

Purification of Aldose Reductase and Decolorization of Dye by the Enzyme

– Research Note –

Mi Jang and Kyungsoon Kim[†]

Department of Chemistry, Myongji University, Gyeonggi 449-728, Korea

Abstract

Aldose reductase was purified to electrophoretic homogeneity from porcine liver. The purified enzyme was a monomer of 36 kDa. The enzyme was strongly inhibited by Cu^{2+} and Mg^{2+} ions. Incubation of the enzyme with pyridoxal 5'-phosphate led to complete inhibition of enzymatic activity, suggesting that lysine residue is involved at or near the active site of the enzyme. The enzyme exhibited a broad substrate specificity. Furthermore, the enzyme was capable of decolorizing Alizarin, an anthraquinone dye.

Key words: aldose reductase, purification, property, color removal

INTRODUCTION

Aldose reductases (alditol : NAD(P)⁺ oxidoreductase, EC 1.1.1. 21) are widely distributed in man and animals, yeasts, filamentous fungi, and other microorganisms (1-6). Aldose reductase catalyzes the reduction of a wide range of aldehydes, including aldoses to the corresponding alcohols (7). The enzyme has been thought to be involved in the detoxification of reactive compounds in the liver and the brain, both endogenous and xenobiotic aldehydes (8,9). Recently, mammalian aldose reductase has been studied extensively, probably due to its potential role in the development of diabetic complications such as retinopathy and neuropathy (10-12).

Increasing amounts of synthetic dyes are being manufactured and consumed mainly in pulp and paper, textile and tannery industries. Textile, dyestuff and tannery industries also consume large quantities of water, and release large volumes of colored effluents. These industrial effluents often contain residual dyes which affect water quality and are therefore a source of considerable color pollution (13-16). The discharge of industrial colorants into the environment has become one of the major environmental concerns in wastewater treatment.

Color in dye and textile effluents needs to be treated before discharge, and decolorization of textile dye wastewater has recently become one of major scientific interest (17-19).

Among the important chemical chromophores of dye classes, the most common are the anthraquinone and azo chromophores. Textile and tannery dyestuffs are difficult to decolorize due to their stability, complex structure and recalcitrant nature. In particular, anthraquinone dyes are the most difficult to decolorize, due to their fused ar-

omatic structure (20,21). Some physicochemical methods such as adsorption, ion-exchange, electrochemical oxidation and coagulation/ precipitation are available for the decolorization of dye-containing effluents. However, these methods have some drawbacks, such as the significant amounts of sludge that is generated, the high operation costs, the complex processes and the secondary pollution caused.

Problem with sludge disposal, high costs, or longer contact times has restricted their widespread acceptance. Thus, search for an alternative treatment technology that is eco-friendly, cost-effective, and simple has been performed (22). Bioremediation is an environmentally-friendly technology and appears to be a highly attractive option. Enzymes produced by microorganisms, plants, and animals could be used in bioremediation, and enzymatic process requires shorter contact times (23-25).

Aldose reductase has been extensively studied for a potential role in the development of secondary diabetes complications (26). However, there are few reports on the color removal potential of aldose reductase.

In our laboratory, aldose reductase was purified and examined for its ability to remove color. Purified aldose reductase rapidly and efficiently decolourized Alizarin, an anthraquinone dye. In this study, purification and properties of aldose reductase, and the color removal of Alizarin by purified aldose reductase are described.

MATERIALS AND METHODS

Chemicals

Alizarin, azobenzene, anthraquinone, N-ethylmaleimide,

[†]Corresponding author. E-mail: kskim@mju.ac.kr
Phone: +82-31-330-6183, Fax: +82-31-335-7248

4,4'-azoxyanisole, 4-nitrobenzaldehyde, octyl aldehyde, N-acetylimidazole, naphthol green B, disperse orange 11, disperse blue 3 were purchased from Aldrich (Milwaukee, USA).

N-bromosuccinimide, NADH, NADPH, pyridoxal 5'-phosphate, DEAE-sepharose, bromophenol blue, molecular weight standard, N,N'-methylenebisacrylamide, Coomassie brilliant blue R-250, bovine serum albumin, Sephacryl, lauryl sulfate were obtained from Sigma (St. Louis, USA). Coomassie brilliant blue G-250 was from Bio-rad (Richmond, USA). Centriprep-10 concentrator was obtained from Amicon (Beverly, USA). All other chemicals were of the highest purity grade commercially available.

