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Enzymatic metabolism of 3-deoxyglucosone, a Maillard intermediate

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Summary. In order to verify the formation of endogenous 3-deoxyglucosone (3-DG), an intermediate compound in the Maillard reaction, we tried to detect 3-deoxyfructose (3-DF) which is main metabolite of 3-DG. Endogenous 3-DF was detected in the urine of normal and diabetic rats by the oral administration of 3-DG-free feed. Metabolizing activities of crude extracts prepared from porcine organs were examined using methylglyoxal (MG) and 3-DG as substrates. NAD- or NADP-dependent 2-oxoaldehyde dehydrogenase activity was detected in liver, kidney, small intestine and lung. On the other hand, NADH- or NADPH-dependent 2-oxoaldehyde reductase activity was detected in all porcine organs in which liver and kidney contained higher activity of NADPH-dependent enzyme than the other organs. The reductase which catalyzes the reduction of 3-DG to 3-DF and MG to acetol, was purified and characterized from porcine kidney. The enzyme was the same to NADPH-dependent-2-oxoaldehyde reductase from porcine liver, which is speculated to prevent the advanced stage of the Maillard reaction as a self-defense enzyme.

Keywords: Amino acids – Maillard reaction product metabolism – 3-Deoxyglucosone – 3-Deoxyfructose – 2-Oxoaldehyde reductase - Dicarbonyl metabolism

Introduction

The Amadori rearrangement compound, the product in the early stage of the Maillard reaction of proteins with glucose, is known to be degraded into 3-deoxyglucosone (3-DG). 2-Oxoaldehydes such as methylglyoxal (MG) and 3-DG were reported to be highly reactive and cytotoxic compounds (Kato et al., 1987b; Együd and Szent-Györgyi, 1966; Szent-Györgyi et al., 1963 and 1967). In the advanced stage of the Maillard reaction, proteins are modified into colored, fluorescent and cross-linked molecules. This process has been hypothesized to play an important role in aging and diabetic complications (Monnier et al., 1986; Cerami, 1985). Our group speculates that 3-DG reacts as cross-linker responsi-

ble for the polymerization of proteins induced with glucose under physiological conditions (Kato et al., 1987a, 1987b, 1989; Shin et al., 1988).

3-DG was detected in some foodstuffs such as soy source and soy paste (Kato et al., 1961) and fruit juice (Kato et al., 1963). 3-DG also was tentatively identified in calf and rabbit livers (Kato et al., 1970). We are interested in whether it is exogenous 3-DG derived from feed or endogenous 3-DG from glycated proteins *in vivo*.

We revealed that 3-DG was metabolized in rats to 3-deoxyfructose (3-DF) (Kato et al., 1990). Accordingly, we have proposed the self-defense mechanism by the enzymatic inactivation of 2-oxoaldehydes such as 3-DG. Recently, we have found a new enzyme which catalyzes the reduction of 3-DG to 3-DF and MG to acetol in parsley (Liang et al., 1990a), chicken (Shin et. al., 1991) and porcine (Liang et al., 1991).

In this paper, we investigate on the formation of endogenous 3-DG *in vivo* and the distribution of 2-oxoaldehyde-metabolizing enzymes in porcine. Then NADPH-dependent 2-oxoaldehyde reductase was isolated and characterized from porcine kidney, as a self-defense enzyme preventing the advanced stage of the Maillard reaction.

Materials and methods

Chemicals

Methylglyoxal (MG) and NADPH were obtained from Nacalai Tesque, Inc. DL-Lactaldehyde was prepared from DL-threonine by the method of Huff and Rudney (1959). Glucosone was prepared by the method of Hamilton and Smith (1952). 3-Deoxyglucosone (3-DG) was prepared by the method of Kato et al. (1987a or 1990), and its concentration was measured as described in our previous paper (Liang et al., 1990a). Other chemicals used were analytical reagents.

Experimental animals and diets

Two male rats of Wistar strain weighing 210g on average were divided into control and diabetic rats. Diabetic rat was induced with streptozotosine (1mg/100g rat). Rats were fed by ad-libitum for a period of 20 days in each metabolic cage. Diet was prepared from the mixture of 15% milk casein, 5% soybean oil, 5% cellulose, 25% sucrose, 4% mineral mixture, 1% vitamin mixture and 45% α-corn starch. 3-DG did not detect in the diet. After 10 days, urine was collected in each bottle cooled at 0°C and lyophilized.

