Note

Purification and Properties of L-Gulono-1,4-lactone Oxidase from *Grifola frondosa*

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Summary L-Gulono-1,4-lactone oxidase activity was detected in *G. frondosa*; therefore its properties were studied after purification. A 766-fold purified preparation of the enzyme from fresh fruit bodies was obtained by means of a seven-step procedure, the overall yield being 14%. The purified enzyme gave a single band on polyacrylamide gel electrophoresis and its absorption spectrum exhibited the characteristic of a flavoenzyme. The enzyme produced L-ascorbic acid and H₂O₂, with L-gulono-1,4-lactone (GL) as the substrate and oxygen as the electron acceptor, and was optimally active at around pH 7.0 and 45°C. Its molecular mass was determined to be 250 kDa on gel filtration, while the dissociated enzyme exhibited a molecular mass of 69 kDa on SDS-polyacrylamide gel electrophoresis, but the true molecular weight is unknown because of the trypsin treatment in the purification process. The apparent *K_m* value for GL was 24 ± 1 mM. Its substrate specificity was extremely high and, assuming that for GL to be 100, the following results were obtained: D-mannono-, 25; D-glucono-, 4; L-idono-, 3; L-galactono-1,4-lactone, 2; and 15 other lactones tested, 0. It is presumed that this enzyme is similar to animal GL-oxidase, ascomycetes D-arabinonolactone oxidase, etc.

Key Words L-gulono-1,4-lactone oxidase, *Grifola frondosa*, basidiomycetes, L-ascorbic acid synthesis, 6-deoxy-L-ascorbic acid

Ascorbic acid (AA) exists in the form of AA analogs in ascomycetes and basidiomycetes. In ascomycetes, D-arabo-AA exists in *Penicillium* sp. (1), and D-erythro-AA in *Candida* sp. (2), *Neurospora crassa* (3), *Saccharomyces cerevisiae* (4), and *Lypomyces starkeyi* (4). D-Glucanone dehydrogenase (which is effectively an oxidase since oxygen is an electron acceptor) and D-arabinonolactone oxidase catalyze the last steps of these biosyntheses, respectively. They were purified and various characteristics were investigated in detail (5–8). They are all flavoenzymes using oxygen as the electron acceptor and are similar to animal L-gulono-1,4-lactone oxidase (GLO). These enzymes are named after the substances that could be their best substrates. The AA analogs present in ascomycetes and the substrate specificities of the oxidases exhibit a close relationship.

On the other hand, in the fruit bodies of basidiomycetes, there are D-erythro-AA, 6-deoxy-L-AA and associated glycosides, in which glucose or xylose is combined with C-5 of the AA analogs (9, 10). Thus, the AA-related substances present in basidiomycetes are more diverse than those in ascomycetes, and the biosynthesis pathway for the AA analogs and the sugar bond reaction are very interesting, but there has been no report on these enzymes. Determination of the characteristics of the enzyme that catalyzes the last step of AA analog biosynthesis is expected to provide clues as to the biosynthesis pathway, as well to provide interesting information on the evolution of fungi. Accordingly, sev-

eral kinds of basidiomycetes were investigated, among which *G. frondosa* showed GLO activity. The enzyme was purified and its properties were studied.

Experimental

Chemicals. L-Gulono-1,4-lactone (GL) was purchased from Aldrich (USA), and L-galactono- and D-mannono- from P-L Biochemicals (USA), D-gulono- from Sigma (USA), and D-galactono-, D-glucono- and D-glucurono-1,4-lactone from Wako Pure Chemical Industries (Japan), respectively. The other lactones used were obtained by oxidizing the corresponding aldoses with bromine to produce aldonic acids and crystallized in their 1,4-lactone forms according to Green (11). D-Lyxose was purchased from Tokyo Kasei (Japan), D-xylose and D-ribose from Wako Pure Chemical Industries, and L-idose and L-talose from Sigma. Nonion (polyoxyethylene nonylphenol ester) NS-210 was obtained from Nippon Yushi Co. (Japan). All other chemicals were of analytical grade.

