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SHORT COMMUNICATION

A simple method for identification of native collagen by reversed-polarity electrophoresis: Short report

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Abstract: The high molecular weight of collagen and the high uncommon amino acid composition (proline and hydroxyproline) make the protein particular at structural and physicochemical levels compared to others. Polyacrylamide gel electrophoresis (PAGE) is a simple and inexpensive method to identify collagen integrity; however, native forms of proteins generally show low quality bands. In this work, we considered the charge of the protein to perform a very simple method to identify the native form of type I collagen, exhibiting an appropriate electrophoretic resolution. First, we determined the collagen charge at different pHs and then modified a previously published method by changing the gel buffer and reversing the polarity of the electrophoresis chamber by turning the power cords; now the protein was moved from the anode to the cathode. The result was well-resolved protein bands that maintained their classical structure without degradation after PAGE, which were confirmed by extracting the protein from the native-PAGE and electrophoresing it in a sodium dodecyl sulphate-PAGE. This advantage could be useful when the electrophoresed native collagen is used by Western blotting for recognition with antibodies.

Keywords: extracellular matrix; native protein separation methods; polyacrylamide gel electrophoresis; protein electric charge.

INTRODUCTION

Collagen is a superfamily of proteins characterised by triple-α helical domains, where the most abundant member in vertebrates is type I. Type I collagen

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plays structural and informational roles in connective tissues, and its heterotrimeric structure $[\alpha_1(I)]_2,\alpha_2(I)]$, gives collagen its particular helical shape with the Gly-X-Y sequence (where X and Y are frequently proline and hydroxyproline, respectively).[1] Furthermore, the protein is considered to be among the fibril-forming colloid group of the superfamily,[2] therefore its physicochemical properties should be considered when handling it. The salting-out effect is evident for collagens; the high molecular weight of the protein and the electrostatic interactions between collagen fibres can be easily modified by neutralising the surface charge of the protein.[3,4] For example, NaCl can dissolve or precipitate neutral collagen solutions at concentrations less than or greater than 1 M/l, respectively,[3] where ionic strength and pH contribute to fibrillogenesis *in vitro*.[5] Among the different methods for the identification, semiquantification and separation of collagens, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used,[6] because it is easy and inexpensive to perform and the required device is regularly part of a laboratory of general biochemistry. However, the method is based on the ability of collagen to be negatively charged by SDS, which ultimately also denatures the protein.[7] So, when it is necessary to identify collagen under native conditions, a non-denaturing PAGE method is the choice. In this process, SDS is excluded and acidic conditions must be used.[8] Unfortunately, non-denaturing or native-PAGE shows some limitations due to the resolution of the collagen bands in the gel, contrary to the excellent images observed after SDS-PAGE. Unlike SDS-PAGE protocols, the use of native gel electrophoresis typically requires the optimisation of the separation conditions for specific samples (*i.e.*, collagens).[9] In this work, we introduce a simple method for performing native-PAGE to enhance the resolution of native collagen molecules by reversing the polarity of the electrophoretic chamber.

EXPERIMENTAL

We used type I pepsinized collagen (10 mg ml^{-1}), obtained from porcine sources (DSM Branch Pentapharm, Aesch, Switzerland) that was dialysed against 5 mM acetic acid to have a lower strength sample; collagen samples were diluted with 50 mM acetic acid. To measure the protein charge, 0.66 mg ml^{-1} collagen were evaluated in triplicates in a pH range of 3–7, with Nanotrac Wave equipment (Verder Scientific, Haan, Germany). Denaturing SDS-PAGE was conducted following the Laemmli method.[10] Briefly, 5.5 μg of heat-denatured collagen were loaded onto Mini Protean III Cell (Bio-Rad, Hercules, CA, USA) in a 0.75 mm thick discontinuous gel (4 % stacking gel, pH 6.8, and 6 % resolving gel, pH 8.8). The gel was stained with a 0.2 % Coomassie brilliant blue R-250 solution, 50 % methanol and 10 % glacial acetic acid for 2 h, and destained with a solution of 20 % methanol and 10 % glacial acetic acid for at least 4 h. Images of the bands were captured using the Gel Doc XR Gel Documentation System (Bio-Rad).

