Indonesian Journal of Biotechnology and Biodiversity
Volume 4, Issue 2 (2020) : page 118-127

Critical Aspects to Produce Low-Cost Protein Molecular Weight Marker : A Review

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ABSTRACT

Proteomic studies usually begin with characterizing protein profile, such as the molecular weight of protein, which can be done by the SDS-PAGE technique followed by Western Blot analysis. These methods need a standard protein called molecular weight marker (MWM). In this review, the important, fundamental aspects of either recombinant or native MWM production were discussed, including the type and effect of dyes used to prepare a prestained MWM. Moreover, buffer and polyols used in the formulation can also affect the quality and stability. Also, another adjuvant may be needed to increase the robustness of MWM to lower the risk of protease contamination that can breakdown the protein inside the MWM during storage. Understanding those critical aspects will help to produce or formulate a good quality of MWM.

Keywords: *protein ladder, protein stability, electrophoresis*

INTRODUCTION

Many biotechnology applications have become more important in human life lately. It replaced the conventional methods to produce commercial products efficiently and precisely. Biotechnology has evolved into a powerful tool that contributes to a sustainable industry through research and development activities. One of the most advanced and applicative biotechnology techniques is a genetically modified organism and protein engineering to produce protein products that are usually active biomolecules, such as hormones and enzymes. These products can be used in many fields, including food, health, environmental, bioprocess technology, and agriculture [1].

Based on the applications, biological products can be used as a crude extract or purified quality, but it still needs a product characterization step. The sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique is commonly employed to analyze or to estimate the relative molecular of a specific protein within the sample. The method is very popular and widely used because of its simplicity, reproducibility, and relatively lower price than other methods, and it also can be applied to analyze other macromolecules [2, 3]. The characterization of protein in a sample is important since protein may change because of post-translational modification or even determine whether the sample contains our target protein and its purity. For analyzing protein samples with the SDS-PAGE technique, a molecular weight marker (MWM) is needed. Unfortunately, for some researchers in a developing country, like Indonesia, the need of

MWM is still dependent on imported products with a high price and quite a long waiting period to order the item. The data showed in 2014, the global MWM market was valued USD 265.36 Million and was predicted to reach USD 472.47 × 10^6 Million in 2019 [4]. The fact shows that making MWM on our own can help reduce research costs and promising business opportunities. In this review, the type of protein and 'excipient' that compose protein MWM will be discussed to get a better understanding of the making of MWM.

METHODS

This paper is an integrative review from selected related articles published in the last 15 years (2006-2019), although few older articles were also included for specific discussion. Keywords used for the article search from databases were (protein ladder OR protein marker OR molecular weight marker) AND (SDS PAGE OR PAGE electrophoresis OR protein blotting OR protein analysis OR protein detection OR protein bands) AND (protein stability OR stained protein OR colored protein OR marker resolution OR band resolution). Database-based used are Springerlink, Science Direct and PubMed.

RESULTS AND DISCUSSION

1. Use of Protein MWM

Protein Electrophoresis such as SDS-PAGE will include a comparison between protein bands of the samples with those of protein ladder or so-called MWM. Protein MWM can be used as a standard because it contains a set of defined molecular weight proteins [5]. Commercial protein MWM come in different versions, giving a narrow until broad range MW protein. The ladder can give different resolutions that may affect the interpretation of SDS-PAGE results.

Although the function of MWM seems to be simple, its role is irreplaceable until now. The MWM is also used widely in Western Blot (WB) Technique, mainly to observe whether the protein transfer from the gel to the membrane is succeeded and completely done before continuing to the next step [6]. In a certain situation, defined and known protein in MWM could play a role as a positive control.

2. Types of Protein Molecular Weight Marker

2.1. Natural vs. Recombinant Molecular Weight Marker

A molecular weight marker (MWM) or protein ladder (PL) is basically a set of known molecular mass proteins (in kDa). There are some MWM made from natural proteins and some other MWN made from recombinant protein. The natural MWM is commonly cheaper than the recombinant one, but the recombinant proteins offer sharper bands than the natural ones (Figure 1).