Preparation of TMS-derivatives of urine sample

Proteins and high-molecular weight substances were removed from lyophilized urine with three types of centricon (cut Mw = 30,000, 10,000 and 3,000) in order. The low-molecular weight fractions were eluted with CM-cellulose (Pharmacia) and lyophilized. The powder (50 mg) was dissolved with 200 μl of 21% trimethylsilyl (TMS) imidazole (GL Sciences) in pyridine and incubated for 15 min at room temperature. The samples (1 μl) were injected with gas chromatography. Glucose, fructose, 3-DG and 3-DF were used as standard sample. 3-DF was prepared according to the previous paper (Kato et. al., 1990).

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

GC

A Shimadzu GC-8A gas chromatograph with a flame inonization detector and a Shimadzu CR-2A data processing system were used. The GC conditions were as follows: column:

50 × 0.25 min i.d. fused silica capillary column with chemically bonded Carbowax 20M (GL Sciences), carrier gas: N_2, flow rate: 1.56 ml/min and split ratio: 40.6:1. The oven temperature was held at 110°C and then programmed to 190°C at 4°C/min. The injection port and the detector temperature were kept at 200°C.

GC-MS

JEOL-DX303 (Japan Electron Optic Laboratory Inc.) was used. Helium was used as the carrier gas. The other GC conditions were the same as described above, and MS analyses of electron impact (EI) at 70eV and chemical ionization (CI) using isobutane were done.

Extraction from porcine organs and standard assay system for enzyme from porcine

Fresh porcine organs were homogenized with 2 volumes 20 mM sodium phosphate buffer (pH 7.2) containing 10 mM 2-mercaptoethanol at four times (for 40 sec/one time). The suspension was centrifuged for 20 min at 12,000 × g. After the supernatant was dialyzed against the same buffer, the crude extracts were obtained.

For the assay of NAD- or NADP-dependent 2-oxoaldehyde dehydrogenase, the mixture contained 100 mM Tris buffer (pH 8.6), 1 mM NAD or NADP, 10 mM 3-DG or MG and a suitable amount of the enzyme in a total volume of 1 ml.

The assay mixture for NADH- or NADPH-dependent 2-oxoaldehyde reductase and NADH- or NADPH-dependent aldehyde reductase (alcohol dehydrogenase, EC 1.1.1.1 and 1.1.1.2) contained 100 mM sodium phosphate buffer (pH 7.0), 0.3 mM NADH or NADPH, 10 mM 3-DG, MG or acetaldehyde and a suitable amount of the enzyme in a total volume of 1 ml.

Enzyme activity was measured at 25°C by the rate of decrease in the absorbance at 340 nm. One activity unit (IU) was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol coenzyme/min.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis

SDS/PAGE was performed using a polyacrylamide concentration of 10% as described by Weber and Osborn (1989). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250.

Molecular mass measurement

The molecular mass of the enzyme was estimated by SDS. Cytochrome *c* (12.5 kDa), chymotrypsinogen A (25 kDa), ovalbumin (45 kDa) and bovine serum albumin (67 kDa) were used as marker proteins.

Protein measurements and investigation of the reaction product from methylglyoxal

Their methods were performed according to a previous paper (Liang et al., 1991).

Extraction and purification of the enzyme

Extraction

Fresh porcine kidney (100 g) was homogenized for 2 min with 500 ml of acetone previously cooled at −20°C, and sucked dry in a Buchner funnel. After a second similar treatment with acetone, the cake was scrumbled to a powder and dried in air, and kept at room temperature overnight. 20 g acetone powder was obtained. 6 g acetone powder was suspended at 4°C for 90 min with 100 ml of 20 mM sodium phosphate buffer (pH 7.2) containing 10 mM 2-mercaptoethanol (designated buffer A). The suspension was squeezed through the gauze

and centrifuged for 20 min at 9,000 × g. After the supernatant was dialyzed against buffer A, the crude extract was obtained.

Ammonium sulfate fractionation

Ammonium sulfate was added to the crude extract to give 45% saturation. After stirring for an additional 15 min, the mixture was centrifuged for 20 min at 9000 × g. The concentration of ammonium sulfate of the supernatant obtained above was brought to 70% saturation with solid ammonium sulfate. After stirring for 15 min, the precipitated proteins were collected by centrifugation and dissolved in a minimal amount of buffer A, and dialyzed against the same buffer.

