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Bioconversion of 1,5-Anhydro-D-fructose to 1,5-Anhydro-D-glucitol and 1,5-Anhydro-D-mannitol Using *Saccharomyces cerevisiae*

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Abstract: 1,5-Anhydro-D-fructose (AF) was added to cultures of *Saccharomyces cerevisiae* and converted to 1,5-anhydro-D-glucitol (AG) and 1,5-anhydro-D-mannitol (AM) by two NADPH-dependent 1,5-anhydro-D-fructose reductase (AFR). The partial amino acid sequence of AFR (AG-forming; AFR-AG) matched with that of D-arabinose dehydrogenase (ArDH). AFR-AG showed reducing activities toward several organic compounds with highest reducing activity toward AF. In addition, it showed oxidizing activity toward several monosaccharides such as D-arabinose and L-xylose. The substrate specificity (reducing activity) and several amino acid sequence motifs of the enzyme resembled those of AFR from mammals. From these enzymatic properties, we concluded that this enzyme should be called AFR rather than ArDH. AFR (AM-forming; AFR-AM) was classified as an oxidoreductase encoded by the *ymr315w* gene. There was no conserved sequence between AFR-AG and AFR-AM suggesting the existence of two different systems in yeast for converting AF. The AFR-AM (YMR315W-protein) enzyme showed reducing activity only for AF, did not have any oxidizing activity, and has sequence homology only to enzymes of bacterial origin. Finally, we demonstrated an *in vitro* NADP–NADPH cycling system using the AFR-AG ability to reduce AF and oxidize L-xylose. AG production using low concentration of NADPH increased with the addition of L-xylose.

Key words: 1,5-anhydro-D-fructose reductase, NADP–NADPH-recycling, D-arabinose dehydrogenase, YMR315W, aldo–keto reductase family, GFO/IDH/MocA family

1,5-Anhydro-D-fructose (AF) is a monosaccharide produced by the action of α -1,4-glucanlyase (EC 4.2.2.13) on α -1,4-glucans.^{1,2)} AF is a useful food ingredient in our daily life because of its antioxidative and antibacterial activities.^{3,4)} In recent years, a method has been developed for the mass production of AF, thereby enabling its supply to food producers.⁵⁾

While AF is identified as an intermediate of *in vivo* metabolism of glycogen and starch,^{1,6)} several AF metabolites have antibacterial activities, including microthecin and ascopyrone P, which are pyrone compounds from *Morchella vulgaris* and *Anthracoelia melaloma*, respectively.^{7,8)} The NADPH-dependent reductase from *Sinorhizobium morelense* reduces AF to 1,5-anhydro-D-mannitol (AM).⁹⁾ Another AF derivative, 1,5-Anhydro-D-glucitol (AG), is synthesized

from AF by chemical or enzymatic reduction.¹⁰⁾ This molecule occurs naturally in microorganisms, plants and animals at microgram levels per gram of tissue weight.^{11–13)} In mammals, AG is synthesized by 1,5-anhydro-D-fructose reductase (AFR).¹⁴⁾ In *Escherichia coli*, AF is synthesized from glycogen, as in mammals, and then converted to AG.¹⁵⁾ Hence, natural AG is synthesized from AF, which is produced by the action of α -1,4-glucanlyase on α -1,4-glucans such as glycogen and starch in animals and plants,^{12,13)} and by glucosidase in yeast.¹⁶⁾

The AG content of human blood is maintained at approximately 20 μ g/mL, although in cases of high blood glucose concentrations, such as in diabetic patients, AG levels are decreased by competitive reabsorption of AG and glucose in the kidney.¹⁷⁾ This decrease reflects short term (48 h to two weeks) glucose levels, and thereby provides a clinical marker of glycemic control.¹⁸⁾ Radioactivity expelled in the breath of rats and mice administered ¹⁴C labeled-AG was shown to be less than 1% of the dose administered over 48 h,¹⁹⁾ indicating that AG is metabolically stable. Meat, cereals, beans and vegetables contain 0.5–22 μ g/g AG, with soybeans having the highest content (22 μ g/g).¹³⁾ AG at a concentration of 0.61 mM in a medium was also shown to stimulate insulin release from insulinoma-derived cells when these cells were

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Abbreviations: AF, 1,5-anhydro-D-fructose; AG, 1,5-anhydro-D-glucitol; AM, 1,5-anhydro-D-mannitol; AFR, 1,5-anhydro-D-fructose reductase; AFR-AG, 1,5-anhydro-D-fructose reductase AG-forming; AFR-AM, 1,5-anhydro-D-fructose reductase AM-forming; ArDH, D-arabinose dehydrogenase; HPAEC-PAD, high performance anion exchange chromatography using a pulsed amperometric detector; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

stimulated by 20 mM glucose.²⁰⁾ Based on the properties described above, AG may be used as a new food material. We are therefore interested in developing a method for producing AG as a food material from AF. In general, sugar alcohols are produced on an industrial scale by a hydrogen addition method that uses Pd or Ni as catalysts.²¹⁾ Andersen *et al.* reported that hydrogen reduction of AF using Pd as a catalyst resulted in production of AG and AM, a C2 epimer of AG, at a ratio of 1:4.²²⁾ Chemical reduction is therefore an inefficient method for producing AG. Accordingly, we searched for microorganisms with the ability to convert AF to AG and found that *Saccharomyces cerevisiae*, used in the food industry, produces AG from AF.

