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Author(s)	Nakano, Keiichi; Tamura, Shogo; Otuka, Kohei; Niizeki, Noriyasu; Shigemura, Masahiko; Shimizu, Chikara; Matsuno, Kazuhiko; Kobayashi, Seiichi; Moriyama, Takanori
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**Fort>Title: Development of a high-sensitive three-dimensional gel electrophoresis method for
characterization of monoclonal protein-heterogeneity**

Keiichi Nakano¹, Shogo Tamura^{2,3}, Kohei Otuka², Noriyasu Niizeki⁴, Masahiko Shigemura¹, Chikara Shimizu¹, Kazuhiko Matsuno¹, Seiichi Kobayashi⁵ and Takanori Moriyama^{5*}

¹Clinical Laboratory, Hokkaido University Hospital, Sapporo, Japan

²Division of Health Sciences, Graduate School of Health Sciences, Hokkaido University, Sapporo, Japan

³Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan

⁴Asahikawa Medical University Hospital, Department of Medical Laboratory and Blood Center, Asahikawa, Japan

⁵Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University, Sapporo, Japan

*Corresponding author

Kita-12 Nishi-5, Kita-ku, Sapporo 060-0812, Japan

Tel, Fax: +81-11-706-3413; E-mail: moriyama@hs.hokudai.ac.jp

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Abstract

Three-dimensional gel electrophoresis (3-DE), which combines agarose gel electrophoresis and IEF/SDS-PAGE, was developed to characterize monoclonal proteins (M-proteins). However, original 3-DE method has not been optimized and its specificity has not been demonstrated. The main goal of this study was to optimize the procedure of 3-DE and then compare it with 2-DE. We developed a high-sensitive 3DE method in which M-proteins extracted from first dimension, agarose gel, by diffusing into 150mM NaCl and the recovery of M-proteins was 90.6%. To validate the utility of the high-sensitive 3DE, we compared it with the original 3-DE method. We found that high-sensitive 3DE provided for greater M-proteins recovery and was more effective in terms of detecting spots on SDS-PAGE gels than the original 3-DE. Moreover, high-sensitive 3-DE separates residual normal IgG from M-proteins which could not be done by 2-DE. Applying the high-sensitive 3-DE to clinical samples, we found that the characteristics of M-proteins vary tremendously between individuals. We believe that our high-sensitive 3-DE method described here will prove useful in further studies of the heterogeneity of M-proteins.

Keywords, three-dimensional gel electrophoresis; monoclonal proteins; heterogeneity

1. Introduction

Monoclonal immunoglobulins, termed M-proteins, are produced by a single B cell clone. Each M-protein is composed of two identical heavy chains and two identical light chains. M-proteins have been detected in the serum and urine of patients with multiple myeloma (MM) [1; 2; 3; 4; 5; 6], Waldenström macroglobulinemia [7; 8; 9], heavy chain disease [10; 11; 12], and immunoglobulin light chain amyloidosis [13; 14; 15]. For the past several decades, researchers have isolated M-proteins using methods based on high-resolution agarose gel electrophoresis (HRE) [1; 16; 17]. While HRE is a fast and simple technique for screening M-proteins, it lacks sensitivity. Immunoelectrophoresis (IEP) is another technique that has been employed to identify M-proteins. However, IEP also lacks sufficient sensitivity for detailed analyses of M-proteins [17]. Immunofixation electrophoresis (IFE) has been described as an alternative to HRE and IEP because it enables detection of M-proteins at lower concentrations than HRE and IEP [18]. Because it enables determination of the isotype of an immunoglobulin with high sensitivity, IFE is now the standard method for routine monitoring of M-proteins in clinical practice.

Two-dimensional gel electrophoresis (2-DE), described by O'Farrel in 1975 [19], is commonly used in the analysis of M-proteins [20; 21; 22; 23; 24; 25]. These studies demonstrated that the heavy and light chains of immunoglobulins as well as Bence Jones protein (BJP) separate into heterogeneous spots on 2-DE gels [26; 27; 28; 29]. The heterogeneous pattern produced by M-proteins on 2-DE gels was thought to be the result of post-translational modification of the proteins through glycosylation or phosphorylation, etc. However, proteomic analyses of M-proteins using 2-DE is complicated by the presence of residual normal immunoglobulins and other proteins.

Harrison et al. [30] first reported the combination of three different electrophoresis methods (agarose gel electrophoresis, IEF, and SDS-PAGE) for the characterization of M-proteins. M-proteins were separated using agarose gel electrophoresis, extracted from the gel film using solutions containing SDS, and subsequently subjected to 2-DE [30; 31; 32]. Vu et al. developed methods for extracting M-proteins from commercially available agarose film using centrifugation rather than solutions, and they termed this technique three-dimensional gel electrophoresis (3-DE) [33]. However, this method has not been optimized, especially with regard to the M-protein extraction from the first dimension. Moreover, the pattern of M-proteins on 3-DE gels was visualized using only silver staining, which limits further proteomic analysis of the proteins. Therefore, the main goal of this study was to optimize the procedure of the high-sensitive 3-DE that could detect the spots of M-proteins by using coomassie brilliant blue (CBB) staining and then compare it with 2-DE. Moreover, we studied the heterogeneity of M-proteins isolated from the serum of MM patients using high-sensitive 3-DE methods.

2. Materials and Methods

2.1. Subjects

The study involved 7 patients diagnosed with MM at Hokkaido University Hospital. The median age of study subjects at the time of blood sample collection was 59 years (range, 55-69 years). The patients' characteristics are summarized in Supplementary Table 1. The Institutional Review Board of Hokkaido University Hospital approved the study, and informed consent was obtained from all subjects. All samples were preserved at -80°C until analyzed.

2.2. Measurement of total protein

The total protein concentration in samples was quantified using a Micro BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA) with human serum albumin (Wako, Osaka, Japan) as a standard.

2.3. Quantification of M-protein concentration

The amount of M-protein in each sample was quantified using a densitometric method based on the electrophoretic technique. Briefly, serum proteins were separated using cellulose acetate membranes (Titan III Lipo, Helena Labs, Beaumont, TX, USA) with Electra HR buffer (Helena Labs) for 25 min at a constant voltage of 180 V. Proteins were then visualized by staining the membranes with Ponceau 3R solution (0.8% Ponceau 3R/6% trichloroacetic acid). The intensity of the monoclonal band (M-band) was measured densitometrically using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The concentration of M-proteins in the serum was calculated by the method.

