Structural and kinetic characterization of a maize aldose reductase

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Abstract

The aldo-keto reductases (AKRs) are classified as oxidoreductases and are found in organisms from prokaryotes to eukaryotes. The AKR superfamily consists of more than 120 proteins that are distributed throughout 14 families. Very few plant AKRs have been characterized and their biological functions remain largely unknown. Previous work suggests that AKRs may participate in stress tolerance by detoxifying reactive aldehyde species. In maize endosperm, the presence of an aldose reductase (AR; EC 1.1.1.21) enzyme has also been hypothesized based on the extensive metabolism of sorbitol. This manuscript identifies and characterizes an AR from maize (Zea mays L.) with features of an AR. The cDNA clone, classified as AKR4C7, was expressed as a recombinant His-tag fusion protein in Escherichia coli. The product was purified by immobilized metal affinity chromatography followed by anion exchange chromatography. Circular dichroism spectrometry and SAXS analysis indicated that the AKR4C7 protein was stable, remained folded throughout the purification process, and formed monomers of a globular shape, with a molecular envelope similar to human AR. Maize AKR4C7 could utilize α-glyceraldehyde and some pentoses as substrates. Although the maize AKR4C7 was able to convert sorbitol to glucose, the low affinity for this substrate indicated that AKR4C7 was probably a minimal contributor to sorbitol metabolism in maize seeds. Polyclonal antiserum raised against AKR4C7 recognized at least three AR-like polypeptides in maize kernels, consistent with the presence of a small gene family. Diverse functions may have evolved for maize AKRs in association with specific physiological requirements of kernel development.

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1. Introduction

Analysis of maize phloem has shown sucrose to be the form in which carbon is translocated from leaf to ear in maize plants (Zea mays L.) [1]. The first step of sucrose metabolism in the endosperm tissue of maize kernels produces fructose, and a portion of this fructose can be converted to sorbitol by the ketoreductase activity of a sorbitol dehydrogenase (SDH) enzyme [2,3]. Maize endosperm was shown to have high levels of SDH activity, thus suggesting that a significant amount of fructose could be used for sorbitol synthesis. In some Rosaceous species (e.g. Malus and Prunus) SDH is highly active in developing fruits and has a central role in the import and storage of sorbitol [4–8]. Maize kernels, on the other hand, do not accumulate sorbitol to a great extent [9]. The possibility was suggested that sorbitol could be an intermediate in starch biosynthesis if aldose reductase (AR) (EC 1.1.1.21) were present and catalyzing a sorbitol-to-glucose conversion [10]. However, neither AR nor any other sorbitol-metabolizing enzyme could be detected in earlier studies [10], so the basis of sorbitol synthesis in maize endosperm has remained equivocal for the last 20 years.

To identify potential avenues of sorbitol metabolism in maize endosperm, we searched the MAIZEST database [11] and other public databases for evidence of sequences encoding sorbitol-handling
enzymes in addition to AR, such as NADP-dependent sorbitol dehydrogenase, NADP-dependent 6-P-sorbitol dehydrogenase and sorbitol oxidase. Transcripts were identified for only an AR. In this manuscript we present the cloning, expression, and purification of such a maize AR, as well as its initial structural characterization by circular dichroism spectrometry and SAXS analyses. Structural features and enzymatic activity indicated that the maize AR clone was a new member of the AKR4C subfamily, and was designated AKR4C7. The enzymatic properties of the maize AKR4C7 were investigated to identify its putative biological targets. The recombinant maize

**Fig. 1.** Comparison of amino acid sequences of the aldose reductase protein from *Zea mays* (AKR4C7, DQ517521) to those of the same protein subfamily in *Hordeum vulgare* (AKR4C1, CAA88322), *Bromus inermis* (AKR4C2, AA21751), *Avena fatua* (AKR4C3, AAC49138), *Xerophyta viscosa* (AKR4C4, AAD22264), *Digitalis purpurea* (AKR4C5, CAC32834) and *Digitalis purpurea* 2 (AKR4C6, CAC32835). The multiple sequences were aligned using the CLUSTALW program and visualized using BOXSHADE. Letters shaded in black indicate amino acids that are identical. Conserved substitutions are shaded in gray. Secondary structure elements (E, β-sheet; H, α-helix) are indicated below the predicted structure deduced from the DQ517521 sequence (by PSIPRED program).
2. Results and discussion

2.1. Classifying maize aldose reductases

Currently, sequence identity indicates an aldo-keto reductase superfAMILY of more than 120 proteins distributed throughout 14 families (AKR1–AKR14) [12]. Members within a given family share less than 40% amino acid sequence identity with other families, but members within the same subfamily have greater than 60% sequence identity [12]. The amino acid sequence of the recombinant maize AR protein described here (DQ517521) was aligned with similar sequences from several plant species. The highest identity was seen with Avena fatua (60%) and Bromus inermis (62%), Hordeum vulgare (61%), Avena fatu (60%) and Bromus inermis (59%) (Fig. 1).