In this study, we purified the enzyme responsible for the conversion of AF to AG in *S. cerevisiae* and investigated its properties. In addition, we developed a method for preparing AG using the isolated enzyme.

MATERIALS AND METHODS

Chemicals. AF was prepared as described previously.³⁾ AM was synthesized by the method reported by Andersen *et al.* with some modifications: Aqueous AF (0.6 mM) was hydrogenated at 70°C for 1 h under 0.8 MPa pressure using 20% Ni as a catalyst.²²⁾ The resulting AM was purified by HPLC (Jasco Corporation, Tokyo, Japan). AG, coenzymes, D- and L-forms of reducing sugars, and organic chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TOYOPEARL-DEAE 650M, TOYOPEARL-Super Q 650M, TOYOPEARL-AF-Red 650M, TOYOPEARL-AF-Blue HC 650M, TSKgel Phenyl-5PW and TSKgel Super SW 3000 were purchased from Tosoh Corporation (Tokyo, Japan), and YMC-Pack PROTEIN-RP and Hydrosphere C 18 columns were purchased from YMC Co., Ltd. (Kyoto, Japan). All the other chemicals used in this study were of analytical grade.

Microbes and growth conditions. *S. cerevisiae* NBRC 0210 was provided by the National Institute of Technology and Evaluation, Japan. Yeast were cultivated in 5 mL of YPD medium (1% yeast extract, 2% peptone and 2% glucose) in a test tube at 30°C for 24 h, and later transferred to 100 mL of the same media in a shaking flask. After 10 h of cultivation, AF was added to the medium to a final concentration of 60 mM for efficient conversion of AF to AG and AM. Cultivation was performed at 30°C for 48 h, and growth of yeast was monitored by measuring turbidity at 600 nm.

Analytical methods. Analysis of sugars in the medium was performed using HPLC with a refractive index detector (Jasco Corporation) and a MITSUBISHI MCI GEL CK 08 S column (Mitsubishi Chemical Corporation, Tokyo, Japan). The column was eluted with water at 40°C with a flow rate of 1.0 mL/min. The products of the enzyme reaction were analyzed by high performance anion exchange chromatography using a pulsed amperometric detector system (HPAEC-PAD) and a Carbo Pack MA 1 column (Nihon Dionex K.K., Osaka, Japan), eluting with 0.5 M NaOH at 35°C and a flow rate of 0.4 mL/min. The ¹H-NMR spectra were recorded in D₂O using a UNITY INOVA 500 spectrometer (VALIAN Medical Systems, Inc., Palo Alto, USA) at 500 MHz and 300 K.

Isolation of two AF derivatives. *S. cerevisiae* NBRC 0210

were cultivated as described above. After cultivation, the cells were removed by centrifugation at 4,000 × G, supernatants were desalted using cation and anion exchange resins (SK-1B: 250 mL and SA 10 AP: 500 mL, Mitsubishi Chemical Corporation), followed by concentration using an evaporator. The two AF derivatives were separated by ligand-exchange chromatography on a UBK 530 column (Na⁺ form, 30 × 700 mm, Mitsubishi Chemical Corporation) and were then purified by HPLC.

Reductive and oxidative activities. Enzyme activity was determined by measuring the concentration of NADPH. Reductive activity was measured as follows: A reaction mixture consisting of 50 mM Bis-Tris buffer (pH 7.0), 25 mM AF, 0.15 mM NADPH, and an appropriate amount of enzyme in a total volume of 2 mL was incubated at 30°C for 20 min. The decrease in NADPH concentration was determined by measuring absorbance at 340 nm. One unit (U) of the enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min. Oxidative activity was measured as follows: A reaction mixture containing 50 mM Tris-HCl buffer (pH 9.0), 100 mM substrate, 0.5 mM NADP⁺, and an appropriate amount of enzyme in a total volume of 2 mL was incubated at 30°C for 20 min. The resulting NADPH was determined by absorbance at 340 nm. One unit (U) of the enzyme was defined as the amount of enzyme required to reduce 1 μmol of NADP⁺ per min.

Properties of AFRs. The activity for various substrates was measured using the above standard assay conditions, with the substances listed in Table 1 as substrates instead of AF. The optimal temperature and stability was measured under standard conditions at 25–50°C. To measure optimal pH and pH stability, three kinds of buffers (pH 4.0–6.0, sodium acetate; pH 5.5–7.5, Bis-Tris; pH 7.0–9.0; Tris-HCl) were used. The effect of metal ions on the AFR-AM activity was evaluated as follows: To the enzyme solution, EDTA solution was added to give a final concentration of 2 mM, and the mixture was incubated at 30°C for 30 min. Then, the activity was measured in the presence of 5 mM metal salts (BaCl₂, HgCl₂, ZnCl₂, MnCl₂, MgCl₂, CaCl₂, CuCl₂ or CdCl₂).

