Cloning and Identification of a cDNA from Rat Liver Coding for a Portion of L-Gulonolactone Oxidase[†]

Bum-Shik HONG, Paul A. SEIB,* H.-C. YANG
and George L. MARCHIN**

Institute of Biotechnology, Korea University, Seoul, Korea

**Department of Grain Science and Industry, and*

***Bioserve Space Technologies (a NASA CDS Center), Section on Microbiology,
Division of Biology, Kansas State University,
Manhattan, Kansas 66506, U.S.A.*

Received September 30, 1988

An enzyme, L-gulono- γ -lactone oxidase, was purified from the livers of male albino rats and used to prepare a polyclonal rabbit antiserum. Reaction of the enzyme in cell-free extracts with the antiserum gave a single precipitin line when tested by the double diffusion method on agarose plates. Using the purified enzyme, the antiserum could detect nanogram quantities of the enzyme in dot-blotting procedures. This antiserum was used to screen a rat liver cDNA library in the vector λ gt11. Positively reacting clones could easily be identified. One clone contained a cDNA insert of 800 bp and produced a β -galactosidase fusion protein with a MW 30,000 greater than the native molecule.

In 1979 Jaffe¹⁾ estimated that world production of L-ascorbic acid was 32,750 metric tons. To date, the commercial synthesis of L-ascorbic acid is done by the Reichstein and Grusser scheme starting from D-glucose.^{1,2)} All five steps in that scheme occur in greater than 90% yield; the overall yield from D-glucose is 50~65%. A second method of preparing L-ascorbic acid from D-glucose is being developed in Japan.³⁾ The process consists of a two-stage fermentation: oxidative fermentation of D-glucose to calcium D-threohex-2,5-dinlosonate by a mutant strain of *Erwinia* and then its reductive fermentation to calcium 2-keto-L-gulonate by a mutant of *Corynebacterium*. The 2-keto-L-gulonate, which is produced in ~50% yield from D-glucose is converted to L-ascorbic acid using hot acids under special conditions.²⁾

In 1985, recombinant DNA technology was used to create a new organism that ferments glucose to 2-keto-L-gulonic acid.⁴⁾ This metabolic trait of the *Corynebacterium* used in the

Japanese fermentation method was transferred to an *Erwinia* strain. The new organism converts glucose to 2-keto-L-gulonic acid in a single fermentation step rather than the dual fermentative step in the Japanese process.

The pathway of ascorbic acid biosynthesis has been elaborated in animals^{5,6)} and plants.^{7,8)} In ascorbic-acid-synthesizing animals, such as the rat, this process involves the following steps: D-glucose $\rightarrow$ D-glucuronate $\rightarrow$ L-gulonate $\rightarrow$ L-gulono- γ -lactone $\rightarrow$ L-ascorbate.

Thus, the terminal step in the biosynthesis of ascorbic acid in mammals has been shown to be the oxidation of L-gulono- δ -lactone by L-gulono- γ -oxidase (EC 1.1.3.8).¹⁾ L-Gulono- γ -lactone oxidase has been purified and characterized in several laboratories.^{9,10)} Rat enzymes were found to have a monomeric molecular weight of 51,000 by SDS-polyacrylamide gel electrophoresis.^{9,10)}

Enzymatic synthesis of L-ascorbic acid may offer advantages over current microbiological-chemical methods. Enzymatic synthesis reac-

[†] This is contribution no. 89-85-J from the Kansas Agricultural Experiment Station, Manhattan, Kansas 66506, U.S.A.

tions are specific, giving few if any by-products, so purification of the desired product is simplified. Also, fermentation generally must be done on more dilute concentrations of substrates than enzymic conversions. We propose here to construct strains of *Escherichia coli* that contain the rat gene for this enzyme and subsequently produce the enzyme in high yield.

We report here the purification of L-gulonolactone oxidase from rat liver and preparation of a polyclonal rabbit antiserum against the enzyme. Rat liver cDNA libraries in the vector λ gt11 then were screened using the antiserum. One-positive-reacting clone was examined and found to contain an approximately 800 bp insert. A fusion protein that was capable of reacting with antibody was identified. The molecular weight of the fusion protein was 30,000 larger than that of β -galactosidase. These isolated clones confirm the utility of the procedure and provide probes for isolation of the complete gulonolactone-coding sequence.

