Noncanonical interactions between serpin and β-amylase in barley grain improve β-amylase activity in vitro

Maja Cohen | Robert Fluhr

Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel

Correspondence
Robert Fluhr, Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel.
Email: Robert.Fluhr@weizmann.ac.il

Funding information
US-Israel Binational Agricultural Research and Development Fund for Research IS-4915-16.

Abstract
Serpin protease inhibitors and β-amylase starch hydrolases are very abundant seed proteins in the endosperm of grasses. β-amylase is a crucial enzyme in the beer industry providing maltose for fermenting yeast. In animals and plants, inhibitory serpins form covalent linkages that inactivate their cognate proteases. Additionally, in animals, noninhibitory functions for serpins are observed such as metabolite carriers and chaperones. The function of serpins in seeds has yet to be unveiled. In developing endosperm, serpin Z4 and β-amylase showed similar in vivo spatio-temporal accumulation properties and colocalize in the cytosol of transformed tobacco leaves. A molecular interaction between recombinant proteins of serpin Z4 and β-amylase was revealed by surface plasmon resonance and microscale thermophoresis yielding a dissociation constant of 10^{-7} M. Importantly, the addition of serpin Z4 significantly changes β-amylase enzymatic properties by increasing its maximal catalytic velocity. The presence of serpin Z4 stabilizes β-amylase activity during heat treatment without affecting its critical denaturing temperature. Oxidative stress, simulated by the addition of CuCl_{2}, leads to the formation of high molecular weight polymers of β-amylase similar to those detected in vivo. The polymers were cross-linked through disulfide bonds, the formation of which was repressed when serpin Z4 was present. The results suggest an unprecedented function for a plant seed serpin as a β-amylase-specific chaperone-like partner that could optimize β-amylase activity upon germination. This report is the first to describe a noninhibitory function for a serpin in plants.

KEYWORDS
amylose, barley, enzyme stability, Hordeum vulgare, oxidative stress, protein interaction, serpin

INTRODUCTION

Serpin proteins are part of a well-conserved superfamily of proteins in plants and animals. All serpin types share a similar secondary structure, although their specific amino acids sequence can differ. Serpins are metastable proteins and undergo conformational changes, from "stressed" to "relaxed" transition to complete their various functions (Huntington, Read, & Carrell, 2000). They are mostly known as serine protease inhibitors in animal cells (Gettins, 2002) and as cysteine protease inhibitors controlling cell death in plant cells (Fluhr, Lampl, & Roberts, 2012; Lampl, Alkan, Davydov, & Fluhr, 2013; Lampl et al., 2010; Lema Asqui et al., 2017; Vercammen et al., 2006). The secondary structure of a serpin includes a reactive center loop (RCL) domain that acts as bait for its cognate protease
by mimicking the preferred P1-P′ digestion site (Khan et al., 2011). Other functions for serpins were discovered in animal systems. The noninhibitory serpins do not exhibit protease inhibitory properties and instead have a panel of activities including: hormone transport by the serpin thyroxine-binding globulin (Pemberton, Stein, Pepys, Potter, & Carrell, 1988); chaperone-like functions by SERPINH1 binding to collagen (Sauk, Nikitakis, & Siavash, 2005) and as storage proteins for ovalbumin, in egg white (Stein et al., 1990). In contrast, the myeloid and erythroid nuclear termination (MENT) serpin retains protease inhibition but also functions in chromatin remodeling (Irving et al., 2002). In plants, noninhibitory functions for serpins have yet to be characterized.

Serpins are present in seeds of cereals such as barley, wheat, oat, and rye (Hejgaard, 2001; Hejgaard & Hauge, 2002; Østergaard, Finnie, Laugesen, Roepstorff, & Svensson, 2004; Rasmussen, Dahl, Nørgård, & Hejgaard, 1996; Rosenkrands, Hejgaard, Rasmussen, & Bjørn, 1994). In barley grains, the two major types, serpin Z4 and serpin Z7, represent up to 5% of the total seed proteome (Evans & Hejgaard, 1999). The amounts of the two proteins vary among the various cultivars (Evans & Hejgaard, 1999; Finnie et al., 2004). In wheat and oat, the RCL sequence of the grain serpins contains glutamines, a very abundant amino acid in storage proteins (Østergaard, Rasmussen, Roberts, & Hejgaard, 2000). Therefore, it was suggested that serpins could protect grain storage proteins by inhibiting predator’s proteases adapted to the proteolysis of glutenin-rich substrates. However in barley, the RCL sequence of serpin Z4 and Z7 is not similar to any of the main storage protein sequences, and, although both serpins are protease inhibitors of cathepsin G and trypsin, their inhibitory activity appears to be less potent than other plant serpins (Dahl, Rasmussen, & Hejgaard, 1996). Alternatively, considering their abundance in the endosperm, serpins were suggested to have a role as storage proteins. However, serpins in barley grains are relatively resistant to degradation by proteases during germination. Indeed, the level of the soluble serpin continues to increase 2 days after seed imbibition (Bønsager, Finnie, Roepstorff, & Svensson, 2007). Hence, the function of barley grains serpins remains an open question.

In seeds, serpins exist as two distinct forms, the free serpins, extractable with water-based buffer and the bound fraction of serpins, extractable only with thiol enriched buffers (Evans & Hejgaard, 1999). β-amylase, another very abundant endosperm protein, also exists in these two forms (Evans, Wallace, Lance, & MacLeod, 1997; Evans, MacLeod, et al., 1997). The ratio between bound and free β-amylase can be modulated by the presence of a cysteine at position 115 (Li et al., 2002). This position is also critical for conferring distinct IEF band patterns (Ma, Langridge, Logue, & Evans, 2002) and protein thermostability (Ma, Evans, Logue, & Langridge, 2001; Ma, Stewart, et al., 2000). β-amylase accumulates as a water-insoluble protein during grain ripening but during germination or malting becomes readily extractable (Finnie, Melchior, Roepstorff, & Svensson, 2002; Finnie et al., 2004). It is thought that cysteines play a key role in these changes. For example during germination, the C-terminal region of β-amylase that contains a cysteine is removed by limited proteolysis (Guerin, Lance, & Wallace, 1992; Lundgard & Svensson, 1986). In addition, two cysteines are targeted and reduced by thioredoxins (Hagglund et al., 2010). The combined action of proteases and reducing agent released by the aleurone layer convert the bound form of β-amylase in the endosperm to its free form. Serpin and β-amylase are both found in the endosperm tissue (Finnie & Svensson, 2003; Roberts, Marttila, Rasmussen, & Hejgaard, 2003) adhering to the border of starch granules (Borén, Larsson, Falk, & Jansson, 2004; Hara-Nishimura, Nishimura, & Daussant, 1986; Wang et al., 2011). Upon gel filtration of salt extract from barley dry grain, serpin and β-amylase eluted in high molecular weight fractions prompting speculation of their tight association (Hejgaard & Carlsen, 1977).

