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Detection of L-Xylosone and its Physiological Effects in *Saccharomyces cerevisiae*

Yeong-Jae Seok, Kap-Seok Yang, Ju-Gyeong Kang, Seong-Tae Kim, Won-Ki Huh, and Sa-Ouk Kang*

Laboratory of Biophysics, Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Republic of Korea

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L-Xylosone was detected as its quinoxaline derivative in the degradation solution of dehydro-L-ascorbic acid. The production rate of L-xylosone was much faster in aerated phosphate-citrate buffer (pH 5.6) than in pure water. L-Xylosone and dehydro-L-ascorbic acid were identified in the crude extracts of *Saccharomyces cerevisiae*. The concentration of L-xylosone in the crude cell extracts was calculated to be about 0.2 nmol (mg protein)$^{-1}$. When L-xylosone was added to asynchronous culture of *S. cerevisiae*, it inhibited primarily the synthesis of protein and RNA. Examination of the effect of L-xylosone on synchronous culture of the yeast indicated that L-xylosone inhibited the initiation of DNA replication and that the cells were arrested at G_1 stage of cell division cycle. These results suggested a possibility that L-xylosone can act as an inhibitor of cell growth.

Key words: L-xylosone, α-ketoaldehyde, L-ascorbic acid, dehydro-L-ascorbic acid, cell growth, *Saccharomyces cerevisiae*

L-Ascorbic acid is known to be involved in a great variety of biological phenomena such as the immune system, the cytochrome P450 system, neurological function, atherosclerosis and free radical reactions in biological samples. But none of these proposals have led to well defined and acceptable physiological roles of L-ascorbic acid. Identifications of the metabolic pathways and their intermediates are expected to bring us much more information about the physiological roles of L-ascorbic acid.

Dehydro-L-ascorbic acid is the first relatively stable oxidation product of L-ascorbic acid. In neutral or alkaline solutions, dehydro-L-ascorbic acid is very rapidly delactonized to 2,3-diketogulonic acid, which is subsequently decarboxylated to pentonic acids via pentosones in several organisms (6,8). Of these pentosones, L-xylosone (*L-threo*-pentos-2-ulose), the precursor of L-xylonic acid, is produced nonenzymatically from dehydro-L-ascorbic acid (6) or enzymatically in bacteria (7). From further investigation on these discoveries with nuclear magnetic resonance spectroscopy, a model for the possible catabolic pathway of L-ascorbic acid has been suggested (18,9).

In the present study, we detected L-xylosone as its quinoxaline derivative in the degradation solution of dehydro-L-ascorbic acid and in the crude extracts of *Saccharomyces cerevisiae*. Then, we observed its effects on the cell growth and the synthesis of macromolecules by adding it to the asynchronous and synchronous culture of *S. cerevisiae*. Through these experiments, we intended to determine the metabolic roles of L-xylosone, one of the degradation products of L-ascorbic acid.

Materials and Methods

Chemicals

Dehydro-L-ascorbic acid was prepared from L-ascorbic acid according to the method proposed by Ohmori *et al.* (15) and L-xylosone was prepared freshly from L-xylose in each experiment according to the method proposed by Vuorinen and Serianni (20). All other reagents used were of the highest quality generally available.

Organism and culture conditions

S. cerevisiae ATCC 26786 (haploid, a-type) was used

* To whom correspondence should be addressed

throughout the experiments. Cells were grown in YEPD medium containing 1% yeast extract, 2% peptone and 2% glucose. Starvation medium contained 10 mM KCl, 2.4 mM $CaCl_2$ and 2.5 mM $MgCl_2$. The synthetic medium employed in asynchronous or synchronous culture contained 5 g NaOH, 8.3 g succinic acid, 10 g glucose, 1 g $(NH_4)_2SO_4$, 0.87 g K_2HPO_4, 0.12 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 10 μg $FeCl_3 \cdot 6H_2O$, 4 mg inositol, 0.8 mg calcium pantothenic acid, 0.8 mg pyridoxine, 0.8 mg thiamine dihydrochloride and 20 μg biotin in 1 l distilled water. For the induction of L-ascorbic acid synthesis, 1-day cultured cells in YEPD medium were harvested, washed with 0.1% aqueous dithiothreitol solution and transferred to a synthetic medium containing 20 mM L-galactono-1,4-lactone instead of glucose, and incubated for 22 h.

Preparation of crude cell extracts

Exponentially growing cells in YEPD medium were harvested by centrifugation at 2000 × g for 10 min, washed twice with distilled water, resuspended in 10 mM Tris-HCl buffer (pH 7.6) containing 50 mM KCl, and homogenized with Bead Beater (Biospec Co.) using glass beads for 5 min at 4℃. The homogenized suspension was centrifuged at 35000 × g for 15 min and the supernatant was used for detection of L-xylosone.