Materials and Methods

Biochemicals, immunoreagents, and bacterial strains. L-Gulonolactone was a gift from Dr. T. C. Crawford of Pfizer, Inc., Groton, CT. Tween 20, ammonium sulfate, Brij 35, trypsin (Type I), 4-chloro-1-naphthol, 2,4-dinitrophenylhydrazine, and isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Sigma Chemical Company, St. Louis, MO. Sephadex G-150 and DEAE-Sephadex A-50 were products of Pharmacia Fine Chemicals, Inc., Piscataway, NJ. Bio-Gel HTP was obtained from Bio-Rad Laboratories, Richmond, CA. Eco RI was purchased from BRL, Bethesda, MD. Biotinylated anti-rabbit goat IgG, biotinylated horseradish peroxidase, avidin, and *E. coli* Y1089 were obtained from Clonetech, Palo Alto, CA.

Enzyme assay and PAGE. L-Gulonolactone oxidase was assayed by the method of Roe and Kuether¹¹ as modified by Ayaz *et al.*¹² One ml of 2% 2,4-dinitrophenylhydrazine cupric ion reagent was added to a 4-ml sample solution, and the mixture was incubated at 47°C for 90 min. The reaction mixture was cooled by swirling in an ice bath, while 5 ml of H₂SO₄ (9 vol conc. H₂SO₄ + 1 vol H₂O) was added. The absorbance was read at 540 nm in a Gilford Model 2220 spectrophotometer.

Polyacrylamide gel electrophoresis (8%) was done as

described by Davis.¹³ Tween 20 (nonionic detergent, 0.1%) was added to the gels and the electrophoresis buffer. Protein was stained with Coomassie brilliant blue. To measure the enzyme activity, gels were sliced in 2-mm pieces and each slice was crushed in the reaction mixture described above for the enzyme assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 8% gels as described by Laemmli.¹⁴

Purification of L-gulonolactone oxidase from rat liver.

The enzyme was purified by the method of Nishikimi *et al.*⁴ with minor modifications. Livers were obtained from male albino rats after decapitation and were stored at -20°C. The livers were homogenized using a Polytron homogenizer (Kinematica GmbH, Switzerland) in 4 ml of 0.25 M sucrose for each gram of liver and the homogenate was centrifuged for 15 min at 10,000 $\times g$. The supernatant was centrifuged for 60 min at 100,000 $\times g$. The microsomes were suspended in 1.15% KCl containing 10 mM EDTA (pH 7.4) and stored at -20°C. The microsomes (2-3 g of protein) were suspended at a protein concentration of 10 mg/ml in 20 mM Tris-acetate buffer (pH 8.0), containing trypsin (0.3 mg/ml) and 1 mM EDTA and were kept overnight at 4°C. The suspension was centrifuged for 90 min at 100,000 $\times g$, and the precipitate was suspended in a solution containing 1.5% Tween 20 (polyoxyethylene sorbitan monolaurate), 20 mM Tris-acetate buffer (pH 8), and 1 mM EDTA. After standing for 30 min at 4°C, the suspension was centrifuged for 90 min at 100,000 $\times g$ and the clear supernatant layer was collected. Solid ammonium sulfate then was added to the supernatant to give a concentration of 161 mg/ml, 15%. The mixture was centrifuged for 10 min at 10,000 $\times g$, and the pellicle formed was discarded. Additional ammonium sulfate (148 mg/ml) was added to the solution to give a 30% solution. The resulting precipitate was dissolved in a minimal volume (7.5 ml) of 20 mM Tris-acetate buffer (pH 8), containing 10 mM KCl, 1 mM EDTA, and 0.4% Brij 35 (polyoxyethylene 23-lauryl ether). The sample solution was passed through a Sephadex G-150 column (2.5 $\times$ 37 cm). The proteins were eluted with the Tris-acetate buffer (pH 8), containing 10 mM KCl, 1 mM EDTA, and 0.4% Brij 35, and the fractions containing the enzyme activity were combined. The pooled fractions were placed on a DEAE-Sephadex A-50 column (2.5 $\times$ 11.5 cm), and the proteins were eluted with a 100-ml linear gradient of KCl (10-300 mM), containing 20 mM Tris-acetate buffer (pH 8), 1 mM EDTA, and 0.4% Brij 35. The fractions (approximately 20 ml) containing high activity were combined and dialyzed overnight against 2 l of 10 mM potassium phosphate buffer (pH 7), containing 1 mM EDTA and 0.4% Brij 35. The dialyzed solutions were put on a hydroxylapatite (Bio-Gel HTP, Bio-Rad Lab, Richmond, CA.) column (2.5 $\times$ 5.0 cm). The enzyme was eluted with 30 mM potassium phosphate buffer (pH 7), containing 1 mM EDTA and 0.4% Brij 35.