The maize AKR4C7 was also found to share a 47% sequence identity with the human ALR2 enzyme. The comparison further showed that in both enzymes one of the catalytic sub pockets, responsible for the cavity binding, was formed by side chains having a conserved Tyr48, His110 and Trp111 [13]. Crystallographic structures of the human ALR2 have also shown these active site residues to be critical for the interaction between the protein and some AR inhibitors (mainly through hydrogen bonds). Other residues to be critical for the interaction between the protein and some AR inhibitors (mainly through hydrogen bonds).

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2.2. Cloning, expression and purification of the maize AKR4C7 enzyme

PCR products of the AKR4C7 transcript were cloned in a pET28a vector and subsequently transformed into E. coli DH5α and BL21(DE3) pRIL strains. The transformed strain was tested under various conditions to optimize the expression of the recombinant protein. Protein expression was most efficient when induced for 6 h at 37°C from the pRIL promoter. Purification of the recombinant maize AKR4C7 was in the flow-through fraction. The SDSPAGE analysis indicated that the expressed protein was purified in two steps by immobilized metal affinity chromatography (Fig. 2). The amount of recovered protein was similar to that reported for other AKR members [18,19].

2.3. Structural characterization of the maize AKR4C7 enzyme

A molecular mass of 34,561 Da was determined by mass spectrometry for the recombinant maize AKR4C7 protein. This value was nearly identical to the predicted 34,561 Da calculated from the primary amino acid sequence. The maize AKR4C7 showed a molecular mass similar to, but slightly smaller than that found for the Hordeum vulgare (barley) [18], Xerophyta viscose [20], Medicago sativa (alfalfa) [21], and Digitalis purpurea [22] ARs. The peptide mass fingerprint (PMF) determined by mass spectrometry of the digested maize AKR4C7 was compared to a theoretical digestion of the protein. Nineteen different peptides were characterized from the AKR4C7 sample. The molecular masses of these peptides matched the expected peptides with a difference of less than 50 ppm. Results supported the proposed protein identity. The N-terminal sequence of this protein was found to be: MARHFVLNT-GAK, further confirming its identity.

Integrity and folding of the purified recombinant maize AKR4C7 was analyzed by CD spectroscopy. The protein presented a predominant signal from α-helices, with a maximum ellipticity at 190 nm and minimum at 208 and 222 nm (data not shown). This result was consistent with the secondary structure predicted from the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred) that estimated approximately 40% α-helices and 12% β-strands, as shown in Fig. 1. It is interesting to note that the maize AKR4C7 has a region of α-helices between amino acids 5 and 40, which seem to be absent among other ARs studied previously (Fig. 1). This could be responsible for unique properties of the maize AKR4C7. The CD spectrum indicated that the protein was stable and remained folded throughout the purification process (data not shown).

Members of the AKRs superfAMILY are typically monomeric (α/β)8-barrel proteins, but several dimers and tetramers have been reported [23,24]. The corrected experimental intensity data of the SAXS analysis of maize AKR4C7 and the corresponding GNOM curve fitting are shown in Fig. 3A. A Guinier plot of this data indicated a linear region (inset of Fig. 3A), indicating monodispersity of the protein sample in solution. The p(r) function, resulting from the GNOM fitting procedure, is shown in Fig. 3B. The maximum dimension value obtained for the maize AKR4C7 molecule was 70 Å. Guinier approximation (in the domain qRg < 1.2) and...
the p(r) function provided the same $R_g$ value: $(21.1 \pm 0.2) \text{Å}$. The resulting value for the molecular mass was 40 kDa. This value confirmed a monomeric state for this protein in solution, since this molecular mass was in close agreement with that predicted from the primary sequence: 34.56 kDa. These overall parameters and the shape of the p(r) function suggested that the protein possessed a globular shape. In addition, as shown in Fig. 3C, the scattering data of maize AKR4C7 was in close agreement with the scattering curve calculated from the crystallographic structure of human AR, therefore indicating that both proteins may have similar molecular envelopes.