2.4. Purification of IgG

IgG was purified using a HiTrap Protein G column (7 × 25 mm; GE Healthcare, Buckinghamshire, UK). Briefly, 100 µL of serum was diluted with 2 mL of binding buffer (20 mM sodium phosphate, pH 7.0) and then applied to the column. Protein was eluted from the column with elution buffer (100 mM glycine-HCl, pH 2.7). The elution profile was monitored at 280 nm using an Akta Explorer System (GE Healthcare). The eluted sample was dialyzed against 150 mM NaCl in order to exchange the buffer, and then concentrated using an ultrafiltration device (Minicon B15, Millipore, Tokyo, Japan).

2.5. 2-DE

A flowchart illustrating 2-DE analysis of serum IgG is summarized in Supplementary Figure 1A. A total of 12.5 µg of protein G-purified IgG was desalted and concentrated by precipitation with cold 100% acetone. The protein pellet was dissolved in protein solubilizer (Invitrogen, Tokyo, Japan), 0.65% carrier ampholytes 3–10 (Invitrogen), and 3.2 mM dithiothreitol (DTT). The IPG strips dimension designed as mini-gel format was used for 2-DE experiments. The protein solution was then applied to an IPG Zoom strip gel (pH 3–10, 7cm, Invitrogen) and incubated overnight. Isoelectric focusing (IEF) was carried out as the manufacture's recommend conditions (175 V constant for 20 min, linear increase from 175 to 2000 V over 45 min, followed by 2000 V constant for 30 min) [34; 35; 36]. IEF standards (Bio-Rad Laboratories, Tokyo, Japan) were used as pI markers. The proteins resolved in the IPG strip were reduced with 50 mM DTT in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and alkylated with 125 mM iodoacetamide in LDS sample buffer. After alkylation, the gel was transferred onto a precast NuPAGE 4%–12% Bis-Tris polyacrylamide gel (Invitrogen), and NuPAGE MES SDS running buffer (Invitrogen) was applied. The second dimension SDS-PAGE separation was performed at a constant voltage of 200 V. Mark12 unstained protein standards (Invitrogen) were used as molecular weight markers. After electrophoresis, the gel was stained with CBB R-250 (0.25% CBB R-250/50% methanol/10% acetic acid).

2.6. High-sensitive 3-DE

The flowchart of high-sensitive 3-DE method, which combines agarose gel electrophoresis, IEF, and SDS-PAGE, is illustrated in Supplementary Figure 1B. Samples were also analyzed using the original

3-DE described by Vu et al. [33] to validate the utility of the high-sensitive 3-DE.

2.6.1. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out at 15 V/cm using a 1% agarose gel (type HAS, Litex, Copenhagen, Denmark) in 0.05 M barbital buffer, pH 8.6. The agarose gel plate was prepared by hand using commercially available slide glass (76 × 26 mm).

2.6.2. Extraction of M-proteins

Two agarose gels were prepared per sample, and the gels were simultaneously electrophoresed in parallel. One gel from each pair was fixed in a 5.2 mM picric acid/20% acetic acid solution to visualize the M-band, and this gel was used as a template to locate the position of the M-band on the other agarose gel. The M-band on the unfixed gel was excised using a picking tool guided by the location of the M-band on the fixed gel. The excised gel strip was transferred into a 1.5-mL microcentrifuge tube and homogenized using a microspatula. M-proteins were extracted from the band using five solutions: deionized water, 150 mM NaCl, 100 mM acetate-buffered saline (ABS, pH 4.8), 16 mM phosphate-buffered saline (PBS, pH 7.4), and 20 mM Tris-buffered saline (TBS, pH 8.0). Some M-proteins were extracted by sonicating for 5 min or vortexing for 30 min in order to increase the extraction efficiency. Following extraction, all samples were stored overnight at 4°C to facilitate diffusion of M-proteins from the gel. The gel slurry containing extracted protein was then transferred onto an Ultrafree-MC centrifugal filter (Millipore). On the side of these treatments, the M-bands which were disposed without any solutions were subjected to transfer directly onto an Ultrafree-MC centrifugal filter. After centrifugation at 12,000 × *g* for 5 min, the extract was collected and the total protein concentration

was determined.

2.6.3. IEF/SDS-PAGE

A total of 12.5 µg of M-protein extracted from the agarose gel was subjected to 2-DE as described above.

2.7. Image analysis

Protein spots detected on gels were analyzed using an ImageMaster 2D Platinum 6.0 (GE Healthcare). Corrected pI values were calculated using the following formula in order to take into account the volume of each spot:

$$\text{Corrected pI} = \frac{\sum_1^N pI \times volume}{\sum_1^N volume} .$$

2.8. Statistical analysis

Values for the percentage of M-protein extracted from gel slices and the corrected pI values for heavy chains and/or light chains are shown as the mean ± SD. Statistical significance was determined using one-factor ANOVA and Mann-Whitney's U-test. The level of significance was set at $P < 0.05$.

Results

3.1. Overview of high-sensitive 3-DE method

As the step of M-proteins extraction from agarose gel has not been optimized, we first established the most suitable methods for extracting M-proteins from the agarose gel. We used IgG₁-κ for

all M-protein extraction recovery experiments. The M-protein concentration was 1.73 g/dL. Various aqueous solvents (deionized water, saline, ABS, PBS, or TBS) were tested to determine the optimal solution for M-protein extraction. We then examined the effect of extraction solution volume (0, 180, or 360 μ L) on M-protein extraction efficiency.

3.1.1. Type of extraction solution

In determining the optimal solution for M-protein extraction, the volume was fixed at 360 μ L, which corresponded to 10 times the volume of the excised gel band. The percent recoveries were $79.1 \pm 7.2\%$, $90.6 \pm 4.9\%$, $78.8 \pm 12.9\%$, $89.0 \pm 21.5\%$, and $89.2 \pm 9.8\%$ in deionized water, saline, ABS, PBS, and TBS, respectively (Figure 1A), indicating that saline is the most suitable solution for M-protein extraction. We also examined the effect of sonication or vortexing on the efficiency of M-protein extraction in saline, but neither treatment had an appreciable effect on recovery (data not shown).

3.1.2. Volume of extraction solution

Next, we determined the optimal volume of extraction solution. Recovery of M-protein was examined using 0, 180, and 360 μ L of saline (150 mM NaCl) as the extraction solution. The recovery of M-protein using either 180 or 360 μ L was significantly higher ($P < 0.01$ or $P < 0.001$, respectively) than in extractions carried out in the absence of extraction solution (Figure 1B). The data thus indicated that extraction in 360 μ L of saline provided the greatest recovery.