Protein determination and electrophoresis

Protein concentration was determined according to the method of Bradford (27), using bovine serum albumin as the standard. The protein content in fractions collected during each chromatographic procedure was determined by absorbance at 280 nm. Polyacrylamide gel electrophoresis was carried out according to the procedure described by Laemmli (28).

The gels were stained with Coomassie brilliant blue R-250.

Activity measurement

Standard reaction mixtures consisted of 90 mM sodium phosphate buffer (pH 7.0), 10 mM glucose, 150 μ M NAD(P)H and enzyme. Reactions were initiated by the addition of the enzyme. The decrease in absorbance at 340 nm due to NAD(P)H oxidation was monitored spectrophotometrically. Conditions for the specific reactions are presented in the related table or figure legends. One unit of enzyme was defined as the amount that catalyzed the oxidation of 1 μ mol of NADH per min.

Enzyme purification

All subsequent steps were carried out at 4°C unless otherwise indicated. The aldose reductase was purified to electrophoretic homogeneity from porcine liver. Porcine liver was homogenized in 20 mM sodium phosphate buffer, pH 7.0. The homogenate was centrifuged at 15,000 $\times g$ for 30 min and the supernatant was subjected to fractional precipitation. The extract was brought to 40% saturation by slowly adding ammonium sulfate. The suspension was stirred for additional 1h and then centrifuged at 12,000 $\times g$ for 20 min at 4°C. The supernatant was decanted and adjusted to 75% saturation by adding ammonium sulfate, and centrifuged as before. The pellet was then resuspended in a minimum volume of 20 mM Tris-HCl buffer, pH 7.5, and dialyzed against

the same buffer. After centrifugation, the sample was applied to a column of DEAE-Sepharose resin, which was previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was washed with the same buffer and then aldose reductase was eluted from the column with a linear gradient of 0~0.5 M NaCl in the same buffer. Fractions were assayed for protein concentration and reductase activities as indicated. The active fractions were pooled, concentrated, and then applied to a Sephacryl S-200-HR column equilibrated with 20 mM Tris-HCl buffer, pH 7.5. Proteins were eluted, and the active fractions were pooled and used for further study.

Spectroscopy and kinetics

UV-visible spectroscopy was performed using a Shimadzu Model 3100 UV-NIR Spectrophotometer. Kinetic constants were determined by monitoring the disappearance of NADH at 340 nm.

Estimation of molecular weight by size exclusion chromatography

Molecular weight of the enzyme was determined by size exclusion chromatography. A Sephacryl S-200 column was standardized with the following proteins ; β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa), and void volume of the column was determined using blue dextran.

RESULTS AND DISCUSSION

Aldose reductase was purified to electrophoretic homogeneity from porcine liver by a combination of ammonium sulfate fractionation, ion-exchange and gel permeation chromatographies. Details of the purification procedure have been described under Materials and Methods. On polyacrylamide gel electrophoresis, the purified aldose reductase revealed a single band, and the molecular mass of the enzyme was estimated to be 36 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with standard protein markers (Fig. 1). To determine its native molecular mass by gel filtration, the purified enzyme was applied to a Sephacryl S-200 column. Aldose reductase eluted from this column as a peak corresponding to 36.3 kDa. The subunit molecular weight was estimated by SDS-PAGE to be 36 kDa (Fig. 1). These data suggest that the enzyme is monomeric. In contrast, Mayr et al. (29) have isolated a *Candida intermedia* aldose reductase that is apparently composed of two subunits; a dimer with a subunit molecular mass of 36 kDa.

The effects of various metal ions on the aldose reductase activity were also examined.

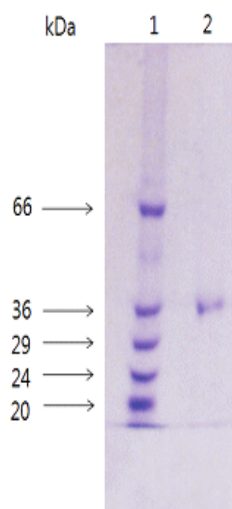


Fig. 1. SDS-polyacrylamide gel electrophoresis. Lane 1: molecular weight marker proteins containing bovine albumin (66 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa) and soybean trypsin inhibitor (20 kDa), Lane 2: purified enzyme.

Table 1. Effect of chemical modifiers on aldose reductase activity

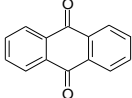
Chemical modifier	Residual activity (%)
None	100
Pyridoxal 5'-phosphate	0
N-ethylmaleimide	71
N-bromosuccinimide	114
N-acetylimidazole	28

1 mM concentrations of CuCl_2 and MgCl_2 inhibited the enzyme 100% and 94%, respectively, whereas the enzyme was not inhibited by ZnCl_2 (data not shown). To examine the amino acid residues at or near the active sites of the enzyme, the residual activity was determined after incubation with the group-specific potential inactivators.

