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Design of a molecular weight analysis system for proteins using sodium dodecyl sulfate-agarose gel

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SUMMARY

Methods for separating proteins according to molecular weight are usually based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, the toxicity of acrylamide and its handling during gel preparation constitute a significant problem. Therefore, we determined the molecular weights of proteins using agarose gel instead of polyacrylamide. We used the Mupid electrophoretic apparatus, because it is small, easy to use, and time-saving. We evaluated different types of agarose and buffer solutions, and parameters such as gel concentration, buffer SDS concentration, and quantity of gel (central thickness). The optimal gel was made from 5 ml (central gel thickness 2 mm) 4% NuSieve 3:1 agarose. An electrophoresis buffer containing 25 mM Tris, 190 mM glycine (pH 8.3), and 0.1% SDS is optimal for protein separation. With this method, the standard curve was linear for reference proteins in the range of molecular weights from 14.4×10^3 to 97×10^3 .

Key words: SDS agarose gel, electrophoresis, molecular weight.

INTRODUCTION

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to measure the molecular weights of proteins. In some clinical laboratories, SDS-PAGE is used to distinguish between glomerular dysfunction and tubular dysfunction. However, polyacrylamide is highly toxic and the preparation of polyacrylamide is time-consuming. Agarose gel is an appropriate support gel with which to replace polyacrylamide in this context. Agarose gel is a polymer of sugar extracted from seaweed and forms a comparatively large reticulated structure.

Bricon et al.¹⁾ and Sakakibara et al.²⁾ have previously reported the separation of proteins of different molecular weight using SDS-agarose gel. In 1998, Bricon et al. used agarose gel to separate some proteins in urine, especially Bence Jones protein. Sakakibara et al. purified human chorionic gonadotrophin, and produced its antibody in 1987.

We assembled a horizontal SDS-agarose gel electrophoretic apparatus using the Mupid equipment (Advance, Co., Ltd., Tokyo, Japan), an electrophoresis device that is easy and time-saving to use in a clinical laboratory.

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; β_2 -m, β_2 -microglobulin; RBP, retinol binding protein; α_1 -m, α_1 -microglobulin; HSA, human serum albumin; TF, transferrin; ALB, albumin; IgG, immunoglobulin G; TSA solution, trichloroacetic acid and sulfosalicylic acid solution; LMW, low molecular weight.

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MATERIALS AND METHODS

1. Preparation of reagents and samples

Reagents

As shown in Table 1, NuSieve 3:1 (BMA, Rockland, ME, USA), SeaPlaque (BMA), SeaKem LE (BMA), and Agarose S (Wako Pure Chemical Industries Ltd., Osaka, Japan) were used. A 25 mM Tris, 190 mM glycine buffer containing 0.1% SDS (pH 8.3) was prepared according to the method of Laemmli.³⁾ A 50 mM imidazole buffer (pH 9.5) and a 100 mM phosphate buffer (pH 7.0) were prepared, using the protocols of Bricon et al.¹⁾ and Weber and Osborn,⁴⁾ respectively. Both buffers contained 0.1% SDS. A solution (TSA) containing 0.4 M trichloroacetic acid and 0.03 M sulfosalicylic acid was used to fix proteins, and Quick CBB (Wako Pure Chemical Industries Ltd.) was used to stain proteins.

Preparation of samples

Samples of sera from two healthy subjects and a LMW marker (Low Molecular Weight Calibration Kit for electrophoresis, Amersham Biosciences Co. Ltd., Piscataway, NJ, USA) were used. Samples were diluted with each buffer and bromophenol blue (BPB) was added to a final concentration of 0.1%. The protein standards β_2 -microglobulin (β_2 -m; Sigma Chemical Co., Ltd., St. Louis, MO, USA), retinol binding protein (RBP; Sigma Chemical Co., Ltd.), α_1 -microglobulin (α_1 -m; Cortex Biochem Inc., San Leandro, CA, USA), human serum albumin (HSA; Wako Pure Chemical Industries Ltd.), transferrin (TF; Wako Pure Chemical Industries Ltd.), and IgG (Oriental Yeast Co., Ltd., Tokyo, Japan) were used to characterize the separation according to molecular weight.