DEAE-cellulose column

The non-dialyzable fraction was applied to a DEAE-cellulose (Whatman DE-52) column (1.8 cm × 30 cm) previously equilibrated with buffer A, and eluted with 100 ml buffer A. The active fractions were concentrated by ultrafiltration on an Ultra Filter UK-10 membrane.

Hydroxyapatite column

The concentrated solution was applied to a hydroxyapatite column (1.5 cm × 17 cm) equilibrated with buffer A then the enzyme was eluted with 80 ml buffer A. The enzyme fractions (fractions 13–17) were pooled.

Results

Detection of 3-deoxyglucosone in urine of rats

Authentic GC peaks of TMS derivatives of 3-DF gave five optical isomers. GC-EI/MS of these peaks showed M/Z = 362, 349, 272, 259 and 169, and these GC-CI/MS showed a peak of $M + H^+$ = 453. GC-EI/MS of TMS derivatives in urine of diabetic rats were identical to the MS data of TMS derivatives of authentic 3-DF (Fig. 1). Table 1 shows the amounts of 3-DF in urine of normal and diabetic rats. The amount of 3-DG in diabetic urine shows a tendency to increase compared with that in control subject. Verification for the preliminary experimental results is now in progress to calculate the significant differences between control and diabetic groups.

These results (Fig. 1 and Table 1) indicate the formation of endogenous 3-DG.

Distribution of 2-oxoaldehyde-metabolizing enzymes

Table 2 shows the distribution of 2-oxoaldehyde-metabolizing enzyme activity, by using 3-DG and MG as substrates, in porcine organs. NAD-dependent dehydrogenase activity for both substrates at pH 8.6 was detected in liver, kidney and small intestine, NADP-dependent one being in liver, kidney, lung, heart and small intestine. Of these activities, NAD-dependent dehydrogenase in liver showed highest activity.

On the other hand, NADH- or NADPH-dependent reductase activity at pH 7.0 was detected in all organs in the extent of 0.9–395U/100 mg tissue. The highest activity of NADPH-dependent reducing enzyme was detected in liver

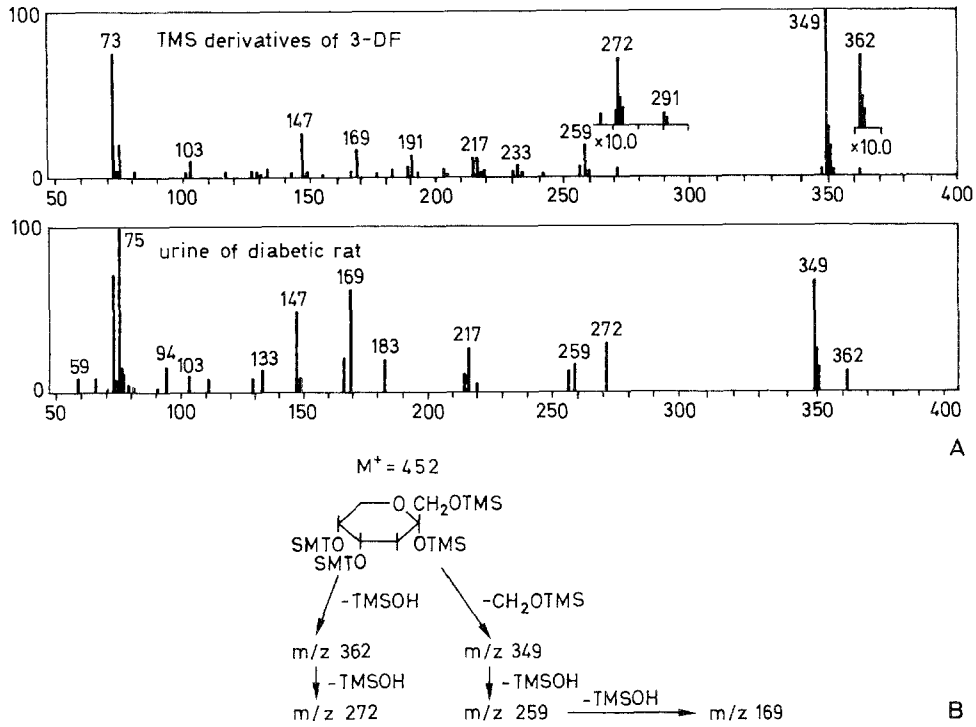

Fig. 1. **A** GC-EI/MS of TMS derivatives of 3-deoxyfructose (3-DF) and 3-DF in urine of diabetic rats. **B** M/Z and fragmentation for TMS derivatives of 3-DF