Detection of L-xylosone

L-Xylosone was detected as quinoxaline derivative prepared according to the method proposed by Ohmori *et al.* (16) with some modifications: 2 ml of 10 mM *o*-phenylenediamine and 0.2 ml of 5 M perchloric acid were added to 2 ml of 50 mM dehydro-L-ascorbic acid solution or crude cell extracts, and incubated at 35℃ for 40 min. After extracting three times with the same volume of diethylether, diethylether layer was acidified with 0.5 ml 5 M HCl, and dried in vacuo. Dried sample was dissolved in 2 ml of acetonitrile and this acetonitrile solution was analyzed with a high-performance liquid chromatography (HPLC) system (Waters). The samples were separated on a μBondapak C18 column (300×3.9 mm), and isocratically eluted at a rate of 1 ml min^{-1} with the mixture of 10 mM KH_2PO_4 in *o*-phosphoric acid and acetonitrile (85:15, v/v). Chromatograms were recorded at 313 nm.

Determination of cell density and cellular contents of DNA, RNA and protein

Cell density was determined by turbidity in asynchronous culture, and by hemacytometer in synchronous culture. The cellular content of DNA was determined according to the method proposed by Gurney and Gurney (4), and those of RNA and protein according to the method proposed by Herbert *et al.* (5).

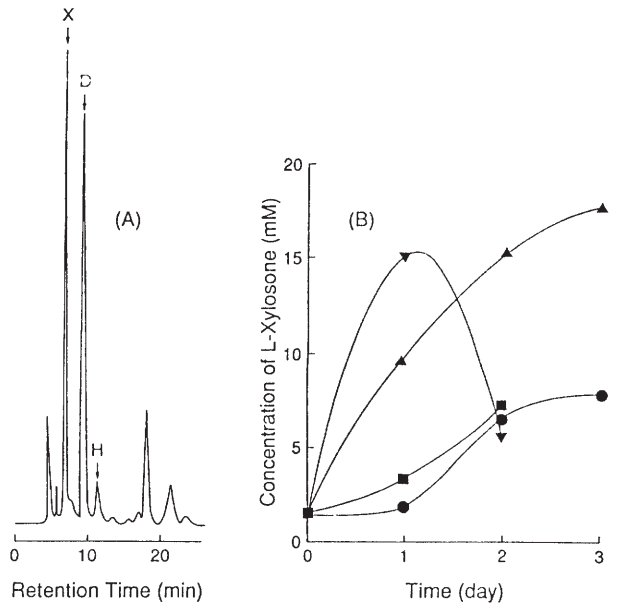

Fig. 1. Detection of L-xylosone as quinoxaline derivative in the degradation solution of dehydro-L-ascorbic acid. (A), High-performance liquid chromatogram of the degradation products of dehydro-L-ascorbic acid. 50 mM dehydro-L-ascorbic acid was dissolved in 20 mM phosphate-citrate buffer, pH 5.6, incubated at 25℃ with aeration for 1 day, treated with *o*-phenylenediamine and analyzed with high-performance liquid chromatography. The chromatogram was recorded at 313 nm. X, D and H indicate the quinoxaline derivatives of L-xylosone, dehydro-L-ascorbic acid and hydroxypyruvaldehyde, respectively. (B), Changes of L-xylosone concentration in the degradation products of 50 mM dehydro-L-ascorbic acid in pure water without aeration (●) or with aeration (■) and in 20 mM phosphate-citrate buffer, pH 5.6, without aeration (▲) or with aeration (▼). The data presented here are the results representative of three independent experiments.

Synchronization procedure

Cells were synchronized according to the method proposed by Williamson and Scopes (21) with some modifications: single colony was inoculated to 100 ml synthetic medium in a 500 ml baffled flask and cultivated at 25℃ for 4 days with vigorous shaking. Cells were harvested and washed twice with sterilized distilled water. About 1×10^9 cells were loaded onto the top of 20~40% sorbitol gradient in starvation medium. The tubes were spun with swinging-bucket centrifuge (IEC) until the cell band reached half the height of the tube, then the center layer was taken, harvested and washed three times with starvation medium. This preparation of uniform-sized cells was subjected to starvation procedure of vigorous shaking for 6 h in 200 ml starvation medium. After the starvation period, cells were collected,