Enzyme purification of AFR-AG. Purification of AFR-AG from *S. cerevisiae* NBRC 0210 was performed as follows. Cells (100 g wet weight) were grown under the same conditions described above except that the volume of the culture was 10 L. Cells were then harvested and resuspended in 400 mL of 20 mM Bis-Tris (pH 7.0; buffer A) containing 1.0 M sorbitol, 2 mM PMSF and 1 mM DTT, followed by lysis with 2 mg Zymolyase® 100T at 30°C for 1 h. After sonication, cell debris was removed by centrifugation at 24,000 × G for 15 min and the supernatant was then dialyzed against buffer A. Solid ammonium sulfate was added to the dialysate to give 30% saturation. The precipitate was removed by centrifugation and the supernatant was brought to 80% saturation. The resulting precipitate was collected by centrifugation, dissolved in buffer A and dialyzed against buffer A at 4°C overnight. The solution was then applied to a TOYOPEARL-DEAE 650M column (22 × 300 mm) and the active fraction eluted using a linear gradient from 0 to 0.5 M NaCl in buffer A. The reductase fraction was collected, desalted, applied to a TOYOPEARL-AF-Red 650M (30 × 100 mm) column, and active fractions were eluted and

collected using a linear gradient from 0 to 2.0 M NaCl in buffer A. Ammonium sulfate was added to the active fraction to give 30% saturation and the solution was applied to a TSKgel Phenyl-5PW (7.5 × 75 mm) column equilibrated with buffer A containing 30% ammonium sulfate. After the column had been washed, the enzyme protein was eluted using a linear gradient of 30% ammonium sulfate from 30 to 0% in buffer A. The active fractions were collected and concentrated using Amicon Ultra-15 Ultracel-10 k (Nihon Millipore K.K., Tokyo, Japan). The desalted solution was applied to a Mono-Q column (4.6 × 100 mm; GE Healthcare UK Ltd., Little Chalfont, UK), washed with buffer A, and the active fractions were eluted using a linear gradient from 0 to 0.5 M NaCl in buffer A.

Enzyme purification of AFR-AM. Purification of AFR-AM was performed as follows: The crude extract from *S. cerevisiae* NBRC 0210 was obtained as described above. The supernatant was applied to a TOYOPEARL-Super Q 650M (16 × 100 mm) column and eluted using a linear gradient from 0 to 0.5 M NaCl in buffer A. The active fractions were applied to a TOYOPEARL-AF-Blue HC 650M (16 × 50 mm) column and eluted using a linear gradient from 0 to 2.0 M NaCl in buffer A. The active fractions were re-chromatographed, the reductase fraction was applied to a TOYOPEARL-AF-Red 650M (16 × 25 mm) column, and the active fractions were eluted using a linear gradient from 0 to 3.0 M NaCl in buffer A.

Protein analysis. Mr of the enzymes was estimated by SDS-PAGE. The mass of the protein under these non-denaturing conditions was determined by gel chromatography using a TSKgel Super SW 3000 column (4.6 × 300 mm) equilibrated with buffer A containing 0.3 M NaCl. The molecular weight and size of AFRs was determined using a Gel Filtration Calibration Kit, aldolase (158,000), conalbumin (75,000), ovalbumin (43,000), carbonic anhydrase (29,000), ribonuclease A (13,700) and aprotinin (6,500) (GE Healthcare UK Ltd.). Protein concentrations were determined using Quick Start Bradford reagent (Nihon Bio-Rad Laboratories K.K., Tokyo, Japan) with bovine serum albumin as the protein standard.

Partial amino acid sequencing. The enzymes AFR-AG and AFR-AM were purified by reversed-phase HPLC on a YMC-Pack PROTEIN-RP column (2.0 × 150 mm). For the following separation, each protein was digested with lysyl endopeptidase. The resulting peptides were collected separately and then purified and sequenced using a Procise 49 X-HT Protein Sequencer system (Applied Biosystems Inc., Foster City, USA).

Enzymatic production of AG. A reaction mixture containing 25 mM AF, 0.15 mM NADPH, 4 mU/mL AFR-AG and 20 mM Bis-Tris buffer (pH 7.5) in a total volume of 7.0 mL was incubated at 30°C for 4 h. AG produced was determined by HPAEC-PAD. In the case of the NADP–NADPH cycling reaction, L-xylose was added to the same reaction mixture described above at a final concentration of 100 mM for 60 min.

RESULTS AND DISCUSSION

Bioconversion of AF to AG and AM by *S. cerevisiae*.

At a concentration of 60 mM, AF had no apparent effect

Fig. 1. Growth of *S. cerevisiae* NBRC 0210 and the bioconversion of AF to AG and AM in YPD media.

Growth (closed triangles) was monitored at 600 nm and concentrations of glucose (closed circles), AF (open circles), AG (closed squares) and AM (open squares) were determined by HPLC. Error bars show the standard error values for triplicate determinations.

on the growth of *S. cerevisiae* NBRC 0210 and was converted to its derivatives after glucose had disappeared from the medium. To prepare AF derivatives by *S. cerevisiae*, AF was added to the culture media after cultivation for 10 h, a time at which glucose would have been almost completely assimilated. This resulted in accumulation of several products in the medium. These products were isolated, purified and identified by ¹H-NMR as AG and AM. The spectrum of the two polyols matched those of authentic AG and AM. The decrease in the amount of AF was almost similar to that of the amount of AG and AM produced in the medium, with an almost equimolar ratio of AG and AM being observed (Fig. 1). Concentrations of these polyols did not decrease from 30 to 50 h despite the absence of glucose and AF in medium, indicating that *S. cerevisiae* did not assimilate AG and AM. Furthermore, when 60 mM AG or AM were added to *S. cerevisiae* cultures and cultivated for 48 h, no conversion was detected.