3.2. Validation of the utility of the high-sensitive 3-DE method

In order to validate the utility of the high-sensitive 3-DE method, we first compared the percentage of M-protein recovered in the agarose gel electrophoresis with the high-sensitive method and

the original method developed by Vu et al. [33]. As shown in Figure 2A, recovery of M-protein was significantly higher with the high-sensitive method than with the original method. ($90.6 \pm 4.9\%$ vs. $14.8 \pm 1.0\%$, $P < 0.001$).

We then compared the pattern of M-protein spots obtained with each 3-DE method. Spots were easily detected with CBB staining with the high-sensitive 3-DE method (Figure 2B). In contrast, spots were barely detectable using the original 3-DE method with CBB staining (Figure 2C) due to relatively poor M-protein recovery from the first dimension. Spots could be clearly detected on gels prepared using the original method by double staining with CBB and silver stain (Figure 2D). These results indicated that high-sensitive 3-DE method is more effective in terms of detecting spots with CBB staining.

3.3. The comparison of the high-sensitive 3-DE with 2DE

Although 2-DE has been employed for the characterization of M-proteins, the results of analyses of M-proteins isolated from serum, plasma, urine, and even immunoglobulin purified with protein A or protein G have been less than satisfactory, as M-proteins are often masked in 2-DE gels by residual immunoglobulins or other proteins. To further confirm the utility of the 3-DE method, we compared between high-sensitive 3-DE and 2-DE method.

For high-sensitive 3-DE analysis, 12.5 μg of M-protein extracted from an agarose gel was subjected to IEF/SDS-PAGE, and the resulting gel was stained with CBB. The spot pattern obtained using the high-sensitive 3-DE method is shown in Figure 3A. Only monoclonal heavy chains (solid arrows) and light chains (solid arrowheads) were detected. For the 2-DE analysis, purified IgG was subjected to a first dimension separation involving IEF, followed by SDS-PAGE in the second dimension. The 2-DE spot

pattern is shown in Figure 3B. Polyclonal γ heavy chains (open arrows), monoclonal heavy chains (solid arrows), and monoclonal light chains (solid arrowheads) were detected on the 2-DE gels upon CBB staining. These results indicated that the high-sensitive 3-DE method is capable of separating M-proteins from residual normal IgG.

3.4. Analysis of M-protein heterogeneity using the high-sensitive 3-DE method

The serum samples collected from patients diagnosed with MM were subjected to 3-DE analysis. Upon first dimension agarose gel electrophoresis, the electrophoretic mobility of the M-bands from the point of sample application ranged from -4.82 to 2.74 mm. These M-proteins were extracted from the agarose gel and resolved into several spots using IEF/SDS-PAGE. As shown in Figure 4A, in all cases, the monoclonal heavy and/or light chains separated into heterogeneous spots according to charge. As shown in Figure 4B, the monoclonal light chains separated into heterogeneous spots according to mass in only one case.

We also examined the corrected pI values, which were calculated taking into account the volume of each spot on the 3-DE pattern. The corrected pI value reflects the charge variation of an M-protein more so than the uncorrected pI. The mean heavy chain corrected pI was 7.86 ± 0.45 . In contrast, the mean κ chain corrected pI of 7.53 ± 0.34 was significantly higher than that of the λ chain (6.73 ± 0.48 , $P < 0.05$). The results of the 3-DE analyses are summarized in Table 1.

4. Discussion

In this study, we developed a method for high-sensitive 3-DE analysis of M-proteins that provides extremely high recovery of protein from the agarose gel using saline extraction. M-proteins are then concentrated by acetone precipitation and subjected to IEF and SDS-PAGE. The high-sensitive 3-DE method we describe is more effective than the originally described 3-DE method for detecting M-protein spots on gels stained with CBB. The high-sensitive 3-DE method we developed also separates M-proteins from residual normal IgG which could not be done by 2-DE. Furthermore, high-sensitive 3-DE analysis of clinically derived serum samples demonstrated that M-proteins are highly heterogeneous and vary considerably between individuals.

There have been only a limited number of reports of studies combining agarose gel electrophoresis and 2-DE [30; 31; 32; 33], all of which focused on characterizing M-proteins. However, the methods used in these studies varied. In particular, the process of extracting M-proteins from the agarose gel and preparing them for IEF/SDS-PAGE was challenged. We initially determined the recovery of M-protein extracted from agarose gels in order to evaluate the optimal extraction conditions for the high-sensitive 3-DE. Our experiments indicated that saline provides the highest percent recovery of M-protein, at 90.6% (Figure 1A). The use of saline permits extraction of M-proteins under physiological conditions, thereby minimizing the potential for degradation.

To validate the utility of the high-sensitive 3-DE method, we compared it to the method described by Vu et al. [33]. The recovery of M-proteins with the high-sensitive method was clearly higher than with the method of Vu et al. (Figure 2A). In the high-sensitive method, M-bands were picked from handmade agarose gels, homogenized, and the gel fragments were incubated in saline to facilitate

diffusion of protein out of the agarose. In the method of Vu et al., however, the M-bands are scraped from the commercially available gel film and centrifuged without liquid extraction. Our results indicate that allowing for diffusion into an aqueous solvent results in a significant increase in the percent recovery of M-proteins. These results agree with the 3-DE patterns. Using the high-sensitive 3-DE method, spots of M-proteins can be clearly detected with CBB staining alone (Figure 2B). In contrast, M-protein spots are difficult to visualize with CBB staining alone using the original 3-DE method (Figure 2C), which requires silver staining in order to detect M-proteins (Figure 2D). The few 3-DE studies that have been published to date involved the use of only silver staining, presumably due to low recovery of M-protein from the agarose gel. We believe that the high-sensitive 3-DE method described here substitute for the previous 3-DE methods because M-protein spots can be clearly detected with CBB staining alone, suggesting that the high-sensitive 3-DE represents a powerful new tool for characterizing M-proteins in proteomic studies.

We also examined M-proteins using the high-sensitive 3-DE method and purified IgG using the traditional 2-DE. Only monoclonal heavy and light chains were detected on 3-DE gels (Figure 3A), whereas polyclonal γ heavy chains were detected in addition to heavy and light monoclonal chains on 2-DE gels (Figure 3B). To the best of our knowledge, this is the first report to confirm that 3-DE can clearly separate M-proteins from residual normal IgG, in contrast to 2-DE.