Preparation of antisera to rat L-gulonolactone oxidase.

The purified enzyme was used for immunization of young New Zealand white rabbits. A 50~100 μ protein sample of the enzyme was emulsified in 0.5 ml of Freund's complete adjuvant (Sigma Chemical Company, St. Louis, MO.) and was then injected subcutaneously into the backs of the rabbits. Two additional injections were given at 2-week intervals. The rabbits were bled from a lateral ear vein 2 weeks after the last injection. The immunological reactivity of the antisera to purified gulonolactone oxidase was tested by the double diffusion agarose plates. A 1% ultra-pure agarose solution in 0.089 M Tris-borate buffer was layered on the hydrophilic side of Gel Bond film (FMC Corporation, Rockland, ME.) with 0.15 cm thickness of the agarose.

Screening libraries with antibody probe. A commercially available λ gt11 rat liver cDNA library containing 6.8×10^5 independent clones with an average insert size of 1.1 kb, (Clontech, Palo Alto, Ca.) was screened with an anti-gulonolactone oxidase antibody probe as plaques on a lawn of *E. coli* Y1090 as described in Young and Davis.¹⁵⁾ A 0.2-ml sample of the Y1090 culture was mixed with 0.1 ml of phage buffer containing the λ gt11 phage with recombinant DNA. Phage was allowed to adsorb to the cells at 37°C for 15 min and then was poured onto LB plates (15 g of agar, 10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 10 g of NaCl in 1 l). Plates were incubated at 42°C for 3.5 hr. A dry nitrocellulose filter previously saturated in 10 mM IPTG was overlayed on each plate, and plates were incubated 3.5 hr more at 37°C. The filters then were incubated in [TBST 50 mM Tris (pH 7.9), 150 mM NaCl, 0.05% Tween 20] plus rabbit anti-gulonolactone oxidase serum for 1 hr at room temperature. After being washed, the filters were incubated with biotinylated anti-rabbit goat IgG for 30 min and then with the avidin-biotinylated horseradish peroxidase complex for 30 min. Finally, the filters were incubated in a peroxidase substrate solution (4-chloro-1-naphthol, 3 mg/ml). Plaques corresponding to strongly purple spots were picked up from the agar plate with a sterile Pasteur pipette,

diluted in phage buffer, and replated. Successive platings were done until all plaques were positive to the antibody probe.

Size characterization of a λ gt11 recombinant. λ gt11 recombinant phage DNA was prepared by the plate lysate method with a modification of the procedure of Maniatis *et al.*¹⁶⁾ The inserted cDNAs were separated from EcoRI-digested phage DNA and their sizes estimated by gel electrophoresis. To produce the recombinant fusion protein, Y1089 was lysogenized with a positive-reacting λ gt11 recombinant phage. Single colonies were tested for temperature sensitivity at 42°C. Clones that grew at 32°C but not at 42°C were assumed to be lysogens. SDS-PAGE was done to detect the fusion protein, using a Bio-Rad 360 mini vertical slab-cell apparatus. The crude lysate was solubilized in electrophoresis sample buffer containing 2% SDS, 5% mercaptoethanol, and 10% glycerol in 0.5 M Tris-HCl, pH 6.8. Approximately 10 μ g of protein were put on an 8% polyacrylamide gel and electrophoresed by a 6-mA constant current for approximately 4 hr or until the tracking dye reached the bottom of the gel. Reference proteins used as standards in the electrophoresis system were β -galactosidase, phosphorylase B, and ovalbumin. The gels were either stained with Coomassie brilliant blue or analyzed by immunoblotting.

