DETECTION OF L-GULONO-γ-LACTONE OXIDASE ON SDS–POLYACRYLAMIDE GELS BY THE FLUORESCENCE OF ITS COVALENTLY BOUND FLAVIN

Morimitsu NISHIKIMI, Kazutoshi KIUCHI and Kunio YAGI
Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan

Received 25 July 1977

1. Introduction

L-Gulono-γ-lactone oxidase (L-gulono-γ-lactone: oxygen 2-oxidoreductase, EC 1.1.3.8) is a microsomal enzyme that catalyzes the terminal step of L-ascorbic acid biosynthesis in animals, and is missing in primates and guinea pigs which require L-ascorbic acid in diet to prevent scurvy (for reviews, [1,2]). This enzyme has recently been purified near to homogeneity from rat and goat liver [3], and has been shown to possess a flavin prosthetic group covalently bound to the apoprotein [4]. In the course of a study on the enzyme, we found that it can be localized on sodium dodecylsulfate (SDS)–polyacrylamide gels by the intrinsic fluorescence of its covalently bound flavin. In the present study, this method was used to test microsomal preparations of the liver and the kidney of various animals for the occurrence of L-gulono-γ-lactone oxidase.

2. Materials and methods

Rat liver L-gulono-γ-lactone oxidase was purified by the method of Nishikimi et al. [3]. Microsomal preparations of tissues of various animals were prepared as described previously [3]. Human liver and kidney were obtained at autopsy from a patient who died of brain cancer.

SDS–polyacrylamide gel electrophoresis was carried out on 7.5% gels by the method of Fairbanks et al. [5] with some modifications [6]. All reagents used for SDS–polyacrylamide gel electrophoresis was purchased from Nakarai Chemical Co., Kyoto. Cytochrome *c*, chymotrypsinogen and bovine serum albumin were obtained from Boehringer, Mannheim and used as molecular weight markers. Protein samples were dissociated in a solution containing 1% SDS, 10% sucrose, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 1% β-mercaptoethanol by heating at 100°C for 3 min. One hundred micrograms of the microsomal proteins or 3 μg purified L-gulono-γ-lactone oxidase was subjected to electrophoresis until the tracking dye reached the bottom of gels. After the gels were soaked in 7% acetic acid, fluorescence was observed on them under ultraviolet illumination (λ 365 nm). Protein was stained with Coomassie Blue.

3. Results and discussion

A nearly homogeneous preparation of L-gulono-γ-lactone oxidase of rat liver was found to fluoresce as a yellow band on gels soaked in 7% acetic acid. The fluorescent band corresponded to the protein band stained with Coomassie Blue as shown in fig.1. By applying various amounts of the purified enzyme to gels, it was found that the fluorescent band was visible with a lower detection limit of 0.3 μg. The same fluorescent band was observed when rat liver microsomes were used as sample*.

* The fluorescent band of the microsomes tended to migrate slightly faster than that of the purified enzyme; however, a single fluorescent band was observed when these two samples were dissociated together and subjected to electrophoresis. The difference in migration distance of the fluorescent bands between the microsomes and the purified enzyme may be caused by that in the amounts of SDS bound to the enzyme in these samples. Thus the fluorescent band of the microsomes is ascribed to L-gulono-γ-lactone oxidase