Purification of GLO from *G. frondosa*. The following operations were carried out at 0–10°C. One kilogram of fresh fruit bodies of *G. frondosa* was ground with a Juicer JC-6306G (Fuji Electric Co., Japan) to obtain a porridge-like consistency, and then the homogenate was mixed well with 2 L of 50 mM potassium phosphate buffer, pH 7.0 (PB). The mixture was further homogenized well with a glass homogenizer and then filtered through a fine nylon screen. Solid ammonium sulfate was added

to the resulting filtrate to 65% saturation. After standing for 30 min, the precipitate was collected by centrifugation at $5,000\times g$ for 15 min. Five hundred milliliters of 50 mM PB was added and mixed well with the precipitate, and then the mixture was centrifuged. Since the target enzyme was in the gelled precipitate obtained on centrifugation, one more washing was performed. Five hundred milliliters of 50 mM PB containing 2% Nonion-NS-210 was added and mixed well with the precipitate, and then the mixture was centrifuged at $5,000\times g$ after standing for 30 min. After washing with 500 mL of 50 mM PB, the precipitate was resuspended in 200 mL of 10 mM PB containing 400 IU trypsin, incubated for 3 h at 37°C to solubilize it, and then centrifuged at $5,000\times g$ for 15 min. The resulting supernatant was applied to a hydroxylapatite column, 2.5×10 cm, equilibrated with 25 mM PB beforehand. After washing with the same buffer, the proteins were eluted with 50 mM PB containing 10% ammonium sulfate. The fractions containing enzyme activity were combined and dialyzed overnight against 5 L of 5 mM PB. The dialyzed sample was applied to a DEAE-cellulose column, 2×5 cm, equilibrated with 5 mM PB beforehand. After washing with the same buffer, the proteins were eluted with 100 mM PB. The fraction containing enzyme activity (3 mL) was loaded onto a Sephadex G-150 (Amersham Pharmacia, Sweden) column, 2.5×100 cm, equilibrated with 5 mM PB, and then the proteins were eluted with the same buffer. The fractions containing enzyme activity were combined and concentrated on a DEAE-cellulose column. That is, the enzyme was adsorbed to a DEAE-cellulose column, 0.5×1.5 cm, equilibrated with 5 mM PB and then eluted with 2 mL of 100 mM PB.

Enzyme assay. The reaction mixture (2 mL) comprised 50 mM PB, 2 mM GL and the enzyme. After incubation in air at 37°C for 15 min, the reaction was stopped by adding 0.5 mL of 25% trichloroacetic acid or 25% metaphosphoric acid, and standing in ice water for 10 min, followed by centrifugation. Then L-AA of the supernatant was measured. One unit of the enzyme was defined as the amount of enzyme which produced 1 μ mol L-AA per min under the conditions described above.

Determination of L-AA, AA analogs, and protein. The α,α' -dipyridyl method (12) or osazone-HPLC method (10) was used to measure L-AA and AA analogs. The Lowry method (13) or absorbance at 280 nm was used

to measure protein, with bovine serum albumin as a standard protein.

Molecular mass measurement. The gel filtration and SDS-polyacrylamide gel electrophoresis (PAGE) methods were used. For the gel filtration method, a column of Sephadex G-200, 2.5×100 cm, was used, which was eluted with 100 mM PB containing 100 mM KCl. As standard marker proteins, bovine liver glutamate dehydrogenase (350 kDa), bovine liver catalase (240 kDa), and yeast alcohol dehydrogenase (141 kDa) were used. For SDS-PAGE, a 7.5% polyacrylamide gel containing 1% SDS was used, an LMW electrophoresis calibration kit (Amersham Pharmacia) being used as the molecular mass markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α -lactalbumin, 14.4 kDa).

Results and Discussion

The purification process and changes in enzyme activity are summarized in Table 1. A crude extract of 1 kg of fresh fruit bodies exhibited 23 U activity and approximately 90% of it was recovered as a precipitate on salting out. When this precipitate was washed with 50 mM PB, the target enzyme was not dissolved but could be obtained as a gelled precipitate (approximately 15 mL). The enzyme was not soluble on Nonion treatment. The gelled precipitate was slightly viscous, containing 27% sugar, as measured by the phenol-sulfuric acid method. The enzyme was purified 766 times (yield, 14%), was pale yellow in color, and emitted fluorescence on UV radiation (365 nm). The activity of the purified enzyme was inhibited by 40% on the addition of acriflavine (1.5 mM) as a flavoenzyme inhibitor.

