JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 96, No. 1, 89–91. 2003

A Novel Inhibitory Effect of D-Allose on Production of Reactive Oxygen Species from Neutrophils

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Received 18 November 2002/Accepted 3 April 2003

The rare sugar D-allose produced from D-psicose using an immobilized L-rhamnose isomerase bioreactor was shown to have weak scavenging activity toward reactive oxygen species (ROS) and potent inhibitory effect on production of ROS from stimulated neutrophils. These findings may have important implications in understanding the ameliorative effect of D-allose in transplantation and ischemia/reperfusion injury.

[**Key words:** D-allose, rare sugars, reactive oxygen species, antioxidant, neutrophils]

Rare sugars are defined by the International Society of Rare Sugars (ISRS) as monosaccharides and their derivatives that are rare in nature (The 1st International Symposium of ISRS, Takamatsu, Japan, 2002). They have received increasing attention in recent years for a variety of usages such as low-calorie carbohydrate sweeteners and bulking agents (1). However, biological functions and physiological implications of rare sugars have been little known so far. D-Allose, an aldo-hexose, is one of the exceptions among rare sugars whose biological function has been suggested. Arnold *et al.* reported that D-allose substantially inhibited segmented neutrophil production and lowered platelet count without other detrimental clinical effects (US patent no. 5620960, 1997). A research group at Kagawa Medical University has recently studied the immunosuppressive effect of D-allose and compared it with that of FK506, a frequently used potent immunosuppressant, on the basis of neutrophil count and animal survival in liver transplantation experiments using rats (2). Their study showed that rate of six month allograft survival was significantly increased with less tissue damage when low-dose of FK506 was administered in combination with D-allose as compared to the administration of each drug separately (2). The same group also performed a series of experiments using hepatic ischemia/reperfusion in a rat model to evaluate protective effect of D-allose, and found that the ameliorative effect was achieved mainly by reducing the number of total neutrophils during or after reperfusion (3). Since neutrophils have been considered mostly to be responsible for the pathophysiological changes occurring after liver transplantation as well as hepatic ischemia/reperfusion, suppression of neutrophils has been reported to be critical to reduce these injuries (4, 5).

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In view of the above findings concerning the protective effect of D-allose following trauma, in the present study, we investigated whether D-allose had any effect on reactive oxygen species (ROS) in addition to its activity to reduce the number of infiltrated neutrophils. For this purpose, highly purified D-allose was required in large quantity. Izumori *et al.* have established the method of production of D-allose from D-psicose using immobilized L-rhamnose isomerase (6), and that of mass production of D-psicose from D-fructose using an immobilized D-tagatose 3-epimerase bio-reactor (7). The total scheme of enzymatic conversion of all six-carbon monosaccharides was recently summarized as "Izumoring" (8) which allows the production of all six-carbon rare sugars from inexpensive monosaccharides such as D-glucose and D-fructose. The schematic structures of seven monosaccharides examined in the present study are shown in Fig 1. The four rare sugars were prepared from D-psicose. The purity of all sugars used was more than 99%.

In order to clarify the role of D-allose as an anti-oxidant, we first examined its scavenging activity together with that of D-psicose, D-glucose and D-fructose using electron spin resonance (ESR) method. ESR spin trapping of oxygen free radicals was performed in 50 mM phosphate buffer (pH 7.4) containing 0.5 mM diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid (DTPA), 0.3 mM hypoxanthine (Sigma Chemical, Tokyo) and 50 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Dojindo Lab., Kumamoto) as described previously (9). Various concentrations of sugars ranging from 0–50 mM were added to the reaction mixture. Reactions were started by adding xanthine oxidase (Boehringer Mannheim, Mannheim, Germany), and the ESR spectra were recorded at room temperature with a JEOL JES-RE1X spectrometer. Instrument settings were: scan speed, 5 mT/min; time constant, 0.1; microwave power, 8 mW; microwave frequency, 9.45 GHz; modulation amplitude, 0.1 mT; and receiver gain,