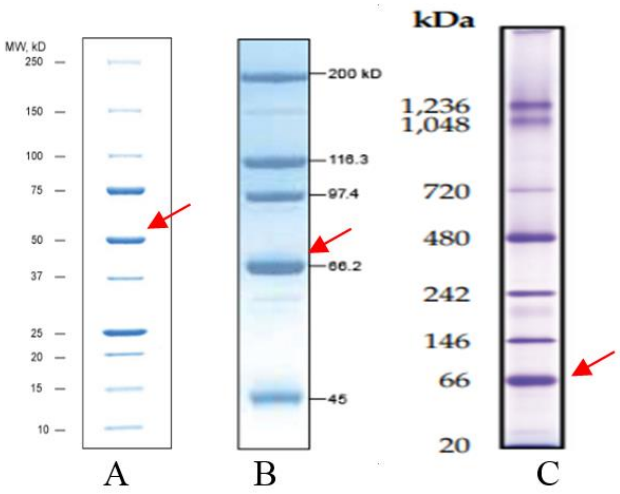

Fig. 1. Comparison of commercial unstained recombinant, natural, and native molecular weight marker. A: Precision Plus ProteinTM Standards Recombinant (Biorad Inc.); B: Unstained SDS-PAGE Standards Natural (Biorad Inc.); C: Native Mark Unstained Protein Standards (Thermo Fisher Scientific). The red arrows show different protein bands profile, while in the A the protein bands look sharper, while in the B and C they look broader.

The recombinant protein is produced by an engineered organism contained a gene of interest. The protein products were usually purified with affinity chromatography (e.g. polyhistidine protein purification used immobilized metal-ion chromatography or widely named IMAC technique since the metal ion serve ligand affinity for the Histidine), so the

obtained protein is specifically only His-tagged protein, while the natural one usually used traditional chromatography techniques, e.g. size exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), or anion/cation exchange chromatography [7]. Using SEC or IEC raises the possibility of obtaining another protein that is very similar in molecular weight and charge, so the bands are broader and the lot-to-lot variant is higher. In the recombinant MWM, special attributes may also be added, like affinity tags for better detection.

2.2. Unstained vs. Prestained MWM

The SDS-PAGE gel containing protein bands can be visualized by staining the gel using a dye solution. Post-electrophoresis (PE) staining produced monocolored protein bands. The most famous dyes used to PE staining is colloidal Coomassie Brilliant Blue (R- and G-250) followed by silver staining, Amido Black, Ponceau S, Procion Blue, Alcian Blue, and other fluorescence dyes, i.e., SYPRO and Deep Purple [10-12]. The unstained type of protein MWM (natural and recombinant) can be visualized along with the sample bands staining process, so both MWM and protein bands will look in the same color (Figure 2). This technique offers better molecular weight estimation because the PE staining will not affect the migration rate of the protein. The disadvantage is that monocolored protein bands could make some ambiguous interpretations due to a different percentage of acrylamide gel or the success of the electrophoresis process. To overcome this issue, some prestained MWM has been made. The pre-electrophoresis protein coloring technique was introduced by Griffith and become popular to manage immediate protein visualization [13]. Up to now, a lot of commercial prestained MWM use this technique with some modification.

