Bull. Mukogawa Women's Univ. Nat. Sci., **47**, 35－37(1999)
武庫川女子大紀要（自然科学）

An Improved Convenient Molecular Weight-determination Method for Active Stainable-Enzyme after SDS Electrophoresis

Tokumitsu Okamura, Noboru Horie and Masahiro Ohsugi

Department of Food Science and Nutrition,
School of Human Environmental Sciences,
Mukogawa Women's University, 6-46, Ikebiraki-cho, Nishinomiya 663-8558, Japan.

An improved method to determine the molecular weight of alcohol dehydrogenase after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been developed. This method was based on the finding that on a gel, which was washed with a buffer to remove SDS after SDS-PAGE, stained with an enzymatic activity staining mixture and then stained with coomassie blue, there appeared two active stained bands with apparent molecular weights of 148,000 (tetramer) and 35,000 (monomer) on the SDS-PAGE gel. The method developed here may be applicable to a wide range of active stainable-enzymes as a rapid and simple molecular weight determination method after SDS-PAGE.

Introduction

Electrophoretic techniques are one of the premier methods for the separation and analysis of proteins. In this technique, proteins react with the anionic detergent, sodium dodecylsulfate (SDS or sodium lauryl sulfate), to form negatively charged complexes. The amount of SDS bound by a protein, and therefore the charge of the complex, is roughly proportional to its size (mass).

Many studies have been made on staining methods based on enzyme activity after polyacrylamide gel electrophoresis (PAGE)[10]. Among these, methods using tetrazolium salts have been widely used for detection of NAD^+- and $NADP^+$ requiring enzymes such as hexokinase (EC 2.7.1.1)[1], phosphoglucomutase (EC 2.7.5.1)[2], glucose-6-phosphate dehydrogenase (EC 1.1.1.49)[3], phosphogluconate dehydrogenase (EC 1.1.1.43)[4], malate dehydrogenase (EC 1.1.1.37)[5], glutamate dehydrogenase (EC 1.4.1.4)[6], alcohol dehydrogenase (EC 1.1.1.1)[7], lactate dehydrogenase (EC 1.1.1.27)[8] and fumarase (EC 4.2.1.2)[9].

In general laboratories, researchers determine enzyme activities based on the reduction of tetrazolium salts, which in the presence of phenazine methosulfate acts as an electron carrier during polyacrylamide electrophoresis (PAGE)[10], using an active staining solution, and then determines the molecular weight on SDS-PAGE. To our knowledge, direct staining method for NAD^+- and $NADP^+$ requiring enzymes on SDS-PAGE have not been reported.

In this paper, we report the improved convenient molecular weight-determination method for active stainable-enzymes such as alcohol dehydrogenase after SDS electrophoresis.

Materials and Methods

Alcohol dehydrogenase (ADH) (EC 1.1.1.1) (obtained from yeast)[11] and NAD^+ (β-Nicotinamide-adenine dinucleotide, oxidized

form)were purchased from Oriental yeast Co. (Tokyo, Japan). Acrylamide, ammonium persulfate and sodium dodecyl sulfate(SDS)were purchased from Bio-Rad(München, Germany) ;coomassie brilliant blue R-250 was from Merck AG(Carmstadt, Germany);and bromophenol blue was from Sigma Chemical Co. (St. Louis, MO).

SDS-PAGE was done on a 12.0 X 10.2 X 0.15cm slab according to Laemmli[12], with a 2.5% stacking gel and a 7.5% separating gel. The sample buffer contained no β-mercaptoethanol. One milligram of ADH mixed with 20μl of 10mM Tris-HCl buffer(pH 7.5) was electrophoresed at 20mA per gel until the dye front reached the bottom of the gel. After electrophoresis, the gel was washed with 10mM Tris-HCl buffer(pH 7.5) containing 2.5% Triton X-100 for 20min, 0.1M glycine·NaOH(pH 7.5) containing 2.5% Triton X-100 for 20min, 10mM Tris-HCl buffer(pH 7.5)containing 0.1M glycine·NaOH for 20min, and 10mM Tris-HCl buffer(pH 7.5)for 20min to remove SDS, respectively.

The enzymatic activity staining mixture contains 50mM Tris-HCl buffer(pH 7.5), 1.25mM NAD^+, 10mM ethyl alcohol, 0.4mM phenazine methosulfate and 0.5mM nitro blue tetrazolium. The gel, which was removed SDS, was stained with the mixture.

Molecular weight calibration was performed using proteins of known molecular weight under identical conditions. The gel was stained with coomassie blue.

Results and Discussion

After SDS-PAGE of ADH, two stained bands were detected with ADH activity specific staining mixture on SDS-PAGE gel(Fig. 1(a)). The same two main stained band was also stained with ADH activity specific staining mixture and then coomassie blue on SDS-PAGE gel(Fig. 1(b))). Fig. 1(c)shows one stained band with ADH activity specific staining mixture on PAGE gel. The band on PAGE gel did not show the molecular weight(Fig. 1(c)), however, the two stained bands on SDS-PAGE gel was present in tetramer(M. W. 148,000)and monomer(M. W. 35,000)of ADH by molecular weight(Fig. 1(a)and(b))[11].