Although the exact function of β-amylase in natural seed physiology is not known, it is a critical enzyme in the beer industry; it produces highly fermentable maltose disaccharides through the hydrolysis of the α-1,4-glycosidic bond of polysaccharide chains (MacGregor, Bazin, Macri, & Babb, 1999). Moreover, as one of the most abundant barley protein and as one of the only residual beer proteins, serpin has been investigated for its impact on beer quality (Iinure, Nankaku, Kihara, Yamada, & Sato, 2012; Iinure & Sato, 2013; Specker, Niessen, & Vogel, 2014). In the results presented here, we show that serpin and β-amylase interact in a quantifiable manner. The interaction enhances β-amylase enzymatic properties and increases β-amylase stability to heat and oxidative stress. This research suggests an unprecedented noncanonical function for serpin in plants as a β-amylase-specific molecular chaperone.

2 | MATERIALS AND METHODS

2.1 | Plant material

Barley cv. Harrington was grown in the greenhouse under 24/20°C (day/night) at natural day length conditions. For sampling of developing grain, flowering kernels were marked and sampled at the indicated times. For germination experiments, barley grains were imbibed in water for 24 h and placed on wet filter paper at 22°C under natural day length conditions. The grains were sampled at the indicated time without the coleoptile and the radicle.

2.2 | Gel fractionation and immunoblots

Grains at different growth stages were ground in liquid nitrogen. Tissue (100 mg) was dissolved in 1 ml of Dulbecco’s phosphate-buffered saline (DPBS without calcium and magnesium) and plant protease inhibitor cocktail (Sigma P9599) with or without 20 mM DTT. The extracted tissue was left shaking for 15 min at room temperature and centrifuged for 15 min at 17,000 g at 4°C. Gel fractionation on denaturing or non-denaturing gels was as described (Lampl et al., 2010). The membrane was developed with β-amylase antibodies (1:1000) and secondary anti-rabbit horseradish peroxidase (1:3000) or with serpin Z4 antibodies (1:1000), and secondary anti-rabbit horseradish peroxidase (1:3000). For purified proteins, the proteins were first dialyzed in PBS and then mixed with reducing or
oxidizing buffers, left at room temperature for 10 min, and then fractionated onto 10% SDS-PAGE. Purified serpin Z4 was used to generate antibodies from rat serum. Antibodies against β-amylose were purchased from antibody-online (cat # ABIN285322).

2.3 Cloning and transient expression in Nicotiana benthamiana

Serpino Z4 and β-amylose were cloned from cDNA of barley cv. Harrington. The serpin and the amylose were fused to an RFP on the N terminus and a GFP on the C-terminus, respectively, under a 35S promoter in vector pART27 and introduced into the plant transformation vector pML-BART (Pogorelko, Fursova, Ogarkova, & Tarasov, 2007). The primers used for the cloning are shown in Table S1. The plasmid was expressed in Agrobacterium GV301 and infiltrated into 1-month-old leaves of N. benthamiana. All images were taken with a model no. A1 Confocal Microscope (Nikon, Melville, NY) GFP emission/excitation 525/488 and RFP emission/excitation 595/561.

2.4 Protein expression and purification

A construct encoding β-amylose (WT and mutants) or serpin Z4 isolated from barley cv. Harrington was cloned into the pET28-(a) vector (Novagen) with a C-terminal hexahistidine tag. The primers used for the cloning are shown in Table S1. Expression was induced in Escherichia coli SHuffle cells (Lobstein et al., 2012) grown to an OD600 of 0.6–0.7 at 28°C then supplied with 1 mM isopropyl-β-d-thiogalactoside and cultured for 16 h at 16°C. Cells were resuspended in lysis buffer (0.2 M NaH2PO4, 500 mM NaCl, 10 mM imidazole, 1 mM DTT, or CuCl2 and left at room temperature for 10 min. Results were analyzed by linear regression software provided by Thermo Fisher Scientific.

2.5 Chymotrypsin inhibition assay

Peptidase activity of 70 ng of chymotrypsin (Sigma C4129) was tested on casein substrate with serpin BsZx or Z4 at a molar ratio of 2:1, serpin:protease. Proteolytic activity was measured using Pierce™ Fluorescent Protease Assay Kit (cat# 23266).

2.6 Biacore and Microscale thermophoresis assays

Purified proteins (serpin Z4 and β-amylose) were first immobilized via amine coupling to the free carboxyl groups on the CM5 chip surface following standard BIAcore protocols. Next, the reciprocal protein (either serpin Z4 or β-amylose) was injected at various concentrations to generate kinetics sensorsgrams. The experiments were carried out in PBS buffer on a Biacore T200 instrument (GE Healthcare Life Sciences).

Microscale thermophoresis analysis was performed on a NanoTemper Monolith NT.115 instrument (NanoTemper Technologies). β-amylose and serpin Z4 were fluorescently labeled with a NHS-Ester Fluorescent Dye. Labeled β-amylose (70 nM) was mixed with varying concentration of unlabeled serpin Z4 (40 µM to 1 nM) and 70 nM of labeled serpin Z4 with 16 µM to 0.5 nM of unlabeled β-amylose. Each solution was transferred into a standard glass capillary soon after mixing and immediately analyzed using settings of 20% power and 50% excitation power. For each set of binding experiments, three independent MST measurements were carried out. Data analysis was performed by the Nanotemper analysis software.

2.7 β-amylose activity assays

Purified β-amylose was first dialyzed in PBS to remove DTT using centrifugal filter (Amicon ultra, millipore). β-amylose (2 or 2.5 pmol) in 10 µl PBS was mixed with 20 µl of 2% starch in 25 mM NaAc pH 4.8 and incubated for 10 min at 25°C. The reaction was terminated by the addition of 30 µl of 3,5-dinitrosalicylic acid (DNS) solution (Miller, 1959) then boiled for 5 min. After cooling, 250 µl of distilled water was added, and the absorbance was measured at 540 nm (microplate reader TECAN Infinite 200) with maltose as the standard. Amylose activity is expressed in nmol maltose per minutes. For additives, 2 or 2.5 pmol of β-amylose in 5 µl was mixed with 5 µl of 2× solution containing either serpin Z4, DTT, or CuCl2 and left at room temperature for 10 min. Results were analyzed by linear regression software performed by GraphPad Prism software. The specific measurement of β-amylose activity in grains was measured using a megazyme® β-amylose assay kit (Betamyl-3; Megazyme, Australia) following the manufacturer’s instructions.

2.8 Thermal shift assay

β-amylose (5-0.5 µM) in 2.5 µl was mixed with 2.5 µl of a 1:5 molar ratio of serpin Z4. PBS buffer (10 µl) and 5 µl of 4× sypro-orange solution (S5692; Sigma) were added to the protein and inserted into a real-time polymerase chain reaction device (StepOne™ Real-time PCR System) using 96-well plate in four replicates. The settings were as follows: Starting temperature was 25°C, and the fluorescence data were collected every 2°C until 99°C. The results were analyzed using protein thermal shift software provided by Thermo Fisher Scientific.

