Trypsin Inhibitor from Wheat Endosperm: Purification and Characterization

S. BOISEN and R. DJURTOFT, Department of Biochemistry and Nutrition, Technical University of Denmark, DK-2800 Lyngby, Denmark

ABSTRACT

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A trypsin inhibitor was purified from wheat flour by extraction with 0.1 *M* sodium acetate buffer (pH 5.0), fractionation with ammonium sulfate, heat treatment, anion and cation exchange chromatography on diethylaminoethyl-Sephadex and SP-Sephadex, respectively, and gel filtration in Ultrogel AcA 54. The final preparation was homogeneous by electrophoretic analyses. The isolated inhibitor was a basic protein with a pI of about 9.0. Molecular weights of 12,500 and 12,300 were calculated from sodium dodecyl sulfate polyacrylamide gel electrophoresis and from amino acid composition, respectively. Inhibition of bovine trypsin was linear and stoichiometric up to 90% inhibition with benzoyl-L-arginine-*p*-nitroanilide as substrate and to 70% with casein as substrate. The preparation was also found to have a weak inhibitory action on bovine chymotrypsin. This action was not stoichiometric. The inhibitor was very stable to pepsin at pH 2.0 and to heat at 100°C. The purified inhibitor is immunochemically different from other known cereal trypsin inhibitors in wheat, barley, and rye.

Wheat seeds contain at least four trypsin inhibitors in the embryo (Hochstrasser and Werle 1969; Mistunaga 1974, 1979) and three in the endosperm (Mistunaga 1974).

Using gel filtration on Sephadex G-75 of an extract from wheat seeds, Mikola and Kirsi (1972) found two inhibitor peaks, a small one originating from the embryo (with an elution volume corresponding to a molecular weight of 18,500) and a more dominant one, mainly from the endosperm, with a higher elution volume (the molecular weight was not estimated). Petrucci et al (1974) found that gel filtration on Sephadex G-100 of a wheat albumin fraction gave a comparable elution profile having two active peaks. In their experiments they calculated the corresponding molecular weights to be 24,000 and 12,500.

Three different inhibitors are reported to have been purified from the embryo (Hochstrasser and Werle 1969, Mistunaga 1979). However, none of the inhibitors from the endosperm have as yet been purified and further investigated.

The purpose of the present work is to describe the purification and the chemical and inhibitory properties of the dominant trypsin inhibitor in wheat endosperm.

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MATERIALS AND METHODS

A sample of wheat (*Triticum vulgare*) flour was obtained from Schulstad Bread Factory, Glostrup, Denmark. The flour was produced from imported French wheat, and the husk and embryo had been nearly removed during the milling process.

Assay of Enzyme and Inhibitor Activity

The activity or inhibition of trypsin (NOVO, crystalline preparation from bovine pancreas) was routinely assayed with benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) as substrate. The method of Erlanger et al (1961) was used with some modifications as previously described (Boisen and Djurtoft 1981). The inhibitory activity towards chymotrypsin (NOVO, crystalline preparation from bovine pancreas) was determined with glutaryl-L-phenylalanine-*p*-nitroanilide as substrate, essentially by the method of Erlanger et al (1966). The inhibitor activity toward trypsin was also measured using casein as substrate by the method previously described (Boisen and Djurtoft 1981). The same method was used for estimating the activity of elastase (Boehringer, suspension from porcine pancreas).

One inhibitor unit (U) is defined as the amount of inhibitor necessary to inhibit 1 mg of active trypsin in the assay with L-BAPA

as substrate. One inhibitor equivalent is defined as double the amount of inhibitor necessary for 50% inhibition in the trypsin assay using L-BAPA as substrate. The concentration of active trypsin was estimated by titration with pure bovine pancreas trypsin inhibitor (Kunitz).

Preparation of Inhibitor

Extraction of Trypsin-Inhibiting Activity. Wheat flour (0.5 kg/L) was suspended in 0.1 *M* sodium acetate buffer, pH 5.0. The suspension was allowed to stand overnight at 4°C, and the extract was then isolated by centrifugation (13,000 × *g* for 15 min).

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the extract with vigorous stirring to obtain 50% saturation, and the solution was left for 2 hr at 4°C. The precipitate containing the inhibitor activity was collected by centrifugation and suspended in 90 ml of 0.1 *M* sodium acetate buffer, pH 5.0.

Heat Treatment. The suspension was heated for 5 min in a water bath at 80°C and cooled in an ice bath. The coagulated proteins were then removed by centrifugation.