2. Procedures

Each type of agarose shown in Table 1 was prepared at 2%, 3%, 4%, and 5% concentrations using each buffer solution. Five, 7.5, and 10 ml of each gel solution was poured into a 5×6 cm² gel tray of the Mupid unit (Advance, Co., Ltd.) and left to set for about 20 min at room temperature. Samples of LMW protein markers (14.4 μ g), human serum (35 μ g), and protein standard (5 μ g), were applied to the wells of the agarose gels and separated electrophoretically for 30 min at a constant voltage of 100 V. After electrophoresis, the proteins in the agarose gels were fixed with TSA solution for 1 h. The proteins were then stained with Quick CBB for 2 h

and destained with distilled water. Photographs of the agarose gels were taken using Pictro Stat (Fuji Photo Film Co., Ltd., Tokyo, Japan).

The relative mobilities of the proteins in the LMW marker sample and the protein standards were calculated by the comparing the migration distance with that of the BPB marker.

RESULTS

Selection of agarose

All four kinds of agarose, NuSieve 3:1, SeaPlaque, SeaKem LE, and Agarose S, were dissolved to a concentration of 4%. Five milliliters of each agarose (giving a central gel thickness of 2 mm) was prepared and used for electrophoresis in 25 mM Tris, 190 mM glycine buffer (pH 8.3). Protein electrophoretic patterns produced with the four kinds of agarose gel are shown in Fig. 1 a, and the standard curve for the LMW marker is shown in Fig. 1 b. Because the melting temperature of SeaPlaque is low, gel preparation was easier than for the other gels, but the resolution was very poor and no standard curve could be plotted. Moreover, the gel strength of SeaPlaque was low. Although the separation capacity of SeaKem LE and Agarose S did not differ significantly from that of NuSieve 3:1, the sharpness of the protein bands on these gels was inferior to that achieved with NuSieve 3:1. NuSieve 3:1 is a low viscosity and is easy to prepare at high concentrations (4-5%). Although the gel strength of NuSieve 3:1 is slightly lower than that of the other gels, separation of the LMW marker was most effective. Therefore, we decided to examine the other gel parameters using NuSieve 3:1. Standard curves for each agarose gel, except SeaPlaque, showed good linearity in the molecular weight range from 14.4×10^3 to 97×10^3 .

Selection of buffer solution

Buffer solutions were examined using 4% NuSieve 3:1. Three kinds of buffer solution were tested: 25 mM Tris, 190 mM glycine buffer (pH 8.3), 50 mM imidazole buffer (pH 9.5), and 100 mM phosphate buffer (pH 7.0). The SDS concentration in the buffer solutions was 0.1%. The relative mobilities of the protein fractions in 100 mM phosphate buffer (pH 7.0) were very low, even though the electrophoretic period was identical for all buffers (Fig. 2). In 50 mM imidazole buffer (pH 9.5), the bands of the LMW markers became broad and the separation was poor. The separation in 25 mM Tris, 190

Table 1. Kind of agarose.

Product name (manufacturer)	EEO	Re-melting temperature	Gel strength
SeaKem LE (BMA Inc.)	0.09-0.13	≤100°C	≥1200 g/cm ² (1%)
NuSieve 3:1 (BMA Inc.)	≤0.13	≤90°C (4%)	≥1400 g/cm ² (4%)
Agarose S (Wako Pure Chemical Industries, Ltd.)	0.1	≤90°C (1.5%)	≥1200 g/cm ² (1.5%)
SeaPlaque (BMA Inc.)	≤0.13	≤65°C (1.5%)	≥200 g/cm ² (1%)

Fig. 1. Effects of different kinds of agarose on protein band patterns and standard curves of each LMW marker.

a: Patterns of electrophoresis. 1. SeaKem LE; 2. NuSieve 3:1; 3. Agarose S; 4. SeaPlaque. ① LMW marker; ② human serum albumin standard; ③④ healthy human serum. b: Standard curve for each LMW marker.