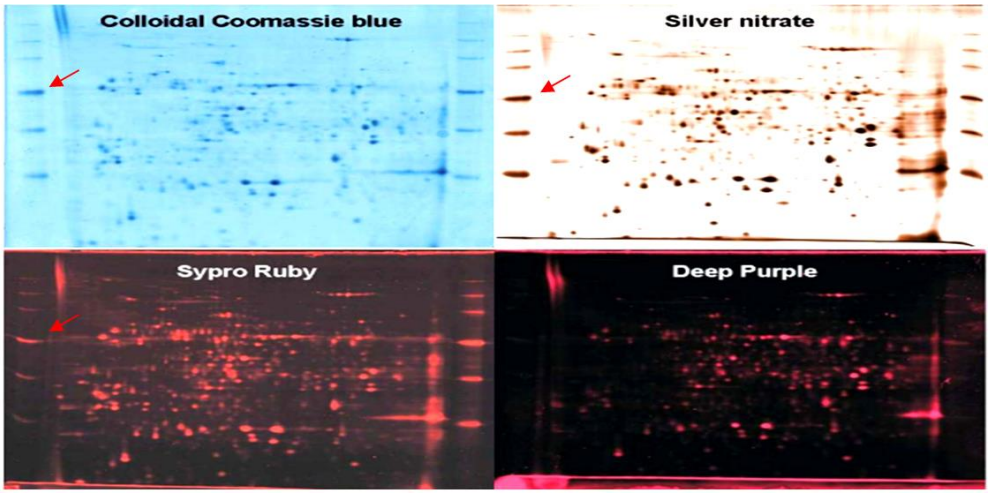

Fig. 2. Post-Staining isoelectric electrophoresis with various dyes [12]. Red arrows indicate the same color of unstained MWM with samples.

The prestained MWM offers several advantages, i.e., it permits one to visualize protein separation during electrophoresis and overview transfer efficiency from gel to membrane. Commonly, commercially available prestained MWM used Remazol® as a dye [5]. Remazol dyes provide an excellent stability linkage with proteins through a covalent bond [13] and offer a good availability because it is also used as a textile dye with a relatively low price. The vinyl sulfone derivates of Remazol molecules could bind to primary, secondary amine groups of the protein and to alcohol or sulphydryl groups under alkaline conditions. The challenge in the making prestained MWM using kind of reactive dyes like Remazol is the modification on protein relative mobility (Rf), since each type of dyes have a propensity to modifying protein mobility on gel, i.e., diffusion broadening and the change of Rf when trypsin

inhibitor coupled with Remazol Blue (Figure 3) [14].

Nowadays, the development of prestained MWM becomes advanced with chemiluminescence/fluorescence quantitative analysis of targeted proteins expression in WB technique. The chemiluminescent method is generally used because of its simplicity, low interfering emissions, low consumption of reagent, and allows an extensive range of protein concentration. Chemiluminescent also provides one of the highest sensitivity, like HRP conjugate (based on the substrate to product). The main problem is that the chemiluminescent technique only provides detection of one protein at one time. Another limitation to this technique is the needed instrument that can capture the light that is specifically designed for this purpose, also poor quantitative reproducibility because it depends on protein transfer from the gel as well as the continuous reaction of chemiluminescent. In contrast to chemiluminescent, the fluorescent technique can detect multiple targets is because it uses proteins labeled with a dye molecule, which has different excitation wavelengths, like Cy3 (554-568 nm) and Cy5 (649-666 nm) (Figure 4), so the detection can be done in one time without reprobe process. Although the sensitivity of the fluorescent technique is basically lower than chemiluminescent, the fluorescence is more quantitatively proved by the linearity (r) with protein amount is 0,997 with a coefficient of variation (CV) of 17 %. In comparison, the chemiluminescent WB is 39 % [15], because the ratio is 1:1 for protein amount: signal, with better signal stability.

To increase the sensitivity of the fluorescent technique, a high resolution and merging image technique has been done to the level of comparable result with chemiluminescent one [16]. Besides using a chemical dye like cy3/cy5, another variant from recombinant MWM is using a famous protein, a green fluorescent protein (GFP). Using *E. coli* clones with a plasmid containing GFP fusion with the protein under the T7 promoter, the specific protein products easily purified with His-Bind column, producing a set of desired molecular weight proteins [17]. The strategy is quite a success since the linearity (R^2) of MWM Rf produced is 0.97 compared with unstained MWM. However, the fluorescent signal of GFP-protein MWM can be seen under visible and UV light (Figure 5). This strategy offers an effective production process since the protein expression under certain promoter could be enhanced by chemical induction like IPTG. Still, the limitation is the relative intensity of fluorescence is declined by about 50% after transfer to a membrane, which means that it is more suitable only for SDS-PAGE.