Incubation of the enzyme with 1 mM pyridoxal 5'-phosphate led to 100% inhibition (Table 1). Even when the enzyme was incubated with 100 μM pyridoxal 5'-phosphate, the enzyme was completely inhibited. Accordingly, these results suggest that a lysine residue is involved at or near the active sites of the enzyme.

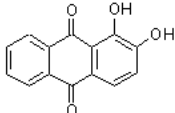
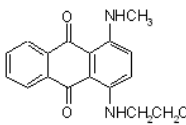
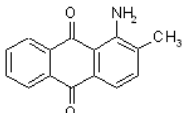
Several different electron acceptors were tested as substrates for the purified enzyme (Table 2). In addition to the variety of aldoses and aldehydes, the enzyme was also capable of reducing some dyes, for example, Alizarin, an anthraquinone dye (Table 3). Fig. 2 shows the change of absorption spectra of Alizarin treated with porcine liver aldose reductase. After aldose reductase treatment, the intensity of the local maximum absorbance, corresponding to Alizarin, was almost completely

Table 2. Substrate specificity of aldose reductase

Substrate	Structure	Relative enzyme activity (% of control)
Glucose		100
Mannose		70
Galactose		110
4-Nitrobenzaldehyde		274
Benzaldehyde		110
Heptaldehyde		58
Octyl aldehyde		3
2-Hexanone		0
2-Octanone		0
Anthraquinone		263

The reaction mixture consisted of 100 μM of the indicated substrate, 200 μM NADH, 100 mM sodium phosphate buffer (pH 7.0) and the purified enzyme.

Table 3. Activity of the purified aldose reductase toward some dyes

Dye	Structure	Relative activity (%)
Glucose		100
Alizarin		87
Azobenzene		30
4,4'-azoxyanisole		0
Disperse blue 3		38
Disperse orange 11		52

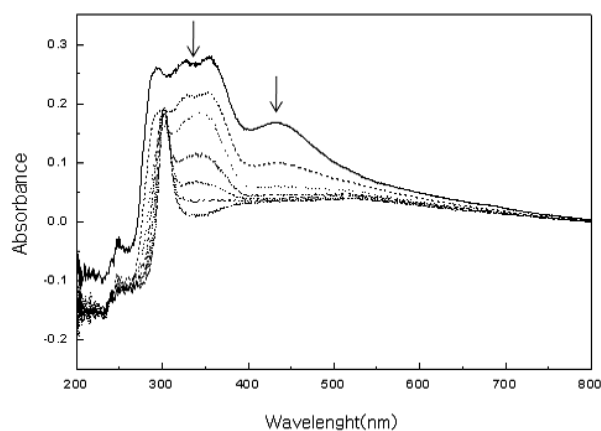


Fig. 2. Spectrophotometric analysis of decolorization of Alizarin by aldose reductase at different time intervals. —: 0 min,: 1 min,: 5 min,: 10 min,: 15 min,: 20 min,: 25 min. The decrease of the absorption band of Alizarin was indicated by the arrow.

decreased. The overall intensity in the visible range has also been decreased. This implies that the chemical structure of Alizarin is broken down by aldose reductase. Anthraquinone dyes have been used extensively in textile and dyestuff industries. Decolorization catalyzed by the enzyme is presently under investigation in our laboratory.