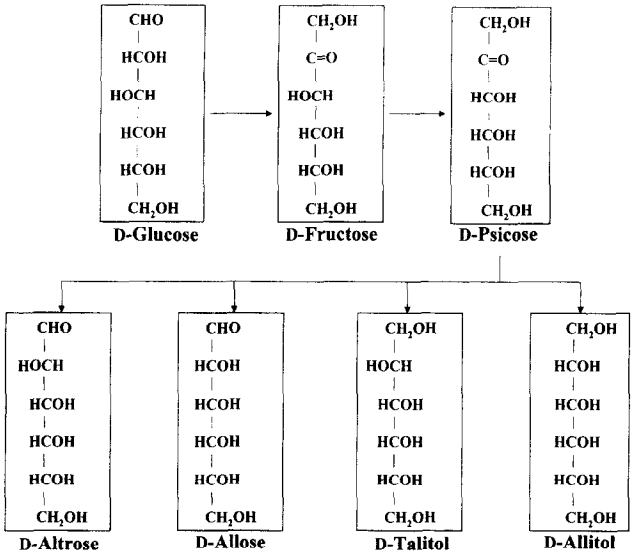

FIG. 1. Schematic structures of seven monosaccharides used in the present study. D-Glucose and D-fructose are abundantly present in nature, whereas D-psicose, D-altrose, D-allose, D-talitol and D-allitol are rare sugars.

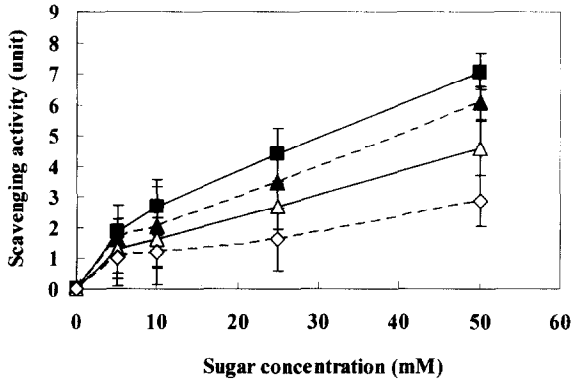

FIG. 2. Electron spin resonance study of scavenging activity of various sugars. Rare sugars, D-psicose (solid squares) and D-allose (solid triangles), showed higher scavenging activity against ROS than D-glucose (open diamonds) and D-fructose (open triangles). Values are mean±SD of three independent experiments.

400. The results are shown in Fig. 2. All four sugars examined showed dose-dependent scavenging activity with rare sugars (D-allose and D-psicose) having a higher activity than that of D-glucose and D-fructose. However, the scavenging activity of these sugars was much weaker (about 1/20th or less) than the other common scavengers such as superoxide dismutase (SOD) and carotinoids (data not shown).

Next, we analyzed the effect of D-allose and other sugars on production of ROS from rat blood neutrophils stimulated by the addition of opsonized zymosan (OZ). As described previously (10), blood samples (10–50 μl) were incubated in 0.5 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing 0.9% NaCl, 10 mM phosphate buffer, 6 mM KCl, and 6 mM $MgCl_2$ in the presence of 400 μM 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(*2H,3H*)dione (L-012). The reaction was started by adding 2 mg/ml of OZ. Monosaccharides at various concentrations were added

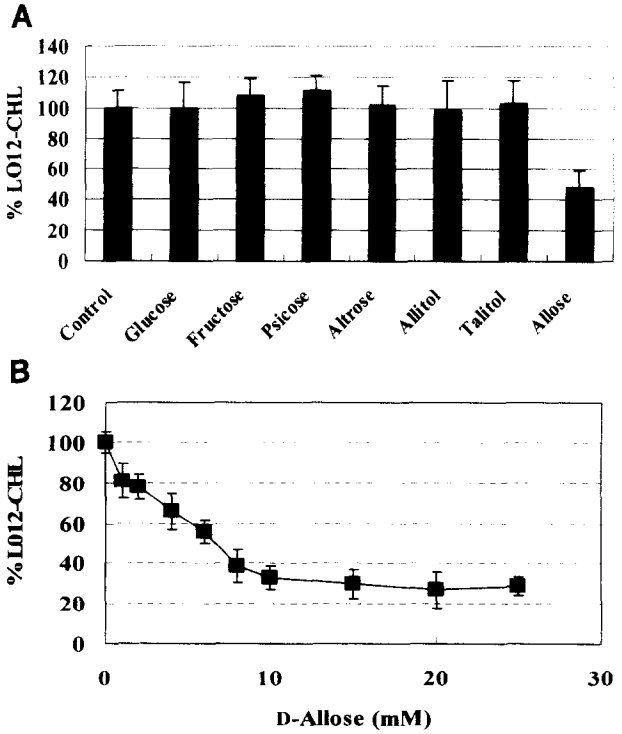

FIG. 3. Effect of sugars on production of ROS. Neutrophils were stimulated to produce ROS by the addition of OZ and CHL of L-012 was measured. L-012-CHL values for each sugar (A) or each concentration of D-allose (B) were calculated as percentage of the control (no sugar added) in each case which was taken as 100%. Values are shown as mean±SD of five independent assays. (A) Pre-incubation of sugars with neutrophils for 15 min allowed only D-allose to inhibit the production of ROS. (B) The inhibition of ROS by D-allose was dose-dependent with IC_{50} of 6.7 mM.

