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Divergent biochemical and enzymatic properties of oxalate oxidase isoforms encoded by four similar genes in rice

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ABSTRACT

The biochemical and enzymatic properties of four highly similar rice oxalate oxidase proteins (OsOxO1–4) were compared after their purification from the leaves of transgenic plants each overexpressing the respective *OsOxO1–4* genes. Although alignment of their amino acid sequences has revealed divergence mainly in the signal peptides and they catalyze the same enzymic (oxalate oxidase) reaction, divergence in apparent molecular mass, K_m, optimum pH, stability and responses to inhibitors and activators was uncovered by biochemical characterization of the purified OsOxO1–4 proteins. The apparent molecular mass of oligomer OsOxO1 was found to be similar to that of OsOxO3 but lower than the other two. The molecular mass of the subunit of OsOxO1 was lower than that of OsOxO3. The K_m value of OsOxO3 was higher than the other three which had similar K_m. OsOxO1 and OsOxO4 possessed peak activity at pH 8.5 which was close to that at the optimum pH 4.0. The activity of OsOxO2 at pH 8.5 was only 65% of that at its optimum pH 3.5, while the activity of OsOxO3 did not vary much at pH 6–9 and was also much lower than that at its optimum pH 3. OsOxO2 and OsOxO3 still maintained all their activities after being heated at 70 °C for 1 h while OsOxO1 and OsOxO4 lost about 30% of their activities. Pyruvate and oxaloacetic acid inhibited the activity of OsOxO3 more strongly than the other three. Interestingly, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-biphosphate related to photosynthetic assimilation of triose phosphate greatly increased the activities of OsOxO3 and OsOxO4. In addition to the differences in the biochemical properties of the four OsOxO proteins, an intriguing finding is that the purified OsOxO1–4 exhibited substrate inhibition, which is a typical of the classical Michaelis–Menten enzyme kinetics exhibited by a majority of other enzymes.

1. Introduction

There has been considerable interest in oxalate oxidase (OxO, EC 1.2.3.4) which catalyzes the oxidative breakdown of oxalate to H_2O_2 and CO_2. From a practical viewpoint, OxO is a useful analytical enzyme for determination of oxalate in clinical urine and blood samples as oxalate is a risk factor for kidney stone formation (Berckmans and Boer, 1988; Ladwig et al., 2005). OxOs from different plants are of particular interest in studies on their enzymatic and biochemical properties related to this application.

In plants, OxO appears to play important roles in several developmental processes. Wheat germin, a protein marker for the onset of wheat seed germination, has OxO activity (Lane et al., 1993). The germins of other cereals, for example, germinating barley (Dumas et al., 1993), corn (Vuletić and Šukalović, 2000), rice, oat and rye (Lane, 2000) have also been found to possess OxO activity. During wheat seed germination, OxO activity was first observed in the coleorhiza, epiblast and scutellum, then root and coleoptile (Caliskan and Cuming, 1998). Likewise, OxO activity was also found to exhibit differential temporal and spatial distributions in developing wheat grains (Lane, 2000). In ryegrass, OxO activity increased dramatically during ageing of leaf sheaths (Piquery et al., 2000; Davoine et al., 2001).

Many proteins have been shown to share sequence similarity with wheat germin and are known as germin-like proteins (Dunwell, 1998). In plants, barley OxO has been one of the most extensively characterized germins (Suguira et al., 1979; Kotsira and Clonis, 1997, 1998; Requena and Bornemann, 1999) and shown to have *ca* 96% identity at the amino acid sequence level with a wheat germin (Dumas et al., 1993; Lane et al., 1993). Purification and characterization of many germin-like proteins have yet

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to be carried out, and it is not known if they possess OxO activity or not. Expression of most germins and germin-like proteins are induced by pathogenic and environmental factors (Davidson et al., 2009). Transcription of a germin-like oxalate oxidase gene in barley and wheat leaves was shown to increase following powdery mildew attack (Zhou et al., 1998; Hurkman and Tanaka, 1996a). In addition, expression of the OxO gene from wheat or barley in several transgenic plants improved defense against oxalic acid secreted by *Sclerotinia sclerotiorum* (Hu et al., 2003; Livingstone et al., 2005; Dong et al., 2008; Zhang et al., 2013a). In rice, a member of the OxO gene family, *OsOxO4* could be induced by *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *Oryzae* (Carrillo et al., 2009). OxO gene expression and/or OxO activation could also be increased by abiotic stresses including heat (Valentovičová et al., 2009), salinity (Hurkman and Tanaka, 1996b) and heavy metal ions (Berna and Bernier, 1999; Valentovičová et al., 2009). All of these studies support a role for OxO in plant response to abiotic and biotic stress.

In the rice genome, there is a family of four *OsOxO1–4* genes tandemly located in chromosome 3. *OsOxO1–4* share >90% nucleotide sequence identity but each gene has its own distinct regulatory sequences as well as temporal and spatial patterns of expression (Carrillo et al., 2009). OsOxO1 shares 89%, 91% and 90% amino acid sequence identity with OsOxO2, OsOxO3, OsOxO4, respectively. The amino acid sequence identity between OsOxO2 and OsOxO4 is up to 98%, 97% between OsOxO3 and OsOxO4, 96% between OsOxO2 and OsOxO3. Divergences in amino acid sequences of the OsOxO1–4 proteins have been found mainly in the deduced signal peptides (Fig. 1). Apart from the highly similar amino acid sequences of the four OsOxO proteins, there is a paucity of information about their enzymatic, biochemical and regulatory properties. Only OsOxO1 and OsOxO4 have been unambiguously shown to possess OxO activity (Molla et al., 2013; Zhang et al., 2013b).

