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Inactivation of barley limit dextrinase inhibitor by thioredoxin-catalysed disulfide reduction

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ABSTRACT

Barley limit dextrinase (LD) that catalyses hydrolysis of α-1,6 glucosidic linkages in starch-derived dextrins is inhibited by limit dextrinase inhibitor (LDI) found in mature seeds. LDI belongs to the chloroform/methanol soluble protein family (CM-protein family) and has four disulfide bridges and one glutathionylated cysteine. Here, thioredoxin is shown to progressively reduce disulfide bonds in LDI accompanied by loss of activity. A preferential reduction of the glutathionylated cysteine, as indicated by thiol quantification and molecular mass analysis using electrospray ionisation mass spectrometry, was not related to LDI inactivation. LDI reduction is proposed to cause conformational destabilisation leading to loss of function.

1. Introduction

Barley limit dextrinase (LD) is one of the few enzymes of glycoside hydrolase family 13 that are regulated by a proteinaceous inhibitor [1]. LD is the sole enzyme involved in starch debranching during seed germination and has high activity towards α-1,6 glucosidic linkages in α-limit dextrins and pullulan [2,3] albeit very low activity on amylopectin [4]. Germinating seeds contain both active LD and LD inactivated in complex with the tight-binding endogenous limit dextrinase inhibitor (LDI) [5,6]. LDI belongs to a family of small (approx. 12 kDa) proteins with 4–5 disulfide bonds called CM-proteins (chloroform/methanol soluble proteins) or cereal-type inhibitors, including inhibitors of bacterial, fungal, and insect serine proteases and α-amylases [7]. LDI is a monomer having four disulfide bridges and one cysteine residue disulfide-linked to glutathione or cysteine (Fig. 1) [8]. LDI is stable in a remarkably broad pH range and at exceptionally high temperatures [9]. The LDI gene is only expressed during seed development and from about two days after the onset of germination the LDI protein level decreases concomitant with an increase of LD activity [5,10]. The loss of LDI has been proposed to stem from degradation by cysteine proteases appearing at germination [5,11] and also from disulfide reduction by thioredoxin (Trx) [12]. Thus overexpression of wheat Trxh in endosperm of germinating barley led to fourfold increase of LD activity [13].

Trx reduces target protein disulfide bonds with concomitant oxidation of its active site CXXC motif [14]. Regeneration of reduced Trx is coupled to electron transfer *via* NADPH-dependent thioredoxin reductase (NTR). In cereal seeds, reduction of disulfides in proteinaceous hydrolase inhibitors by h-type Trx was proposed to facilitate starch mobilisation [15]. Structural and biochemical properties of barley (*Hordeum vulgare*) Trxh (HvTrxh) have been characterised [16–20] and a large number of potential target proteins were identified using proteomics approaches, including α-amylase inhibitors of the CM-protein family [21–24]. No correlation, however, of the extent of disulfide reduction and loss of inhibitor activity has been reported. Breakthroughs in production of recombinant LDI [9] and LD [25] now made detailed investigation possible of HvTrxh-mediated disulfide reduction and its impact on the activity of LDI.

Abbreviations: CM-protein, chloroform/methanol soluble protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ESI MS, electrospray ionisation mass spectrometry; GSH, glutathione; HvNTR, barley NADPH-dependent thioredoxin reductase; HvTrxh, barley thioredoxin h; IAM, iodoacetamide; LD, limit dextrinase; LDI, limit dextrinase inhibitor; LMW, low molecular weight; nanoES, nano-electrospray; Trx, thioredoxin

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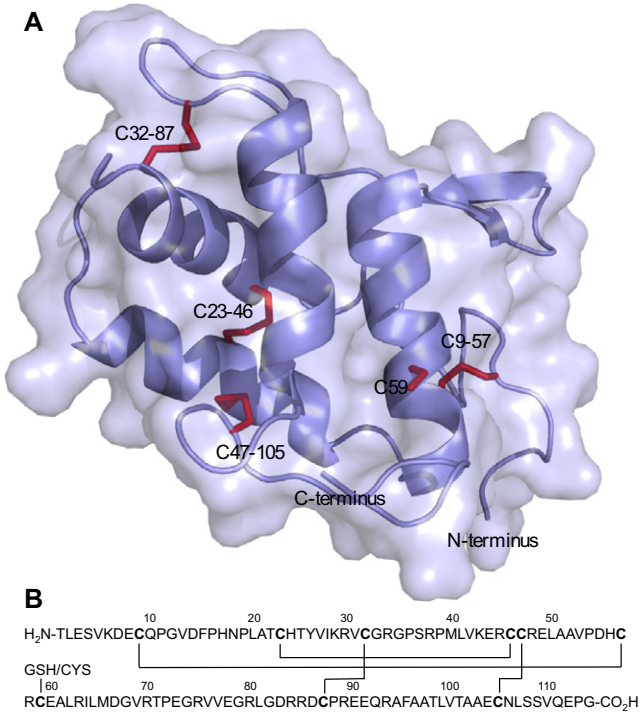

Fig. 1. (A) Homology model of LDI based on the crystal structure of the bifunctional α-amylase/trypsin inhibitor from ragi seeds (PDB ID: 1B1U). (B) Predicted cysteine connectivity based on the structure of the inhibitor from ragi.

2. Materials and methods

2.1. Proteins

Recombinant LD [25] and C-terminally His_6-tagged LDI [9] were produced in *Pichia pastoris*. N-terminally His_6-tagged HvTrxh1, HvTrxh2, and HvNTR2 were produced in *Escherichia coli* [17,19]. Protein concentrations were determined spectrophotometrically at 280 nm using molar extinction coefficients determined by aid of amino acid analysis [9,17,19,25].