Fig. 2. Effects of buffer solutions on protein band patterns and standard curves for each LMW marker.

a: Patterns of electrophoresis. 1. 50 mM imidazole buffer (pH 9.5); 2. 100 mM phosphate buffer (pH 7.0); 3. 25 mM Tris, 190 mM glycine buffer (pH 8.3). ① LMW marker; ② human serum albumin standard; ③④ healthy human serum. b: Standard curves for each LMW marker.

mM glycine buffer (pH 8.3) was the best of the three buffer solutions. The bands of the LMW markers in 25 mM Tris, 190 mM glycine buffer (pH 8.3) were sharp and clear. The above results indicate that when the buffer pH is near neutrality, protein bands migrate with difficulty. Conversely, when buffers are highly alkaline (pH 9.5), the protein bands become blurred. Therefore, we selected 25 mM Tris, 190 mM glycine buffer (pH 8.3) to test the other gel parameters.

Selection of gel concentration

The gel concentration of NuSieve 3:1 was examined using 25 mM Tris, 190 mM glycine buffer containing 0.1% SDS (pH 8.3) (Fig. 3). Gel concentrations of 2%, 3%, 4%, and 5% were prepared. At 2%, the separation of the protein bands was poor and all the bands were inclined towards the anode. This phenomenon occurred because at low gel concentrations, the pore size is coarse. At 5%, the protein bands were indistinct and the relative mobility of each protein band was low. Comparing the 3% and 4% gels, the LMW marker pattern on the 4% gel was sharper than on the 3% gel. Therefore, we selected 4% gel as the optimal concentration.

Selection of SDS concentration in the buffer solution

Having selected 4% NuSieve 3:1 and 25 mM Tris, 190 mM glycine buffer (pH 8.3) as optimal for electrophoresis, we next examined the appropriate SDS concentration to be added to the buffer. Gels with final SDS concentrations of 0, 0.05, 0.1, and 0.2% were prepared. At 0% (no SDS), separation according to molecular weight did not occur. At 0.2%, separation of the lower molecular weight bands of the LMW markers was poor

and the protein bands were comparatively broad. The protein bands were separated more clearly in buffers containing 0.05% and 0.1% SDS. Linearity of the standard curves was achieved in the range of molecular weights from 14.4×10^3 to 97×10^3 with SDS concentrations of both 0.05% and 0.1%. However, when these two SDS concentrations were compared, the serum protein bands were separated more clearly with a buffer concentration of 0.1% SDS. For this reason, we selected 0.1% as the optimal SDS concentration (Fig. 4).

Selection of gel thickness

We compared 5-ml, 7.5-ml, and 10-ml 4% NuSieve 3:1 gels. Gels with a central thickness of 2 mm, 3 mm, and 4 mm were also prepared. A minimum of 5 ml gel solution was required when preparing the gels with the gel maker. The same protein patterns and standard curves for the LMW markers were achieved with the 7.5-ml and 10-ml gels. On the other hand, extended linearity of the standard curve was achieved with the 5-ml gel (Fig. 5).

Identification of the separation of some protein standards

Under the optimum conditions for SDS-agarose gel electrophoresis of a 5-ml 4% NuSieve 3:1 gel (gel size, $5 \times 6 \times 0.2 \text{ cm}^3$) run in 25 mM Tris, 190 mM glycine buffer containing 0.1% SDS (pH 8.3), we investigated whether protein bands are separated according to molecular weight under these conditions (Fig. 6). We used the protein standards: β_2 -m, RBP, α_1 -m, HSA, TF, and IgG. The reference values and our measured values

Fig. 3. Effects of gel concentrations on protein band patterns and standard curves for each LMW marker. a: Patterns of electrophoresis. 1. 2%; 2. 3%; 3. 4%; 4. 5%. ① LMW marker; ② human serum albumin standard; ③④ healthy human serum. b: Standard curves for each LMW marker.