Anion Exchange Chromatography. The supernatant from the heat treatment was dialyzed overnight against 5 L of 0.01 *M* Tris buffer, pH 8.0. A small amount of precipitate was removed by centrifugation, and the clear supernatant was applied to a 2.5 × 30-cm column of diethylaminoethyl Sephadex equilibrated with the same buffer. All the trypsin-inhibiting activity passed through with the buffer, whereas most of the colored polyphenolic components remained on the column.

Cation Exchange Chromatography. The active effluent from the diethylaminoethyl column was dialyzed overnight against 0.1 *M* sodium acetate buffer, pH 5.0, and then applied to a 2.5 × 30-cm column of SP-Sephadex equilibrated with the same buffer. The activity was eluted with a linear salt concentration gradient.

Gel Filtration. The active peak (fractions 45–55 in Fig. 1) from the preceding step was concentrated by ultrafiltration to a volume of 13.5 ml. The concentrated preparation was chromatographed on a 2.5 × 90-cm column of Ultrogel AcA 54 equilibrated with 0.1 *M* NaCl in a 0.1 *M* sodium acetate buffer, pH 5.0. All inhibitory activity was found in one symmetrical peak. The active fractions were stored in the buffer at −20°C.

Immunochemical Methods

Specific antibodies against the purified inhibitor were obtained after polymerization of the antigen with glutardialdehyde as previously described (Boisen and Djurtoft 1981). Double immunodiffusion was performed as described by Ouchterlony (1959).

Other Analytical Methods

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed as described by Jensen et al (1980). Molecular weights were calculated using ovalbumin (mol wt 45,000), carboanhydrase (mol wt 30,000), cytochrome C (mol wt 12,400), and bovine pancreas trypsin inhibitor (Kunitz, mol wt 6,500) as standard proteins.

Isoelectric focusing was done in 2% Ampholine (LKB), pH 3–10, in 74 × 2.7-mm acrylamide gel rods. Experimental conditions have previously been described in detail (Bruhn and Djurtoft 1977).

Samples for amino acid analyses were hydrolyzed in constantly boiling HCl containing 0.1% phenol at 110°C for 20 or 72 hr and analyzed with a Durrum D-500 analyzer. Half-cystine was estimated after oxidation with performic acid. Tryptophan was estimated spectrophotometrically (Wetlaufer 1962).

RESULTS AND DISCUSSION

A pure preparation of the wheat endospermal trypsin inhibitor was obtained from a wheat flour extract by ammonium sulfate fractionation, heat treatment, anion and cation exchange chromatography, and gel filtration as described above. The results

TABLE I
Purification of Wheat Trypsin Inhibitor

Fraction	Volume (ml)	Activity (U)[a]	Specific Activity (U/E_{280})	Recovery (%)
Extract	710	74.5	0.010	100
Ammonium sulfate precipitate, 50%	103	72.6	0.016	97
Heat treatment	103	67.2	0.036	90
Dialysate	124	65.1	0.041	87
DEAE[b]-Sephadex (unbound material)	200	52.2	0.094	70
SP-Sephadex peak	75	36.2	0.92	49
AcA 54 peak	45	30.6	1.96	41

[a]Inhibitor unit, the amount of inhibitor necessary to inhibit 1 mg of active trypsin.
[b]Diethylaminoethyl.

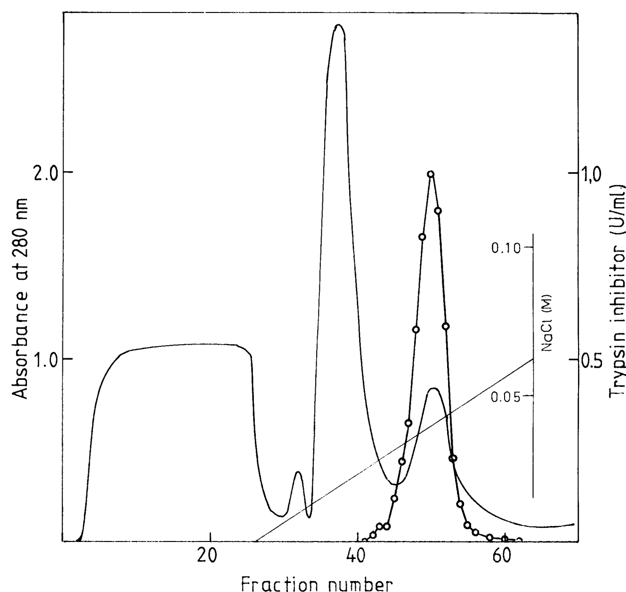

Fig. 1. SP-Sephadex chromatography of the crude wheat trypsin inhibitor from the diethylaminoethyl chromatography step. Bound material was eluted with a linear NaCl gradient. Absorbance at 280 nm (—) was measured continuously, and trypsin inhibitor activity (o—o) was assayed in small samples from 8.5-ml fractions of the eluate.