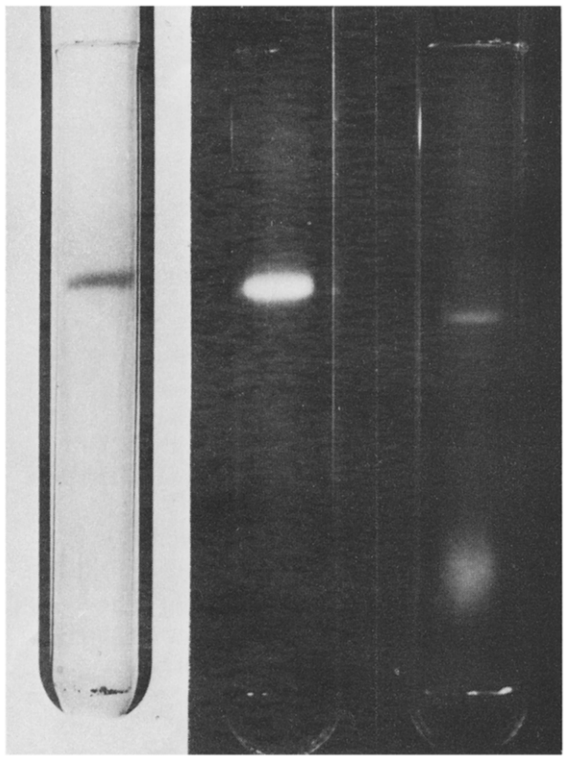

Fig.1. SDS–polyacrylamide gel electrophoresis of rat L-gulono-γ-lactone oxidase. The purified enzyme (3 μg) was subjected to electrophoresis on a 7.5% polyacrylamide gel in the presence of 0.1% SDS. The enzyme was stained with Coomassie Blue (left), or localized as a fluorescent band under ultraviolet illumination (middle). Rat liver microsomes (100 μg protein) were subjected to the electrophoresis and the enzyme was detected by the fluorescence (right).

The soaking of gels in an acetic acid solution is important to detect the fluorescence. The flavin of the native enzyme does not show any detectable fluorescence at a neutral pH [3]; however, the flavinyl peptides obtained by digestion with pronase or trypsin and chymotrypsin are reported to fluoresce at an acidic pH [4,7]. The enzyme denatured by SDS also shows fluorescence in an acetic acid solution.

The molecular weight of the enzyme of rat liver was estimated to be 51 000. The value is in good accord with the one obtained previously with a different SDS–polyacrylamide gel system [3].

We tested for the occurrence of the fluorescent band on gels with the microsomal preparations of tissues of various animals. The microsomes of rabbit liver, chicken kidney and bullfrog kidney, which are known to contain L-gulono-γ-lactone oxidase [8], all gave a fluorescent band. The migration distance of each band was the same within the experimental error. These findings indicate that L-gulono-γ-lactone oxidase of vertebrates of three classes, Mammalia, Aves and Amphibia, have a covalently bound flavin and that the molecular weight of each enzyme is almost the same. On the other hand, no fluorescent band was observed with the microsomes of rat kidney, rat brain, rabbit kidney, chicken liver and frog liver. This was also the case for the microsomes of the liver and the kidney of humans and guinea pigs. The microsomal preparations of carp hepatopancreas and kidney gave no fluorescent band, either. Since all these microsomal preparations that did not give the fluorescent band are reported to have no enzymic activity [8], localization of L-gulono-γ-lactone oxidase on SDS–polyacrylamide gels by its intrinsic fluorescence seems to provide a means of detecting the enzyme with crude preparations.

Nishikimi and Udenfriend [9], using the immunological technique, showed that the microsomes of guinea pig and monkey liver have no cross-reacting protein related to L-gulono-γ-lactone oxidase, and suggested that the gene for L-gulono-γ-lactone oxidase is not expressed in animals subject to scurvy. The present study supports this view by showing that tissues of these animals do not contain any flavinyl polypeptide homologous to L-gulono-γ-lactone oxidase.

The method of detecting L-gulono-γ-lactone oxidase in the present investigation would be applied to the detection of other enzymes which possess covalently bound flavins.

References

[1] Burn, J. J. (1967) in: Metabolic Pathways (Greenberg, D. M. ed) Vol. 1, 3rd edn, pp. 394–411, Academic Press, New York.
[2] Nishikimi, M. and Udenfriend, S. (1977) Trends Biochem. Sci. 2, 111–113.
[3] Nishikimi, M., Tolbert, B. and Udenfriend, S. (1976) Arch. Biochem. Biophys. 175, 427–435.
[4] Nakagawa, H., Asano, A. and Sato, R. (1975) J. Biochem. (Tokyo) 77, 221–232.

[5] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[6] Ikehara, Y. and Kato, K. (1974) in: Seitaimaku Jikkenho (Akamatsu, U., Asano, A., Anraku, Y., Ohmura, T., Fujita, M. and Mizushima, S. eds) Part 1, pp. 230–237, Kyoritsu Shuppan, Tokyo.
[7] Kenney, W. C., Edmondson, D. E., Singer, T. P., Nakagawa, H., Asano, A. and Sato, R. (1976) Biochem. Biophys. Res. Commun. 71, 1194–1200.
[8] Chatterjee, I. B. (1973) Science 182, 1271–1272.
[9] Nishikimi, M. and Udenfriend, S. (1976) Proc. Natl. Acad. Sci. USA 73, 2066–2068.