The conversion of additional AF into AG has been reported in *E. coli* C600¹⁵⁾ and the erythroleukemia cell line K-562.²³⁾ *E. coli* converted 3.0 mg/L of 0.02 mM AF to 1.5 mg/L of 0.01 mM AG, although AG did not accumulate in the medium. This indicated the reabsorption of AG for energy metabolism. Erythroleukemia cells converted 70 mg/L of 0.43 mM AF to 25 mg/L of 0.15 mM AG, whereas *S. cerevisiae* converted 10 g/L of 62 mM AF to 5 g/L of 31 mM AG. These results showed that the conversion efficiency of *S. cerevisiae* is 200-fold greater than that of erythroleukemia cells. Therefore, the production of AG from AF using *S. cerevisiae* has several advantages, including high conversion and production rates. In addition, AG is released into the medium and is no longer utilized as an energy source.

Kühn *et al.* reported that *S. morelense* converted AF to AM, which was then oxidized to mannose.⁹⁾ As mentioned above, the conversion of AF to either AG or AM has been shown in living cells. Therefore this is the first report of a double conversion of AF to AG and AM by one species, and also the first to demonstrate AM production by a eukaryote. Animal tissues, plants and food contain AG,^{11–13)} whereas AM has been discovered in only one bacterium.⁹⁾ Highly-sensitive analysis of AM may lead to its discovery in animals,

plants and foods, and further research into its roles in eukaryotic cells.

Purification and amino acid sequences of AFR-AG.

We attempted to isolate the enzymes involved in AG and AM production. Potentially, AG and AM are converted from AF by two specific reductases. Preliminary examination of culture medium and cell homogenates of *S. cerevisiae* showed enzyme activity only in cell homogenates, which states that these enzymes are intracellular in nature. The reaction requires only NADPH as a coenzyme, thus proving that NADPH is the sole cofactor required for the reaction and is suitable for use in the enzyme assay. The assay described above did not discriminate between AG and AM. Therefore, the reaction products of individual purification steps needed to be analyzed using HPAEC-PAD. Before purification, it was possible to detect both AG and AM after the crude enzyme had acted on AF. However, AFR-AM activity was lost following ammonium sulfate precipitation. We then used five steps that achieved 300-fold purification AFR-AG with a yield of 1.3%. Total activity, total protein and the specific activity of the purified enzyme were 3.8 U, 0.16 mg and 24.4 U/mg, respectively.

Purified AFR-AG was eluted as a single peak of 78 kDa by gel filtration chromatography. Further analysis using SDS-PAGE showed two bands of purified enzyme at 37 and 38 kDa (Fig. 2(a)). Reverse phase HPLC was then used to isolate the 38 kDa subunit. As the N-terminal of the subunit was blocked, we obtained five fragments following digestion with lysyl endopeptidase. The amino acid sequences of these fragments matched NADP-dependent D-arabinose dehydrogenase (EC 1.1.1.117, ArDH). The ratio of the amount of the two bands was not constant on SDS-PAGE analysis and varied between different lots of AFR-AG purification. Kim *et al.* showed the enzyme was a heterodimer with a dimeric structure,²⁴⁾ whereas Bergen *et al.* using ArDH expression in *E. coli*, showed that the enzyme formed a 38-kDa single band on SDS-PAGE.²⁵⁾ Amako *et al.* used Western blotting and peptide mass fingerprinting analyses to confirm that this

enzyme had a homodimer structure, with its N-terminal region undergoing post-translational degradation.²⁶⁾ Based on these results, we concluded that AFR-AG (ArDH) has a homodimeric structure.

Enzyme properties of AFR-AG.

Oxidation of reducing sugars and reduction of organic compounds with two keto groups by ArDH has been reported, whereas there is no evidence of AF reduction by this enzyme. As shown in Table 1, when the oxidation and reduction activities for various substrates were compared, AF was found to have the highest reducing activity. The K_m and k_{cat} values for AF reduction were 1.1 mM and 3.8 s⁻¹, respectively. Bergen *et al.* reported that the K_m and k_{cat} values for the reduction of 2,3-butandione at 25°C were 7.7 mM and 6.9 s⁻¹, respectively,²⁵⁾ whereas Kim *et al.* showed that the values for the oxidation of D-arabinose at 30°C were 161 mM and 194 s⁻¹, respectively.²⁴⁾ The K_m value for the reduction of AF in the present study was therefore low, and the selectivity for AF was higher than for the substrates in the aforementioned reports. The optimum temperature was 40°C and the optimum pH values for the reduction of AF and the oxidation of D-arabinose were 7.0 and 9.0, respectively.