3 RESULTS

3.1 Serpin and β-amylose coexpress during development and colocalize to the cytoplasm

Specific antibodies were used to concurrently analyze by immunoblot the accumulation of serpin and β-amylose during grain
development and germination in barley cv. Harrington. In developing grain, under fractionation in reducing conditions (1,4-dithiothreitol, DTT), polypeptides of approximately 40 and 60 kDa for serpin Z4 and β-amylase (Figure 1a and b lower blots) were detected, respectively. A gradual increase in both proteins was observed during the first 30 days followed by a plateau phase lasting until the grain was dry. In nonreducing conditions, in which pre-existing disulfide bonds are maintained, the monomeric forms of both serpin Z4 and β-amylase (Figure 1a and b upper blots) accumulated from 8 DAP to 28 DAP. They decreased from 28 DAP to the stage of “dry grains” while higher molecular weight forms of these polypeptides accumulated (100–200 kDa). Enzymatic activity of β-amylase was determined in the free (water soluble) and total (thiol extractable) fraction of the developing grains and is represented by the ratio of free β-amylase activity over total β-amylase activity. A decrease in the free/total β-amylase activity was recorded during grain development that parallels the accumulation of bound β-amylase and the appearance of higher molecular weight complexes.

In a similar manner, the content of serpin and β-amylase was investigated during germination. Under reducing conditions (Figure 1d and e; lower blots), the full-length protein and a processed form are observed. The processed size of serpin Z4 corresponds to the size of a spent serpin that has interacted with an unknown protease at the RCL (Gettins, 2002). The size of the processed β-amylase (55 kDa) coincides with the size of β-amylase after partial proteolysis of the C-terminal portion. Indeed, proteases released from the aleurone layer are known to target the β-amylase C-terminus (Guerin et al., 1992; Lundgard & Svensson, 1986). Fractionation of proteins without reducing agent show that the 200 kDa size migrating polypeptides disappeared between 5 and 24 HAI (Figure 1d and e; upper blots). The disappearance of high molecular weight proteins is consistent with reports of increasing accumulation of thioredoxin reductive activity (Hagglund et al., 2010; Lundgard & Svensson, 1986). In a reciprocal manner to what was observed in developing seeds, the ratio of free over total enzymatic activity of β-amylase increased in parallel with the disappearance of higher molecular weight forms (Figure 1f).

**FIGURE 1** Accumulation of β-amylase and serpin Z4 during grain maturation and germination. Each Western blot was replicated at least three times using independent biological replicates. (a–c) Proteins were extracted from developing grains of barley cv. Harrington with either PBS (upper blots) or PBS with 20 mM DTT (lower blots). In a and b, the proteins were fractionated on a nonreducing SDS-PAGE gel transferred to a PVDF membrane and blotted against serpin Z4 in a or against β-amylase in b. Lanes are days after pollination (DAP) Lanes 1–8; 8, 13, 18, 22, 28, 32, 39, dry grains. (c) β-amylase activity was assayed on the same samples using the Betamyl-3 method, shown is the ratio of free β-amylase activity over total β-amylase activity. (d–f) Proteins were extracted from 100 mg of germinating grains of barley cv. Harrington with 1 ml of either PBS (upper blots) or PBS with 20 mM DTT (lower blots). Lanes are hour after imbibition (HAI) from left to right 1–6; 0, 5, 24, 30, 48, and 120 HAI. (d and e) Proteins were loaded on a nonreducing SDS-PAGE gel transferred to a PVDF membrane and blotted against serpin Z4 in d and against β-amylase in e, f. β-amylase activity was assayed on the same grain samples as carried out in (c). The sizes of the proteins are indicated in kDaltons (kDa). The asterisk indicates the monomer size of serpin and β-amylase, and the arrow indicates high molecular weight aggregates of the proteins.
The results underscore correlation between the accumulation patterns of serpin Z4 and β-amylase during seed development and germination. Therefore, it was of interest to examine their cellular localization as well. Plant serpins are known to reside either in the cytoplasm or are secreted to extracellular spaces (Fluhr et al., 2012), while β-amylase can localize to the chloroplast (amyloplast) or cytoplasm in Arabidopsis leaves (Mita, Suzuki-Fujii, & Nakamura, 1995; Sparla, Costa, Schiavo, Pupillo, & Trost, 2006). Agrobacterium harboring constructs with serpin Z4 fused to RFP and β-amylase fused to GFP were coinfiltrated into N. benthamiana plants. To verify the reliability of this assay, immunoblot analysis showed that the expressed protein sizes were commiserated with their expected fused size (Fig. S1). As shown in Figure 2, the confocal images of both serpin Z4 and β-amylase colocalized to the cytoplasm surrounding the chloroplasts of spongy mesophyll (Figure 2a). They are also expressed in the cytoplasmic peripheral regions of leaf epidermal cells (Figure 2b).

3.2 Serpin Z4 and β-amylase exhibit quantifiable protein–protein interactions

To examine for possible interaction between serpin Z4 and β-amylase, their recombinant proteins were first purified by affinity chromatography and size exclusion chromatography. The gel image of the proteins is shown in Fig. S2. The proteins were further tested for conventional activity. The activity of serpin as protease inhibitor was established by calculating its inhibition factor, $X_{inh}$. This factor measures the fraction of complex forming serpin in equilibrium with the protease as determined by the residual peptidase activity. As shown in Fig. S3a, recombinant serpin Z4 inhibits chymotrypsin with a $X_{inh}$ of 0.35 while a different barley recombinant serpin, BsZx inhibited the protease with a ratio of 0.88. The results are consistent with that measured previously for isolated barley serpins (Dahl et al., 1996). Recombinant amylase activity was measured using two independent assays: the DNSA reducing sugar method (Miller, 1959) and the Betamyl-3 method as described in the Material and Methods. Figure S3c and d shows that recombinant β-amylase activity in both methods was linear from 1 to 8 pmol of enzyme, similar to what was reported previously (Lundgard & Svensson, 1987).

Serpin Z4 and β-amylase activities were reported to comigrate during gel filtration of barley seed extracts (Hejgaard & Carlsen, 1977). This could be a result of their interaction or stem from similar molecular weights due to self-oligomerization. To examine this directly, we employed microscale thermophoresis as shown in Figure 3. Microscale thermophoresis measures the motion of molecules along a microscopic temperature gradient (Jerabek-Willemsen,
Wienken, Braun, Baaske, & Duhr, 2011). A fluorescent label was attached covalently, to one of the proteins to be tested and added to a serial dilution of the unlabeled cognate protein (X axis in log [M]; see Materials and Methods; Figure 3). The change in motion is presented as normalized fluorescence intensity (Y axis). Typical MST traces and capillary shapes are shown in Fig. S4. The KD of this interaction was found to be 2 \times 10^{-7} M or 1 \times 10^{-7} M depending on the direction of the reciprocal labeling (Figure 3a and b). The results quantify a physical interaction between serpin and \( \beta \)-amylase in native nonreducing conditions with an affinity of approximately \( 10^{-7} M \).

The interaction of \( \beta \)-amylase and serpin Z4 was further measured by surface plasmon resonance using a BiAcore sensor chip (see Materials and Methods; Figure 3c and d). When serpin was immobilized on the surface and \( \beta \)-amylase was the analyte the recorded sensogram yielded a binding affinity constant (KD) of 3.16 \times 10^{-7} M. Likewise, when the proteins were interchanged and \( \beta \)-amylase was immobilized the affinity measured was 1.42 \times 10^{-7} M. As shown in Figure 3e, the KD is the result of the ratio of the two rate constants, where Ka and Kdiss were \( \sim 10^4 \) and \( 10^{-3} \), respectively. The relatively low Ka indicates a slow association rate and the relatively high Kdiss, a fast dissociation process. Thus, two diverse biophysical methods of measurement show that \( \beta \)-amylase and serpin Z4 display a significant measurable interaction with a binding affinity constant greater than \( 10^{-7} M \). Although the motivation for this experiment relied on the observation that serpin Z4 and \( \beta \)-amylase comigrate during gel filtration, the consensus KD of \( 10^{-7} M \) measured here would be too low to form stable complexes during gel filtration. Hence, it is possible that previous observation of comigration emanated from the formation of homomultimers yielding similar filtration qualities (see below).