On the other hand, native-PAGE was performed according to the method described by Ramshaw JA and Werkmeister JA,[11] with some modifications. The collagen sample was dissolved in 0.1 M aqueous lactic acid containing 10 % sucrose. Samples were dissolved in 5 X

loading buffer (5 ml of 0.1 M lactic acid buffer with 10 ml of glycerol). Then, 8.3 μg of native or 1 h heat hydrolysed collagen samples were loaded into Mini Protean III Cell (Bio-Rad) in a 1.0 mm thick continuous gel, 3.2 %, pH 6.6. Before loading the samples, the gel was run for 30 min at 70 mA in 0.1 M lactic acid as cathode buffer and 50 mM Tris-HCl pH 6.6 as anode buffer. The loaded gel was run with running buffer (50 mM Tris-HCl pH 6.6) and reversed the chamber polarity for 6.5 h and 70 V in a cold room (3–4 °C). The gel was stained and destained as before. Finally, after staining, protein bands were excised and isolated from gel using 200 μl of elution buffer (50 mM Tris, pH 7.9, 0.1 mM EDTA, 0.15 M NaCl, 0.1 % SDS) for 24 h at 30 °C in a water bath. The eluate was concentrated five times using a CentriVap Concentrator (Labconco, Kansas City, MO, USA) and electrophoresed by SDS-PAGE.

RESULTS AND DISCUSSION

Native collagen evaluation by PAGE is much less frequent as a result of the particular physicochemical properties of the protein or the need for sophisticated devices. Our group has been working with native collagen and collagen copolymers,[12] so we face the need to evaluate collagen using a simple method such as PAGE. When attempting to replicate previously published methods, we encountered challenges, such as difficulty in reproduction, as a result of the lack of reagents or devices, and issues such as the resolution of bands in the published gel photographs.

In an attempt to adapt the Ramshaw JA and Werkmeister JA methods, by modifying the gel buffer, we observed the absence of collagen bands, leading us to suspect the involvement of the protein charge. Consequently, we conducted collagen charge determinations at different pHs. Our findings revealed a positive charge for the protein in the acidic range (Table I).

TABLE I. Relationship between pH and collagen charge; charge = average of triplicates, fC = femtocoulombs, *SD* = standard deviation

pH	Charge, fC	*SD*
3.06	+3.07	0.81
4.05	+3.22	0.89
4.95	+5.20	1.20
6.50	+5.71	0.41
7.12	+6.91	0.08

With this insight, we decided to reverse the polarity of the chamber by simply switching the power cords in the power supply. The result was a well-defined band of commercially purified type I collagen, as was also observed in the SDS-PAGE bands (Fig. 1a and b). The rationale for the polarity reversal was based on the positive charge of collagen, which facilitates protein migration from the anode (+) to the cathode (–) under acidic conditions (loading buffer, gel matrix and running buffer).

To demonstrate possible structural modifications in the protein as a result of the method, we recovered it from the native-PAGE gel and subjected it to extended