We also applied the high-sensitive 3-DE method to the analysis of M-proteins heterogeneity. The high-sensitive 3-DE analysis showed that M-proteins form heterogeneous spots according to charge and/or mass (Figures 4A and 4B). These data agree with the results of previous 3-DE studies [30; 31; 32;

33], which demonstrated that like M-proteins, BJP and monoclonal free light chains also resolve into heterogeneous spots upon 2-DE or IEF [20; 21; 22; 23; 24; 25; 26; 27; 28; 29; 37; 38; 39; 40]. Harrison [30] examined whether storage produces artifacts with respect to heterogeneity in the pattern of immunoglobulin spots, and suggested that charge heterogeneity of monoclonal light chains reflects case-specific differences in the physiology of M-protein metabolism rather than artifacts introduced by degradation occurring during collection, storage, or analysis. Mimura et al. [41] showed that charge heterogeneity of mouse monoclonal IgG (1B7-11) can be accounted for by intracellular modification and extracellular chemical modification that reflect aging of its molecules. Fully analyzing the heterogeneity of M-proteins thus necessitates that they be analyzed inside the cells that produce them.

We also examined the corrected pI of heavy chains and light chains. In contrast to a previous study [33], we found that the λ chain corrected pI is significantly lower than that of the κ chain. Mimura et al. [42] and Hashimoto et al. [43] reported that light chains from MM patients are abnormally glycosylated. It is known that glycosylation affects the charge of glycoprotein molecules; in particular, attachment of sialic acid to the nonreducing terminal results in a negative charge. Glycomics studies involving samples from a large number of clinical cases are needed in order to clearly determine if there is significant variation in the corrected pI of light chain isotypes.

In conclusion, we developed a high-sensitive 3-DE method that is more efficient than existing methods in terms of M-protein recovery, resulting in greater detection sensitivity. We also demonstrated that high-sensitive 3-DE separates M-proteins from residual normal IgG. An analysis of clinical samples using the high-sensitive 3-DE method demonstrated that the characteristics of M-proteins vary

tremendously between individuals. We believe that the high-sensitive 3-DE method described here will prove useful in further studies of the heterogeneity of M-proteins.

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The authors have no conflicts of interest to declare.

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Table 1.

The characteristics of M-proteins

Case	Isotype of M-proteins	Electrophoretic mobility of M-bands (mm) ^{a)}	Spots heterogeneity	Spots numbers		Corrected pI	
				Heavy chain	Light chain	Heavy chain	Light chain
1	IgG ₁ -κ	-4.82	Charge and mass	1	2	8.27	7.34
2	IgG ₁ -κ	-1.70	Charge	4	2	7.30	7.93
3	IgG ₁ -κ	0.57	Charge	5	3	7.46	7.68
4	IgG ₁ -λ	-1.86	Charge	1	2	8.20	7.14
5	IgG ₁ -κ	-2.50	Charge	2	2	7.84	7.17
6	IgG ₁ -λ	2.74	Charge	3	2	7.52	6.84
7	IgG ₁ -λ	-2.50	Charge	1	2	8.44	6.20

a) Electrophoretic mobility of M-bands on agarose gels was measured from the point of sample application.

Figure legends

Figure 1.

Comparison study on extraction recovery of M-proteins from agarose gels. M-proteins were extracted with (A) 360 μ l of deionized water (dH₂O), saline, acetate-buffered saline (ABS), PBS or TBS and (B) 180 or 360 μ l of saline or without extract solution (0 μ l). Statistical analysis was performed by one factor ANOVA. The level of significance was set at $p < 0.05$. All samples were analyzed in triplicate (error bars indicate standard deviations).

Figure 2.

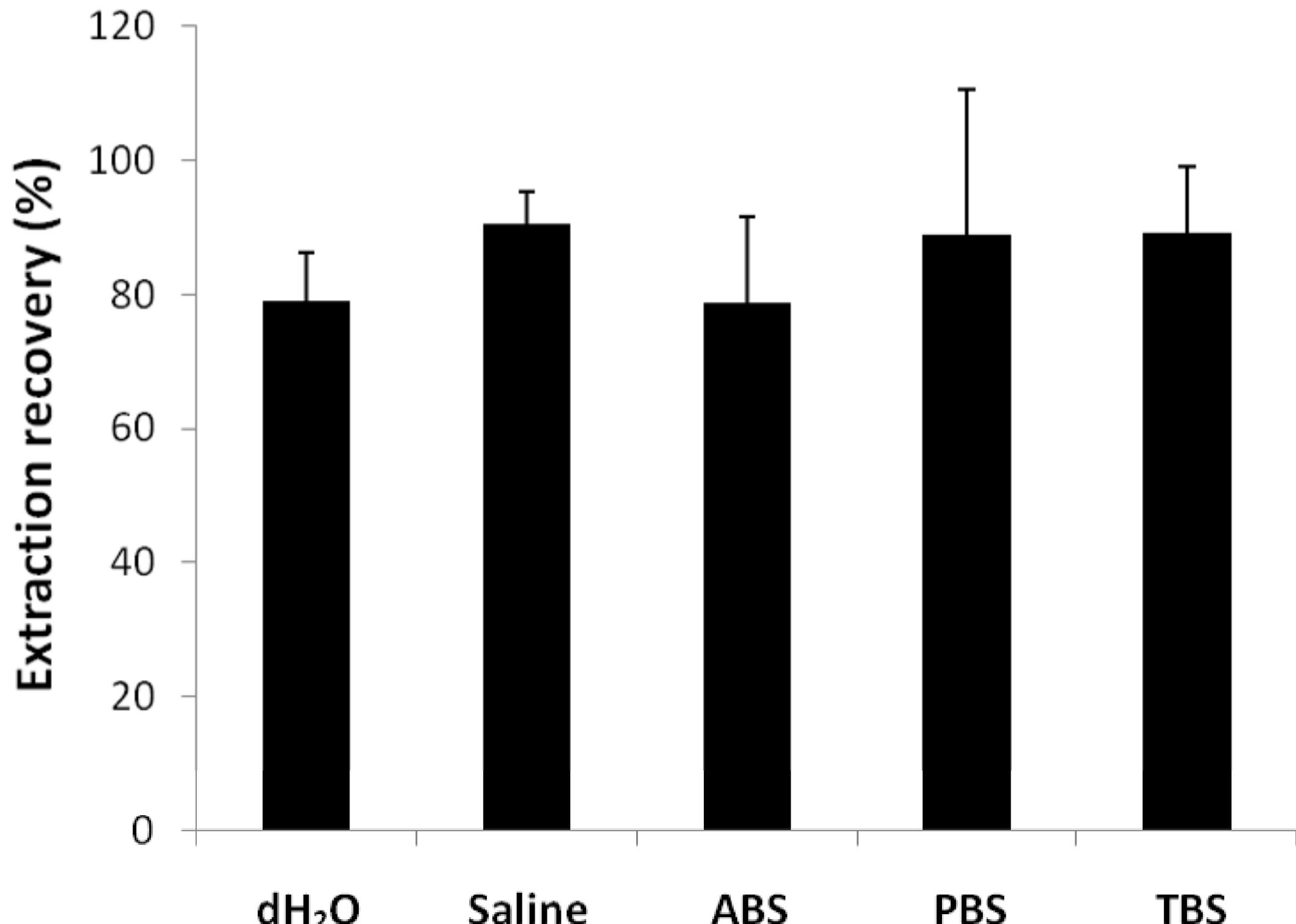
Validation study between high-sensitive 3-DE and original 3-DE method. (A) M-proteins were extracted using our method with 360 μ l of saline (High-sensitive) and the original method described by Vu et al. [33] (Original). Statistical analysis was performed by one factor ANOVA. The level of significance was set at $p < 0.05$. All samples were analyzed in triplicate (error bars indicate standard deviations). (B) The high-sensitive 3-DE spot pattern with CBB staining. The original 3-DE spot patterns (C) with CBB staining and (D) double staining with CBB and silver stain.