Table 1. Amounts of 3-deoxyfructose (3-DF) in the low-molecular weight fractions derived from urine of rats

urine	3-DF (μg/ml)
in control rat	15
in diabetic rat	28

and kidney. As we found that NAD-dependent alcohol dehydrogenase showed considerable activity toward MG and fairly low activity toward 3-DG (Liang et al., 1990b), NADH- and NADPH-dependent aldehyde reductase (alcohol dehydrogenases, EC 1.1.1.1 and 1.1.1.2) activities were also measured using acetaldehyde as substrate. Aldehyde reductase activity in liver was 30–50 times as stronger as in the case of kidney (Table 2), indicating the abundance of the NADPH-dependent 2-oxoaldehyde reductase in kidney.

Purification of NADPH-dependent 2-oxoaldehyde reductase

Figure 2 shows elution patterns of porcine kidney 2-oxoaldehyde reductase on DEAE-cellulose and hydroxyapatite columns. The enzyme was purified approx-

Table 2. Distribution of 2-oxaldehyde-metabolizing enzyme activity in internal organs of porcine

	Metabolizing activity (μM/min/100 mg tissue)									
	NAD-dependent		NADP-dependent		NADH-dependent		NADPH-dependent		NADH-dependent	NADPH-dependent
	MG	3DG	MG	3DG	MG	3DG	MG	3DG	acetaldehyde	acetaldehyde
Liver	41.1	38.9	2.1	2.6	192.1	31.3	395.3	191.2	41.4	148.9
Kidney	6.4	6.1	0.8	2.7	18.6	20.1	321.0	358.7	1.4	3.4
Lung	0	0.6	0.5	0.3	12.2	4.3	41.7	42.9	1.1	1.9
Heart	0	0.9	0.3	0.3	4.3	3.5	19.9	16.2	1.1	0.8
Pancreas	0	0	0	0	4.2	3.5	51.2	71.3	1.8	1.4
Spleen	0	0	1.6	0	4.3	4.7	41.4	59.5	1.3	0.9
S. intestine	0.9	2.4	1.3	2.4	4.5	4.7	53.0	68.6	0.5	0.8
L. intestine	0	0	0	0	3.4	1.3	11.1	12.9	0.5	0.6
Stomach	0	0	0	0	2.6	2.6	18.8	24.6	0	0.6
Muscle	0	1.1	0	0	4.5	3.4	9.2	6.7	0.5	0.8
Brain	0	0	0	0	2.6	2.7	13.3	12.2	0.5	0.5
Eyeball	0	0	0	0	2.7	0.9	4.7	3.7	1.1	0.5

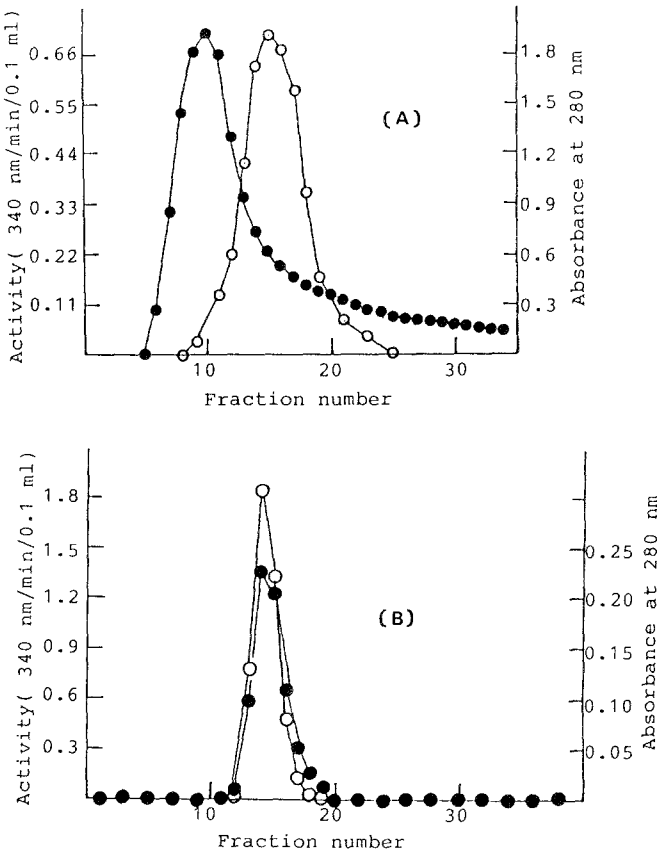

Fig. 2. Elution patterns of the enzyme on DEAE-cellulose column (**A**) and a hydroxyapatite column (**B**). The conditions for column chromatography are described under Materials and methods. (•) Absorbance at 280 nm; (○) enzymatic activity towards 3-deoxyglucosone