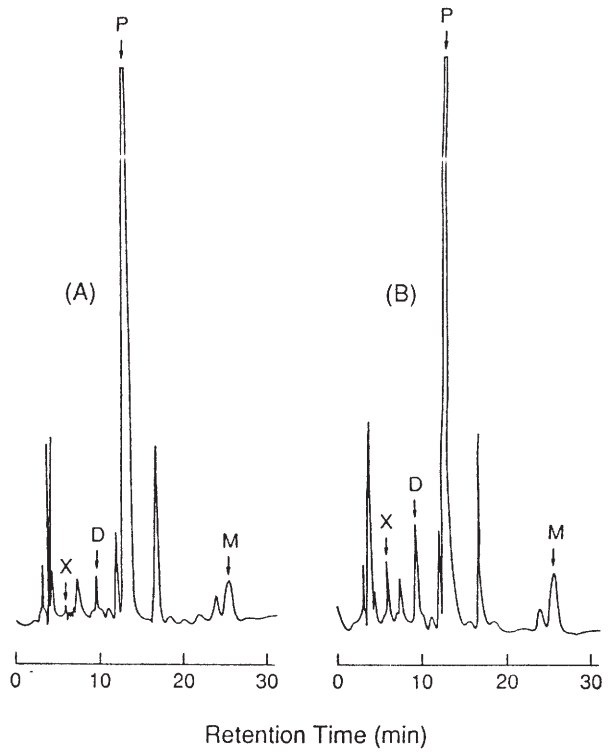

Fig. 2. Detection of L-xylosone as quinoxaline derivative in the crude extracts of *S. cerevisiae*. High-performance liquid chromatogram of quinoxaline derivatives were obtained from the crude extracts of the yeast (A) and spiked with the mixture of the reference quinoxaline derivatives of L-xylosone, dehydro-L-ascorbic acid and methylglyoxal (B). X, D, P and M indicate the quinoxaline derivatives of L-xylosone, dehydro-L-ascorbic acid, pyruvic acid and methylglyoxal, respectively. The chromatograms were recorded at 313 nm. The data presented here are the results representative of three independent experiments.

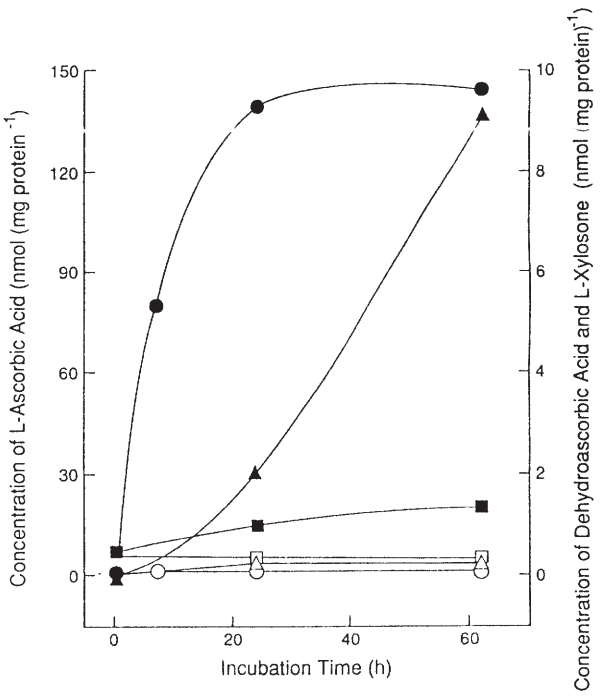

Fig. 3. Changes of intracellular concentrations of L-ascorbic acid, dehydro-L-ascorbic acid and L-xylosone in *S. cerevisiae*. The intracellular level of L-ascorbic acid (circle), dehydro-L-ascorbic acid (triangle) and L-xylosone (square) were measured with high-performance liquid chromatography before (open symbols) and after (closed symbols) the induction of L-ascorbic acid synthesis. L-Ascorbic acid synthesis was induced by incubating the yeast in synthetic media containing 20 mM L-galactono-1,4-lactone as a sole carbon source. The data presented here are the results representative of three independent experiments.

resuspended in 100 ml synthetic medium, and incubated for 40 min without shaking. Incubation in synthetic medium and starvation in starvation medium were repeated three times, and the cells were again screened by sorbitol gradient centrifugation. The resulting uniform-sized cells were subjected to synchronous growth.