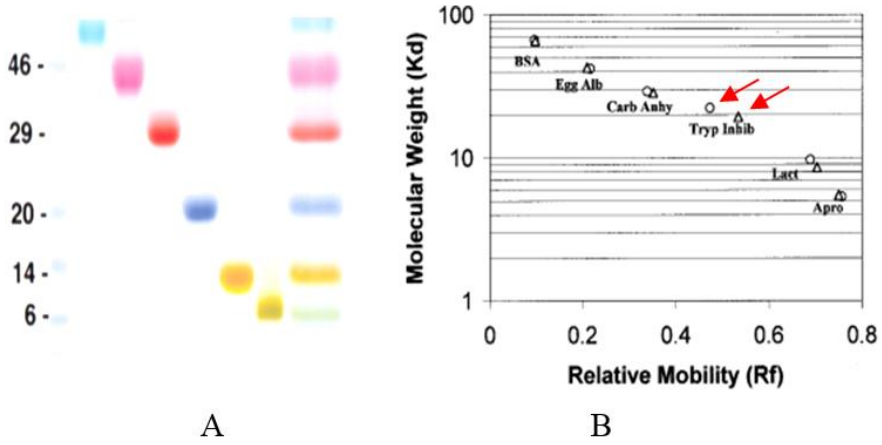

Fig. 3. The visual look of multicolored prestained MWM [14]. A: a set of Remazol prestained MWM on SDS-PAGE gel; B: the red arrows shows Rf modification to certain specific protein due to linkage with dye molecule (o: prestained Trypsin inhibitor with Remazol; Δ: unstained MWM).

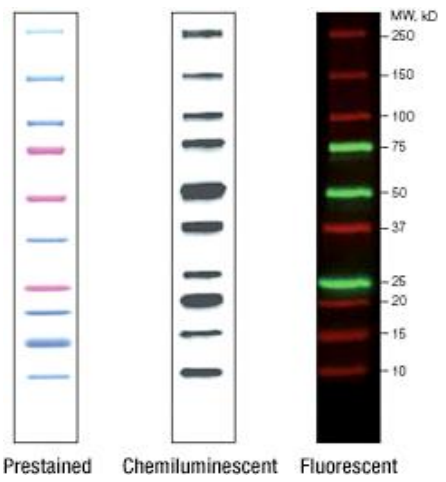

Fig. 4. Prestained, chemiluminescent and fluorescent MWM for the same size range (Biorad Inc.)

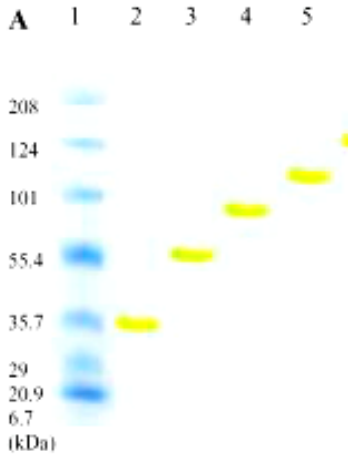

Fig. 5. GFP-Fused Protein as a prestained MWM [17]. The yellows band under visible light, the green bands under UV light.

3. Protein Molecular Weight Marker 'Excipients'

Protein MWM product typically does not only consist of purified protein, but also some other compounds as supporting components or excipients. These components ensure that MWM can work according to its function and have good stability for a relatively long shelf life. In general, the MWM is a liquid consisting of the protein mixture, buffer, denaturing agent, thickening agent, chelating agent, and a tracking dye.

3.1. Choices of Buffer

The acid-base buffer system is the popular one; i.e., Tris-HCl buffer is commonly used in MWM solution, while the other may use Bis-HCl or Tris-H_3PO_4. The main factor that needs to be considered when choosing a buffer is the buffering capacity, which means how well it can maintain the pH. The buffers themselves play a role in protein stabilization (prevent protein degradation), mainly from the freeze-thawing cycle.

During freeze-thaw, the processes, including cooling, freezing, isothermal hold, and thawing, can cause stress to protein. Different physical stresses occur in different steps, and the decrease of temp below the equilibrium leads to freezing concentration stage (formation ice crystal from water component and leave high salt/protein in aqueous phase), and this may cause crystallization of components to a pH change [18]. The pH change can vary depending on buffer composition (Table 1). The wrong choices a buffer coupled with inappropriate storage resulting in spontaneous unfolding protein and protein aggregation (for native protein) and even protein degradation [19, 21].

Table 1. pH changes in the various buffer from room temperature to freezing temperature [18].