### 3.3 Serpin interaction with \( \beta \)-amylase impacts on its enzymatic properties

To investigate possible ramifications of the interaction between \( \beta \)-amylase and serpin Z4, a dose–response activity curve for \( \beta \)-amylase in the presence of increased serpin concentration was performed. Figure 4a shows the effect of serpin on \( \beta \)-amylase enzymatic activity assayed with the Betamyl-3 method. As the
amount of serpin increases (abscissa axis), $\beta$-amylase activity in the presence of excess substrate is significantly enhanced. The Betamyl-3 method uses short-chain substrate (p-nitrophenyl-$\beta$-D-maltotrioside) that requires auxiliary $\beta$-glucosidase activity. Hence, we also examined the effect of serpin on $\beta$-amylase using high MW starch substrate coupled to a direct assay of released reducing sugars. Figure 4b confirms the results using the DNSA technique. The concentration of serpin that was required for half of the maximal effect (EC50) is similar in both assays and was $1.8 \cdot 10^{-7}$ M and $3 \cdot 10^{-7}$ M for the Betamyl-3 and DNSA methods, respectively. The EC50 value was obtained at a molar ratio of 1 $\beta$-amylase to 1 serpin. The maximal increase in activity was obtained at a molar ratio of 1 $\beta$-amylase to 10 serpins (Figure 4). Similar experiments carried out with bovine serum albumin (BSA) did not yield any change in $\beta$-amylase activity indicating specificity (Fig. S5).

Notably, inspection of the dose-response of $\beta$-amylase activation upon addition of serpin yields a curve that is consistent with the KD values obtained for the direct protein–protein interactions (Figure 3). To further dissect the effect of serpin on the kinetic characteristics of $\beta$-amylase, $V_{\text{max}}$ and $K_m$ were estimated. As shown in Figure 4c, the addition of serpin at a 1:5 molar ratio led to a twofold increase in the $V_{\text{max}}$ independent of the presence of the reducing agent, DTT. Serpin Z4 had a slight negative effect on the affinity of $\beta$-amylase to its substrate as indicated by the increase in $K_m$.

Temperature is a critical environmental parameter (e.g., during beer mashing) that affects the enzymatic activity of $\beta$-amylase (Evans, van Wegen, Ma, & Eglinton, 2003). $\beta$-amylase activity was assessed over a temperature gradient ranging from 37°C to 62°C, supplemented or not, with serpin Z4 (ratio of 1:5, $\beta$-amylase: Z4; Figure 5a). Over a critical range of 37–48°C, the presence of serpin significantly improved $\beta$-amylase activity. At 50°C, the activity of $\beta$-amylase alone dropped by 40%, but with the addition of serpin Z4, the enzyme activity was enhanced by 60% compared with $\beta$-amylase alone at optimal temperatures. Above 53°C, $\beta$-amylase lost more than 80% of its activity with or without serpins. Thus, the presence of serpin elevates $\beta$-amylase activity over a critical range of temperatures. However, the denaturing temperature for protein stability as determined by the "Thermal Shift" assay in Figure 5b showed that the intrinsic melting temperature of $\beta$-amylase does not change in the presence of serpin Z4 and remains 55°C.

3.4 Impact of serpin on the redox-related activity and polymerization state of $\beta$-amylase

Oxidative conditions were shown to play a role in $\beta$-amylase activity (Seung et al., 2013; Sparla et al., 2006). It was therefore of interest to examine whether the presence of serpin impacts on the redox reactivity of $\beta$-amylase. Oxidative conditions were simulated by the use of CuCl2 that oxidizes reactive cysteines inducing the formation of cysteine–cysteine bonds (Cavallini, De Marco, Duprê, & Rotilio, 1969) (Figure 6a). Without serpins, $\beta$-amylase activity starts decreasing from 10 $\mu$M, until 50 $\mu$M at which point, less than 20% of the initial activity remains. The presence of serpins improves $\beta$-amylase stability to oxidation, as after treatment with 50 $\mu$M CuCl2, $\beta$-amylase was as active as the control $\beta$-amylase without the oxidative agent. However, a gradual decrease in activity, although less steep, was observed due to CuCl2 even in the presence of serpin.

Grain development and germination seem to affect the polymerization state of $\beta$-amylase (Figure 1), and these changes have been ascribed to shifts in the cellular redox milieu (Niku-Paavola, Skakoun, Nummi, & Daussant, 1973). Therefore, it was of interest to examine the impact of serpins on the polymerization status of $\beta$-amylase upon oxidation. To visualize this, recombinant proteins were combined at molar ratio of 1:0, 1:2, and 1:5 ($\beta$-amylase:serpin Z4). The mixtures were then subjected to treatment with CuCl2. The proteins were fractionated on a SDS-PAGE with or without reducing agent and developed by immunoblot with anti-$\beta$-amylase or antiserpin Z4 antibody (Figure 6b–e). Oxidative treatment caused $\beta$-amylase and serpin Z4 to migrate in multiple higher molecular weight forms (Figure 6b and c). $\beta$-amylase migrated in apparent dimer and trimer size at approximately 120 and 180 kDa, respectively; however, higher molecular weight migration forms also appeared. In the case of serpin Z4, dimeric (80 kDa) and higher order forms were visible, although to a lesser extent. Interestingly, the addition of serpins increased the amount of $\beta$-amylase monomers, inhibiting the formation of high molecular weight $\beta$-amylase. Notably, all polypeptides

![Figure 5](image-url)

**FIGURE 5** The effect of temperature on $\beta$-amylase activity. (a) $\beta$-amylase (2.5 pmol) was assayed by the DNSA method at different temperatures with or without serpin Z4 at a molar ratio of 1:5. The activity is presented in nmol of maltose released per minute of reaction. From 37°C to 53.3°C, the enzymatic activity of $\beta$-amylase in the presence of serpin is significantly different from the control with a $p$-value $\leq .03$. The experiment was repeated three times with three technical replicates. (b) The thermal stability of $\beta$-amylase with and without serpin Z4 was measured by thermal shift assay using sypro-orange dye. The arrow indicates the melting temperature of $\beta$-amylase. The graph is representative of three independent replicates.
were mixed at the indicated ratios and treated with 5, 10, and 20 μM CuCl₂ for 10 min. The percent of residual amylase activity of 2.5 pmol β-amylase was assayed using the DNSA method with or without the addition of serpin Z4 at a 1:5 ratio upon increasing CuCl₂ concentrations. (b–d, e) β-amylase and serpin Z4 were mixed at the indicated ratios and treated with 5, 10, and 20 μM CuCl₂ for 10 min. The proteins were fractionated in 10% SDS-PAGE gel with (b, c) or without the addition of reducing agent, β-mercaptoethanol (d, e) and blotted with antibody against β-amylase (b, d) or against serpin Z4 (c, e). The asterisk indicates β-amylase monomer size and the arrow polymeric forms. Western blots and enzymatic assays were replicated at least three times.