Fig. 4. Effects of concentrations of SDS in buffer solutions on protein band patterns and standard curves for each LMW marker.
a : Patterns of electrophoresis. 1. 0% ; 2. 0.05% ; 3. 0.1% ; 4. 0.2%. ① LMW marker ; ② human serum albumin standard ; ③④ healthy human serum. b : Standard curves for each LMW marker.

Fig. 5. Effects of gel thickness on protein band patterns and standard curves for each LMW marker.
a : Patterns of electrophoresis. 1. 5 ml (2 mm) ; 2. 7.5 ml (3 mm) ; 3. 10 ml (4 mm). ① LMW marker ; ② human serum albumin standard ; ③④ healthy human serum. b : Standard curves for each LMW marker.

for each protein calculated from the standard curve are shown in Table 2. Our value for β_2 -m agrees almost with the reference value, but our values for HSA, TF and RBP are slightly lower than the corresponding reference values, and our values for IgG and α_1 -m are slightly higher than the corresponding reference values.

DISCUSSION

We studied the technique of separating proteins according to their molecular weights using SDS-agarose gel. Bricon et al.¹⁾ used the Hydragel kit and 50 mM imidazole buffer (pH 9.5). We could not obtain the Hydragel kit. We tested the 50 mM imidazole buffer

Fig. 6. Separation of some standard proteins.
① LMW marker; ② IgG; ③ TF; ④ HSA; ⑤ α₁-m; ⑥ RBP;
⑦ β₂-m.

Table 2. Reference molecular weights and measurement molecular weights of each protein standard.

Protein standard	Reference molecular weight (×10 ³)	Measurement molecular weight (×10 ³)
IgG	120	135
TF	76	72
HSA	66	54
α ₁ -m	30	31
RBP	21	19.5
β ₂ -m	14	14.4

(pH 9.5), but could not improve the separation of protein fractions. Laemmli³⁾ and Werber and Osborn⁴⁾ used 25 mM Tris, 190 mM glycine buffer (pH 8.3) and 100 mM phosphate buffer (pH 7.0), for SDS-PAGE respectively. When the pH of the buffer was near neutrality in our study, the protein bands migrated slowly. In contrast, the protein bands became blurred when the buffer was highly alkaline (pH 9.5). Therefore, we selected 25 mM Tris, 190 mM glycine buffer (pH 8.3) as the most effective.

Wu and Kusakawa⁵⁾ and Acevedo et al.⁶⁾ have previously reported the separation of proteins according to molecular weight. Wu and Kusakawa separated proteins using 7% MetaPhor XR agarose (BMA) for the separating gel and 1% SeaKem Gold agarose (BMA) for the stacking gel. Acevedo et al. separated proteins treated with SDS by discontinuous electrophoresis in a vertical 1% agarose gel. We did not try these methods, and therefore cannot evaluate them. However, we separated proteins according to molecular weight by continuous electrophoresis in horizontal 4% agarose gels using NuSieve 3:1. Melted 4% agarose solutions

were easy to pour into the horizontal gel tray and the gel was easy to handle. Therefore, we consider our method is a useful technique for clinical laboratories.

SDS-PAGE is more popular than SDS-agarose gel electrophoresis for the separation of proteins according to molecular weight. However, SDS-agarose gel electrophoresis has many advantages. Wu and Kusakawa⁵⁾ reported that the intensity of protein staining with Coomassie Brilliant Blue was more uniform in agarose gels than in polyacrylamide gels. They also reported that ovalbumin (45 kDa) was resolved better in agarose than in polyacrylamide gels. Furthermore, they reported that the transfer efficiency of a larger (150-kDa) protein was markedly higher from agarose than from polyacrylamide. Acevedo et al.⁶⁾ reported that proteins separated by SDS-agarose gel electrophoresis could be transferred to membranes by capillary blotting. Sakakibara²⁾ reported efficient recovery of proteins from agarose gels. It is also obvious that proteins are easily solubilized from agarose gels.

In this study, we have undertaken fundamental research into the effectiveness of SDS-agarose gel electrophoresis using the Mupid. As mentioned above, SDS-agarose gel electrophoresis is very useful. We are conducting further research into maximizing the efficiency of agarose gel in protein electrophoresis.

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