Results

A summary of the steps in the purification of the rat enzyme is presented in Table I. About 4.3 mg of enzyme were obtained with an approximately 60-fold increase in specific activity over the microsomes. The overall enzyme purification yield was about 3%. Polyacrylamide gel electrophoresis of the purified rat enzyme in the absence of 0.1% Tween 20 showed one broad protein-staining band that had enzyme activity (Fig. 1A). When the detergent was

Table I. SUMMARY OF ENZYME PURIFICATION FOR L-GULONOLACTONE OXIDASE

Ascorbate was read from a standard curve and enzyme activity is expressed as mU (nanomoles of ascorbic acid formed per minute when L-gulonolactone was added as substrate).

	Total protein (mg)	Total activity (m μ)	Specific activity (m μ /mg protein)	Fold purification	Yield (%)
Microsomes	10,200	30,000	2.9	1	100
Tryptic digestion	4,266	19,200	4.5	1.6	64
Tween 20	1,367	8,200	6.0	2.1	27
(NH ₄) ₂ SO ₄	433	5,200	12.0	4.1	17
Sephadex G-150	278	5,010	18.0	6.2	17
DEAE-Sephadex	52	1,500	28.8	9.9	5
Hydroxylapatite	4.3	750	174.4	60.1	3

Fig. 1. Polyacrylamide Gel Electrophoresis of Rat L-Gulonolactone Oxidase.

The purified enzyme was electro phoresed on 8% polyacrylamide gel in the absence of 0.1% Tween 20 (A) and in the presence of 0.1% Tween 20 (B). Gels were stained with Coomassie brilliant blue. A: One protein staining band, indicated by arrow, was observed which had enzyme activity. B: Two protein staining bands were observed, both of which showed enzyme activity.

present in the electrophoresis system, two broad protein bands were observed (Fig. 1B) both of which showed enzyme activity (data not shown).

When the immunological activity of the antisera to purified gulonolactone oxidase was tested by the double diffusion method on agarose plates (Fig. 2), a single line of precipitation was observed. The purified enzyme preparation, as well as crude extracts of rat liver, showed only one precipitation band on Ouchterlony gels. No precipitation lines were obtained with serum from rabbits taken before immunization. Finally, addition of increasing amounts of antiserum to constant amounts of enzyme formed antibody enzyme complexes which could be pelleted by centrifugation, leaving the supernatant free of gulonolactone oxidase activity (data not shown). This indicates that monospecific antiserum to the

Fig. 2. Immunological Reactivity of L-Gulonolactone Oxidase.

Enzyme was put into Well E, and antiserum and normal serum were put into Well 1 and Well 2, respectively. One percent agarose gel was prepared on Gel Bond film (FMC Corporation, Rockland, Maine).

Fig. 3. Agarose Gel Electrophoresis for the Size Estimation of Inserts.

One percent agarose gel electrophoresis was done for 1 hr at 50 V. Lanes 1 and 2: λ gt11 phage DNA was extracted from positive-reacting clones and was digested with EcoRI restriction enzyme to excise the inserts. Phage DNA (20kb) and the inserts (0.8 kb) are shown. Lane 3: Taq I-digested pBR322 was used as size marker (kb). The DNA was made visible using ethidium bromide.

gulonolactone oxidase was obtained.

A rat liver cDNA library in the vector λ gt11 was screened using the antibody mentioned above. Initial plaque screening of about 5×10^5 independent rat liver cDNA recombinants identified seven putative gulonolactone oxidase cDNA clones in blotting procedures, which were used to detect antigen-antibody complexes. Successive platings were done until

Fig. 4. Detection of β -Galactosidase/Gulonolactone Oxidase Fusion Proteins.

