis in columns stabilized by density gradients and direct determination of isoelectric points, Arch Biochem Biophys, 1, 1962, 132-136.

[11] H. Svensson, Isoelectric Focusing, Editors: P.M. Sheeler, D. E. Bianchi, Cell and Molecular Biology (3. Edition), 1967, USA, Wiley.

[12] H. Rilbe, Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients, II, Buffering Capacity and Conductance of Isoionic Ampholytes, A Correction, Acta Chem Scand, 25, 1971, 2768-2769.

[13] Bjellqvist B., Ek K., Rigretti PG., Gianazza E., Görg A., Westermeier R. and Postel W. (1982). Isoelectric Focusing in immobilized pH Gradients: Principle, Methodology and some Applications, J of Biochem and Biophys Methods, 6 (4), 1982, 317-339.

[14] P.G. Rigretti, Laboratory Techniques in Biochemistry and Molecular Biology: Isoelectric Focusing; Theory, Methodology and Applications, 1983, USA, Elsevier Biomedical Press.

[15] A. Görg, W. Postel, S. Günther, The current state of Two-Dimensional Electrophoresis with Immobilized pH Gradients, Electrophoresis, 9(9), 1988, 531-546.

[16] A. Görg, 2-D Proteome Analysis Protocols, Methods in Mol Biol, 112, 1999, 189-195.

[17] E. F. Robertson, H. K. Dannelly, P. J. Malloy, Rapid Isoelectric Focusing in a Vertical Polyacrylamide Minigel System, Anal Biochem, 167(2), 1987, 290-294.

[18] K. Wilson, K.H. Goulding, A Biologist's Guide to Principles and Techniques of Practical Biochemistry, 1986, England, Cambridge University Press.

[19] F. Basaglia, Comparative study of electrophoretic and isoelectrophoretic characteristics of osteichthyan and amphibian hemoglobin, Ital J Zool, 71, 2004, 287-295.

[20] C.R. Bonini-Domoingos, M.B. Silva, R.M. Romero, P.J.A. Zamaro, L.S .Ondei, C.E.S. Zago, S.B. Moreira, C.G. Salgado, Description of electrophoretic and chromatographic hemoglobin profile of *Rhinoclemmys punctularia*, Genet Mol Res, 30;6 (2), 2007, 415-21.

[21] W. J. Mullin, F.H. Wolfe, Disc Gel Electrophoresis of Caseins Treated with Proteolytic and Glycolytic Enzymes, Journal of Dairy Science, 57 (1),1972, 9-14.

[22] R.B. Rucker, J. Goettlich-Riemann, Isolation and Properties of Soluble Elastin from Copper-Deficient Chicks, The Journal of Nutrition, 102 (4),1972, 563-70.

[23] C.R. Parrish, J.J. Marchalonis, A Simple and rapid acrylamide gel method for estimating the molecular weights of proteins and protein subunits. Anal Biochem. 34 (2), 1970, 436-433.

[24] D.M. Bolag, M.D. Rozycki, S.J. Edelstein, Isoelectric Focusing and Two-Dimensional Gel Electrophoresis, Protein Methods (2. edition), 1996, USA, Wiley-Liss.

[25] P.H. O'Farrell, High resolution two-dimensional electrophoresis of proteins. J Biol Chem, 250, 1996, 4007-4021.

[26] I. Smith, Chromatographic and Electrophoretic Techniques: Zone Electrophoresis, (4. Edition), 1976, England, William Heinemann Medical Books.

[27] A. Zöngür, N. Değerli, (2009). Farklı Çevresel Koşullara Uyumlu Bazı Sazan Türlerinde Hemoglobin Proteini Farklılıklarının Elektroforetik Tekniklerle Araştırılması, Yüksek Lisans Tezi, Cumhuriyet Üniversitesi Fen Bilimleri Enstitüsü, 2009.

[28] A. Zöngür, N. Degerli, Investigation of Hemoglobin Protein Differences in some Carps Species Compatible with Different Environment Conditions by Electrophoretic Techniques, TURJAH, 10(3), 2022, 411-448.