FIGURE 6 Enzymatic activity and immuno-detection of recombinant β-amylase and serpin under oxidizing conditions. (a) The activity of 2.5 pmol β-amylase was assayed using the DNSA method with or without the addition of serpin Z4 at a 1:5 ratio upon increasing CuCl₂ concentrations. (b–d, e) β-amylase and serpin Z4 were mixed at the indicated ratios and treated with 5, 10, and 20 μM CuCl₂ for 10 min. The proteins were fractionated in 10% SDS-PAGE gel with (b, c) or without the addition of reducing agent, β-mercaptoethanol (d, e) and blotted with antibody against β-amylase (b, d) or against serpin Z4 (c, e). The asterisk indicates β-amylase monomer size and the arrow polymeric forms. Western blots and enzymatic assays were replicated at least three times.

3.5 β-amylase mutants exhibit a differential response to oxidation

To investigate the participation of specific cysteines residues in β-amylase contributing to its polymerization, two modified β-amylases were generated. The β-amylase WT used in this study originates from cv. Harrington and contains a cysteine at position 115 (Chiapparino, Donini, Reeves, Tuberosa, & O’Sullivan, 2006). It was modified by a C115R replacement. The second modification was the removal of the C-terminus of β-amylase from the glycine at position 489 (Trunc β-amylase). This removes a cysteine at position C503. These cysteines were chosen as they are known to impact on the thermostability and enzymatic properties of β-amylase (Ma, Eglinton, Evans, Logue, & Langridge, 2000; Ma et al., 2001). Moreover, C503 was found to be the target of thioredoxins released in the endosperm upon germination (Hagglund et al., 2010).

The effect of serpin Z4 was evaluated on β-amylase activity of WT, C115R, and Trunc β-amylase (Figure 7a). As shown in Figure 7a, WT and all the variants displayed similar activity enhancement with no significant statistical difference as result of the presence of serpin Z4. For example, at a 5:1 ratio (arrow; Figure 7a), the addition of serpin Z4 improves all β-amylase to the same degree. Hence, the action of serpin on β-amylase activity was unaffected by modifications of these cysteines.

To investigate the ramifications of the mutations on the polymerization patterns, 5–20 μM of CuCl₂ was applied to the β-amylase variants. Without CuCl₂, each polypeptide migrated at the expected molecular weight size of its monomeric form (Fig. S6). However, as observed in Figure 7b, while β-amylase WT rapidly polymerized due to increasing CuCl₂, the C115R variant remains mainly in its monomeric form even at the highest concentration of CuCl₂ (20 μM). Although WT β-amylase appears smaller than expected, LC/MS/MS analysis was conducted on the protein and showed the expected size value (Fig. S7). The Trunc β-amylase variant also showed polymerization patterns that were distinct from the WT. Beside the monomeric form, one additional band is spotted on the Western blot as a result of oxidation. However, its polymeric migration size as determined by nondenaturing gel fractionation is 140 kDa, a value that is larger than the predicted dimer size (117 kDa) but smaller than the expected trimer size (176 kDa). In addition, the Trunc β-amylase variant appeared to be less susceptible to oxidation than the WT as it remained as a monomer at 5 μM CuCl₂. When the percent of residual amylase activity in the presence of CuCl₂ was measured, both WT and Trunc β-amylase showed a decrease in activity but not the C115R variant (Figure 7c). Thus, the C115R β-amylase, that is less inclined to form polymers, shows less sensitivity to oxidation indicating that the decrease in enzymatic activity might be a consequence of the polymerization process. Although the three forms of β-amylase exhibited distinct and differential responses to CuCl₂, they all responded to serpin as the WT, with enhanced enzymatic activity as measured in Figure 7a. Hence, serpin can improve β-amylase enzymatic activity independent of its ability to modify its oligomerization state.

4 DISCUSSION

Serpin Z4 and β-amylase are highly abundant seed proteins in many barley cultivars (Finnie et al., 2004). Similar to storage proteins, their
Enzymatic activity and immuno-detection of wild type and mutant forms of β-amylose in the presence of serpin Z4. (a) Activity of 2 pmol of β-amylose with increasing concentrations of serpin Z4 assayed by the DNSA method. Assays were on WT, C-terminus truncated β-amylose (Trunc β-amylose) and the C115R amino acid acid replacement. The X axis represents the ratio of serpin Z4 to the β-amyloses. The Y axis expressed the fold change in the β-amylose activity with serpin over the activity of the β-amylose without serpin. Serpin Z4: β-amylose ratios range between 20:1 and 0.04:1. The arrow indicates a ratio of 1:5. (b) Immunoblot analysis of recombinant β-amylose wild type and mutants as in a. Proteins (0.6 μg) were treated with 5, 10, and 20 μM of CuCl₂, for 10 min and fractionated on a 10% SDS nonreducing PAGE gel and immunoblotted with β-amylose antibody. (c) The residual activity of β-amylose compared to no treatment after addition of CuCl₂ as in b assessed by the DNSA method. Capital letters indicate significant differences in β-amylose WT-treated enzyme, lower case letters, significant differences in Trunc β-amylose-treated enzyme, no statistical differences were found in the C115R β-amylose-treated enzyme. The results were analyzed by performing Tukey-Kramer HSD tests. The asterisk indicates the monomeric forms of the three β-amyloses and the arrow, the polymers of β-amylose WT and Trunc β-amylose. Western blots and enzymatic assays were replicated at least three times.

FIGURE 7

content increases in the endosperm of developing grains (Sreenivasulu et al., 2006) and accumulate as matrix-bound proteins. Here, we show that both proteins display comparable accumulation profiles during seed maturation and germination and colocalize to the cytosol in a heterologous expression system (Figures 1 and 2). As the grain ripens and cell death proceeds in the endosperm (Young & Gallie, 2000), cytosolic proteins agglutinate to coat the amyloplast-localized starch granules (Borén et al., 2004; Wang et al., 2011). Consistent with our results, this coating would include serpin Z4 and β-amylose. During the later stages of grain filling and the early stages of germination, both β-amylose and serpin Z4 are detected mostly as a bound nonfree fraction or as high molecular weight protein in the free fraction (Figure 1). As they accumulate, the polymerization profiles of these two barley proteins are consistent with the creation of disulfides bridges to form homopolymers as has been reported for proteins in ripening wheat grains (De Gara, de Pinto, Moliterni, & D’Egidio, 2003). Significantly, serpin Z4 and β-amylose were detected among the multiple putative targets of thioredoxins, that is, reducing agents released from the aleurone layer during germination (Hagglund et al., 2010). Thus, serpin Z4 and β-amylose share common spatio-temporal localization characteristics that would promote interaction during grain development and germination.