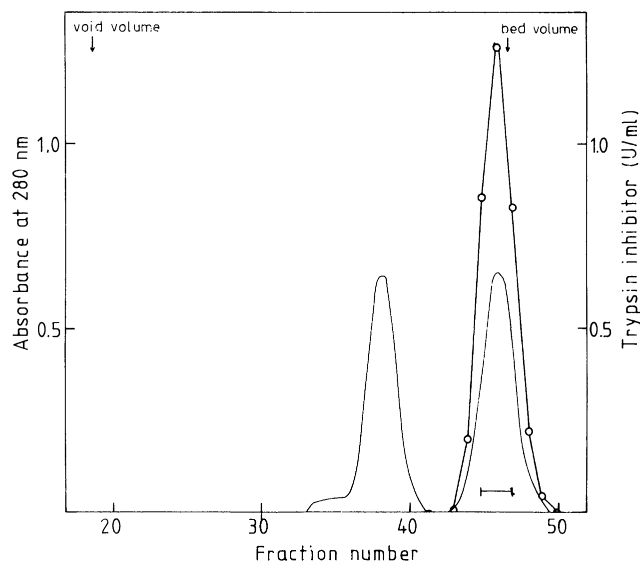

Fig. 2. Ultrogel AcA 54 chromatography of the partly purified wheat trypsin inhibitor from the SP chromatography step. Absorbance at 280 nm (—) was measured continuously, and trypsin inhibitor activity (o—o) was assayed in small samples from 9.5-ml fractions of the eluate. Active peak fractions used for further studies are indicated by a bar at the baseline.

from the purification are summarized in Table I.

In the SP-Sephadex chromatography step (Fig. 1) only one active peak was observed. The initial extract was examined by gel filtration performed on a small aliquot (15 ml) under the same conditions as described for the purified inhibitor. In this case also, only one active peak was found, and the elution volume was exactly the same as that found in the preparative run (Fig. 2). We therefore conclude that wheat endosperm contains only one main trypsin inhibitor.

In the gel filtration step (Fig. 2) the wheat trypsin inhibitor adsorbed markedly to the gel material, even at a relatively high ionic strength (0.2*M*). This resulted in a rather high elution volume for the active peak. The elution volume corresponds to an apparent molecular weight of about 6,000. However, in SDS-gel electrophoresis, the purified inhibitor gave a single zone corresponding to a molecular weight of 12,500. This value is the same as found by Petrucci et al (1974) for the slowest moving inhibitory component in gel filtration of a wheat albumin fraction on Sephadex G-100.

Isoelectric focusing in polyacrylamide gel gave only one zone. The location of the zone corresponds to a pI of about 9.0. The result agrees with that of Petrucci et al (1974), who found that in disc electrophoresis at pH 8.5, all the trypsin inhibitors in a crude fraction of wheat migrated towards the cathode.

The results of the amino acid analyses of the purified inhibitor are shown in Table II. Average numbers of amino acid residues per mole of the inhibitor were calculated on the basis of three phenylalanine residues. The total number of residues, calculated from the nearest integer was 112, and the formula weight was 12,301, which is in good agreement with the value obtained from SDS-gel electrophoresis. The composition of the purified inhibitor from wheat endosperm differs markedly from those reported for the wheat embryo (Hochstrasser and Werle 1969, Mistunaga 1979). Of special interest is the high content of half-cystine, which might indicate a very stable molecule.

TABLE II
Amino Acid Composition of Wheat Trypsin Inhibitor

Amino Acid	Residues per mole[a]	Nearest Integer
Aspartic acid	9.2	9
Threonine	9.0	9
Serine	4.9	5
Glutamic acid	9.0	9
Proline	10.8	11
Glycine	7.3	7
Alanine	7.3	7
Half-cystine	18.4[b]	18
Valine	5.2	5
Methionine	4.0	4
Isoleucine	3.2	3
Leucine	3.9	4
Tyrosine	0.8	1
Phenylalanine	3	3
Histidine	1.5	2
Lysine	3.8	4
Arginine	10.4	10
Tryptophan[c]	0.9	1
Total		112
Molecular weight		12.301

[a]Composition calculated by extrapolation or from the average values estimated on samples after 24 and 72 hr of hydrolysis.
[b]Determined by analyzing the performic acid oxidation product of the inhibitor.
[c]Method of Beaven and Holiday (1952), as modified by Wetlaufer (1962).