Buffer Solution	pH at 25 °C	pH at 0 °C	pH at -30 °C	Lowest pH Measured	Highest pH Measured	Net Change in pH (25 °C to -30 °C)
50 mM Sodium Phospate	7.00	7.02	3.36	3.36 at -30°C	7.00 at 25 °C	3.64
20 mM Sodium Acetate	5.63	5.66	6.14	5.61 at 5 °C	6.14 at -30 °C	0.51
20 mM L-Histidine-HCl	5.37	5.86	6.19	5.37 at 25 °C	6.14 at -30 °C	0.82
20 mM Histidine-Acetate	5.52	5.97	6.48	5.52 at 25 °C	6.48 at -30 °C	0.96
20 mM Sodium Citrate	6.16	6.49	5.93	5.69 at -10 °C	6.17 at 20 °C	0.23
20 mM Tris-HCl	7.37	7.93	8.54	7.37 at 5 °C	8.54 at -30 °C	1.17
20 mM Sodium Succinate	5.55	5.60	5.85	5.49 at -10 °C	5.85 at -30 °C	0.3

3.2. Denaturing Agent

The denaturing agent is the key to make MWM so that we can see the protein bands based on their molecular size (except for native PAGE). The denaturing agent caused protein denaturation by reducing disulfide bridges and also preventing aggregates from forming. There are three reducing agents commonly used in protein preparation, β-mercaptoethanol (BME), dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine (TCEP). The most popular one is DTT since it shows a strong reduction capability. However, a reducing agent can also become an oxidizing agent, e.g., DTT can undergo oxidation when interacting with air, and the oxidation is catalyzed by metal ions [22]. Before the DTT era, BME was very effective as a denaturing agent and widely used. But it has a strong odor with a high volatile rate, makes it replaced by DTT. However, until now, BME is still a good choice as a denaturing agent since it is cheaper and its activity does not depend too much on pH (DTT activities are sluggish on near-neutral pH) [23].

The other denaturing agent is TCEP. It is quite promising one because it is more stable than DTT for long term storage, even without metal chelates in the buffer. Several advantages were also shown for TCEP since it is not pungent and works effectively at a wider pH range (1.5 to 8.5). But TCEP is not stable when used with phosphate buffer, especially at neutral pH. It is also not suitable for use in isoelectric focusing applications because it is charged in solution. Dithiobutylamine (DTBA) is the new reducing agent synthesized from L-Aspartic acid. DTBA is claimed as a potent and versatile denaturing agent with activity 14 times faster than DTT could [23]. Above all, denaturing agent benefits and drawbacks, the important aspect is to add the denaturing agent with the appropriate concentration to prevent protein MWM from reoxidation.

4. Adjuvant for Molecular Weight Marker Excipient

The MWM solution may contain buffer and denaturing agents and several adjuvant components to make it more stable and perform well. One of them is glycerol, which is added to increase the solution density, so it will not diffuse out of the gel wells. Furthermore, glycerol also plays a role as a co-solvent in protecting the protein from physical stress from a freeze-thawing cycle. The glycerol, in this case, acts as a cryo-protectant to stabilize the structure by preventing the formation of ice crystals that may destroy protein structure with 10-50% $^{w}/_{v}$ [24]. The use of glycerol is very significant to native protein because it can inhibit protein aggregation [25]. Few polyols like mannitol, sorbitol, sometimes also used to increase protein stability without increase its density. The typical concentration for glycerol in MWM solution is about 10% to 30%.

The protein degradation by freezing and thawing is not the only one we need to concern since the microbial contamination to MWM solution could also lead to protein degradation. The polyol like glycerol in MWM is a carbon source for microbial growth. The microbial can excrete protease that degrades protein. Anti-microbial agents such as NaN_3 (sodium azide) can be added to MWM solution at a final concentration of 0.02% to 0.05% $^{w}/_{v}$. NaN_3 will bind to heme-iron, leading to asphyxiation, but it is more effective against Gram-negative bacteria [26]. Thimerozal is another preservative for microbial contamination, but because of the toxic property, it is prohibited in many countries nowadays [27].