Using different techniques, serpin Z4 was shown here to interact with β-amylose with a binding constant (KD) of approximately 10⁻⁷ M (Figure 3). This interaction is in the range measured for a variety of chaperones (Schweiger, Soll, Jung, Heermann, & Schwenkert, 2013). Importantly, in the presence of serpin Z4, the enzymatic activity of β-amylose is improved significantly. The improvement was specific as the addition of BSA had no effect (Fig. S3). Thus, β-amylose activity exhibits a sigmoidal curve of activity upon the addition of serpin (Figure 4); a curve that typifies agonistic interactions between proteins (DeLean, Munson, & Rodbard, 1978). A ratio of 1 β-amylose to 5 serpins increased the maximal velocity by twofold and protected β-amylose activity from heat or oxidation stress (Figures 5 and 6). It was previously reported that a serpin Z4-deficient barley line showed more diastatic power, that is, was more efficient in converting starch (Iimure, Kimura, et al., 2012). However, as diastatic power is a gross measurement taking into consideration multiple enzymatic activities including β-amylose, α-amyloses and limit dextrinase activity, it may be that, in that case, other qualities compensated for the reduced level of serpin.

Under oxidizing conditions, β-amylose WT rapidly polymerized and lost significant activity (Figure 7). The kinetics of polymerization shows that while the C115R mutation stabilized β-amylose in its monomeric form, the Trunc β-amylose mutant still polymerized but in a manner distinct from the WT. A number of β-amylose variants exist in barley. The SD1 and SD2 β-amylose isotypes are commonly found in malting-type barley (Allison & Swanston, 1974; Evans, Wallace, et al., 1997). The substitution of cysteine at position 115 by an arginine results in the conversion of SD1 to the SD2 type (Ma et al., 2002). SD1 is more thermostable than SD2 but has lower affinity to its substrate (Ma et al., 2001). Moreover, a SD1 type β-amylose from
cv. Pirkka was shown to polymerize spontaneously (Niku-Paavola et al., 1973) in a manner similar to the WT described in this work (also a SD1 type). The C115R mutation transforms the SD1 β-amylase into a SD2 type and as shown here prevents the formation of high molecular weight moieties (Figure 7) identifying a cysteine critical for higher order polymerization. In this scenario, C115 could serve as a hub for cysteine bonds formation. Engineered removal of the C-terminus eliminates C503 yielding a more stable β-amylase (Figure 7). This is consistent with the finding that reduction of the C503 by thioredoxins (Hagglund et al., 2010) or its removal by proteases (Guerin et al., 1992; Lundgard & Svensson, 1987) efficiently solubilizes the bound β-amylase. The resulting processed β-amylase was found to be more thermostatable but otherwise retained the same kinetic parameters (Ma, Eglington, et al., 2000). The decrease in β-amylase activity due to oxidation reported here differs from that reported for the redox-sensitive Arabidopsis, trbAMY. The Arabidopsis trbAMY, a thioredoxin regulated β-amylase, does not form high molecular weight complexes, suggesting that in that case intramolecular disulfide bonds account for the control of enzymatic activity (SpaI et al., 2006).

Under oxidizing conditions, serpin Z4 interaction impacts on the enzymatic activity of WT β-amylase in two ways. Firstly, the interaction between β-amylase and serpin Z4 represses the formation of multimeric β-amylase (Figure 6b and c). Presumably such polymers are less active (Figure 7c). Secondly, its intrinsic ability to activate β-amylase is evidenced in the mutant C115 that lacks the ability to polymerize but is nevertheless enhanced by serpin. How serpin and β-amylase interactions interfere with polymerization is not known. Based on β-amylase structure (PDB ID: 2XFR), C115 is located on the outer surface far from the active site. One can speculate that in the topography of its interaction, serpin Z4 specifically interferes with the ability of C115 to form cysteine bonds perhaps by physical contact in that area.

The function for serpin Z4 in grains of barley as a chaperone-like stabilizer of β-amylase that enhances enzyme activity and favors monomeric forms differs from classical chaperones that operate in protein folding pathways. Yet, in mammals, the serpin family was reported for the redox-sensitive Arabidopsis, trbAMY. The Arabidopsis trbAMY, a thioredoxin regulated β-amylase, does not form high molecular weight complexes, suggesting that in that case intramolecular disulfide bonds account for the control of enzymatic activity (SpaI et al., 2006). Under oxidizing conditions, serpin Z4 interaction impacts on the enzymatic activity of WT β-amylase in two ways. Firstly, the interaction between β-amylase and serpin Z4 represses the formation of multimeric β-amylase (Figure 6b and c). Presumably such polymers are less active (Figure 7c). Secondly, its intrinsic ability to activate β-amylase is evidenced in the mutant C115 that lacks the ability to polymerize but is nevertheless enhanced by serpin. How serpin and β-amylase interactions interfere with polymerization is not known. Based on β-amylase structure (PDB ID: 2XFR), C115 is located on the outer surface far from the active site. One can speculate that in the topography of its interaction, serpin Z4 specifically interferes with the ability of C115 to form cysteine bonds perhaps by physical contact in that area.

The function for serpin Z4 in grains of barley as a chaperone-like stabilizer of β-amylase that enhances enzyme activity and favors monomeric forms differs from classical chaperones that operate in protein folding pathways. Yet, in mammals, the serpin family was reported for the redox-sensitive Arabidopsis, trbAMY. The Arabidopsis trbAMY, a thioredoxin regulated β-amylase, does not form high molecular weight complexes, suggesting that in that case intramolecular disulfide bonds account for the control of enzymatic activity (SpaI et al., 2006).

The authors thank Dr Irina Shin for her help in the microscale thermophoresis experiments, Dr. Aharon Rabinkov for his expertise in the Surface Plasmon Resonance technology, and Dr Olga Davidov for her support in general laboratory work. R.F. is grateful to the US-Israel Binational Agricultural Research and Development Fund for Research Project IS-4915-16.

**REFERENCES**

Allison, M. J., & Swanston, J. S. (1974). Relationships between β-amylase polymorphisms in developing, mature and germinating grains of barley. *Journal of the Institute of Brewing, 80*, 285–291. https://doi.org/10.1002/j.2050-0416.1974.tb03618.x

Bønsager, B. C., Finnie, C., Roepstorff, P., & Svensson, B. (2007). Spatio-temporal changes in germination and radical elongation of barley seeds tracked by proteome analysis of dissected embryo, aleurone layer, and endosperm tissues. *Proteomics, 7*, 4528–4540. https://doi.org/10.1002/pmic.200600217

Borén, M., Larsson, H., Falk, A., & Jansson, C. (2004). The barley starch granule proteome internalized granule polypeptides of the mature endosperm. *Plant Science, 166*, 617–626. https://doi.org/10.1016/j.plantsci.2003.10.028

Cavallini, D., De Marco, C., Duprè, S., & Rotilio, G. (1969). The copper catalyzed oxidation of cysteine to cystine. *Arch Biochem Biophys.*, *130*, 354–361. https://doi.org/10.1016/0003-9861(69)90044-7

Chiapparino, E., Donini, P., Reeves, J., Tuberosa, R., & O’Sullivan, D. M. (2006). Distribution of β-amylase I haplotypes among European cultivated barleys. *Molecular Breeding, 18*, 341–354. https://doi.org/10.1007/s11032-006-9035-0

Dahl, S. W., Rasmussen, S. K., & Hejgaard, J. (1996). Heterologous expression of three plant serpins with distinct inhibitory specificities. *Journal of Biological Chemistry, 271*, 25083–25088. https://doi.org/10.1074/jbc.271.41.25083

