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Effect of Thioredoxin-Linked Reduction on the Activity and Stability of the Kunitz and Bowman–Birk Soybean Trypsin Inhibitor Proteins

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Protease inhibitors, including the Kunitz and Bowman–Birk trypsin inhibitors of soybean, can be reduced by the NADP/thioredoxin system (NADPH, thioredoxin, and NADP-thioredoxin reductase) from either *Escherichia coli* or wheat germ. In the present study, we report that, when reduced by thioredoxin, the Kunitz and Bowman–Birk soybean trypsin inhibitors lose their ability to inhibit trypsin. Moreover, the reduced form of the inhibitors showed increased susceptibility to heat and proteolysis by either subtilisin or a protease preparation from germinating wheat seeds. These findings, on the one hand, pave the way for complete in vitro inactivation of trypsin inhibitory activity in soybean products and, on the other, provide new evidence that thioredoxin functions in vivo in mobilizing proteins during seed germination.

INTRODUCTION

Certain proteins have the ability to inhibit proteases that are often derived from exogenous sources. The common features of the inhibitor proteins from plants are their content of intramolecular disulfides (cystine groups) and their abundance in storage tissues, notably seeds and tubers (Birk, 1976; Garicia-Olmedo et al., 1987; Ryan, 1981). In seeds, the inhibitory proteins are synthesized de novo during development and deposited in storage tissues such as the endosperm (cereals) or cotyledons (legumes).

The best known of the inhibitor proteins are the Kunitz trypsin inhibitor (KTI) and the Bowman–Birk trypsin inhibitor (BBTI) of soybean (Birk, 1976; Garicia-Olmedo et al., 1987; Wilson, 1988). Owing to their abundance and ability to inhibit the digestive proteases of humans and domesticated animals, these two inhibitors have long been known as antinutrients. To minimize the adverse effects caused by the inhibitors (i.e., improve nutritional quality), soybean products are usually heated during processing. The heat treatment, however, does not fully eliminate inhibitor activity. For example, while 30 min at 120 °C leads to complete inactivation of BBTI of soy flour, about 20% of the original KTI activity remains (Friedman et al., 1991). The prolonged or higher temperature treatments required for full inactivation of inhibitors results in destruction of important amino acids such as cystine, arginine, and lysine (Chae et al., 1984; Skrede and Krogdahl, 1985). Development of a method that could completely inactivate the protease inhibitors would be desirable, especially if it were effective at lower temperature.

In a previous study, we identified KTI and BBTI as target proteins of the NADP/thioredoxin system, consisting of thioredoxin *h*, NADPH, and NADP-thioredoxin reductase (NTR) (Kobrehel et al., 1991). We now report that reduction by thioredoxin, or dithiothreitol (DTT), leads to inactivation of both proteins and to an increase in their heat and protease susceptibility. The results indicate that thioredoxin-linked reduction of the inhibitor proteins is relevant both to their industrial processing and to seed germination.

MATERIALS AND METHODS

Plant Materials. Durum wheat (*Triticum durum*, Desf. cv. Monroe) was a kind gift of Dr. K. Kahn. Wheat germ was obtained from Sigma Chemical Co. (St. Louis, MO).

Chemicals and Enzymes. Reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA), and DTT was from Boehringer Mannheim Biochemicals (Indianapolis, IN). L-1-(Tosylamide)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (type XIII, T8640), subtilisin (type VIII: bacterial subtilisin Carbsberg, P5380), KTI (T9003), BBTI (T9777), azocasein, and other chemicals were purchased from Sigma. *Escherichia coli* thioredoxin and NTR were isolated from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid, pFPI, was kindly provided by Dr. J.-P. Jacquot (de La Motte-Guery et al., 1991). The NTR strain containing the recombinant plasmid, pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel and Model, 1988). The isolation procedures used for these proteins were as described in those studies with the following changes: cells were broken in a Ribi cell fractionator at 25 000 psi, and NTR was purified as described by Florencio et al. (1988) without the red agarose step. The *E. coli* thioredoxin and NTR were, respectively, 100 and 90% pure as determined by SDS–polyacrylamide gel electrophoresis. Wheat thioredoxin *h* was purified as previously described (Johnson et al., 1987).

Germination of Wheat Seeds. Wheat seeds were sterilized by steeping in 50% (v/v) generic bleach for 1 h at room temperature, followed by a thorough wash with distilled water. The sterilized seeds were placed in a plastic Petri dish on two layers of Whatman filter paper moistened with distilled water containing 100 μg/mL chloramphenicol. Germination was continued at room temperature in a dark chamber for up to 5 days.