Results and discussion

L-Xylosone was detected as its quinoxaline derivative by means of HPLC. In the preliminary study, *o*-phenylenediamine reacted selectively with α-dicarbonyl compounds such as α-ketoaldehyde or α-ketoacid, and the production yield of the quinoxaline derivatives formed from various α-ketoaldehydes and *o*-phenylenediamine were above 80%, when compared with commercial quinoxaline derivatives (data not shown). When 50 mM dehydro-L-ascorbic acid solution in 20 mM phosphate-citrate buffer (pH 5.6) was incubated at 25°C for 1 day and treated with *o*-phenylenediamine and analyzed with HPLC, L-xylosone and hydroxypyruvaldehyde were found in the degradation products of dehydro-L-ascorbic acid (Fig. 1A). The concentration of L-xylosone was about five times higher in phosphate-citrate buffer (pH 5.6) than in unbuffered aqueous solution (final pH 3.3) (Fig. 1B). Aeration accelerated the production of L-xylosone. The concentration of L-xylosone reached its maximum value after 1 day of incubation in phosphate-citrate buffer, and L-xylosone seemed to be degraded further to hydroxypyruvaldehyde and other unidentified substances of retention time (t_R) 18.9 and 20.9.

In the crude extracts of yeast cells exponentially growing in YEPD medium, dehydro-L-ascorbic acid, L-xylosone and methylglyoxal were detected as their quinoxaline derivatives (Fig. 2A). These quinoxaline derivatives showed typical absorption spectra of quinoxalines (data not shown). When the crude extracts were mixed with the reference quinoxaline derivatives of L-xylosone, dehydro-L-ascorbic acid and methylglyoxal, and sub-

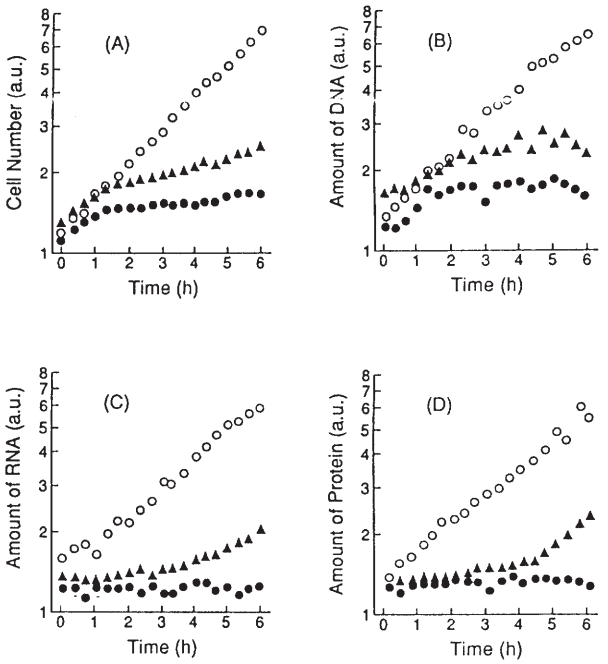

Fig. 4. Effects of L-xylosone and methylglyoxal on the rates of cell growth and macromolecular synthesis in asynchronous culture of *S. cerevisiae*. Inhibitory effects of 30 mM L-xylosone (▲) and 10 mM methylglyoxal (●) on the rate of cell growth (A), DNA synthesis (B), RNA synthesis (C) and protein synthesis (D) were determined in asynchronous culture of the yeast growing exponentially in synthetic media in comparison with the reference culture (○) without any inhibitor. a.u. represents arbitrary unit. The data presented here are the results representative of three independent experiments.

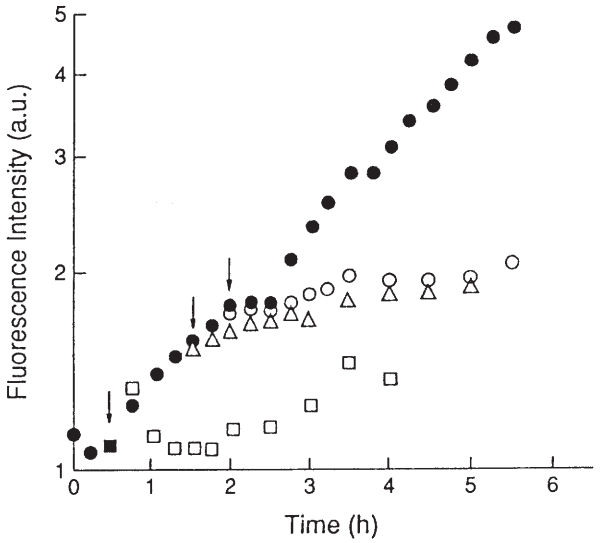

Fig. 5. Effect of L-xylosone on the rate of DNA synthesis in synchronous culture of *S. cerevisiae*. 30 mM L-xylosone was added to the culture prior to (□), during (▲) and after DNA synthesis (○), and its effect on the rate of DNA synthesis was observed in comparison with the reference culture (●) without L-xylosone. Arrows indicate the application time of L-xylosone and a.u. represents arbitrary unit. The data presented here are the results representative of three independent experiments.

jected to HPLC analysis, the peaks of t_R 6.0, t_R 9.1 and t_R 26.5 increased (Fig. 2B). The concentration of L-xylosone in the crude cell extracts was calculated to be about 0.2 nmol (mg protein)$^{-1}$1.