De Gara, L., de Pinto, M. C., Moliterni, V. M. C., & D’Egiscio, M. G. (2003). Redox regulation and storage processes during maturation in kernels of Triticum durum. *Journal of Experimental Botany*, *54*, 249–258. https://doi.org/10.1093/jxb/erg021

DeLean, A., Munson, P. J., & Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose-response curves. *American Journal of Physiology, 235*, 97–102.
Evans, D. E., & Hejgaard, J. (1999). The impact of malt derived proteins on beer foam quality. Part I: The Effect of germination and kilning on the level of protein Z4, Protein Z7 and LTP1. *Journal of the Institute of Brewing*, 105, 159–170. https://doi.org/10.1002/jisn.2050-0416

Evans, D. E., MacLeod, L. C., Eglington, J. K., Gibson, C. E., Zhang, X., Wallace, W., … Lance, R. C. M. (1997). Measurement of beta-amylase in malting barley (Hordeum vulgare L.). I. Development of a quantitative ELISA for beta-amylase. *Journal of Cereal Science*, 26, 229–239. https://doi.org/10.1016/j.jcres.1997.0119

Evans, E., van Wegen, B., Ma, Y., & Eglington, J. (2003). The impact of the level of protein Z4, Protein Z7 and LTP1. *Journal of the Institute of Brewing*, 109, 241–250. https://doi.org/10.1002/jisn.1997.0120

Finnie, C., Maeda, K., Østergaard, O., Bak-Jensen, K. S., Larsen, J., & Svensson, B. (2004). Aspects of the barley seed proteome during development and germination. *Biochemical Society Transactions*, 32, 517–519. https://doi.org/10.1042/bst0320517

Finnie, C., Melchior, S., Roepstorff, P., & Svensson, B. (2002). Proteome analysis of grain filling and seed maturation in barley. *Plant Physiology*, 129, 1308–1319. https://doi.org/10.1104/pp.003681

Finnie, C., & Svensson, B. (2003). Feasibility study of a tissue-specific approach to barley proteome analysis: Aleurone layer, endosperm, embryo and single seeds. *Journal of Cereal Science*, 38, 217–227. https://doi.org/10.1016/S0733-5210(02)00033-X

Fluhr, R., Lampl, N., & Roberts, T. H. (2012). Serpin protease inhibitors in plant biology. *Physiologia Plantarum*, 145, 95–102. https://doi.org/10.1111/j.1399-3054.2011.01540.x

Gettins, P. G. W. (2002). Serpin structure, mechanism, and function. *Chemical Reviews*, 102, 4751–4804. https://doi.org/10.1021/cr010170+

Guerin, J. R., Lance, R. C. M., & Wallace, W. (1992). Release and activation of barley beta-amylase by malt endopeptidases. *Journal of Cereal Science*, 15, 5–14. https://doi.org/10.1016/S0733-5210(92)80052-0

Hagglund, P., Bunkenborg, J., Yang, F., Harder, L. M., Finnie, C., & Svensson, B. (2010). Identification of thioerodoxin target disulfides in proteins released from barley aleurone layers. *Journal of Proteomics*, 73, 1133–1136. https://doi.org/10.1016/j.jprot.2010.01.007

Hara-Nishimura, I., Nishimura, M., & Daussant, J. (1986). Conversion of free beta amylase to bound beta amylase on starch granules in the barley endosperm during desiccation phase of seed development. *Protoplasma*, 134, 149–153. https://doi.org/10.1007/BF01275713

Hejgaard, J. (2001). Inhibitory serpins from rye grain with glutamine as C-terminal region of barley (Hordeum vulgare L.). II. The effect of germination and kilning. *Journal of Cereal Science*, 26, 241–250. https://doi.org/10.1002/jcres.1997.0120

Khan, M. S., Singh, P., Ashar, A., Naseem, A., Rashid, Q., Kabir, M. A., & Jairajpuri, M. A. (2011). Serpin inhibition mechanism: A delicate balance between native metastable state and polymerization. *Journal of Amino Acids*, 2011, 606779.

Kihara, M., Kaneko, T., Ito, K., Aida, Y., & Takeda, K. (1999). Geographical variation of beta-amylase thermostability among varieties of barley (Hordeum vulgare) and beta-amylase deficiency. *Plant Breeding*, 118, 453–455. https://doi.org/10.1046/j.1439-0523.1999.00397.x

Koide, T., Nishikawa, Y., Asada, S., Yamazaki, C. M., Takahara, Y., Homma, D. L., … Kitagawa, K. (2006). Specific recognition of the collagen triple helix by chaperone HSP47: II. The HSP47-binding structural motif in collagens and related proteins. *Journal of Biological Chemistry*, 281, 11177–11185. https://doi.org/10.1074/jbc.M601369200

Lamp, N., Alkan, N., Davydov, O., & Fluhr, R. (2013). Set-point control of RD21 protease activity atAtSerpin1 controls cell death in Arabidopsis. *Plant Journal*, 74, 498–510. https://doi.org/10.1111/tpj.12141

Lamp, N., Budai-Hadrian, O., Davydov, O., Joss, T. V., Harrop, S. J., Curmi, P. M., … Fluhr, R. (2010). Arabidopsis AtSerpin1, crystal structure and in vivo interaction with its target protease Responsive to Desication-21 (RD21). *Journal of Biological Chemistry*, 285, 13550–13560. https://doi.org/10.1074/jbc.M109.095075

Lema Asqui, S., Vercaemen, D., Serrano, I., Valls, M., Rivas, S., Van Breusegem, F., … Coll, N. S. (2017). AtSerpin1 is an inhibitor of the metacaspase AtMCl-mediated cell death and autophagic processing in planta. *New Phytologist*. https://doi.org/10.1111/nph.14446

Li, C. D., Langridge, P., Zhang, X. Q., Eckstein, P. E., Rossnagel, B. G., Lance, R. C. M., … Scoles, G. J. (2002). Mapping of barley (Hordeum vulgare L.) beta-amylase alleles in which an amino acid substitution determines beta-amylase isoenzyme type and the level of free beta-amylase. *Journal of Cereal Science*, 35, 39–50. https://doi.org/10.1006/jcres.2001.0398

Lobstein, J., Emrich, C. A., Jeans, C., Faulkner, M., Riggs, P., & Berkmen, M. (2012). Shuffel, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microbial Cell Factories*, 11, 753. https://doi.org/10.1186/1475-2859-11-56

Lundgard, R., & Svensson, B. (1986). Limited proteolysis in the carboxy-terminal region of barley beta-amylase. *Carlsberg Research Communications*, 51, 487–491. https://doi.org/10.1007/BF02906890

Lundgard, R., & Svensson, B. (1987). The four major forms of barley beta-amylase. Purification, characterization and structural relationship. *Carlsberg Research Communications*, 52, 313–326. https://doi.org/10.1007/BF02907173

Ma, Y. F., Eglington, J. K., Evans, D. E., Logue, S. J., & Langridge, P. (2000). Removal of the four C-terminal glycine-rich repeats enhances the thermostability and substrate binding affinity of barley beta-amylase. *Biochemistry*, 39, 13350–13355. https://doi.org/10.1021/bi000688s
Ma, Y., Evans, D., Logue, S., & Langridge, P. (2001). Mutations of barley β-amylase that improve substrate-binding affinity and thermostability. *Molecular Genetics and Genomics*, 266, 345–352.