Preparation of Wheat Proteases. The endosperm (10–15 g of fresh weight) isolated from 5-day-germinated wheat seeds by excising the roots and shoots was extracted for 30 min at 4 °C with 5 volumes of 200 mM sodium acetate, pH 4.6, containing 10 mM β-mercaptoethanol. The homogenate was centrifuged

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0021-8561/92/1440-2333$03.00/0

for 20 min at 48000g, 4 °C. The pellet was discarded, and the supernatant fluid was fractionated with 30–70% ammonium sulfate. This fraction, which represented the protease preparation, was resuspended in a minimum volume of 20 mM sodium acetate, pH 4.6, containing 10 mM β-mercaptoethanol, and dialyzed against this buffer overnight at 4 °C. When assayed with azocasein as substrate, the protease preparation had an optimal pH of about 4.6 and was stable for at least 1 week at 4 °C.

Reduction and Proteolytic Susceptibility of Trypsin Inhibitors. Unless indicated, the reduction of the trypsin inhibitors (0.4 mg/mL) was carried out in 0.1 mL of 20 mM sodium phosphate buffer, pH 7.9, containing 10 mM EDTA at 30 °C for 2 h. The concentrations of thioredoxin, NTR, and NADPH were 0.024 mg/mL, 0.02 mg/mL, and 0.25 mM, respectively. With DTT as reductant, EDTA and components of the NADP/thioredoxin system were omitted. Following reduction, aliquots of the inhibitor mixture were withdrawn for determination of either trypsin inhibitory activity or proteolytic susceptibility. In the subtilisin tests, the inhibitor mixture (50 μL) was directly mixed with subtilisin and incubated at room temperature for 1 h. With the wheat protease preparation, the pH of the inhibitor mixture (50 μL) was first adjusted to 4.7 by mixing with 35 μL of 200 mM sodium acetate, pH 4.6; 10 μL of the wheat protease preparation was then added, and incubation was continued for 2 h at 37 °C. To stop digestion with subtilisin, 2 μL of 100 mM phenylmethanesulfonyl fluoride (PMSF) and 10 μL of 10% SDS were added to the digestion mixture. With the plant protease preparation, digestion was stopped by adding an equal volume of SDS sample buffer [0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue]. Proteolytic products were analyzed by electrophoresis with 12 or 16% SDS polyacrylamide slab gels (Laemmli, 1970). The dried slab gels were scanned with a laser densitometer (Pharmacia-LKB UltraScan XL), and the peak area of the KTI or BBTI protein band was obtained by integration with a Pharmacia GelScan XL software program.

Assays. Thioredoxin and NTR were assayed as previously described by Florencio et al. (1988). Trypsin activity was measured in 50 mM Tris-HCl, pH 7.9, by following the increase in absorbance at 253 nm with *N*-benzoyl-L-arginine ethyl ester as substrate (Mundy et al., 1984) or by the release of azo dye into the trichloroacetic acid (TCA)-soluble fraction from azocasein substrate (see below). For trypsin inhibition assays, trypsin (5–10 μg) was preincubated with appropriate amounts of KTI or BBTI for 5 min at room temperature in 50 mM Tris-HCl, pH 7.9, and proteolytic activity was then determined. While the two substrates yielded similar data, results are presented with only one substrate.

Wheat protease activity was measured by following the release of azo dye into TCA solution from azocasein substrate at pH 4.7. Fifty microliters of wheat protease in a solution of 20 mM sodium acetate, pH 4.6, and 10 mM β-mercaptoethanol was added to 50 μL of 200 mM sodium acetate, pH 4.6, and 100 μL of 2% azocasein (in 20 mM sodium phosphate, pH 7.0). Following a 1-h incubation at 37 °C, 1 mL of 10% TCA was added and the mixture was allowed to stand for 10 min at room temperature. After centrifugation for 5 min in a microfuge (8000g), 1 mL of the supernatant solution was withdrawn and mixed with 1 mL of 1 N NaOH. The absorbance was read at 440 nm. Protein concentration was determined with Bio-Rad reagent using bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Trypsin Inhibitory Activity. The 20-kDa Kunitz and 8-kDa Bowman–Birk trypsin inhibitors of soybean contain two and seven disulfide groups, respectively (Birk, 1976; Wilson, 1988). Although their physiological functions have not been established, the two types of inhibitors have been extensively investigated owing to their wide distribution in legume seeds and their potential to cause nutritional disorders, e.g., hypertrophy and associated malfunctions of the pancreas. Recently, we found that KTI and BBTI are reduced specifically by the NADP/thioredoxin system from either *E. coli* or plants (Kobrehel et al., 1991). The reduced forms of glutathione and glutaredoxin—a thiol protein capable of replacing thioredoxin in certain animal and bacterial systems but not known to occur in plants (Holmgren, 1985)—were without effect.