To determine whether L-xylosone was produced from L-ascorbic acid *in vivo*, the intracellular level of L-xylosone was measured after the induction of L-ascorbic acid synthesis. *S. cerevisiae* was incubated in a synthetic medium containing 20 mM L-galactono-1,4-lactone, the precursor of L-ascorbic acid, as a sole carbon source, and the changes in concentration of L-ascorbic acid, dehydro-L-ascorbic acid and L-xylosone were observed. The concentration of L-ascorbic acid increased soon, followed by slower increase of dehydro-L-ascorbic acid and L-xylosone contents (Fig. 3). These observations showed good agreement with the previous report that, when L-ascorbic acid was degraded in aqueous solution, the presence of oxygen induced the production of L-xylosone while deoxypentosone predominated in the absence of oxygen (11).

When L-xylosone was added to asynchronous culture of *S. cerevisiae* growing exponentially in synthetic media, cell growth was retarded. Fig. 4 shows the effects of L-xylosone on the cell growth and the synthesis of DNA, RNA and protein. L-Xylosone inhibited the cell growth and the synthesis of the macromolecules, although it was less effective than methylglyoxal. The rate of DNA synthesis decreased remarkably in 1-2 h after the addition of L-xylosone (Fig. 4B). But the rates of RNA and protein synthesis decreased immediately after the addition of L-xylosone (Fig. 4C,D). These results were similar to the findings in liver cell (10), in mouse lymphoma cell (3) and in *E. coli* (2), in which methylglyoxal exhibited a strong inhibition of protein synthesis.

To confirm the effect of L-xylosone on the rate of DNA synthesis, L-xylosone was added to synchronous culture of *S. cerevisiae* (Fig. 5). Addition of L-xylosone after the initiation of DNA synthesis slightly reduced the synthesis rate, and DNA synthesis continued until that cycle was completed. However, the cells failed to initiate a new round of DNA synthesis. Addition of L-xylosone prior to the initiation stage resulted in complete inhibition of the process. Thus, L-xylosone seemed to selectively inhibit the initiation of DNA synthesis rather than DNA synthesis itself. These observations agreed well with the previous report that the addition of methylglyoxal to synchronous culture of *E. coli* blocked the initiation of new round of DNA synthesis (2).

These results, together with the immediate inhibition of protein and RNA synthesis after the addition of L-xylosone, approved the proposal that the blocking effect of α-ketoaldehyde on the initiation of DNA synthesis should be due specifically to inhibition of protein synthesis (2).

Various biological functions suggested for L-ascorbic acid are mainly related to the redox reaction between L-ascorbic acid and its oxidized form, dehydro-L-ascorbic acid. From the study on the nonenzymatic degradation of dehydro-L-ascorbic acid, α-ketoaldehyde has been suggested to be another functional element (1). It has been reported that L-xylosone or 3-deoxythreosone produced in the degradation process of dehydro-L-ascorbic acid is involved in protein modification through reactions with lysine and arginine (13, 14). Likewise, methylglyoxal has been shown to react with and modify proteins under physiological conditions (12,17). And recently, it has been reported that free radicals are generated during the glycation reaction of amino acids by methylglyoxal (22). In the present study, L-xylosone was found in the degradation solution of dehydro-L-ascorbic acid and in the crude extracts of *S. cerevisiae*. In functional aspect, L-xylosone inhibited the synthesis of macromolecules, especially the initiation of DNA synthesis, suggesting possible role as an inhibitor of cell growth. Methylglyoxal is also known as an inhibitor of cell growth and the molecular level mechanisms of its growth-retarding effect have been suggested. Krymkiewicz *et al.* (10) has suggested a hypothesis that methylglyoxal should react with guanosine triphosphate and inhibit DNA replication. It has been also suggested that the growth-retarding effect of methylglyoxal may be due to the blockage of certain thiol groups essential for cell growth and protein synthesis (19). It seems reasonable to suppose that the mechanism of cell growth-retarding effect of L-xylosone resembles that of methylglyoxal. However, the exact regulation mechanism of cell growth by α-ketoaldehydes remains to be elucidated.

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