Bacterial lysates were prepared from Y1089 (lane 1) and lysogens of Y1089 (λ gt11 gulonolactone oxidase) (lane 2) after 42°C induction of fusion proteins in the presence of 5 mM IPTG. Samples were boiled for 2 min in sample buffer and were put on 8% SDS-polyacrylamide gels. Gel was stained with Coomassie brilliant blue. The sizes of reference polypeptides are shown in kDa. Arrow indicates a band unique to the lysogen that specifically reacted with rabbit anti-rat L-gulonolactone oxidase antibody.

all plaques were positive to the antibody probe. Y1089 *E. coli* were infected with recombinant phages and were incubated until the plaques covered almost the entire surface of the plate. After the elution of bacteriophages from the plate, phage DNA was extracted with phenol and was digested with EcoRI restriction enzyme to separate the inserts from phage DNA. The sizes of the inserts from these clones were estimated by agarose gel electrophoresis. One of these clones contained an insert of approximately 800 bp (Fig. 3). Judging from the molecular size of gulonolactone oxidase subunit (approximately 50,000 Da), this is enough information to code for more than half of the gulonolactone oxidase protomer.

Lysogens of the recombinants were pre-

pared in *E. coli* strain Y1089 by the method of Huynh *et al.*,¹⁷⁾ to facilitate characterization of the identified fusion proteins, which are the translated polypeptide from recombinant lacZ-gulonolactone oxidase cDNA genes. Synthesis of the fusion proteins in a lysogen-containing λ gt11 was augmented by IPTG and the proteins of cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Immunoblotting of identical gels with anti-gulonolactone oxidase antibodies indicated (arrow) the presence of gulonolactone oxidase antigenic determinants in a novel fusion protein (immunoblots not shown). The fusion protein molecule was approximately 30 kDa larger than that coded for by the lacZ DNA (114 kDa). This agrees with the size by agarose gel electrophoresis.

Discussion

In the purification procedure for gulonolactone oxidase, tryptic digestion was done to release the enzyme from the membrane. Because of its restricted specificity, trypsin is perhaps the most commonly used proteolytic enzyme for solubilizing membrane components. Although the danger of enzymatic degradation during treatment is inherent in the use of proteinases, other investigators have used this approach to purify membrane enzymes.⁹⁾ The proteinase treatment yielded gulonolactone oxidase preparations with fairly high specific activities.

Tween 20 (polyoxyethylene sorbitan monolaurate) is a nonionic detergent which solubilizes a number of membrane proteins without loss of enzyme activity. Brij 35 (polyoxyethylene 23-lauryl ether), sodium deoxycholate, and Tween 20 were tried for solubilizing membrane enzymes. All yielded enzyme preparations with comparable specific activities.

Eliceiri *et al.*¹⁸⁾ reported two forms of gulonolactone oxidase with molecular masses on the order of 450,000 and 200,000 Da. Our preparation had a broad protein staining band in native gels and both a high molecular mass form (MW $\gg$ 100,000 Da) and a low molec-

ular mass form (MW ~ 60,000 Da) in Tween 20 gels. An explanation for these multiple forms may be that the enzyme is attached to varying amounts of submicrosomal components or that dissociation of an oligomeric enzyme or aggregates may occur in the presence of Tween 20.

When the immunological activity of the antisera to purified gulonolactone oxidase was tested by the double diffusion method on agarose plates, it made a single line of precipitation. Antiserum was also active against purified gulonolactone oxidase in dot-blotting procedures to the nanogram level of sensitivity (data not shown). These data, together with the gulonolactone oxidase activity-precipitating-properties of the antibody, suggested that it would be useful in identifying gulonolactone oxidase recombinants in a rat liver cDNA library.

When the rat liver cDNA library was screened, both the antiserum and the IgG purified from it clearly identified positive plaques. Usually, 5~6 successive platings were done. More and more positive plaques appeared on a plate as successive platings were done. This implies that our antibody probe consistently reacts with the clone we desire.

Immunoblotting of the SDS-polyacrylamide gels containing lysates of the λ gt11 recombinant in Y1089 with anti-gulonolactone oxidase antibodies specifically indicated the presence of gulonolactone oxidase antigenic determinants in a high molecular weight protein. Occasionally, a high background of nonspecific protein signals can appear in immunoblotting gels, since polyvalent antibodies sometimes contain components that bind to antigens normally produced by *E. coli*. However, this background binding activity was removed from our antibody preparation by incubating it with nitrocellulose filters soaked in *E. coli* cell lysates. We postulate, therefore, that this high molecular weight antibody-binding-protein uniquely present in the recombinant-lysogen lysate is the gulonolactone oxidase fusion protein.