2.4. Immunodetection of other putative maize AR polypeptides in maize seeds

Previous studies were unable to identify an aldose reductase activity in maize endosperm extracts [10]. To confirm expression of the AKR4C7 gene at the protein level we raised polyclonal antisera against the recombinant maize AKR4C7 protein and used it in Western blot assays. As shown in Fig. 4, the apparent size of the recombinant AKR4C7 protein was slightly larger than the expected 34 kDa, due to the presence of the histidine-tag. Both the endosperm and embryo of maize kernels presented what appeared to be at least three different polypeptides that reacted with the anti-AKR4C7 sera. The 34 kDa band most likely corresponded to the AKR4C7 protein. One of the other polypeptides was around 36 kDa and was less abundant or less reactive to the anti-AKR4C7 sera. The third polypeptide identified by the anti-AKR4C7 sera was smaller, and about 26 kDa. Although the possibility remains that these peptides arose from degradation of the AKR4C7 protein, their sizes nonetheless match those predicted by cDNAs of related maize ARs.

A search of the recently released maize genome showed the existence of multiple ARs, with three having predicted MW corresponding to the noted bands in the Western blot. Besides AKR4C7, data on http://www.tigr.org also show a clone AZM5-7279 with a predicted MW of 26,412 Da (equivalent to the smaller MW band) and a clone AZM5-1761 that has a predicted MW of 35,659 Da (the approximate size of the larger MW band). These results provide one line of evidence for the possible presence and expression of other AR genes.

The amount of each polypeptide also appeared to differ between endosperm and embryo (Fig. 4, lanes 2 and 3), consistent with potentially different roles for the AR-like polypeptides in these tissues. Multiple AR-like enzymes had also been implicated by earlier work in rice, but had not been recognized by the anti-barley-AR sera used in that study [26]. The expression of the ARs characterized thus far in rice [26] and barley [18,27] appears to be restricted to seeds. In contrast, the maize AKR4C7 is not seed-specific (data not shown). Alignment of the maize AKR4C7 protein with other plant AKR4C family members indicated a closer relationship between the maize AR and the AR proteins identified in Digitalis purpurea than to the seed-specific barley AKR4C1 (Fig. 1). One of the other maize AR-like proteins identified by the Western blot and in silico analysis may represent a maize counterpart to the seed-specific AKR4C1 gene in barley.

2.5. AKR4C7 enzyme activity

The aldo-keto reductases (AKRs) are classified as oxidoreductases and have been found in organisms from prokaryotes to
constitute the first known path for sorbitol. The observed conversion of sorbitol to glucose (sorbitol aldose reductase activity in crude extracts from soybean root tips, and recombinant maize AKR4C7 enzyme was stable with no significant loss of activity during freezing treatments and dehydration conditions. The broad substrate specificity of ARs could be correlated with seed maturation and other maize AR family members will help to clarify their role in maize seeds. If a seed-specific maize AR exists, it will be interesting to investigate whether different substrate specificities have evolved to fulfill the specific physiological requirements of the maize endosperm tissue.

3. Conclusion

The cloned, expressed and purified recombinant maize AR was classified as an AKR and designated AKR4C7. Its identity was confirmed by mass spectrometry. Correct folding of the AKR4C7 was verified by CD spectroscopy that indicated a secondary structure composed mainly of α-helices. The SAXS analyses also indicated that the maize AKR4C7 possesses a globular shape. Although the maize and mammalian AR showed similar molecular envelopes, the maize AKR4C7 activity differed in apparent direction, favoring conversion of sorbitol to glucose. The direction of this reaction in vivo would also depend on the NADP/NADPH ratio, which can show steep gradients within and between plant tissues.