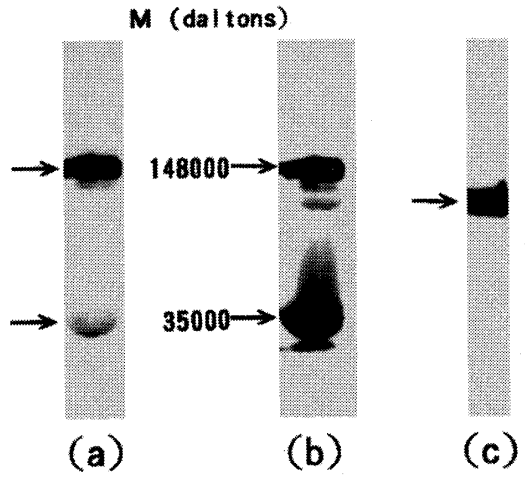

Fig. 1. Polyacrylamide gel electrophoresis of alcohol dehydrogenase(ADH).
(a)SDS-polyacrylamide gel electrophoresis(SDS-PAGE). The gel was stained with ADH activity specific staining mixture.
(b)SDS-PAGE. The gel was stained with ADH activity specific staining mixture and then with coomassie brilliant blue R-250.
(c)Polyacrylamide gel electrophoresis(PAGE). The gel was stained with ADH activity specific staining mixture.
The arrow indicates the position of ADH.

In normal SDS-PAGE, proteins are separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions. The general use mixture of proteins is first dissolved in a sodium dodecyl sulfate(SDS), an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. Anions of SDS bind to main chains at a ratio of about one SDS for every two amino acid residues, which gives a complex of SDS with a denatured protein and a large net negative charge that is roughly proportional to the mass of the protein. However, the protein does

not show enzymatic activity because of its denaturation. Usually, in the case of active stainable-enzymes, electrophoresis must be done twice;one to get an active stain on PAGE, and the other to determinate the molecular weight on SDS-PAGE. However, if the gel is washed and SDS removed with the buffer described above, it is possible to detect the active stainable-enzyme and determine the molecular weight on only one SDS-PAGE gel.

Therefore, this molecular weight determinating method established for alcohol dehydrogenase may be applicable to a wide range of NAD^+- and $NADP^+$ requiring enzymes as a improved and convenient method for detection of their activities directly on SDS-PAGE. And also, identification of a particular enzyme or a group of enzymes is capable even when a crude enzyme solution is used.

Furthermore, specific staining techniques exist for proteins such as phosphoproteins, lipoproteins and glycoproteins. Enzymes can be localized by assaying then for their specific enzymatic activity, e.g. by converting substrates to sparingly soluble products, which can then be coupled chemically to azo dyes. In addition, almost any enzyme for which a suitable assay method exists could be coupled, in theory, to an antibody by cross-linking reagents(e.g. carbodiimide, glutaraldehyde, etc.). The use of these enzyme-labelled antibodies in combination with blotting results in extremely sensitive procedures which can detect as little as 100 pg of protein on a membrane.

Therefore, this improved convenient method can be widely applied to protein estimations as an alternative to the rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are involved.

References

1) L.D. Purich, J.H. Fromm and B.F. Rudolph, *Adv. Enzymol.*, **34**, 249-326(1973).
2) H. Chiba, M. Ueda and M. Hirose, *Agric. Biol. Chem.*, **40**, 2423-2431(1976).
3) A.P. Marks, *Meth. Enzymol.*, **9**, 131-141(1966).
4) A.W. Wood and W.E. Frampton, *Meth. Enzymol.*, **5**, 295-298(1962).
5) J.L. Banaszak and A.R. Bradshaw, *The Enzymes(3rd ed.)*, **11**, 369-396(1975).
6) L.E. Smith, M.B. Austein, M.K. Blumenthal and F.J. Nyc, *The Enzymes(3rd ed.)*, **11A**, 293-367(1975).
7) I.J. Wenger and C. Bernofsky, *Biochim. Biophys. Acta.*, **227**, 479-490(1971).
8) F. Stolzenbach, *Meth. Enzymol.*, **9**, 278-288(1966).
9) S. Yamato and T. Murachi, *Eur. J. Biochem.*, **93**, 189-195(1979).
10) K. Koike, Y. Hakamada, T. Yoshimatsu, T. Kobayashi and S. Ito, *Biosci. Biotech. Biochem.*, **60**, 1764-1767(1996).
11) E.J. Racker, *J. Biol. Chem.*, **184**, 313-319(1950).
12) K.U. Laemmli, *Nature*, **227**, 680-685(1970).