In summary, it has been demonstrated that

L-gulonolactone oxidase was purified to homogeneity from rat liver. The enzyme was used to immunize rabbits and to prepare a polyclonal rabbit antiserum. This antiserum reacted both with purified enzyme and crude rat liver extracts to produce a single precipitation band in Ouchterlony gels.

A rat liver cDNA library in the vector λ gt11 was screened using the antibody mentioned above. One positive-reacting clone contained an insert of approximately 800 bp. A fusion protein that was capable of reacting with anti-gulonolactone oxidase antibody was produced. The fusion protein molecule was 25,000~30,000 daltons larger than that of β -galactosidase.

We are currently in the process of probing full-length cDNA from libraries prepared from immunopurified mRNA. By binding out antibody to polysomes from rat liver, followed by the use of a protein A-Sepharose column as an immunoadsorbent, we were able to enrich the mRNA coding for the gulonolactone oxidase. Furthermore, we intend to use the 800-bp fragment to prepare labeled cDNA, with which we may probe our cDNA libraries by hybridization techniques.

Acknowledgments. The authors are grateful to the Department of Grain Science and Industry for financial support, and to Dr. T. C. Crawford of Pfizer, Inc. for samples of L-gulonolactone. We also thank Dr. K. J. Kramer and Dr. H.-C. Yang for helpful discussions.

References

- 1) G. M. Jaffe, "Kirk-Othmer Encyclopedia Chemical Tech.," 3rd Ed., Vol. 24, ed. by M. Grayson and D. Eckroth, Wiley, New York, pp. 8~40.
- 2) T. C. Crawford and S. A. Crawford, *Adv. Carbohydr. Chem.*, **37**, 79 (1980).
- 3) T. Sonoyama, H. Tani, K. Matsuda, B. Kageyama, M. Tanimoto, K. Kobayashi, S. Yagi, H. Kyotani and K. Mitsushima, *App. Environ. Microbiol.*, **43**, 1064 (1982).
- 4) S. Anderson, C. B. Marks, R. Lazarus, S. Miller, K. Stafford, J. Seymour, D. Light, W. Rastetter and D. Estell, *Science*, **230**, 144 (1985).
- 5) I. B. Chatterjee, *Methods Enzymol.*, **18**, 28 (1970).
- 6) R. Jenness, E. C. Birney and K. L. Ayaz, *Comp. Biochem. Physiol.*, **63**, 195 (1980).

- 7) M. Baig, S. Kelly and F. Loewus, *Plant Physiol.*, **46**, 277 (1970).
 - 8) F. Loewus and J. Helsper, "Metabolism of L-Ascorbic Acid in Plants," ed. by P. A. Seib, and B. M. Tolbert, In "Ascorbic Acid: Chemistry, Metabolism and Uses," American Chemical Society, Washington, D. C., 1981.
 - 9) M. Nishikimi, B. Tolbert and S. Udenfriend, *Arch. Biochem. Biophys.*, **175**, 427 (1976).
 - 10) M. Nishikimi, K. Kiuchi and K. Yagi, *FEBS Lett.*, **81**, 323 (1977).
 - 11) J. H. Roe and C. A. Kuether, *J. Biol. Chem.*, **147**, 399 (1943).
 - 12) K. M. Ayaz, R. Jenness and E. C. Birney, *Anal. Biochem.*, **72**, 161 (1976).
 - 13) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
 - 14) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
 - 15) R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1194 (1983).
 - 16) T. Maniatis, E. F. Fritsch and J. Sambrook, "Molecular Cloning," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
 - 17) T. Huynh, R. A. Young and R. W. Davis, "DNA Cloning," Vol. 1, ed. by D. M. Glover, IRL Press, Washington D. C., 1985, p. 49.
 - 18) G. L. Eliceiri, E. K. Lai and P. B. McCay, *J. Biol. Chem.*, **244**, 2641 (1969).
-