Besides microbial contamination that excretes protease, the protease itself from technical contamination needs to be considered. Repeating aliquot preparation from MWM stock solution could cause protease cross-contamination, i.e., from pipet tips. To prevent it, the chelating agent can be used. The famous chelating agent is EDTA (ethylene diamine tetraacetic acid) or EGTA (ethylene glycol tetraacetic acid). The EDTA is more commonly used because EDTA could bind to Mg^{2+} ions while EGTA binds more specifically to Ca^{2+} ions. Binding of a chelating agent to metal ions can inactivate the enzyme since almost every enzyme needs a metal ion as a cofactor. Some

protease inhibitors could also be added, such as PMSF (Phenyl methyl sulfonyl fluoride) to inhibit serine protease [28], but using EDTA / EGTA seems to be more efficient and cheaper.

5. Low-Cost Protein Molecular Weight Marker

As discussed earlier, the need of protein MWM for researchers working with protein is still primary and massive. But the price for a small amount is relatively high, especially for some developing countries that add tax and shipping costs. This is an important point why protein MWM can be self-produced rather than importing the products with a high price and a long waiting period. This idea can also be a promising business opportunity. From a previous chapter, we agreed that a natural MWM is cheaper than the recombinant one.

The protein itself as the main component can be chosen from abundant and cheap raw material, i.e., lysozyme and ovalbumin from egg white or trypsin inhibitor from soybean. For the initial steps, a protein profile screening from many raw material sources is needed to determine which protein will be used as a component in MWM. The screening process is necessary because the ladder should consist of a number of proteins that cover a broad range of molecular weight. The proteins should be prepurified by IEC or SEC, and the quality control can be done by doing SDS-PAGE to each fraction of IEC or SEC. More advanced techniques are using electro-elution, a method to recover the protein band resolved by electrophoresis. This technique offers a simple, cost-effective, and fast purification than chromatography. It is possible to remove salts, SDS, and dye by dialysis, but the protein load capacity is very limited than chromatography [29, 30]. By doing this, a set of natural proteins could be achieved in a very cost-effective way. If the natural protein does not satisfactory, the recombinant protein with His-tag is the best choice according to the simplicity and selectivity of the purification process, although the resin for His-tag protein purification is relatively quite expensive. Excipients for protein MWM is the last issue that needs attention to make a low-cost MWM. For a prestained MWM, the reactive dye, usually for textile, is easy to get. Other excipients like buffer, denaturing agent and adjuvant can be selected for the best suitability and availability.

A set of protein MWM must meet several requirements as to quality control. The main two essential things are the linearity of protein band migration and stability. Although there is no official provision about the linearity of the migration rate, especially for the prestained marker, the linearity value is ideally above 0.9. The difference with the unstained is not more than 5 %. For the stability of the MWM (no changes in bands profile, migration rates), 6 months until 2 years in a cold condition is relatively ideal shelf life. The transfer efficiency from gel to the membrane is also an exciting feature for application in western blotting.

Table 2. A few published selfmade protein molecular weight marker

Name	Type	Parameter 1	Parameter 2	Parameter 3	Reference
Protein MWM with specific antibody	Natural	Cross-reactivity	Efficiency in Dot-Blot / Western Blot	Exposure Times to Substrate	6
Multicoloured Prestained MWM	Highly Purified Natural	Linearity / Mobility Rates	Stability over Time	Minimum concentration	14
Dye-Free Marker from GFP	Recombinant	Linearity / Mobility Rates	Stability under denaturation	Minimum Concentration to Visible	17
Protein MWM from Heme Subunits	Natural	Linearity / Mobility Rates			31

CONCLUSION

In this review, types of MWM have been discussed with their particular benefit and weaknesses. From the discussion, the protein molecular weight marker actually can be made from several easily purified proteins mixed with other essential components, which is easy enough to obtain, such as buffer, a denaturing agent and adjuvant, in a certain composition. Understanding each role and good formulation could lead to self-made protein MWM or in-country production, which can reduce research costs in countries like Indonesia because it is cheaper than the commercial, imported ones. Mass production scale should be beneficial since almost all MWM marker demand in Indonesia is supplied from overseas.

ACKNOWLEDGMENT

We thank the Faculty of Biotechnology, University of Surabaya for all support and access regarding online resources.

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