Table I. Changes in the Ability of Soybean Trypsin Inhibitors To Inhibit Trypsin following Reduction by the NADP/Thioredoxin System, DTT, or Reduced Lipoic Acid

treatment	relative trypsin activity[a] KTI	BBTI
no inhibitor	100	100
inhibitor		
oxidized	17.0	11.5
reduced by NTS[b]	55.6	70.6
reduced by DTT[c]	68.6	88.9
reduced by LA/Trx *h*[d]	40.5	87.8

[a] The specific activity of the uninhibited control trypsin was 0.018 $\Delta A_{253nm}\ \mu g^{-1}\ min^{-1}$ using *N*-benzoyl-L-arginine ethyl ester as substrate. [b] Reduction by *E. coli* NTS (NADP/thioredoxin system) was conducted at 30 °C for 2 h. [c] Reduction by DTT (1 mM) was conducted at 30 °C for 1 h. [d] Reduction by lipoic acid (LA, 0.4 mM) and wheat thioredoxin *h* (Trx *h*) was conducted at 30 °C for 1 h. In the presence of lipoic acid alone (0.4 mM), trypsin activity was 20.0% for KTI and 12.5% for BBTI.

To determine the consequence of reduction by thioredoxin, we compared the trypsin inhibitory activity of the oxidized and reduced forms of KTI and BBTI. As shown in Table I, preincubation with the NADP/thioredoxin system (NTS) for 2 h at 30 °C resulted in a substantial loss of trypsin inhibitory activity—i.e., there was an increase in trypsin activity relative to the inhibited control. More specifically, the NADP/thioredoxin system effected 3- and 6-fold increases in trypsin activity for KTI and BBTI, respectively. Similar results were obtained with DTT, a nonphysiological substitute for thioredoxin, and with thioredoxin reduced by lipoic acid, a naturally occurring dithiol. Extended incubation with DTT alone (overnight at room temperature) led to complete or almost complete inactivation of both inhibitors (data not shown). Unlike DTT, lipoic acid did not reduce (inactivate) KTI and BBTI significantly in the absence of thioredoxin.

Friedman and colleagues observed that heating soybean flour in the presence of sulfur reductants (sodium sulfite, *N*-acetyl-L-cysteine, reduced glutathione, or L-cysteine) inactivated trypsin inhibitors, presumably as a result of the reduction or interchange of disulfide groups with other proteins in soy flour (Friedman and Gumbmann, 1986; Friedman et al., 1982, 1984). Inactivation of the trypsin inhibitors by these reductants improved the digestibility and nutritive value of flours in tested rats (Friedman and Gumbmann, 1986). Taken together with these earlier observations, the present findings demonstrate that disulfide bonds of both KTI and BBTI targeted by thioredoxin are important to the maintenance of trypsin inhibitory activity.

Heat Stability. Protease inhibitor proteins are typically stable to inactivation treatments such as heat. This stability is attributed, at least in part, to the cross-linking of disulfide bonds (Birk, 1976; Ryan, 1981). It is known that breaking the disulfide bonds by reduction decreases heat stability (Friedman et al., 1982). The question arises as to whether reduction by thioredoxin yields similar results.

The results in Table II provide a positive answer to this question. When heated at 80 °C for 15 min, the thioredoxin-reduced form of KTI completely lost its ability to inhibit trypsin, whereas its oxidized counterpart retained about half of the original activity (Table II). Oxidized BBTI was even more stable, retaining the bulk of its trypsin

Table II. Heat Stability of the Kunitz and Bowman–Birk Trypsin Inhibitors: Oxidized and following Reduction by the *E. coli* NADP/Thioredoxin System

treatment	relative trypsin activity[a] KTI	BBTI
no inhibitor	100	100
inhibitor, unheated		
oxidized	26.6	9.4
reduced	76.4	82.4
inhibitor, heated for 15 min at 80 °C		
oxidized	52.3	nd[b]
reduced	98.7	nd
inhibitor, heated for 25 min at 100 °C		
oxidized	nd	17.2
reduced	nd	98.4

[a] The specific activity of trypsin was 0.319 ΔA_{440nm} mg^{-1} min^{-1} using azocasein as substrate. The temperatures used for inactivation were determined in initial experiments designed to show the heat stability of the trypsin inhibitors under our conditions. [b] nd, not determined.