Ma, Y., Langridge, P., Logue, S. J., & Evans, D. E. (2002). A single amino acid substitution that determines IEF band pattern of barley beta-amylase. *Journal of Cereal Science*, 35, 79–84. https://doi.org/10.1006/jcrs.2001.0421

MacGregor, A., Bazin, S., Macri, L., & Babb, J. (1999). Modelling the contribution of alpha-amylase, beta-amylase and limit dextrinase to starch degradation during mashing. *Journal of Cereal Science*, 29, 161–169. https://doi.org/10.1006/jcrs.1998.0233

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428. https://doi.org/10.1021/ac60147a030

Mita, S., Suzuki-Fujii, K., & Nakamura, K. (1995). Sugar-inducible expression of a gene for beta-amylase in *Arabidopsis thaliana*. *Plant Physiology*, 107, 895–904. https://doi.org/10.1104/pp.107.3.895

Natsume, T., Koide, T., S-i, Yokota, Hirayoshi, K., & Nagata, K. (1994). Interactions between collagen-binding stress protein HSP47 and collagen. Analysis of kinetic parameters by surface plasmon resonance biosensor. *Journal of Biological Chemistry*, 269, 31224–31228.

Niku-Paavola, M. L., Skakoun, A., Nummi, M., & Daussant, J. (1973). The polymorphism of barley β-amylase. *BBA*, 322, 181–184.

Ostergaard, O., Finnie, C., Laugesen, S., Roepstorff, P., & Svennson, B. (2004). Proteome analysis of barley seeds: Identification of major proteins from two-dimensional gels (pI 4–7). *Proteomics*, 4, 2437–2447. https://doi.org/10.1002/1521-688X(200407)4:11<2437::AID-PMET2437>3.0.CO;2-D

Ostergaard, H., Rasmussen, S. K., Roberts, T. H., & Hejgaard, J. (2000). Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins. Cloning and characterization of five major molecular forms. *Journal of Biological Chemistry*, 275, 33272–33279. https://doi.org/10.1074/jbc.M004633200

Pemberton, P. A., Stein, P. E., Pepys, M. B., Potter, J. M., & Carrell, R. W. (1998). Hormone binding globulins undergo serpin conformational change in inflammation. *Nature*, 336, 257–258. https://doi.org/10.1038/336257a0

Pogorelko, G. V., Fursova, O. V., Ogarkova, O. A., & Tarasov, V. A. (2007). New vector system for induction of gene expression in dicotyledonous plants. *Russian Journal of Genetics (Translation of Genetika (Moscow, Russian Federation))*, 43, 136–142.

Rasmussen, S. K., Dahl, S. W., Nargård, Å, & Hejgaard, J. (1996). A recombinant wheat serpin with inhibitory activity. *Plant Molecular Biology*, 30, 673–677. https://doi.org/10.1007/BF00049343

Roberts, T. H., Marttila, S., Rasmussen, S. K., & Hejgaard, J. (2003). Differential gene expression for suicide-substrate serine protease inhibitors (serpins) in vegetative and grain tissues of barley. *Journal of Experimental Botany*, 54, 2251–2263. https://doi.org/10.1093/jxb/erg248

Rosenkranz, I., Hejgaard, J., Rasmussen, S. K., & Bjarn, S. E. (1994). Serpins from wheat grain. *FEBS Letters*, 343, 75–80. https://doi.org/10.1016/0014-5793(94)80610-1

Sauk, J. J., Nikitakis, N., & Siavash, H. (2005). Hsp47 a novel collagen binding serpin chaperone, autoantigen and therapeutic target. *Frontiers in Bioscience*, 10, 107–118. https://doi.org/10.2741/1513

Swieger, R., Soll, J., Jung, K., Heermann, R., & Schwenkert, S. (2013). Quantification of interaction strengths between chaperones and tetratricopeptide repeat domain-containing membrane proteins. *Journal of Biological Chemistry*, 288, 30614–30625. https://doi.org/10.1074/jbc.M113.493015

Seung, D., Thalmann, M., Spanel, F., Hacken, M. A., Lee, S. K., Issakidis-Bourguet, E., … Sankel, D. (2013). *Arabidopsis thaliana* AMY3 is a unique redox-regulated chloroplast α-amylase. *Journal of Biological Chemistry*, 288, 33620–33633. https://doi.org/10.1074/jbc.M113.514794

Spanel, F., Costa, A., Schiavo, F. L., Pupillo, P., & Trost, P. (2006). Redox regulation of a novel plastid-targeted β-amylase of Arabidopsis. *Plant Physiology*, 141, 840–850. https://doi.org/10.1104/pp.106.079186

Specker, C., Niessen, L., & Vogel, R. F. (2014). In vitro studies on the main beer protein Z4 of Hordeum vulgare concerning heat stability, protease inhibition and gushing. *Journal of the Institute of Brewing*, 120, 85–92. https://doi.org/10.1002/jib.118

Sreenivasulu, N., Radvich, V., Strickert, M., Miersch, O., Weschke, W., & Wobus, U. (2006). Gene expression patterns reveal tissue-specific signalling networks controlling programmed cell death and ABA-regulated maturation in developing barley seeds. *Plant Journal*, 47, 310–327. https://doi.org/10.1111/j.1365-313X.2006.02789.x

Stein, P. E., Leslie, A. G. W., Finch, J. T., Turrell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990). Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature*, 347, 99–102. https://doi.org/10.1038/347099a0

Vercammen, D., Belenghi, V., van de Cotte, B., Beunens, T., Gavigan, J.-A., De Rycke, R., … Van Breusegem, F. (2006). Serpin1 of *Arabidopsis thaliana* is a suicide inhibitor for Metacaspase 9. *Journal of Molecular Biology*, 364, 625–636. https://doi.org/10.1016/j.jmb.2006.09.010

Wang, C. P., Pan, Z. F., Nima, Z. X., Tang, Y. W., Cai, P., Liang, J. J., … Yu, M. Q. (2011). Starch granule-associated proteins of hull-less barley (*Hordeum vulgare L*) from the Qinghai-Tibet Plateau in China. *Journal of the Science of Food and Agriculture*, 91, 616–624. https://doi.org/10.1002/jsfa.4223

Widmer, C., Gebauer, J. M., Brunstein, E., Rosenbaum, S., Zauke, F., Drogemüller, C., … Baumann, U. (2012). Molecular basis for the action of the collagen-specific chaperone Hsp47/SerpinH1 and its structure-specific client recognition. *Proceedings of the National Academy of Sciences*, 109, 13243–13247. https://doi.org/10.1073/pnas.1208072109

Young, T. E., & Gallie, D. R. (2000). Programmed cell death during endosperm development. In E. Lam, H. Fukuda, & J. Greenberg (Eds.), *Programmed cell death in higher plants* (pp. 39–57). Netherlands, Dordrecht: Springer. https://doi.org/10.1007/978-94-010-0934-8

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Cohen M, Flurh R. Noncanonical interactions between serpin and β-amylase in barley grain improve β-amylase activity in vitro. *Plant Direct*. 2018;2:1–12. https://doi.org/10.1002/pld3.54