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

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An improved method to determine the molecular weight of the subunit of lactate dehydrogenase and malate 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 one active stained band with apparent molecular weights of 35,500 (monomer) (lactate dehydrogenase) and 31,000 (monomer) (malate dehydrogenase) 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 of the subunit 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)[1]. Among these, methods using tetrazolium salts have been widely used for detection of NAD^+- and $NADP^+$-requiring enzymes such as 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)[1], alcohol dehydrogenase (EC 1.1.1.1)[6], lactate dehydrogenase (EC 1.1.1.27)[7] and fumarase (EC 4.2.1.2)[8].

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), using an active staining solution, and then determine the molecular weight of the subunit on SDS-PAGE. For a long time, it was believed that the protein does not show enzymatic activity on SDS-PAGE because of its denaturation. To our knowledge, direct staining method for NAD^+- and $NADP^+$-requiring enzymes on SDS-PAGE have not yet been reported.

In this paper, we report the improved convenient molecular weight-determination method of the subunit for active stainable-enzymes such lactate dehydrogenase and malate dehydrogenase

as after SDS polyacrylamide electrophoresis.

Materials and Methods

Lactate dehydrogenase(LDH)(EC 1.1.1.27)(obtained from pig heart), malate dehydrogenase(MDH)(EC 1.1.1.37)(obtained from yeast)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(Munich, Germany);Coomassie Blue was Coomassie Brilliant Blue R-250 from Merck AG(Darmstadt, Germany);and Bromophenol Blue was from Sigma Chemical Co. (St. Louis, MO).

SDS-PAGE was done on a 12.0 X 10.2 X 0.15 cm slab according to Laemmli[9], with a 2.5% stacking gel and a 7.5% separating gel. The sample buffer contained no β-mercaptoethanol. One milligram of LDH or MDH mixed with 20μl of 10 mM Tris-HCl buffer (pH 7.5)was electrophoresed at 20 mA per gel until the dye front reached the bottom of the gel. After electrophoresis, the gel was washed with 10 mM Tris-HCl buffer(pH 7.5)containing 2.5% Triton X-100 for 20 min, 0.1 M glycine·NaOH(pH 7.5)containing 2.5% Triton X-100 for 20 min, 10 mM Tris-HCl buffer(pH 7.5)containing 0.1 M glycine·NaOH for 20 min, and 10 mM Tris-HCl buffer(pH 7.5)for 20 min to remove SDS, respectively.

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

Molecular weight calibration was performed using proteins of known molecular weights under identical conditions. The gel was stained with Coomassie Blue.

Results and Discussion

After SDS-PAGE of LDH, one stained band was detected with LDH activity specific staining mixture on SDS-PAGE gel(Fig.1.a). The same stained band was also stained with LDH activity specific staining mixture and then coomassie blue on SDS-PAGE gel(Fig.1.b). And also after normal SDS-PAGE using the perfectly denaturated sample without removing SDS, the same stained band was stained with coomassie blue on SDS-PAGE gel(data was not shown). Fig.1.c shows one stained band(tetramer), with LDH activity specific staining mixture on PAGE gel. The band on PAGE gel did not show the molecular weight(Fig.1.c), but the one stained band on SDS-PAGE gel was present for the monomer(M. W. 35,500)of LDH by molecular weight(Fig.1.a and b). The same results were obtained in the case of MDH(Fig.2.). The one stained band on SDS-PAGE gel was present in monomer(M. W. 31,000)of MDH by molecular weight(Fig.2.a and b), whereas the band(dimer) on PAGE gel did not show the molecular weight(Fig.2.c).

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 of the subunit 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 of the subunit on only one SDS–PAGE gel.

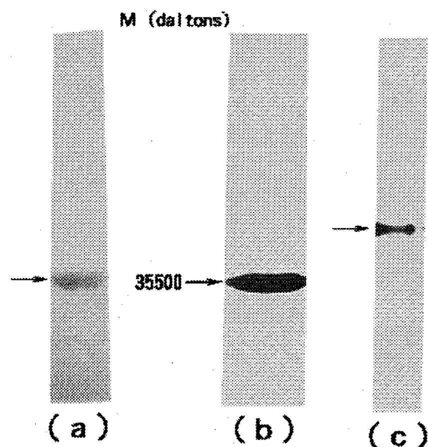

Fig. 1. Polyacrylamide gel electrophoresis of lactate dehydrogenase (LDH).
(a) SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was stained with LDH activity specific staining mixture.
(b) SDS–PAGE. The gel was stained with LDH activity specific staining mixture and then with coomassie brilliant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

Therefore, this molecular weight determinating method of the subunit established for lactate dehydrogenase and malate 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.

Therefore, though all enzymes can not applied, this improved convenient method can be widely applied to the active stainable–enzyme estimations as an alternative to the rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are involved.

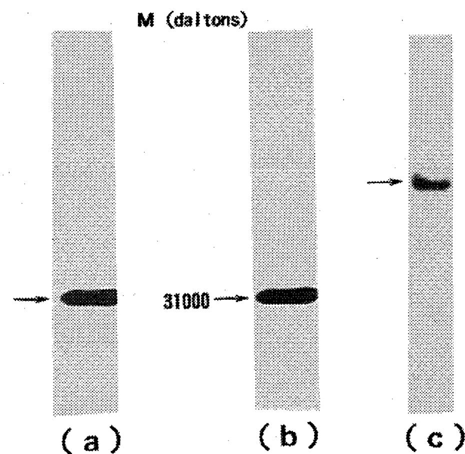

Fig. 2. Polyacrylamide gel electrophoresis of malate dehydrogenase (MDH).
(a) SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was stained with MDH activity specific staining mixture.
(b) SDS–PAGE. The gel was stained with MDH activity specific staining mixture and then with coomassie brilliant blue R–250.
(c) Polyacrylamide gel electrophoresis (PAGE). The gel was stained with MDH activity specific staining mixture.
The arrow indicates the position of MDH.

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