Table III. Effect of Thioredoxin-Linked Reduction on the Susceptibility of Kunitz and Bowman–Birk Trypsin Inhibitors to Proteolysis by a Plant Protease Preparation[a]

treatment	relative abundance[b] KTI	BBTI
no protease	100	100
protease		
no reduction system	97.9	67.2
E. coli NTS[c]	22.1	16.0
NTS minus thioredoxin	90.2	nd[d]
NTS minus NADPH	97.7	nd
NTS minus NTR	97.9	nd

[a] Following reduction by *E. coli* thioredoxin system at 30 °C for 2 h, pH was adjusted to 4.7 by addition of 200 mM sodium acetate, pH 4.6. Wheat protease preparation was then added and incubated at 37 °C for 2 h, followed by SDS–PAGE analyses. [b] Determined by laser densitometer. [c] NTS, NADP/thioredoxin system. [d] nd, not determined.

inhibitory activity after heating at 100 °C for 25 min. Nonetheless, as with KTI, the reduced form of BBTI was fully inactivated by heat (Table II). These results are consistent with prior observations (i) that KTI and BBTI show increased sensitivity to heat on reduction and (ii) that pure BBTI in solution is more heat-stable than pure KTI in solution. The reverse is true for flour—i.e., KTI is more heat-stable than BBTI (Friedman et al., 1982, 1991; DiPietro and Liener, 1989).

Protease Susceptibility. The above results prompt the question of whether the reduced forms of KTI and BBTI show decreased stability to proteases other than trypsin. To test this point, we incubated both the reduced and oxidized forms of KTI and BBTI with a wheat protease preparation or with subtilisin and analyzed the proteolytic products by SDS–PAGE. The extent of proteolysis was determined by measuring the abundance of intact protein on SDS gels by laser densitometer. When tested with a protease preparation from 5-day-germinated wheat seeds, the oxidized form of the Kunitz inhibitor was almost completely resistant to digestion, whereas the thioredoxin-reduced form was susceptible to protease. As shown in Table III, about 80% of KTI was degraded in a reaction that depended on all components of the NADP/thioredoxin system (NTS). BBTI showed the same pattern except that the oxidized protein showed greater proteolytic susceptibility relative to that of KTI. Similar effects were observed with both inhibitors when the plant protease preparation was replaced by subtilisin (data not shown). The nature of the proteolytic reaction was not investigated, but it is noted that peptide products were not detected on SDS gels.

Closing Comments. The results presented in this paper confirm the conclusion that disulfide bonds are essential for the trypsin inhibitory activity of KTI and BBTI (Birk, 1985; Friedman and Gumbmann, 1986; Friedman et al., 1982, 1984). The new information added by the current study is that reduction (inactivation) can take place under physiological conditions—i.e., at low temperature with NADPH-reduced thioredoxin. The ability to inactivate the trypsin inhibitors at lower temperatures provides a potential method for full inactivation of both trypsin inhibitors, thereby improving the quality of soybean products and saving energy. The need for a method for the complete inactivation of KTI is significant since 20% of its activity is consistently retained in soy flour under conditions in which BBTI is fully inactivated (Friedman et al., 1991).

The present results also add new information on the protease susceptibility of KTI and BBTI. Their increase in protease susceptibility following reduction suggests that, if exposed to the protease inhibitors during seed germination, the NADP/thioredoxin system could serve as a mechanism by which the inhibitor proteins are modified (inactivated) and eventually degraded (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978; Orf et al., 1977; Wilson, 1988; Yoshikawa et al., 1979). There is evidence that the NADP/thioredoxin system plays a similar role in mobilizing proteins during the germination of wheat seeds (Kobrehel et al., 1992).

ABBREVIATIONS USED

BBTI, Bowman–Birk soybean trypsin inhibitor; DTT, dithiothreitol; KTI, Kunitz soybean trypsin inhibitor; NTR, NADP-thioredoxin reductase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetatic acid.

ACKNOWLEDGMENT

This research was supported by Grant DMB 88-15980 from the National Science Foundation.

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Received for review May 12, 1992. Accepted August 17, 1992.

Registry No. KTI, 9088-41-9; BBTI, 37330-34-0; NTR, 9074-14-0; NADPH, 53-57-6.