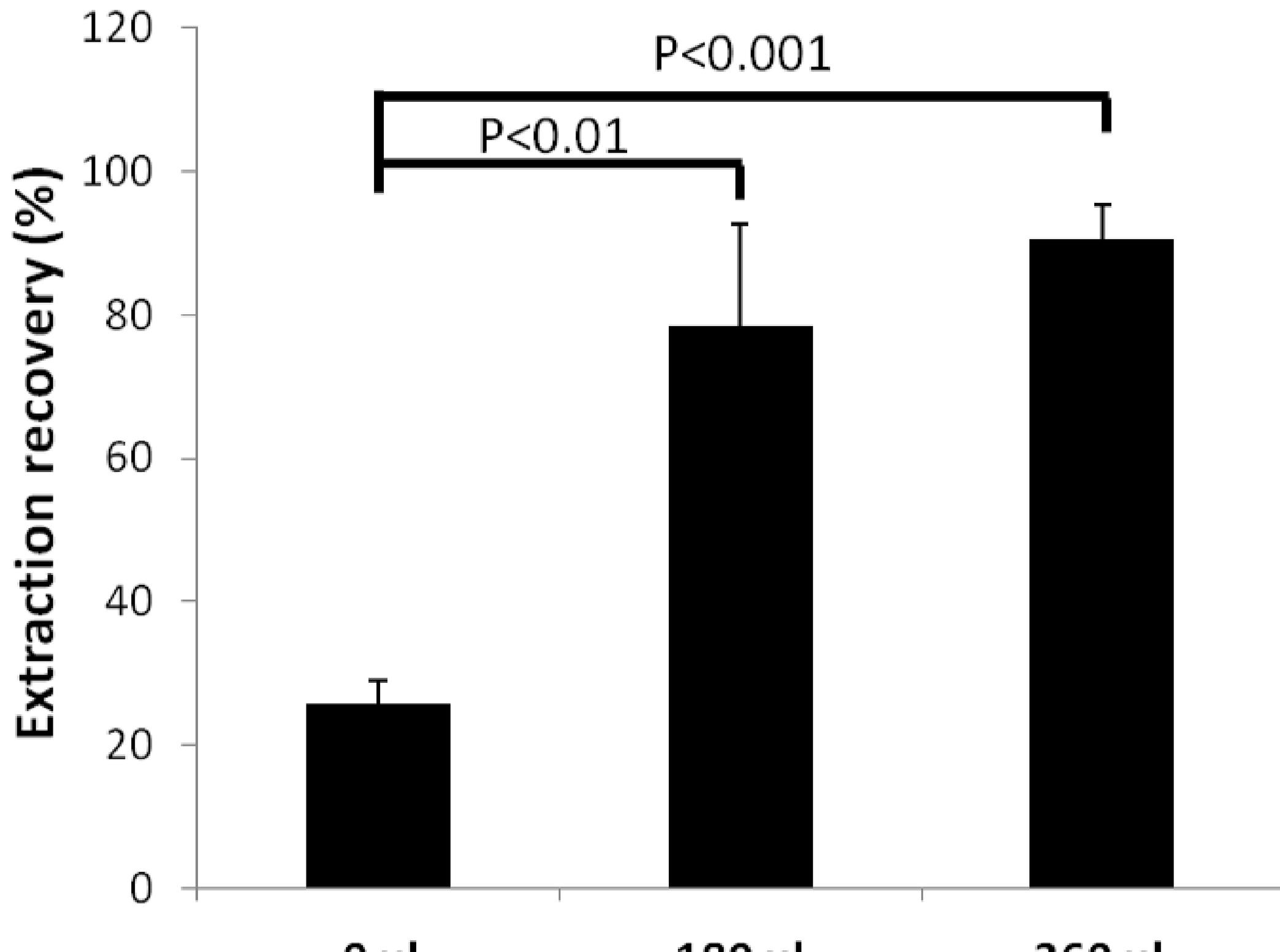
Figure 3.