Table 3. Purification of NADPH-dependent 2-oxoaldehyde reductase from porcine kidney

	Total protein (mg)	Total activity (μM/min)	Specific activity (μM/min/mg)	Recovery of activity (%)	Purity (fold)
Crude extract	1324	64819	48	100	1
$(NH_4)_2SO_4$ ppt	612	42978	70	66.3	1.5
DEAE-cellulose	55.5	31590	569	48.7	11.9
Hydroxyapatite	3.4	28851	8485	44.5	176.8

imately 177-fold from crude extracts with 45% activity yield. Table 3 summarized the results of the enzyme purification.

The enzyme fraction after eluted from the hydroxyapatite column was homogeneous on SDS/PAGE as shown in Fig. 3.

Substrate specificity

Table 4 shows the various carbonyl compounds that were tested as possible substrates for 2-oxoaldehyde reductase. This enzyme was most active for

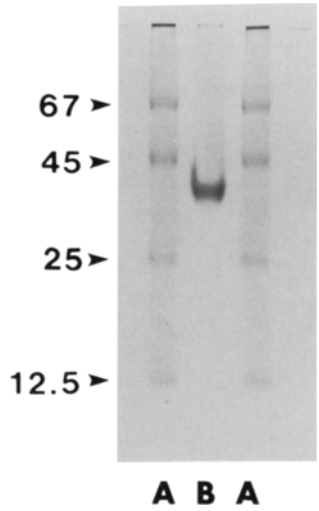

Fig. 3. SDS/PAGE of NADPH-dependent 2-oxoaldehyde reductase purified from porcine kidney. **A** Standard proteins, bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c*. **B** Purified enzyme. The protein was stained with Coomassie brilliant blue

Table 4. Substrate specificity of NADPH-dependent 2-oxoaldehyde reductase from porcine kidney

Substrate	Relative activity (%) of 2-oxoaldehyde reductase
3-Deoxyglucosone	100
Methylglyoxal	74.9
Phenylglyoxal	103.7
Glucosone	33.3
Glyoxal	29.2
Glyceraldehyde	23.2
Diacetyl	4.1
Acetaldehyde	1.1
Hydroxyacetone	0
Dihydroxyacetone	0
Fructose	0
Glucose	0

phenylglyoxal and 3-DG among the carbonyls tested. The enzyme showed considerable activity towards aldehydes such as DL-glyceraldehyde in addition to 2-oxoaldehydes, and no activity with glucose and fructose.

Chemical properties of the enzyme

The purified enzyme has a single band on SDS/PAGE with a mobility in agreement with a molecular mass of approximately 38 kDa as shown in Fig. 3.

The enzyme was most active at pH around 6.5 for both 3-DG and MG in 100 mM sodium phosphate buffer.

The apparent *Km* of the enzyme for 3-DG was 2.9 mM, and that for MG was 3.1 mM at 0.3 mM NADPH.

The effects of various chemicals on the activity of the enzyme were investigated by the methods described in a previous paper (Liang et al., 1991). The enzyme activity was completely lost in the presence of 0.1 mM *p*-chloromercuribenzoate and 53% of the initial activity was inhibited by 1 mM *N*-ethylmaleimide. The relative activity of the enzyme was enhanced to 128 and 132% of the initial activity by 10 mM 2-mercaptoethanol and 0.1 mM dithiothreitol, respectively. These results indicate that the thiol group plays a significant role in the enzyme activity.

The enzyme specifically required NADPH as a coenzyme for activity and substitution of NADPH with NADH reduced the activity to a mere 4% with 3-DG as a substrate.

Identification of reaction products

The reduction product of MG by the enzyme was estimated to be lactaldehyde or acetol. The elution volume of the reaction product of MG by HPLC was identified with that of authentic acetol (Fig. 4), indicating the reduction of aldehyde group of MG by the enzyme.