4. Materials and methods

4.1. Aldose reductase cloning, expression and purification

The complete coding sequence of maize AR was PCR-amplified from an endosperm EST clone [11]. The complete sequence of this template CDNNA was deposited in NCBI together with the corresponding annotation (DQ517521). The primers 5′-gctat ggc cat ggc gcg gca ctt c-3′ (sense) and 5′-ggg att ctt act gga gtt cat ccc aga gc-3′ (antisense) were designed for cloning into the N-terminal region of pET28a. The E. coli strain BL21(DE3) pRil with the recombinant plasmid was cultured overnight at 37 °C and 300 rpm, using 30 mL LB broth, with 50 μg mL⁻¹ kanamycin and 100 μg mL⁻¹ chloramphenicol. This pre-inoculum was transferred to 1.0 L of LB broth containing kanamycin and chloramphenicol at the same concentration, and grown to an OD₆₀₀ of 0.8. At this point, protein expression was induced by adding 100 mM lactose, and incubating for 6 h at 300 rpm and 37 °C. The cells were harvested by centrifugation at 2600 × g at 4 °C for 10 min. Pellets were directly resuspended in 30 mL of affinity buffer (50 mM sodium phosphate buffer, pH 7.2), containing 100 mM NaCl and 5% glycerol. Lysozyme was added to a final concentration of 1.0 mg mL⁻¹. The suspension was incubated for 1 h at 4 °C and sonicated eight times for 15 s at 70% of maximum power in a Sonifier Misonix (Micronson Ultrasonic Cell Disruptor XL). The insoluble debris was removed by centrifugation at 27,500 × g for 15 min at 4 °C and the cleared supernatant was used for protein purification in immobilized metal affinity chromatography (IMAC).

Clariﬁed supernatant was incubated with 400 μL nickel-nitrilotriacetic acid (Ni-NTA) resin, pre-equilibrated with five column volumes (CV) of afﬁnity buffer for 1 h at 4 °C, then passed via gravity through an empty column to pack the resin. The resulting resin-packed column was washed with 5 CV of afﬁnity buffer. Afﬁnity buffer plus increasing concentrations of imidazole (5–200 mM) were used for elution. Fractions were analyzed by SDS-PAGE and also used to estimate total protein concentration.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₐ₉ (mM)</th>
<th>Kₐ₉/K₉ (s⁻¹ mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Sorbitol</td>
<td>399.5 ± 64.3</td>
<td>1.15E−04</td>
</tr>
<tr>
<td>β-Xylose</td>
<td>179.3 ± 64.27</td>
<td>2.49E−05</td>
</tr>
<tr>
<td>Glucose</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>α-Ribose</td>
<td>1081.0 ± 108</td>
<td>1.50E−04</td>
</tr>
<tr>
<td>α-Xylose</td>
<td>466.8 ± 44.1</td>
<td>3.84E−04</td>
</tr>
<tr>
<td>α-Arabinose</td>
<td>448.4 ± 45.3</td>
<td>3.99E−04</td>
</tr>
<tr>
<td>α-Glyceraldehyde</td>
<td>4.29 ± 0.084</td>
<td>4.32</td>
</tr>
</tbody>
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ND, no detectable activity.

a Substrate was oxidized.

b Substrate was reduced.

Table 1 Kinetic parameters of recombinant maize aldose reductase AKR4C7.
Fractions containing maize AR were combined and dialyzed in anion exchange buffer (20 mM Tris–HCl pH 7.5, 5 mM EDTA, 7 mM β-mercaptoethanol, and 20 mM NaCl). The final purification was done using a Q-sepharose FF anion exchange chromatography column (1 mL) (Amersham Biosciences, USA), using an AKTA-FPLC system (Amersham Biosciences, USA). The column was equilibrated with 15 CV of anion exchange buffer, loaded with the dialyzed maize AR and the flow-through proteins collected. Bound proteins were eluted using a gradient established with 1 M NaCl (0–100%) in anion exchange buffer. Both flow-through proteins and eluted fractions were used to estimate total protein concentration and to analyze purity by SDS-PAGE. Purified maize AR was quantified based on absorbance at 280 nm, using a calculated extinction coefficient of 1.824 g L⁻¹ cm⁻¹ [32].

4.2. Mass spectrometry and circular dichroism (CD) spectroscopy

Identity of the recombinant maize AR was analyzed by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Waters-Micromass, Manchester, UK). The trypsin-digested protein and intact protein were characterized using reflectron positive ion mode in the range of m/z 300–6000. The matrices used for trypsin-digested (peptides) and intact protein samples were CHCA and sinapinic acid, respectively. The m/z of monoisotopic peaks with relative intensity greater than 5% were compared to m/z obtained from a theoretical digestion of the protein by trypsin. This comparison was conducted using a Masslynx 4.0v. with Biolyx module.

Circular dichroism (CD) spectroscopy was used to assess the integrity of secondary structure of the recombinant maize AR protein. Far-UV CD spectra were generated using 200 µL of maize AR protein at 7.8 µM in 5 mM sodium phosphate buffer pH 7.0 at 20 °C. Assays were conducted using a 1.0-mm path length cuvette in a Jasco 810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The spectrum was presented as an average of four scans recorded from 190 to 250 nm, at a rate of 20 nm min⁻¹.