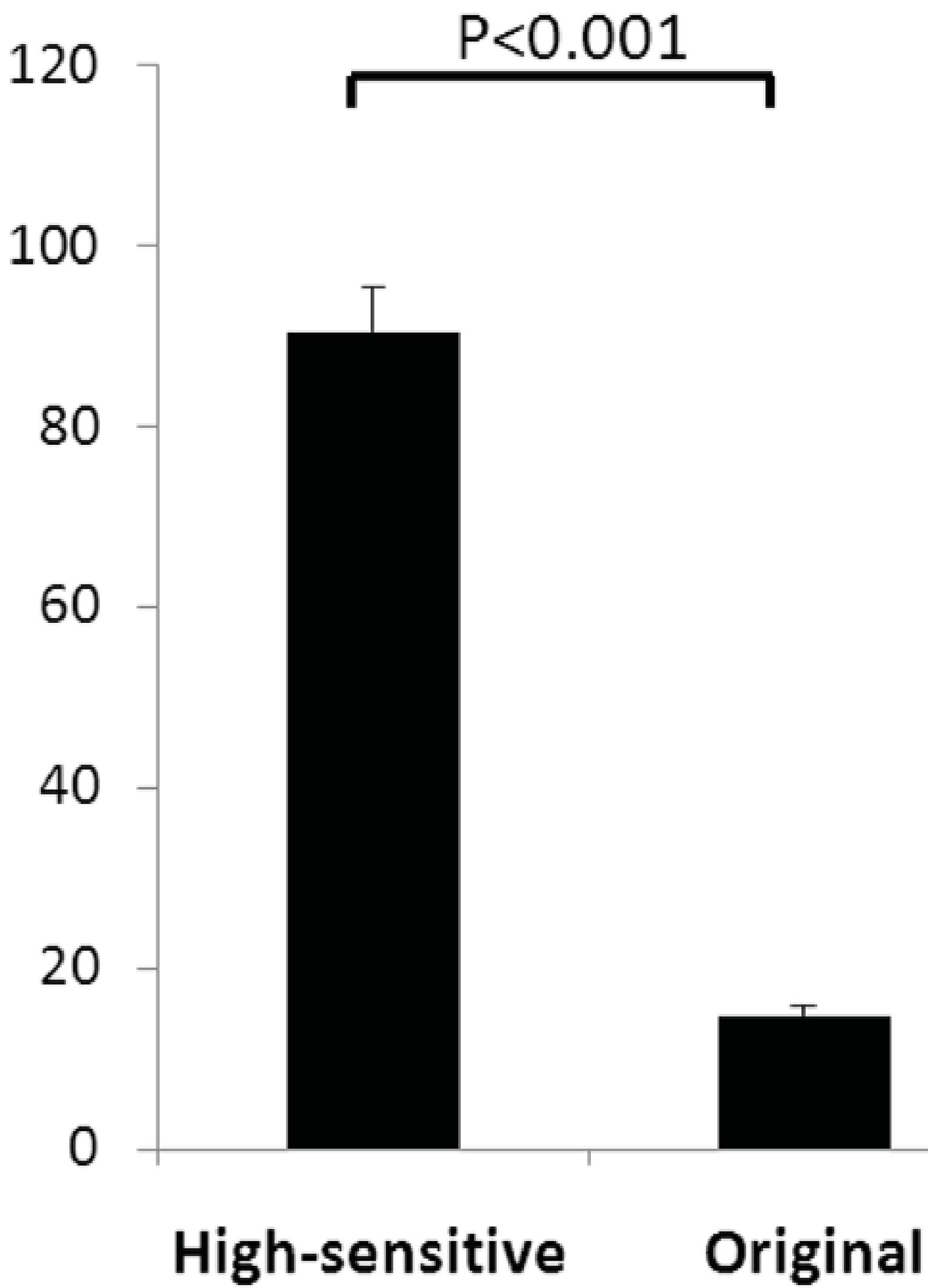
Electrophoretic spot patterns of M-proteins upon high-sensitive 3-DE and 2-DE. (A) High-sensitive 3-DE spot pattern of M-proteins. Only monoclonal heavy chains (solid arrow) and light chains (solid arrowhead) were detected. (B) 2-DE spot pattern of purified IgG. Polyclonal γ heavy chains (open arrows), monoclonal heavy chains (solid arrows), and monoclonal light chains (solid arrowheads) were detected.

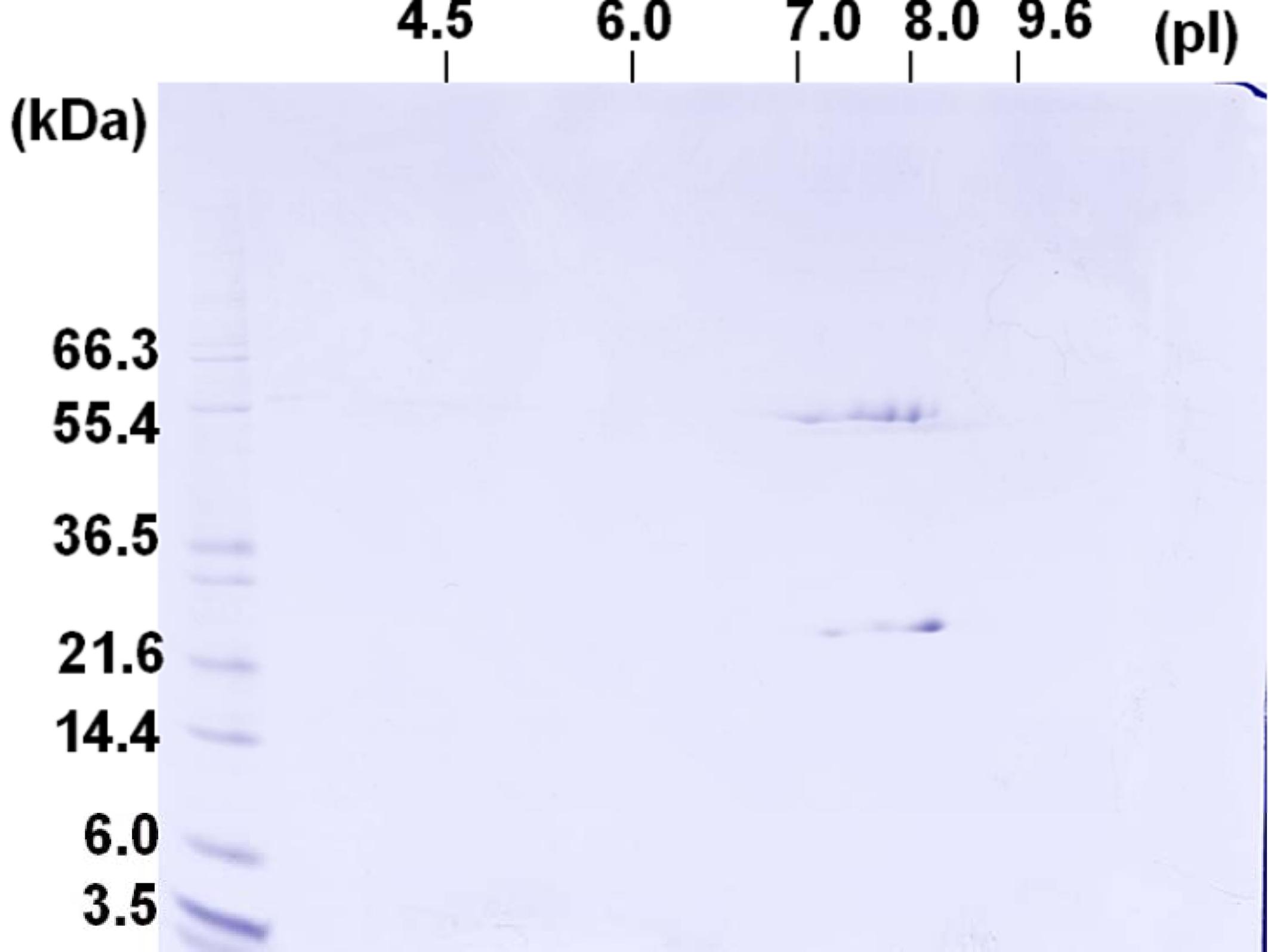
Figure 4.