15 min prior to or at the time of the initiation of reaction. OZ-induced ROS developed strong chemiluminescence (CHL) when reacted with L-012. The CHL intensity was recorded by a Luminescence Reader (Aloka, Tokyo). The rats were deeply anesthetized before being killed, and experiments were performed in accordance with the standard guidelines for animal experimentation at Kagawa Medical University based on the ethical standards formulated in the Helsinki Declaration, 1989.

When sugars at 10 mM concentration were added simultaneously with OZ treatment, there were no significant differences in production of ROS among all sugars tested (data not shown). Only a negligible amount of ROS production was detected without OZ stimulation. We then added sugars prior to the OZ treatment. Neutrophils were incubated with each sugar at 37°C for 15 min, and OZ was added to stimulate ROS production. Significant inhibition of ROS production was detected only when D-allose was added, whereas no difference was detected with other sugars (Fig. 3A). The length of pre-incubation time did not significantly affect the degree of the inhibition as long as it was longer than 10 min (data not shown). When the concentration of D-allose was changed from 0 to 25 mM, the inhibition was found to be dose-dependent with IC_{50} of 6.7 mM (Fig. 3B). Furthermore, the production of ROS was markedly abolished by the addition of deferoxamine and uric acid, an iron chelator

and a scavenger for hydroxyl radical, respectively, and by azide, a specific inhibitor of myeloperoxidase (data not shown). ROS produced from neutrophils by OZ in this experiment were judged to be hydroxyl radical and hypochlorite. The fact that excess amount of D-allose could not completely inhibit ROS production, could be due to the presence of other types of ROS. ROS are produced by NADPH oxidase in neutrophils in accordance with increased NADPH supply via elevated hexose monophosphate shunt (HMP) activity (11). Therefore, it is important to examine the effect of D-allose on HMP activity as well as on the production of other types of ROS such as superoxide, hydrogen peroxide and nitric oxide.

The ameliorative effect of D-allose has previously been observed both following liver transplantation (2) and liver ischemia/reperfusion injury (3). In ischemia/reperfusion injury, reinstitution of oxygen to the ischemic tissues initiates various processes leading to generation of ROS mainly from neutrophils and Kupffer cells, and activates adhesion of neutrophils to the sinusoidal endothelial cells via adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1), leading to generation of inflammatory cytokines (5). Antioxidants such as SOD, and allopurinol have been reported to have protective effect on ischemia/reperfusion injury of the liver (12, 13). Although the scavenging activity of D-allose observed in the present study was not strong as compared to other antioxidants, it showed a strong inhibitory effect on ROS production. This may result in reduced ROS concentration in the ischemic organs. A significant reduction in the number of neutrophils by D-allose (2, 3) may also give rise to a reduction in the amount of ROS in the ischemic tissues. Because of this double effects leading to reduced ROS concentration; D-allose may have the potential as a useful compound in the protection of ischemia/reperfusion injuries.

The inhibitory effect of D-allose on ROS production observed in the present study has not previously been observed. Furthermore, this novel effect was detected only for D-allose among the seven sugars tested including other rare sugars (D-altrose, D-psicose, D-talitol and D-allitol). None of the rare sugars have been previously reported to possess an inhibitory effect on ROS production. Although D-allose also showed a weak scavenging activity toward ROS, this may reflect a general property of all monosaccharides.

Since D-allose also has immunosuppressive effect (US patent no. 5620960, 1997), it may be used as an ideal immunosuppressant with an inhibitory activity on ROS production and without apparent side effects (2, 3). Side effects are common with routinely used immunosuppressants such as FK506 and cyclosporine A (14).

The demonstration of a potent inhibitory effect of D-allose on production of ROS seen in the present study may explain the ameliorative effect of D-allose in transplantation (2) and ischemia/reperfusion injury (3). However, the underlying mechanisms of this action remain to be elucidated. We have started a systematic investigation of genes and proteins to identify these mechanisms using microarray and proteome analyses. In future, it should be possible to produce all rare sugars using the strategic application of Izumoring (8), and we may encounter other rare sugars with more potent inhibitory properties on ROS production than those of D-allose.

We thank Drs. Isuke Imada and Masayasu Inoue (Osaka City University Medical School), and Dr. Najma Janjua (Okayama University) for useful discussion and proofreading of this manuscript, respectively.

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