PAGE of the purified enzyme gave only one protein band (Fig. 1-A). Weak fluorescence was emitted by the part corresponding to the protein under UV radiation (365 nm) when the gel was immersed in 7% acetic acid. When the electrophoretic gel was immersed in a solution comprising GL, horseradish peroxidase and *o*-toluidine under aerobic conditions, *o*-toluidine was oxidized in the part corresponding to the protein (Fig. 1-B). This enzyme was thought to produce H_2O_2 using oxygen as an electron acceptor. When active staining of the enzyme was performed under anaerobic conditions with GL as the substrate and phenazine methosulfate as the electron acceptor, the portion corresponding to the pro-

Table 1. Purification of GLO from *G. frondosa*.

Purification steps	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Fold	Yield (%)
Crude extract	6,117	23,130	3.8	1.0	100
$(NH_4)_2SO_4$ fractionation	4,500	22,380	5.0	1.3	96.8
Washing with PB	228	11,820	51.8	13.8	51.1
Nonion NS-210	80	10,980	137.3	36.1	47.5
Tryptic digestion	—	9,280	—	—	40.1
Hydroxylapatite	7.0	7,020	1,003	276	30.4
DEAE cellulose	4.1	5,510	1,344	340	23.8
Sephadex G-150	1.1	3,200	1,909	766	13.8

Fig. 1. PAGE of *G. frondosa* GLO (10 μ g/one lane). (A) Stained with Coomassie brilliant blue R250. (B) Activity staining of the enzyme with hydrogen peroxide produced. Immersed and stained in 50 mM PB containing 25 mM GL, 1 mg/mL horseradish peroxidase and 2 mM *o*-toluidine under aerobic conditions for 15 min at 20°C in the dark. (C) Activity staining of the enzyme under anaerobic conditions. Immersed and stained in 50 mM PB containing 25 mM GL, 30 mM phenazine methosulfate and 10 mM nitroblue tetrazolium for 15 min at 20°C in the dark.

Fig. 2. SDS-PAGE of *G. frondosa* GLO. Gels were stained with Coomassie brilliant blue R250. (A) The purified enzyme (5 μ g). (B) Molecular mass standards (30 μ g).

tein was stained (Fig. 1-C). Thus, this enzyme is not only an oxidase but also has the characteristics of a dehydrogenase. Since L-AA was not produced even with the use of cytochrome c, NAD or NADP, which usually function as electron acceptors in vivo, and other substances that can be substituted for these had not been discovered at that point, it was determined to be an oxidase. SDS-PAGE on a 7.5% gel gave one major protein band at a position corresponding to 69 kDa and some very weak protein bands (Fig. 2). In spite of several modifications of the purification processes, these weak bands could not be removed. It is presumed that this enzyme is still not completely purified. A value of 250 kDa

Fig. 3. Absorption spectra of *G. frondosa* GLO (1.23 mg/mL) before and after the addition of GL (2 μ mol/mL). (A) Oxidized form. (B) Reduced form (addition of GL under anaerobic conditions).

was obtained on molecular mass measurement by means of gel filtration on Sephadex G-200, but the true molecular weight is unknown because of the trypsin treatment and the impurities.

Absorption spectra of the purified enzyme are shown in Fig. 3. Peaks of the oxidized form were recognized at 455 nm and 358 nm, which are characteristic of a flavoenzyme. When GL was added to this sample to the concentration of 2 mM under anaerobic conditions to obtain the reduced form, the 455 nm peak was not observed.

When GL was used as the substrate, the enzyme was optimally active at around pH 7.0 and 45°C. The *pI* was found to be pH 5.9 with the chromatofocusing method (14). The apparent *K_m* value obtained from a Lineweaver-Burk plot for GL was 24 ± 1 mM, which was much larger than those for isofunctional enzymes such as rat GLO (0.066 mM) (15), goat GLO (0.15 mM) (15), cauliflower L-galactonolactone dehydrogenase (2 mM) (16), and sweet potato L-galactonolactone dehydrogenase (0.12 mM) (14). However, the *K_m* value of the *C. albicans* D-arabinonolactone oxidase (44.1 mM) (8) was larger than that of the *G. frondosa* GLO. The *V_{max}* was 2.1 ± 0.1 μ mol/min/mg (substrate: GL).