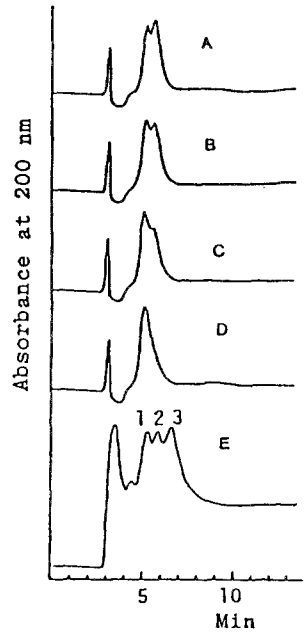

Fig. 4. HPLC of the enzymatic reaction product of methylglyoxal. **A** Incubation for 60 min; **B** incubation for 40 min; **C** incubation for 20 min; **D** control; **E** standard, peak 1, 2 and 3 were methylglyoxal, acetol and lactaldehyde, respectively

Discussion

The enzymatic metabolism of Maillard intermediate, 3-deoxyglucosone (3-DG), was investigated in this paper. Glucose-derived pyrrole aldehyde (named pyrraline) formed during Maillard reaction of proteins has been reported to be detected *in vivo* (Hayase et al., 1989). The pyrraline is considered to be formed from 3-DG and free amino groups in proteins. 3-DG would be generated from Amadori compounds *in vivo* (Hayase et al., 1989), fructose 3-phosphate in the lens of diabetic rats (Szwergold et al., 1990) and some foods. Then, we confirmed indirectly the presence of endogenous 3-DG in normal and diabetic rats by the identification of 3-DF. ^{14}C-3-DF has been also detected in urine by the intravenous and oral administration of ^{14}C-glucose to rats during 7 days (Suzuki et al., 1991). Whether the glucose would be modified to proteins *in vivo* to form Amadori compounds is under way.

As 3-DG inhibits the DNA replication of cells (Shinoda et al., 1990), animals and plants are assumed to possess the self-defense mechanisms against cytotoxic 2-oxoaldehydes.

We propose two defense mechanisms at least. First, we have obtained the evidence for enzymatic inactivation of 2-oxoaldehydes as described below. In fact, our experiments of intravenous and oral administrations of ^{14}C-3-DG to rats showed that the absorbed 3-DG was not biologically utilized by the rats, but was rapidly excreted in the urine as 3-DF (Kato et al., 1990). Secondly, we consider the receptor-mediated endocytosis of proteins modified with 3-DG by macrophage (Shinoda et al., 1991). The endocytosis by macrophage has been also reported on the advanced glycosylation end products produced during Maillard reaction (Vlassala et. al., 1989; Horiuchi et al., 1990).

Extensive distribution of NADH- or NADPH-dependent 2-oxoaldehyde reductase in porcine organs is clarified in the present paper. Especially, the reductase activities were much higher in liver and kidney than in other organs. Kidney enzyme purified and characterized in this paper is suggested to be the same to liver NADPH-dependent 2-oxoaldehyde reductase as described in our previous paper (Liang et al., 1991). The NADPH-dependent 2-oxoaldehyde reductase was also extensively distributed in chicken (Shin et al., 1991), rat (unpublished data) and plant tissues (Liang et al., 1990; Kato et al., 1990).

When the NADPH-dependent-2-oxoaldehyde reductase from porcine liver (Liang et al., 1991) was purified on hydroxyapatite column, the minor peak having activity towards 3-DG was detected. We purified further the minor peak and identified to be aldose reductase (EC 1.1.1.21) which is similar to enzyme isolated from cattle kidney as reported by Attwood and Doughty (1974) (Liang et al., 1990c). Jellum (1968) reported that 2-oxoaldehyde dehydrogenase is involved in the metabolism of 3-DG in rat and sheep livers. In our previous study concerning the metabolism of 3-DG (Kato et al., 1990), minor spot below 3-DG spot detected by TLC autoradiography may be due to enzymatic product by the dehydrogenase. Methylglyoxal reductase of porcine liver is also involved in the metabolism of 3-DG, but its activity is considerable lower (Kato et al., 1988).

The 2-oxoaldehyde reductase among 3-DG metabolizing enzymes has been confirmed to inhibit the advanced Maillard reaction *in vitro* (Liang et al., 1991).

In conclusion, NADPH-dependent 2-oxoaldehyde reductase is speculated to play an important role to prevent the advanced stage of the Maillard reaction as a self-defense enzyme. Aldose reductase, methylglyoxal reductase and 2-oxoaldehyde dehydrogenase are secondarily possible to inhibit the Maillard reaction *in vivo*.

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