AFR-AG showed reducing activity for 2,3-butandione in addition to AF, but not for any other sugar or organic compound (Table 1). This substrate specificity of AFRs (AG-forming) has also been demonstrated in pigs¹⁴⁾ and mice.²⁷⁾ Moreover, multiple sequence alignments of these proteins showed a catalytic tetrad (D66, Y71, K100 and H131), and aldo-keto red motifs I, II and III (Fig. 3), despite the low whole sequence identity of 30%. These motifs are conserved in the aldo-keto reductase family, which participates in the oxidation and reduction of various sugars. Based

Fig. 2. Estimation of relative molecular masses of AFR-AG and AFR-AM by SDS-PAGE.

Lane 1, Precision Plus Protein Standard (Bio-Rad); Lane 2, Purified AFR-AG (a) and AFR-AM (b) from *S. cerevisiae*.

Table 1. Substrate specificity of AFR-AG.

Substrate	Relative activity (%)	
	Reduction	Oxidation
AF	100	0
D-Glucose	1.4	0
L-Glucose	0	11.5
D-Galactose	0	0
L-Galactose	0.6	13.4
D-Mannose	0.9	0
L-Mannose	0	0
D-Fructose	1.0	0
L-Fucose	0	15.5
D-Arabinose	0	10.1
L-Arabinose	0	0
D-Xylose	0	0
L-Xylose	0	26.3
AG	0	0
AM	0	0
2,3-Butandione	31.4	0
Acetone	0	0
DL-Glyceraldehyde	6.5	0
Pyridine-3-aldehyde	5.1	0
p-Nitrobenzaldehyde	5.1	0

Reducing and oxidizing activity were determined as described in the Materials and Methods. Values are expressed relative to the reduction activity of AF.

Fig. 3. Sequence alignment of AFR-AG from *S. cerevisiae* and mammals.

Sequence alignment of AFR-AG (ArDH) from *S. cerevisiae*, mouse (UniProt P82125) and pig (UniProt P82125) was performed by Clustal W. The partial amino acid sequences determined in this study are underlined. Gray amino acid sequence indicates the aldose-ketose reductase motifs I, II and III, and asterisks show identical amino acids. Amino acids Asp66, Tyr71, Lys100 and His131, which form the catalytic tetrad of the putative active site, are boxed.

on these substrate specificities, enzyme kinetics and amino acid sequence homology, we concluded AFR-AG from *S. cerevisiae* should be classified as a reducing enzyme, 1,5-anhydro-D-fructose reductase (AFR), rather than an oxidative enzyme, D-arabinose dehydrogenase (ArDH).

Purification and amino acid sequences of AFR-AM.

Although the presence and function of AG in organisms has been reported previously, very limited information is available on AM. Hence, we isolated an enzyme that produces AM to aid research on the compound. During attempts to stabilize AFR-AM, we found that metal ions, such as Mg^{2+} , Ba^{2+} and Ca^{2+} , maintained enzyme activity. Therefore, we added Ca^{2+} to the buffer in this experiment. To separate AFR-AM from AFR-AG we used ion-exchange chromatography as the first step, followed by enzyme purification by repeat affinity chromatography. Total activity, total protein and the specific activity were 0.18 U, 0.02 mg and 9.8 U/mg, respectively. Although a 200-fold purification was achieved, the yield was only 0.1%, probably because of the unstable nature of AFR-AM. The molecular weight of AFR-AM was 41 kDa in gel-filtration and SDS-PAGE experiments (Fig. 2(b)), which demonstrates that the protein was a monomer. The sequences of the N-terminal and two internal peptides generated by lysyl endopeptidase, showed that the protein was the NADP(H)-specific oxidoreductase YMR315W, which retained the ability to oxidize and reduce several sugars.²⁸⁾ An alignment search of both enzymes showed sequence identity of only 15%, with no consensus sequences in either AFR. Based on these results we conclude that AFR-AG and AFR-AM belong to different enzyme families and that their amino acid sequences are completely different despite the two enzymes were used by *S. cerevisiae* to reduce AF.

Enzyme properties of AFR-AM.

AFR-AM was unstable, and therefore, only a small amount of purified enzyme was obtained. The optimum temperature of the enzyme was 30°C. The thermal stability of the enzyme was low, and its activity was reduced by half after 30 min at 35°C, and inactivated after 30 min at 40°C. The optimum pH was 6.5 with a narrow pH range for stability. Incubation of the enzyme at either pH 5.5 or 8.0 for 30 min reduced the activity by 70%.

Substrate specificity studies, similar to that for AFR-AG, showed that AFR-AM from *S. cerevisiae* reduced AF only at a rate of 1.0 $\mu\text{mol}/\text{min}/\text{mg}$, but did not oxidize any substrate. Hector *et al.* showed that recombinant YMR315W produced by *E. coli* reduced DL-glyceraldehyde at an activity level of 38 nmol/min/mg, in addition to having oxidation activity on monosaccharides (28 nmol/min/mg on L-arabinose).²⁸⁾ These values are considerably smaller than those for AF reduction. These differences may be attributable to the amount of enzyme used, or possible alterations in substrate specificity caused by structural changes due to heterologous expression. In addition, it is possible that there were some impurities in the enzyme preparation, as it is known that certain reductases in *E. coli* actively metabolise and detoxify aldehyde materials such as DL-glyceraldehyde.²⁹⁾ Careful re-examination of the substrate specificity of YMR315W is therefore needed to obtain a better understanding of differences in reducing activity and its physiological role.