Regarding the substrate specificity under aerobic conditions, if the activity toward GL is taken to be 100, that toward D-mannono- was 25, and L-idono-, D-glucono- and L-galactono-1,4-lactone showed only slight reaction. Deoxy-sugar acid lactones such as L-fucono-1,4-lactone (6-deoxy-L-galactono-1,4-lactone) and 6-deoxy-D-glucono-1,4-lactone, and a uronic acid lactone such as D-glucurono-1,4-lactone did not serve as substrates (Table 2). Accordingly, the lactones that can serve as substrates are six carbon sugar acid lactones with hydroxyl groups at C-2 and C-3 in the D arrangement. D-Arabinonolactone oxidase of ascomycetes (8) and GLOs of animals (17) require D arrangement lactones at C-2, but lactones with a hydroxyl group on the opposite side at C-3 also allowed the reaction. Thus, the

Table 2. Substrate specificities of GLOs of *G. frondosa* and *L. edodes*, and isofunctional enzymes of other organisms.

Substrate (1,4-lactone)	<i>G. frondosa</i>	<i>L. edodes</i>	<i>C. albicans</i> ^a	<i>P. cyaneo-fulvum</i> ^b	Yeast ^c	Rat ^d	Cauliflower ^e
Hexono lactone							
L-Gulono-	100	100	25	0	32	100	0
L-Idono-	3	6	—	—	<3	0	—
L-Talono-	0	1	—	—	<3	0	—
L-Galactono-	2	0	88	—	100	87	100
L-Mannono-	0	0	—	—	—	0	—
D-Mannono-	25	24	—	0	<3	68	—
D-Glucono- ^f	4	0	0	100	<3	0	—
D-Allono-	0	0	—	—	<3	0	—
D-Altorono-	0	0	—	—	68	47	15
D-Gulono-	0	0	0	—	0	0	—
D-Galactono-	0	0	1	0	0	0	—
Pentono lactone							
D-Lyxono-	0	0	—	—	—	59	—
D-Xylono-	0	0	—	—	—	0	—
L-Xylono-	0	0	85	—	—	38	—
D-Ribono-	0	0	—	—	—	0	—
L-Ribono-	0	0	—	—	—	12	—
D-Arabinono-	0	0	100	—	—	22	7
Deoxy sugar acid lactone							
L-Fucono-	0	0	—	—	—	38	—
6-Deoxy-D-glucono-	0	0	—	—	—	—	—
Uronic acid lactone							
D-Glucurono-	0	0	2	—	0	0	—

^a From Ref. 8, ^b from Ref. 5, ^c from Ref. 7, ^d from Ref. 17, ^e from Ref. 16, ^f 1,5-lactone was used. Activity is expressed taking the AA production from the most reactive substrate as 100 for each enzyme.

GLO of *G. frondosa* has a high substrate specificity comparable to that of L-galactonolactone dehydrogenases of plants (14, 16). Considering that 6-deoxy-5-O-(α -D-glucopyranosyl)-AA rather than L-AA is present in *G. frondosa*, L-fucono-1,4-lactone was fully expected to produce 6-deoxy-L-AA as the substrate, but there was no reaction at all (Table 2). The reason for this is supposed to be that the hydroxyl group at C-3 is in the D arrangement, which makes it difficult for this enzyme to be a substrate. On the other hand, GL serves as the best substrate and therefore, regarding its pathway, it can be supposed that it is converted into the deoxy-form via L-AA, but the *K_m* value is extremely high, i.e., 24 mM, and there is a possibility that the real substrate may be something else. The name of the enzyme, GLO, will be changed when the real substrate is known. Judging from the hydroxyl group arrangement at C-2 and C-3, 6-deoxy-GL is very possibly a substrate, but this could not be confirmed because this substance was not available at the time. Further study is needed to clarify how this enzyme is related to the biosynthesis of 6-deoxy-L-AA in vivo.