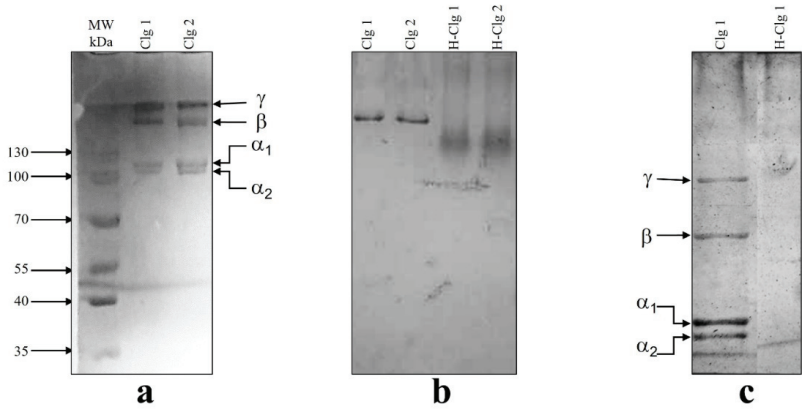

Fig. 1. Images of type I collagen subjected to PAGE. In (**a**), the electrophoretic pattern of collagen is observed under denaturing conditions (SDS-PAGE). The left lane is the molecular weight (MW) ladder in kDa, and the middle and right lanes (Clg 1 and Clg 2) show the pattern of denatured collagen samples. In (b), after the native-PAGE method described here, the first two lanes are single bands corresponding to native collagens (Clg 1 and Clg 2), and the third and fourth lanes exhibit the smear pattern corresponding to the heat-hydrolysed collagen samples (H-Clg 1 and H-Clg 2). Gel image (c) shows the SDS-PAGE of the collagen eluate bands (Clg 1), previously electrophoresed under native conditions. The left lane shows the typical pattern of denatured collagen, whereas the hydrolysed protein was absent in the right lane due to the small size of the protein fragments.

electrophoresis by SDS-PAGE. The goal was to highlight additional protein bands that might be in proximity to the main band, perhaps by degradation. The result revealed the typical electrophoretic pattern of type I collagen, where the α1 and α2 bands were evident, together with the β and γ bands (Fig. 1c). Despite the need to know the integrity of collagens, which has been a concern for researchers long time ago, the different works in which the protein was shown exhibited different issues. For example, in the work of Ramshaw and Werkmeister, 1988, the authors proposed a method for electrophoretic identification of native collagen, although their work only shows the electroblots, leaving out the possibility of knowing the electrophoretic pattern of the native proteins.[11] Furthermore, Ricard-Blum *et al*. published an electrophoretic method to assess various homo- or heterotrimeric collagens using a Phast System apparatus with a reversed polarity electrode assembly.[8] However, they never provided insight into the rationale behind reversing the polarity of the system, and their gel images exhibit blurriness with multiple small protein bands. Otherwise, with the method shown in this work, it was evident that the electrophoresed collagen was not degraded, which is important when the protein was Western blotted for recognition with antibodies. Also, with this approach, it becomes possible to analyse the extracted native collagen from the different tissues, in order to know the real proportion of the entire molecule, without the interference of the contamination of the α bands

present in the continuously turnover collagen. This strategy allows us to easily assess native collagen in cell-derived matrices,[13] to evaluate the real effect of biologically active enzymes from different sources on collagen[14] or to evaluate the collagenolytic enzymes involved in pathological conditions,[15] with the advantage that the analytical method would be focused on obtaining the effects on the real substrate, which can only be observed by native-PAGE, instead of the regular use of the typical denaturing SDS-PAGE. An additional advantage is that by this method, the protein in the native or denatured state could be easily semi-quantified by densitometry.

CONCLUSION

The method shown here was carried out taking advantage of the electrical properties of collagen under acidic conditions. Identification of native collagen by reversed-polarity electrophoresis is now a straightforward method that utilises common reagents and devices readily available to any laboratory conducting basic biochemical analyses.

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ИЗВОД

ЈЕДНОСТАВНА МЕТОДА ЗА ИДЕНТИФИКАЦИЈУ НАТИВНОГ КОЛАГЕНА РЕВЕРСНОМ ЕЛЕКТРОФОРЕЗОМ: КРАТАК ИЗВЕШТАЈ

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Висока молекулска маса и неуобичајени састав аминокиселина (пролин и хидроксипролин) чине колаген посебним међу протеинима у погледу структуре и физичко–хемијских својстава. Електрофореза у полиакриламидном гелу (PAGE) је једноставна и јефтина метода за идентификацију интегритета колагена, међутим, природни облици протеина генерално показују низак квалитет трака. У овом раду је узето у обзир наелектрисање протеина за извођење врло једноставне методе за идентификацију природног облика колагена типа I, који показује одговарајућу електрофоретску резолуцију. Прво је одређено наелектрисање колагена при различитим pH вредностима, а затим је претходно објављена метода модификована променом пуферског гела и променом поларитета коморе за електрофорезу окретањем каблова за напајање услед чега се протеин сада кретао од аноде ка катоди. На овај начин су добијене добро раздвојене протеинске траке које су задржале своју класичну структуру без деградације након PAGE, што је потврђено екстракцијом протеина из нативног PAGE и електрофорезом у натријум-додецил-сулфат–PAGE. Ова предност би могла бити корисна када се природни колаген, подвргнут електрофорези, користи у *Western blot* анализи за препознавање антителима.

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