4.3. SAXS experiments and data analysis

The small angle X-ray scattering experiments were conducted at the D02A-SAXS2 beamline of the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, Brazil. Measurements were performed using a monochromatic X-ray beam with wavelength λ = 1.488 Å. The sample of maize AR in 10 mM Na3PO4 (50 mM NaCl) buffer (pH 7.0) was placed in a 1-mm thick sample cell with mica windows [33]. The scattering intensity curves were recorded using a two-dimensional, position-sensitive MARCCD detector. For the selected sample-to-detector distance of 851.6 mm, the magnitude of the momentum transfer vector q (q = (4πλ)/λ = sinθ, where 2θ is the scattering angle) covered a range from 0.015 Å⁻¹ to 0.3 Å⁻¹. Three successive frames of 300 s each were recorded. The scattering curves were individually normalized by the incident beam intensity and sample absorption. Additional corrections to the intensity were: subtraction of the buffer solution and detector response. The resulting curve was carefully inspected to check for possible radiation-induced damage. The sample scattering patterns were recorded at two different concentrations: 3.02 and 6.03 mg mL⁻¹ in order to check for concentration dependence. To estimate the molecular mass of the recombinant maize AR protein, the scattering intensity produced by a 5.61 mg mL⁻¹ lysozyme (14.32 kDa) solution was also obtained. From the relation: MW_movies = (I(0))_protein/(I(0))_lysozyme × MW_lysozyme, the value of the protein mass was determined using the extrapolated intensity values at the origin [34].

The scattering data of the recombinant maize AR protein was first analyzed using Guinier approximation [35–37]. The monodispersity of the protein solution was confirmed and the radius of gyration (Rg) was evaluated. The Rg was also obtained from the pair distance distribution function p(r), which was calculated using the experimental data and the indirect transformation package GNOM [38]. The p(r) function also provided the maximum dimension (Dmax) of the protein molecule [36,37]. In addition, the experimental scattering curve of the recombinant maize AR protein was compared with that calculated for the crystallographic structure of human AR (recorded under the code 1ads in Protein Data Bank, http://www.rcsb.org) using the program CRYSOL [39].

4.4. Antibody production and immunodetection

The recombinant maize AR protein (150 µg) was homogenized in phosphate-buffered saline (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2) and emulsified with 2 × Freund’s complete adjuvant. The suspension was injected subcutaneously into a female New Zealand rabbit. The subcutaneous injection was repeated twice with recombinant maize AR protein (200 and 300 µg, respectively) emulsified with 2 × Freund’s incomplete adjuvant. A booster intravenous injection of 400 µg of the recombinant protein emulsified with 2 × Freund’s incomplete adjuvant was given on day 42 and the rabbit was bled on day 50.

For Western blotting, whole maize kernels were harvested at 20 DAP, and the embryo and endosperm were dissected. The tissues were ground and homogenized in 50 mM Tris–HCl (pH 7.0) containing 1 mM DTT, at the ratio of 0.1 mg fresh weight mL⁻¹ buffer. Crude homogenates were centrifuged twice at 13,000 rpm for 15 min to pellet debris. The maize kernel proteins (10 µg) and the recombinant maize AR protein (150 ng) were loaded in a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences). The membrane was probed with antiseraum (1:5000 dilution) raised against the His-tagged AR protein followed by an incubation with Goat anti-rabbit IgG(H + L)-HRP conjugated (BioRad). The protein–antibody complex was detected by using the chemiluminescence (ECL) Western blotting detection system from GE Healthcare (Buckinghamshire, UK) according to the manufacturer’s recommendations.

4.5. Enzyme activity assays

Maize AR activity was assayed spectrophotometrically in a thermostated Hewlett-Packard 8453 spectrophotometer at 30 °C. Aldo-keto reductase activity was determined by measuring the reduction of NADP or oxidation of NADPH at 340 nm using a molar extinction coefficient of 6220 M⁻¹ cm⁻¹ and 0–20 mM of d-lyxose, d-ribose, d-arabinose, d-glucose, d-xylitol and d-sorbitol, as substrates. The reactions were assayed in 50 mM sodium phosphate at pH 7.0. The concentration of NADP for d-sorbitol and d-xylitol, and NADPH for the other substrates was kept constant at 0.25 mM in all experiments and the amount of recombinant maize AR was 10 µg for each reaction (400 µL). Blanks contained no substrate. Each assay was replicated three times.

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