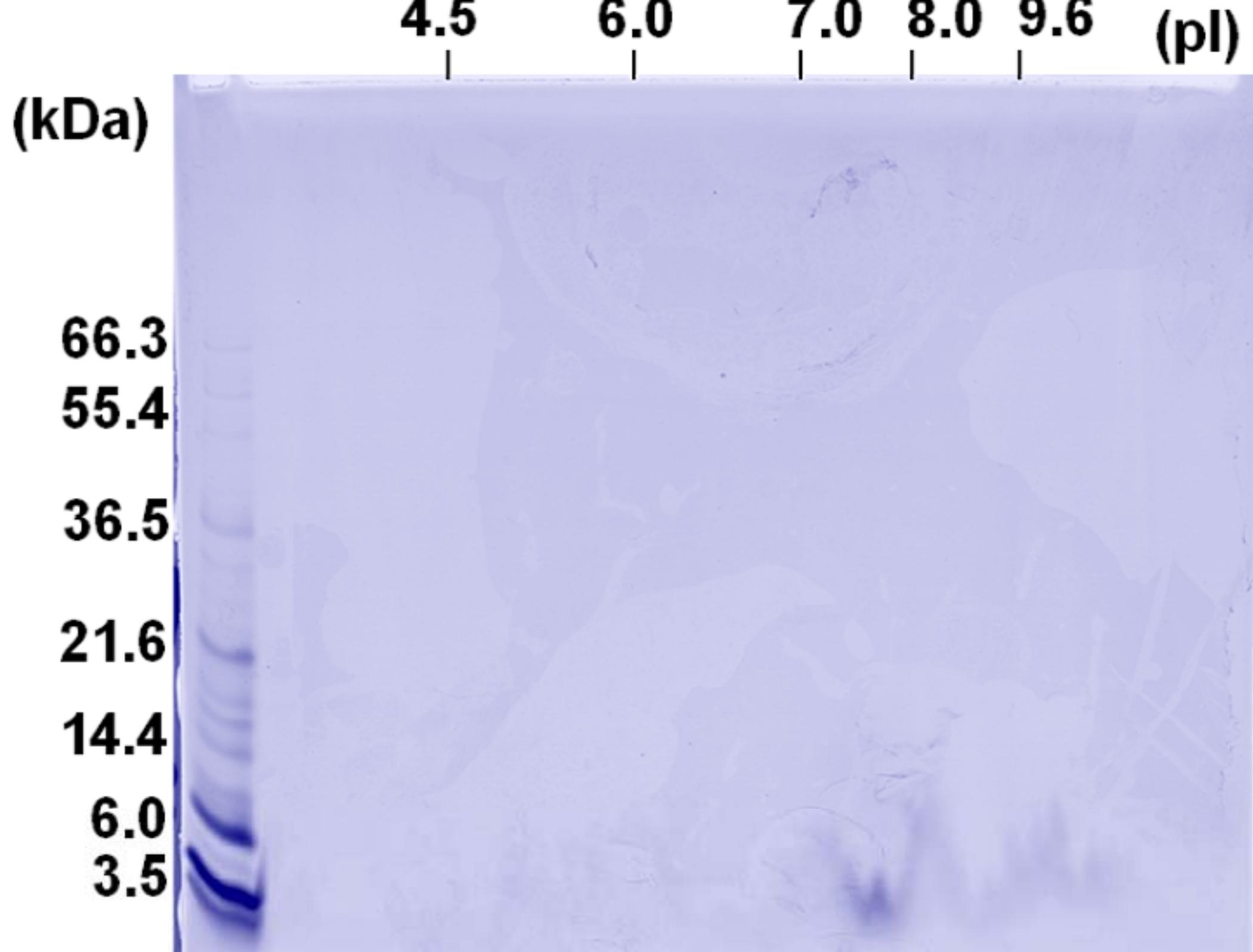
The high-sensitive 3-DE spot patterns of M-proteins with CBB staining. (A) The high-sensitive 3-DE spot pattern of IgG- λ type M-proteins (Case 6). Monoclonal heavy chains and light chains were separated into three and two heterogeneous spots according to charge, respectively. (B) The high-sensitive 3-DE spot pattern of IgG- κ type M-protein (Case 1). Monoclonal heavy chains were separated into one spot. Monoclonal light chains were separated into two heterogeneous spots according to charge and mass. The solid and dashed line circle indicated the charge and mass heterogeneous spots, respectively.