The production of L-AA or AA analogs was examined by means of the same reaction system as for *G. frondosa* using crude extracts of *Lentinus edodes*, *Flammulina velvutipes*, *Pleurotus pulmonarius*, *Hypsizygus marmoreus*, *Pholiota nameko* and *Agrocybe cylindracea*, and with L-gulono-, L-galactono-, D-mannono-, D-glucono-, D-arabinono-, D-ribono- and L-fucono-1,4-lactone as substrates. The same results as those for *G. frondosa* were

only obtained for *L. edodes* (Table 2), i.e., there was no reaction with the other fungi. Why no reaction was observed for the other fungi is currently being examined.

REFERENCES

- 1) Takahashi T, Mitsumoto M, Kayamori H. 1960. Production of D-araboascorbic acid by *Penicillium*. *Nature* **188**: 411–412.
- 2) Murakawa S, Sano S, Yamashita A, Takahashi T. 1977. Biosynthesis of D-erythroascorbic acid by *Candida*. *Agric Biol Chem* **41**: 1799–1800.
- 3) Dumbra V, Pall ML. 1987. Control of nucleotide and erythroascorbic acid pools by cyclic AMP in *Neurospora crassa*. *Biochim Biophys Acta* **926**: 331–338.
- 4) Nick JA, Leung CT, Loewus FA. 1986. Isolation and identification of erythroascorbic acid in *Saccharomyces cerevisiae* and *Lypomyces starkeyi*. *Plant Sci* **46**: 181–187.
- 5) Takahashi T, Yamashita H, Kato E, Mitsumoto M, Murakawa S. 1976. Purification and some properties of D-glucono- γ -lactone dehydrogenase D-erythorbic acid producing enzyme of *Penicillium cyaneo-fulvum*. *Agric Biol Chem* **40**: 121–129.
- 6) Shimizu M, Murakawa S, Takahashi T. 1977. The covalently bond flavine prosthetic group of D-gluconolactone dehydrogenase of *Penicillium cyano-fulvum*. *Agric Biol Chem* **41**: 2107–2108.
- 7) Nishikimi M, Noguchi E, Yagi K. 1978. Occurrence in yeast of L-galactonolactone oxidase which is similar to a key enzyme for ascorbic acid biosynthesis in animals, L-gulonolactone oxidase. *Arch Biochem Biophys* **191**: 479–486.

- 8) Huh W-K, Kim S-T, Yang K-S, Seok Y-J. 1994. Characterization of D-arabinono-1,4-lactone oxidase from *Candida albicans* ATCC 10231. *Eur J Biochem* **225**: 1073–1079.
- 9) Okamura M. 1994. Distribution of ascorbic acid analogs and associated glycosides in mushrooms. *J Nutr Sci Vitaminol* **40**: 81–94.
- 10) Okamura M. 1998. Separative determination of ascorbic acid analogs contained in mushrooms by high-performance liquid chromatography. *J Nutr Sci Vitaminol* **44**: 25–35.
- 11) Green JW. 1948. The halogen oxidation of simple carbohydrates, excluding the action of periodic acid. *Adv Carbohydr Chem* **3**: 129.
- 12) Okamura M. 1980. An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. *Clin Chim Acta* **103**: 259–268.
- 13) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275.
- 14) Oba K, Ishikawa S, Nishikawa M, Mizuno H, Yamamoto T. 1995. Purification and properties of L-galactono- γ -lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *J Biochem* **117**: 120–124.
- 15) Nishikimi M, Tolbert BM, Udenfriend S. 1976. Purification and characterization of L-gulono- γ -lactone oxidase from rat and goat liver. *Arch Biochem Biophys* **175**: 427–435.
- 16) Mapson LW, Breslow E. 1958. Biological synthesis of ascorbic acid: L-Galactono- γ -lactone dehydrogenase. *Biochem J* **68**: 395–406.
- 17) Ashwell G, Kanfer J, Smiley JD, Burns JJ. 1961. Metabolism of ascorbic acid and related uronic acids, aldonic acids, and pentoses. *Ann NY Acad Sci* **92**: 106–114.