The report on AFR (AM-forming) is only from the bacterium *S. morelense* S-30.7.5.⁹⁾ Comparison of the amino acid sequences of the enzymes showed a low identity of 20%. However, the NADPH binding motif (10-GxGxxA-15), the substrate binding motif (92-AGKxVxxEKP-101) and catalytic triad (K100, D183 and H187) were common in both enzymes (Fig. 4).³⁰⁾ These motifs were shown to be well conserved in a glucose-fructose oxidoreductase from *Zymomonas mobilis*,³¹⁾ inositol-2dehydrogenase from *Bacillus subtilis*,³²⁾

<i>S. cerevisiae</i>	MSPLNVGIVG IGIF ARDRLPSYQEFDPDKFKVIAAFNRHKAKALDFAKVADIPENKVYDN 60
<i>S. morelense</i>	MN--RWGLIGASTIAREWVIGAIRATGG--EVVSMSTSAERGAAYATENGIG--KSVTS 54
	* * * * *
<i>S. cerevisiae</i>	LDEILNDPHVDYIDALLPAQFNADIVEKAVK AGKPVILEKP IAANLDQAKEIVKIAESTP 120
<i>S. morelense</i>	VEELVGDPDVAVVYSTTNELHREQLAAIRAGKHVLCEKPLAMTLEDAREMMVAAREAG 114
	* ** *
<i>S. cerevisiae</i>	LPVGVAENWLYLPCIKIAKEQIEKIGPVVAFTHNSTGPFVTQNKYLTTTWKQKEHIGGF 180
<i>S. morelense</i>	VVLGTNHHLRNAAAHRAMDAIAEG-RIGRPAAARVHAVYLPPLQGWRLERPEAGGGV 173
	* * * *
<i>S. cerevisiae</i>	LSDGGV IQ LALVLSLLGEFGSVSALTRQVR--ERSGADDIVFATVQLKNKEVIGSFTYGS 238
<i>S. morelense</i>	ILDITV HD ADTLRFVLNDDPAEAVAISHSAGMGKEGVGDGVMGGVRFQSG-VIAQFHD-- 230
	* ** *
<i>S. cerevisiae</i>	AFGATEKSVFLKVVYKNGTIVTDLSDKKDPVVKVLGGSAEDNGDEQIFK VNDNESFGVN 298
<i>S. morelense</i>	AFTTKFAETGFEVHGTEGSLIGRNVMTQKPGVTTLRNAEGES-----QLPLDPANLYE 284
	** * * * *
<i>S. cerevisiae</i>	AEFLNFHEAVSKDKSLYLGTPTAFHHLACVDAFLKSSAKNGDYVKIEQP- 349
<i>S. morelense</i>	TALAAFHSAIEGHGQPSATGE--DGVWSLATGLAVVKAAT--GQAAEIEITGL 333
	** * * * *

Fig. 4. Sequence alignment of AFR-AM from *S. cerevisiae* and *S. morelense*.

Sequence alignment of AFR-AM from *S. cerevisiae* and *S. morelense* S-30.7.5 (UniProt Q2I8V6) was performed. The amino acid sequences determined in this study are underlined. Gray amino acid sequence indicates the NADPH (10-GxGxxA-15) and substrate (92-AGKxVxxEKP-101) binding sites. Asterisks show identical amino acids. Amino acids Lys100, Asp183 and His187, which form the catalytic triad of the putative active site, are boxed.

Fig. 5. *In vitro* production of AG by AFR-AG from *S. cerevisiae* using NADP redox cycling.

Experimental and analytical methods are described in the Materials and Methods section. AG concentrations in reactions containing AFR-AG, AF and NADPH are shown as closed triangles, and A_{340} measurements are shown as open triangles. The AG concentration of reactions in the presence of L-xylose (indicated by an arrow) are shown as closed squares, and A_{340} measurements as open squares. The average values of two independent experiments were plotted.

and a mammalian D-xylose dehydrogenase,³³⁾ with all enzymes participating in the oxidation and reduction of sugars of the GFO/IDH/MocA family. The presence of AF in *S. cerevisiae*,¹⁶⁾ the unique substrate specificity of the enzyme and the amino acid sequence homology together suggests that AFR-AM from *S. cerevisiae* participates in the reduction of AF *in vivo*. Therefore, we concluded that YMR315W protein is 1,5-anhydro-D-fructose reductase (AM-forming). Hector *et al.* suggested that the YMR315W protein contributes to NADPH production as an alternative source of NADPH.²⁸⁾ As mentioned, AF is the sole substrate for reduction by the YMR315W protein, suggesting that AF may play an important role in controlling NADP–NADPH cycling in yeast.

Applications and enzymatic production of AG.

Figure 5 shows the enzymatic production of AG by AFR-AG following incubation with AF and NADPH. During the reaction, the increase in AG reached a plateau of 50 μ M after 180 min. Although a sufficient amount of NADPH was

added to the reaction mixture, the reaction may have stopped because of lack of NADPH. We therefore tried to recycle NADPH using the NADP–NADPH cycling reaction.