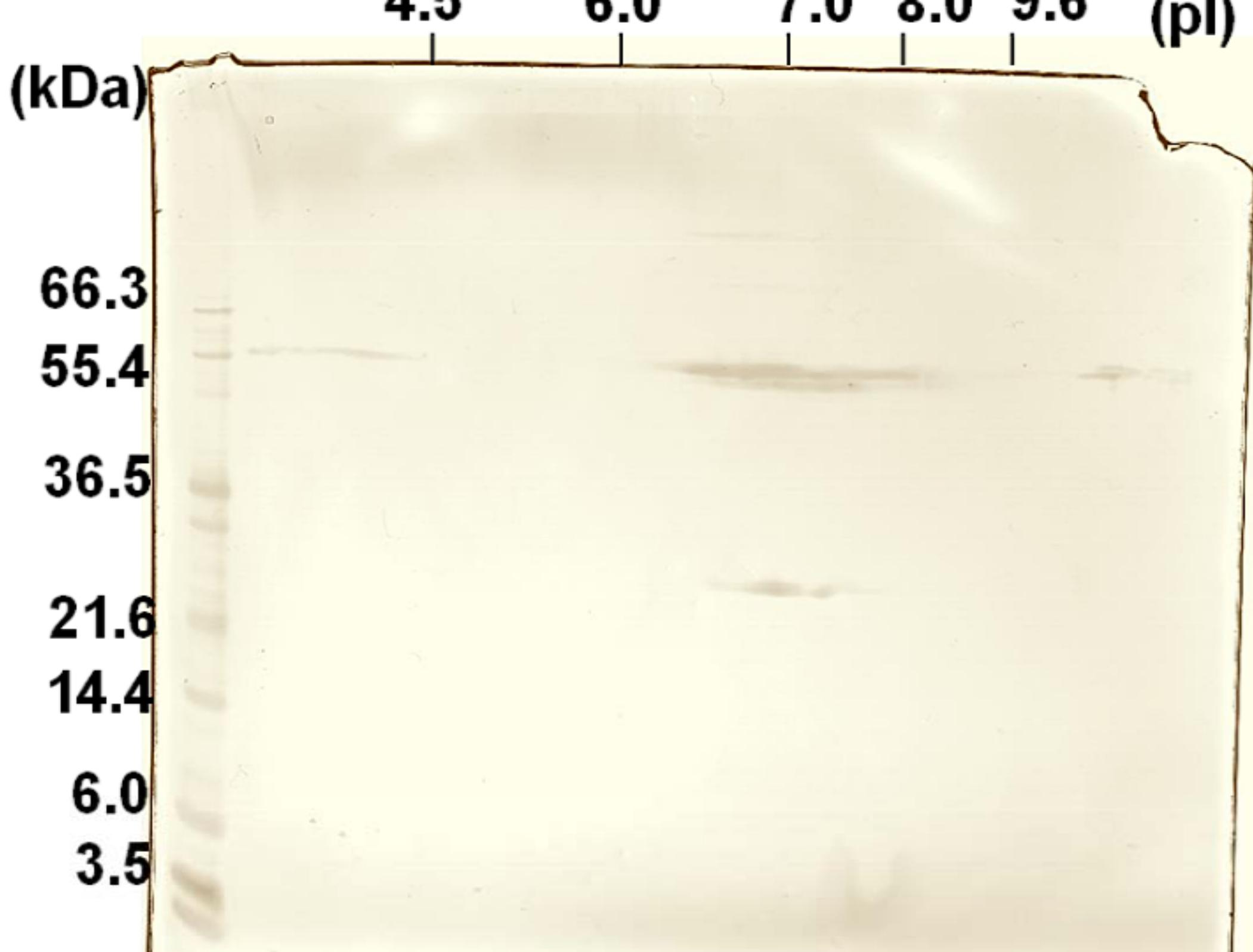


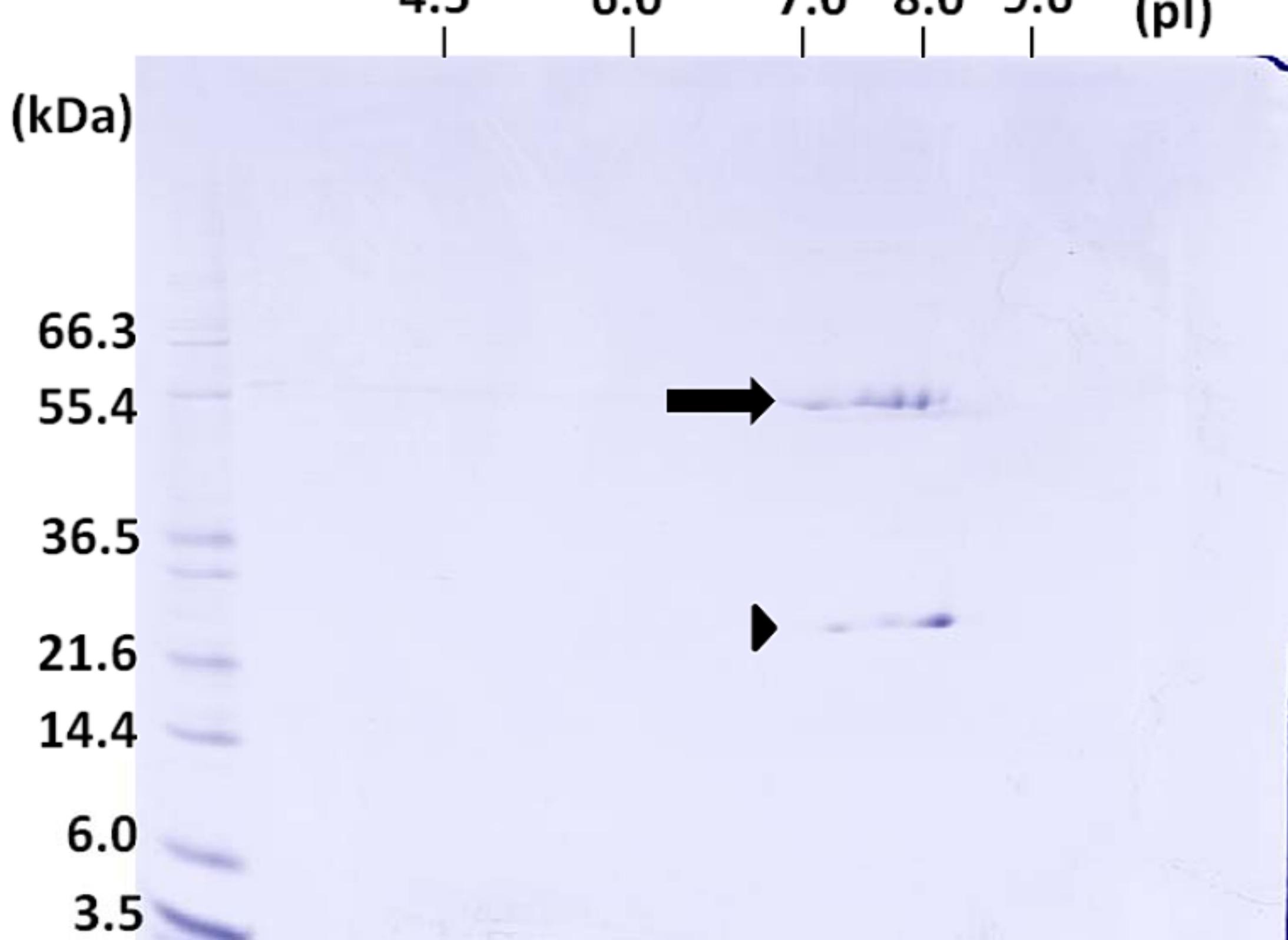












(kDa)

66.3

55.4

36.5

21.6

14.4

6.0

3.5

(pI)



(kDa)

11.0 8.0 7.0 6.0 5.0 (pI)

66.3

55.4

36.5

21.6

14.4

6.0

3.5