REFERENCES

- Azuma M, Ikeuchi T, Kiritani R, Kato J, Ooshima H. 2000. Increase in xylitol production by *Candida tropicalis* upon addition of salt. *Biom Bioener* 19: 129-135.
- Cunha JM, Jolivald CG, Ramos KM, Gregory JA, Calcutt NA, Mizisin AP. 2008. Elevated lipid peroxidation and DNA oxidation in nerve from diabetic rats: effects of aldose reductase inhibition, insulin, and neurotrophic factors. *Meta Clin Exp* 57: 873-881.
- Gross W, Seipold P, Schnarrenberger C. 1997. Characterization and purification of an aldose reductase from the acidophilic and thermophilic red alga *Galdieria sulphuraria*. *Plant Physiol* 114: 231-236.
- Neuhauser W, Haltrich D, Kulbe KD, Nidetzky B. 1997. NAD(P)H-dependent aldose reductase from the xylose-assimilating yeast *Candida tenuis*. *Biochem J* 326: 683-692.
- Stevens MJ, Dananberg J, Feldman EL, Lattimer SA, Kamijo M, Thomas TP, Shindo H, Sima AF, Greene DA. 1994. The linked roles of nitric oxide, aldose reductase and, (Na⁺,K⁺)-ATPase in the slowing of nerve conduction in the streptozotocin diabetic rat. *J Clin Invest* 94: 853-859.
- Zheng YJ, Tao Y, Zhang W, Jordan DB. 2001. Inhibition of fungal aldose reductase. *Prot Pep Lett* 8: 407-412.
- Vincent TE, Mendiratta S, May JM. 1999. Inhibition of aldose reductase in human erythrocytes by vitamin C. *Diab Res Clin Prac* 43: 1-8.
- Dunlop M. 2000. Aldose reductase and the role of the polyol pathway in diabetic nephropathy. *Kidney Inter* 58: S-3-S-12.
- Jagt DV, Kolb NS, Jagt TV, Chino J, Martinez FJ, Hunsaker LA, Royer RE. 1995. Substrate specificity of human aldose reductase: identification of 4-hydroxynonenal as an endogenous substrate. *Biochim Biophys* 1249: 117-126.
- Crabbe MC, Goode D. 1998. Aldose reductase: a window to the treatment of diabetic complications? *Prog Ret Eye Res* 17: 313-383.
- Demiot C, Tartat M, Fromy B, Abraham P, Saumet JL, Sigaudo-Roussel D. 2006. Aldose reductase pathway inhibition improved vascular and C-fiber functions, allowing for pressure-induced vasodilation restoration during severe diabetic neuropathy. *Diabetes* 55: 1478-1483.
- Lorenzi M. 2007. The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. *Exp Diab Res* 2007: 61038.
- Santos AB, Bisschops IE, Cervantes FJ, Lier JB. 2004. Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30°C) and thermophilic (55°C) treatments for decolourisation of textile wastewaters. *Chemosphere* 55: 1149-1157.
- Santos AB, Cervantes FJ, Beas RE, Lier JB. 2003. Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor. *Enz Micro Tech* 33: 942-951.
- Shen ZM, Wu D, Yang J, Yuan T, Wang WH, Jia JP. 2006. Methods to improve electrochemical treatment effect of dye wastewater. *J Hazard Mater* 131: 90-97.
- Zee FP, Bouwman RM, Strik DT, Lettinga G, Field JA. 2001. Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnol Bioeng* 75: 691-701.
- Barros AL, Pizzolato TM, Carissimi E, Schneider IH. 2006. Decolorizing dye wastewater from the agate industry with Fenton oxidation process. *Miner Engin* 19: 87-90.
- McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat IM, Marchant R, Smyth WF. 2001. Microbial decolourisation and degradation of textile dyes. *Appl Microb Biotech* 56: 81-87.
- Rodriguez E, Pickard MA, Vazquez DR. 1999. Industrial dye decolorization by Laccases from ligninolytic fungi. *Curr Microb* 38: 27-32.
- Robinson T, McMullan G, Marchant R, Nigam P. 2001. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Biore Tech* 77: 247-255.
- Zee FP, Bisschops IE, Lettinga G. 2003. Activated carbon as an electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. *Environ Sci Technol* 37: 402-408.
- Palmieri G, Cennamo G, Sannia G. 2005. Remazol brilliant blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enz Microb Technol* 36: 17-24.
- Baldrian P. 2004. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. *Appl Microbiol Biotechnol* 63: 560-563.
- Russ R, Rau J, Stolz A. 2000. The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. *Appl Environ Microbiol* 66: 1429-1434.
- Swamy J, Ramsay JA. 1999. Effects of Mn²⁺ and NH₄⁺ concentrations on laccase and manganese peroxidase production and amaranth decoloration by *Trametes versicolor*. *Appl Microbiol Biotechnol* 51: 391-396.
- Enomoto S, Okada Y, Venc AG, Erdurak CS, Coskun M, Okuyama T. 2004. Inhibitory effect of traditional turkish folk medicines on aldose reductase (AR) and hematological activity, and on AR inhibitory activity of quercetin-3-O-methyl ether isolated from *Cistus laurifolius* L. *Biol Pharm Bull* 27: 1140-1143.
- Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Mayr P, Brgler K, Kulbe KD, Nidetzky B. 2000. D-Xylose metabolism by *Candida intermedia*: isolation and characterisation of two forms of aldose reductase with different coenzyme specificities. *J Chromato B Biomed Sci Appl* 737: 195-202.