The stability of the inhibitor toward heat and pepsin treatment was investigated. The heat stability was examined at 100°C in a 0.1*M* sodium acetate buffer at pH 5.0, and the stability towards pepsin was examined at 37°C in a HCl solution at pH 2.0, with equimolar amounts of inhibitor and porcine pepsin. In both cases the activity of the inhibitor decreased only about 10% after incubation for half an hour.

The effects of the purified inhibitor on the hydrolysis of L-BAPA and casein by pure bovine trypsin are shown in Fig. 3A and B, respectively. The inhibitory action on trypsin with L-BAPA as substrate is both linear and stoichiometric up to 90% inhibition. The same is true for casein as substrate, but only up to about 70%.

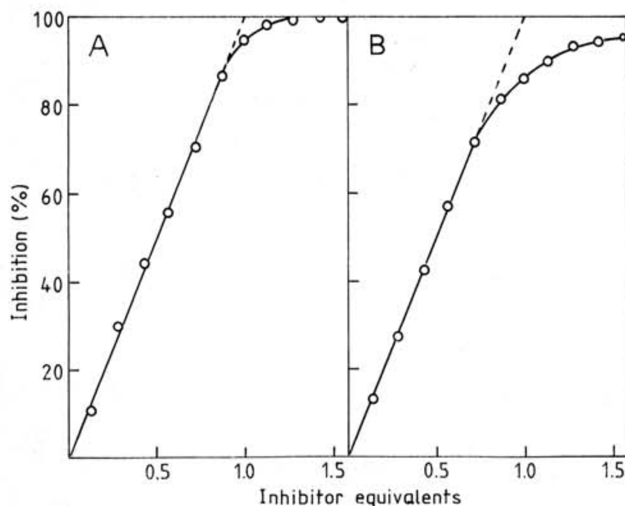

Fig. 3. Inhibition of trypsin by wheat trypsin inhibitor. Hydrolysis of benzoyl-L-arginine-*p*-nitroanilide at pH 8.2 (**A**) and of casein at pH 7.6 (**B**) by bovine trypsin. An inhibitor equivalent is double the amount of inhibitor necessary for 50% inhibition.

The preparation also inhibited bovine chymotrypsin, although this inhibition was weak and nonstoichiometric. The specific activity of the inhibitor was much lower when measured against chymotrypsin (0.44 U/mg of protein) than against trypsin (3.76 U/mg of protein). The high specific activity indicates the presence of two reactive sites against trypsin. No further investigations on this finding were made in this study.

No inhibitory effect was observed on elastase, even when incubation was performed using 5 moles of inhibitor per mole of elastase.

Antibodies against purified wheat endosperm trypsin inhibitor were raised against polymerized inhibitor in the same way as previously described for the rye endosperm trypsin inhibitor (Boisen and Djurtoft 1981). However, in contrast to the rye inhibitor, the wheat inhibitor provoked a relatively weak response in two different rabbits. In immunodiffusion experiments, the antibodies gave precipitates only with the purified wheat endosperm trypsin inhibitor and with whole wheat extract (Fig. 4). No precipitates were obtained with extracts of wheat germ or rye and barley seeds or with purified endosperm trypsin inhibitors from rye, isolated as described by Boisen and Djurtoft (1981), or

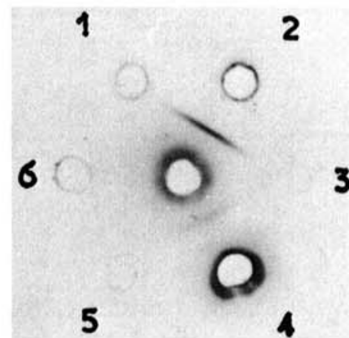

Fig. 4. Double immunodiffusion in 1% agarose gel at pH 8.6. Central well contains specific antiserum against the purified wheat endosperm trypsin inhibitor. Outer wells contain approximately equal amounts of trypsin inhibitor activity from purified endosperm trypsin inhibitors from barley (**1**), wheat (**2**), and rye (**3**) and from extracts of wheat seeds (**4**), isolated wheat germ (**5**), and rye seeds (**6**).

barley (Boisen 1976, Mikola and Suolinna 1969). The same negative result was obtained with the purified wheat trypsin inhibitor against specific immunoglobulins provoked by the rye and barley trypsin inhibitors (not shown). The results agree with those of Mikola and Kirsi (1972), who found that antibodies against the barley endosperm trypsin inhibitor gave precipitates with extracts of rye endosperm but not with extracts of wheat endosperm. The immunogenic properties of purified wheat endosperm inhibitor, therefore, seem to be different not only from the wheat embryo inhibitors but also from the endosperm inhibitors in barley and rye. This indicates that the structure of wheat endosperm trypsin inhibitor is rather specific and different from that of other known cereal trypsin inhibitors.

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