L-Xylose was the most reactive substrate of the sugars tested in the oxidation reaction (Table 1), and was therefore selected as the substrate for the coupling reaction. As shown in Fig. 5, the addition of L-xylose to the reaction mixture after 60 min continually increased AG for 240 min up to a concentration of 75 μ M, which is 1.5-fold greater than that in the control reaction. The enzyme also acted on D-arabinose, with the product of this oxidation being D-arabinolactone, which was then converted to erythroascorbic acid by D-arabinolactone oxidase.²⁴⁾ Therefore, AG, and an ascorbic acid-like substance, can be prepared simultaneously by this recycling reaction of AFR-AG without the loss of NADPH.

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REFERENCES

- 1) M.A. Baute, R. Baute and G. Deffieux: Fungal enzymatic activity degrading 1,4- α -D-glucans to 1,5-anhydrofructose. *Phytochemistry*, **27**, 3401–3403 (1988).
- 2) K. Yoshinaga, M. Fujisue, J. Abe, I. Hanashiro, Y. Takeda, K. Muroya and S. Hizukuri: Characterization of exo-(1,4)-alpha glucan lyase from red alga *Gracilaria chorda*. Activation, inactivation and the kinetic properties of the enzyme. *Biochim. Biophys. Acta*, **1472**, 447–454 (1999).
- 3) M. Fujisue, K. Yoshinaga, K. Muroya, J. Abe and S. Hizukuri: Preparation and antioxidative activity of 1,5-anhydrofructose. *J. Appl. Glycosci.*, **46**, 439–444 (1999).
- 4) R. Fiskesund, K. Abeyama, K. Yoshinaga, J. Abe, Y. Yuan and S. Yu: 1,5-Anhydro-D-fructose and its derivatives: biosynthesis, preparation and potential medical applications. *Planta Med.*, **76**, 1635–1641 (2010).