The objective of this study was to purify individual OsOxO1–4 for a comparative study at the level of enzyme (OxO) properties and regulation. This has not been done before.

2. Results and discussion

2.1. Isolation and purification of OsOxO1–4 and their molecular masses

OsOxO1–4 were purified one at a time in two steps from the leaves of transgenic rice plants over-expressing the respective genes. This was a successful strategy to a large extent as no OxO activity was detected in the leaves of the wild type plants (*Oryza sativa* L. subsp. Japonica Kato, Zhonghua 11) using the method of Zhang et al. (1996). Since the OsOxOs are relatively heat stable, the OxO purification method was coupled with a thermal treatment of the leaf extract at 70 °C for 45 min to precipitate a majority of the cellular proteins before the isolation of the respective OsOxOs using Sephadex G-25 chromatography. SDS–PAGE analysis of the individual purified OsOxO1–4 gave only one band for each of the four OsOxOs (Fig. 2A), suggesting that they had been purified to homogeneity. The apparent molecular masses of the subunits of the four OsOxOs estimated from SDS–PAGE analysis (Fig. 2A) were similar (ranging from 25.9 to 27.4 kDa), but were higher than those calculated based on the amino acid sequences of the respective OsOxO1–4 (ranging from 24.1 to 24.3 kDa) (Table 1), suggesting that posttranslational modifications occurred in OsOxO1–4. Staining after SDS–PAGE with the Schiff's reagent (Fig. 2B) indicated that OsOxO1–4 were glycoproteins. This is consistent with the predictions from the amino acid sequences of the respective OsOxOs that there are two possible N-glycosylation sites in each of the OsOxOs (Fig. 1). The OxOs of rice, barley root, wheat embryo and the *Ceriporiopsis subvermispora* OxO expressed in *Pichia pastoris* are also glycoproteins (Dumas et al., 1993; Lane et al., 1992; Moussatche et al., 2011). It is, however, not clear why the estimated molecular mass of the OsOxO1 subunit was lower by about 5% than those of OsOxO2–4. This may be due to the glycan of OsOxO1 being different from the other three OsOxOs which had different residue in site 84 near the predicted glycosylation sites compared with OsOxO1 (Fig. 1). CN-PAGE analysis (Fig. 2C) established that the molecular masses of native OsOxO1–4 were 235.1, 287.0, 222.7 and 295.7 kDa (Table 1), respectively, indicating that OsOxO3 was a homo-octamer and the other three were homo-decamer enzymes.

2.2. Kinetic properties of purified OsOxO1–4

The substrate specificity of the purified OsOxO1–4 was investigated using 0.4 mM oxalate and a range of related carboxylic acids at optimum pH. All four OsOxOs showed high activity with oxalate, but there was no detectable activity against other related organic acids including citrate, glyoxylate, malonate, succinate, glutarate, malate, glycolate, acetate, lactate and formate. With respect to oxalate as the substrate, the kinetic reaction of the four different OsOxOs showed that the product formation curves rose to a maximum and then declined as the substrate concentration was

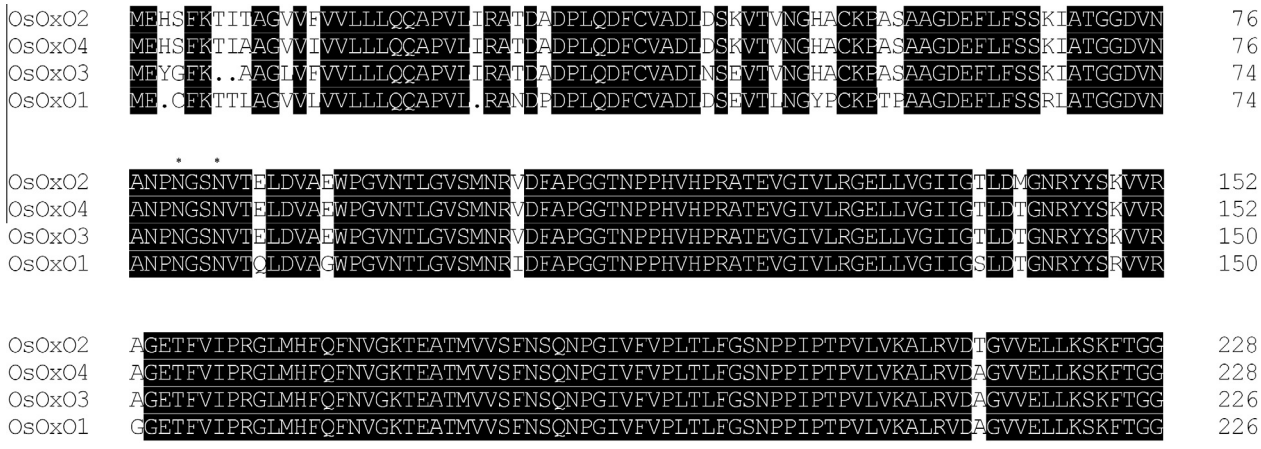

Fig. 1. Amino acid sequence alignment of OsOxO1–4. Grey letters with black background indicate 100% match; grey letters with white background indicate dissimilarity; and dots indicate no amino acid in these positions, * indicates putative N-glycosylation sites.

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