2.2. Analysis of effect of HvTrxh on the LDI and LD activities

HvTrxh1 or HvTrxh2 (4 μM) reacted (at RT) with 1 μM LD, 20 μM LDI, or a mixture of 1 μM LD and 1 μM LDI – forming the LD/LDI complex [9] – in 0.1 μM HvNTR2, 4 mM NADPH, 6 mM EDTA, 100 mM Tris–HCl, pH 8.0 (final volume 50 μl) for 1 h (LDI also 3 and 9 h). In parallel, 1 μM LD, 20 μM LDI, or 1 μM LD/LDI were treated with 10 mM DTT, 100 mM Tris–HCl, pH 8.0 for 2 h (RT). LD-containing reaction mixtures were diluted to 20 nM LD; LDI samples were incubated with 20 nM LD at 1:1 M ratio for 15 min. LD activity was measured towards pullulan essentially as described [25]. Briefly, one Limit-Dextrizyme substrate tablet (Megazyme, Bray, Ireland) was added to 20 nM LD (500 μl) in 100 mM sodium acetate, pH 5.5, 0.005% (v/v) Triton X-100 after 15 min pre-equilibration at 40 °C. The reaction (10 min) was stopped by 5 ml 1% (w/v) Tris base. The suspension was left 10 min (RT), centrifuged (2 ml, 20 000*g*, RT, 10 min), and the absorbance (590 nm) was measured and correlated to a standard curve provided by the manufacturer. One activity unit (U) is defined as the amount of LD that releases one μmole of glucose reducing equivalents per min from pullulan [1]. One LDI inhibitor activity unit is defined as the change in LD U per μg LDI (ΔU/μg LDI) [26] and is reported as percentage of inhibition. Noticeably, at LD/LDI of molar ratio 1:1 the Limit-Dextrizyme method does not show 100% inhibition, probably due to LDI binding to insoluble substrate – as seen for other CM-proteins [27]. The apparent <100% inhibition is not due to loss of LDI functionality as complete inhibition was observed in reducing sugar analysis of soluble pullulan hydrolysed by LD [9]. A reducing sugar assay, however, is incompatible with conditions used in studies of disulfide reduction.

2.3. DTNB analysis of HvTrxh-catalysed LDI thiol release

HvTrxh1 or HvTrxh2 (4 μM) reduction of LDI (20 μM) in 0.1 μM HvNTR2, 4 mM NADPH, 6 mM EDTA, 100 mM Tris–HCl, pH 8.0 (500 μl) at RT in the dark was monitored by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to quantify thiol group formation [28]. Aliquots (50 μl) were removed at time intervals and mixed with 6 M guanidine hydrochloride, 100 mM Tris–HCl, pH 8.0, 0.4 mM DTNB (50 μl) in a 16 × 24 microtiter plate. The absorbance at 412 nm was measured after approximately 5 min (ELISA reader; BioTek, Winooski, VT) and thiol groups were quantified using *N*-acetyl-L-cysteine (0–350 μM) as standard. Controls containing LDI or HvTrxh1/HvTrxh2 and HvNTR2 were used to correct for thiols in the proteins and indicated on average 3.7 and 2.6 free thiol groups per molecule of HvTrxh1 and HvTrxh2, respectively, while LDI gave no free thiols.

2.4. Reduction of LDI by DTT

LDI (200 μM) was incubated (at RT) in 200 mM DTT, 100 mM Tris–HCl, pH 8.0 (110 μl) for 60 min followed by removal of DTT (100 μl) using NAP™-5 columns (GE Healthcare, Uppsala, Sweden) equilibrated with 100 mM Tris–HCl, pH 8.0 (500 μl). Thiol analysis in the eluate using DTNB (as above) and controls of either 200 mM DTT or 200 μM LDI confirmed DTT removal and LDI recovery.

2.5. HvTrxh-mediated release of low molecular weight (LMW) thiol from LDI

HvTrxh (4 μM), 20 μM LDI, 0.1 μM HvNTR2, 4 mM NADPH, 6 mM EDTA in 100 mM Tris–HCl, pH 8.0 (600 μl) reacted (at RT) in the dark and aliquots (80 μl) were mixed at time intervals with 6 M guanidine hydrochloride, 100 mM Tris–HCl, pH 8.0 (80 μl).

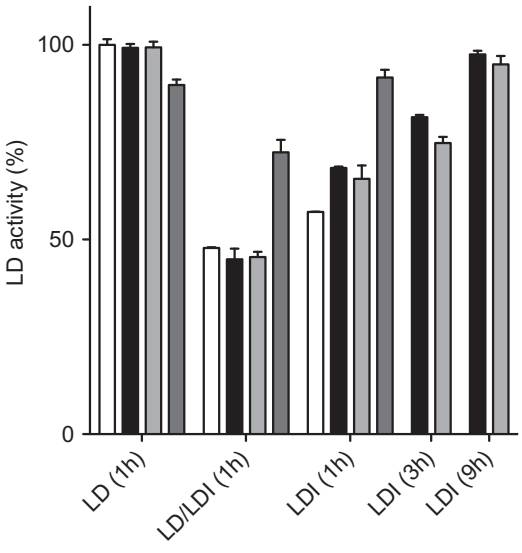

Fig. 2. Percentage LD activity after treatment of LD, LDI, and the LD/LDI complex with HvTrxh1 (black), HvTrxh2 (gray), or DTT (dark gray); control (white) without HvTrxh1/HvTrxh2/DTT (see text for details). Error bars indicate SD in duplicate experiments.