- 5) K. Yoshinaga, H. Ishiba, K. Yoshimoto and T. Katano: Functional research and production technology development of 1,5-anhydrofructose. *Bull. Appl. Glycosci.*, **1**, 70–75 (2011). (in Japanese)
- 6) S. Kametani, Y. Shiga and H. Akanuma: Hepatic production of 1,5-anhydrofructose and 1,5-anhydroglucitol in rat by the third glycogenolytic pathway. *Eur. J. Biochem.*, **242**, 832–838 (1996).
- 7) G. Deffieux, R. Baute, M.A. Baute, M. Atfani, A. Carpy and G. Deffieux: 1,5-D-anhydrofructose, the precursor of the pyrone microthecin in *morchellavulgaris*. *Phytochemistry*, **23**, 261391–261393 (1987).
- 8) M.A. Baute, G. Deffieux, J. Vercauteren, R. Baute and A. Badoc: Enzymic activity degrading 1,4- α -D-glucans to ascopyrones P and T in *Pezizales* and *Tuberales*. *Phytochemistry*, **33**, 41–45 (1993).
- 9) A. Kühn, S. Yu and F. Giffhorn: Catabolism of 1,5-anhydro-D-fructose in *Sinorhizobium morelense* S-30.7.5: discovery, characterization, and overexpression of a new 1,5-anhydro-D-fructose reductase and its application in sugar analysis and rare sugar synthesis. *Appl. Environ. Microbiol.*, **72**, 1248–1257 (2006).
- 10) S.M. Andersen, I. Lundt, J. Marcussen and S. Yu: 1,5-Anhydro-D-fructose; a versatile chiral building block: biochemistry and chemistry. *Carbohydr. Res.*, **337**, 873–890 (2002).
- 11) Y. Shiga, H. Mizuno and H. Akanuma: Conditional synthesis and utilization of 1,5-anhydroglucitol in *Escherichia coli*. *J. Bacteriol.*, **175**, 7138–714 (1993).
- 12) Y. Konishi, K. Hashima and K. Kishida: Increases in 1,5-anhydroglucitol level in germinating amaranth seeds and in ripening banana. *Biosci. Biotechnol. Biochem.*, **64**, 2462–2465 (2000).
- 13) T. Yamanouchi, Y. Tachibana, H. Akanuma, S. Minoda, T. Shinohara, H. Moromizato, H. Miyashita and I. Akaoka: Origin and disposal of 1,5-anhydroglucitol, a major polyol in the human body. *Am. J. Physiol.*, **263**, E268–E273 (1992).
- 14) M. Sakuma, S. Kametani and H. Akanuma: Purification and some properties of a hepatic NADPH-dependent reductase that specifically acts on 1,5-anhydro-D-fructose. *J. Biochem.*, **123**, 189–193 (1998).
- 15) Y. Shiga, S. Kametani, T. Kadokura and H. Akanuma: 1,5-Anhydroglucitol promotes glycogenolysis in *Escherichia coli*. *J. Biochem.*, **125**, 166–172 (1999).
- 16) K. Hirano, M. Ziak, K. Kamoshita, Y. Sukenaga, S. Kametani, Y. Shiga, J. Roth and H. Akanuma: N-linked oligosaccharide processing enzyme glucosidase II produces 1,5-anhydrofructose as a side product. *Glycobiology*, **10**, 1283–1289 (2000).
- 17) Y. Akanuma, M. Morita, N. Fukuzawa, T. Yamanouchi and H. Akanuma: Urinary excretion of 1,5-anhydro-D-glucitol accompanying glucose excretion in diabetic patients. *Diabetologia*, **31**, 831–835 (1988).
- 18) T. Yamanouchi, N. Ogata, T. Tagaya, T. Kawasaki, N. Sekino, H. Funata, I. Akaoka and H. Miyashita: Clinical usefulness of serum 1,5-anhydroglucitol in monitoring glycaemic control. *Lancet*, **347**, 1514–1518 (1996).
- 19) S. Kametani, Y. Hashimoto, T. Yamanouchi, Y. Akamuma and H. Akanuma: Reduce renal reabsorption of 1,5-anhydro-D-glucitol in diabetic rats and mice. *J. Biochem.*, **102**, 1599–1607 (1987).
- 20) T. Yamanouchi, T. Inoue, K. Ichianagi, T. Sakai and N. Ogata: 1,5-Anhydroglucitol stimulates insulin release in insulinoma cell lines. *Biochim. Biophys. Acta*, **1623**, 82–87 (2003).
- 21) B.W. Hoffer, E. Crezee, F. Devred, P.R.M. Mooijman, W.G. Sloof, P.J. Kooyman, A.D. Langeveld, F. Kapteijn and J.A. Moulijn: The role of the active phase of Raney-type Ni catalysts in the selective hydrogenation of D-glucose to D-sorbitol. *Appl. Catal.*, **253**, 437–452 (2003).
- 22) S.M. Andersen, I. Lundt and J. Marcussen: 1,5-Anhydro-D-fructose: stereoselective conversions to 1,5-anhydroalditols and deoxy/amino substituted analogues. *J. Carbohydr. Chem.*, **19**, 717–725 (2000).
- 23) M. Suzuki, S. Kametani, K. Uchida and H. Akanuma: Production of 1,5-anhydroglucitol from 1,5-anhydrofructose in erythroleukemia cells. *Eur. J. Biochem.*, **240**, 23–29 (1996).
- 24) S.T. Kim, W.K. Huh, B.H. Lee and S.O. Kang: D-Arabinose dehydrogenase and its gene *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **1429**, 29–39 (1998).
- 25) B.V. Bergen, R. Strasser, N. Cyr, J.D. Sheppard and A. Jardim: α,β -dicarbonyl reduction by *Saccharomyces* D-arabinose dehydrogenase. *Biochim. Biophys. Acta*, **1760**, 1636–1645 (2006).
- 26) K. Amako, K. Fujita, C. Iwamoto, M. Sengee, K. Fuchigami, J. Fukumoto, Y. Ogishi, R. Kishimoto and K. Goda: NADP⁺-Dependent D-arabinose dehydrogenase shows a limited contribution to erythroascorbic acid biosynthesis and oxidative stress resistance in *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **70**, 3004–3012 (2006).
- 27) M. Sakuma and S. Kubota: Mouse AKR1E1 is an ortholog of pig liver NADPH dependent 1,5-anhydro-D-fructose reductase. *Biosci. Biotechnol. Biochem.*, **72**, 872–876 (2008).
- 28) R.E. Hector, M.J. Bowman, C.D. Skory and M.A. Cotta: The *Saccharomyces cerevisiae* YMR315W gene encodes an NADP(H)-specific oxidoreductase regulated by the transcription factor Stb5p in response to NADPH limitation. *New Biotechnol.*, **26**, 171–180 (2009).
- 29) J. Ko, I. Kim, S. Yoo, B. Min, K. Kim and C. Park: Conversion of methylglyoxal to acetol by *Escherichia coli* aldo-keto reductase. *J. Bacteriol.*, **187**, 5782–5789 (2005).
- 30) T.R. Danbe, A.M. Kühn, T. Brossette, T. Giffhorn and A.J. Scheidig: Crystal structure of NADP(H)-dependent 1,5-anhydro-D-fructose reductase from *Sinorhizobium morelense* at 2.2 Å resolution: Construction of a NADH-accepting mutant and its application in rare sugar synthesis. *Biochemistry*, **45**, 10030–10042 (2006).
- 31) M. Zachariou and R.K. Scopes: Glucose-fructose oxidoreductase, a new enzyme isolated from *Zymomonas mobilis* that is responsible for sorbitol production. *J. Bacteriol.*, **167**, 863–869 (1986).
- 32) Y. Fujita, K. Shindo, Y. Miwa and K. Yoshida: *Bacillus subtilis* inositol dehydrogenase-encoding gene (*idh*): sequence and expression in *Escherichia coli*. *Gene*, **108**, 121–125 (1991).
- 33) S. Aoki, S. Ishikura, Y. Asada, N. Usami and A. Hara: Identity of dimeric dihydrodiol dehydrogenase as NADP(+)-dependent D-xylose dehydrogenase in pig liver. *Chem. Biol. Interact.*, **30**, 775–784 (2001).