Isolation of Trypsin Inhibitors from Rye, Triticale, and Wheat Samples[1]

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ABSTRACT

Trypsin inhibitors (TI) of rye, triticale, hard red winter wheat, and durum wheat were isolated by extraction, affinity chromatography, CM-cellulose ion-exchange chromatography, and gel filtration chromatography. Affinity chromatography produced a relatively high yield (82 and 83%, respectively) for rye and triticale TI but a poor yield (37 and 29%, respectively) for hard red winter wheat and durum wheats TI. The specific activity of the inhibitors was increased 100–200 times by this step. Affinity chromatography resolved predominantly one rye TI (mol wt 17,000), one triticale TI (mol wt 19,500), two hard red winter wheat TI (mol wt 36,800 and 21,000), and two durum wheat TI (mol wt 38,500 and 22,500). CM-cellulose ion-exchange and gel filtration steps further purified the inhibitors to increase their specific activities.

Among the many purification techniques, affinity chromatography has been found the most versatile for isolating proteins and peptides (Cuatrecasas 1971, Cuatrecasas and Anfinsen 1971a). Recently agarose derivatives have been widely used as a solid matrix in purification procedures (Cuatrecasas and Anfinsen, 1971a, 1971b). In a buffer of an ionic strength of $0.05 M$ or greater, agarose exhibits very little nonspecific absorption of proteins. It can be covalently linked to a ligand with a free α-amino or ϵ-amino group by the cyanogen bromide activation method (Cuatrecasas and Anfinsen, 1971a, 1971b). Agarose affinity chromatography would seem well suited for isolating trypsin inhibitors (TI) from various sources including cereals.

Many studies of soy TI have been reported; however, only a few deal with cereal TI, particularly with their isolation. Hochstrasser and Werle (1969) reported the use of polyanionic trypsin resin to isolate TI from many sources, including wheat and rye germs, with affinity chromatography. Shyamala and Lyman (1964) reported a TI that they isolated from whole wheat flour. Madl and Tsen (1974) isolated six protein fractions with inhibitor activity from an acetate extract of triticale flour with gel filtration and electrophoresis. To explore further the nature of cereal TI, we employed agarose affinity chromatography with ion-exchange chromatography and gel filtration to purify and isolate TI from rye, triticale, hard red winter (HRW) wheat, and durum wheat. Results of the purification steps, the number of TI with their molecular weights, and specific activities from these cereals are reported here.

MATERIALS AND METHODS

The HRW wheat and rye were Kansas-grown samples. Durum wheat was from North Dakota. Triticale was from Tribune, KS. Whole grains were milled into flour with an experimental mill.

Trypsin (bovine pancreas type III, twice×crystallized) and CNBr-activated sepharose 4B were purchaseed from Sigma Chemical Co. (St. Louis, MO). CM-32 cation exchanger was from Whatman (Clifton, NJ), and Bio-Gel P-60 (200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA).

Removal of Fatty Materials from Flour

The whole grain flour was defatted with acetone. After 1 hr or immersion in an acetone solvent, the flour was separated by a Büchner funnel with a vacuum. This process was repeated twice. The defatted flour was then air dried overnight.

Preparation of the Affinity Column

Coupling of Trypsin to Agarose (Sepharose) Gel. CNBr-activated sepharose 4B (15g) was suspended in 500 ml of $0.001 N$

HCl for 30 min to wash out any preservatives in the sepharose. The gel was washed and reswollen on a sintered glass filter and then dispersed in a borate buffer of $0.05 N$ at pH 9.0 with added $0.05 N$ $CaCl_2 \cdot 2H_2O$. One gram of bovine pancreatic trypsin was dissolved in 5 ml of $0.01 N$ HCl and added to the sepharose suspension. The mixture was shaken overnight at $4°C$, using a horizontal rotating plate, and filtered through a sintered glass filter. The filtrate was collected for measure trypsin absorbance units at 280 nm. The sepharose gel was further washed alternately with acetate buffer $(0.1 M, \text{pH } 4)$ and borate buffer $(0.1 M, \text{pH } 8)$ for four times to wash out any unbound trypsin. The buffers all contained $1.0 M$ NaCl. The washings were also collected for measuring trypsin absorbance units at 280 nm. The total trypsin units of the filtrate and washings, which represented the amount of the unbound trypsin, was 252 in this study. Because 1 g of trypsin had an absorbance unit of 1,440 at 280 nm ($E_{280 \text{ nm}}^{0.1\%} = 14.4$ as indicated by the trypsin supplier), the amount of trypsin retained on the 15 g of sepharose was figured as follows:

$$1,440 - (252 / 1,440) = 83\% \text{ or } 830 \text{ mg.}$$

Packing the Affinity Column. The trypsin-bound sepharose gel was packed in a short column (14 cm) with a wide diameter (5 cm) and a sintered glass filter on the bottom. The column was equilibrated with tris buffer of $0.05 N$ at pH 7.5 (with the addition of $0.1 N$ $CaCl_2$ and $1.0 N$ NaCl).

Preparation of the Cation Exchange Column

The pretreated CM-32 cation exchanger was equilibrated in $0.05 N$ acetate buffer, pH 5.3, and packed into a chromatographic column (2.5 × 45 cm). The column was thoroughly preequilibrated with the same buffer. A pump was used to control the eluting rate at 60 ml/hr. Absorbance of the eluent was recorded by a LBK 8300 UVCORD monitor.

Preparation of the Gel Filtration Column

Bio-Gel P-60 (200–400 mesh) was the gel filtration medium. Gel preparation was according to Madl and Tsen (1974). Sixteen grams of dry gel was suspended in 800 ml of $0.05 N$ acetate buffer, pH 5.3, and allowed to swell fully. The gel was then poured into a column (2.5 × 45 cm) to assure a uniform packing of the gel particles. The system was equilibrated with buffer at an eluting rate of 9 ml/hr. The column was calibrated by using reference proteins (3 mg each) of known molecular weight. A standard curve was then constructed with elution volume plotted against the logarithm of the molecular weight for each reference protein.

Isolation of TI

Defatted flour (1 g) was suspended in 15 ml of $0.05 N$ tris buffer at pH 7.5 with $0.1 N$ NaCl and $0.02 N$ $CaCl_2$. The extraction was carried out for 1 hr at room temperature. An adjustment of pH back to 7.5 was needed before the slurry was centrifuged at 14,000 rpm for 30 min at $4°C$.

For affinity chromatography, a known amount of trypsin inhibitor extract was applied to the column. Passage of the extract through the affinity gel was by gravity force. TI activity was checked on the effluent to prevent overloading of the extract on the column. The column was washed with the buffer until all the nonbinding proteins were eluted from the column. At this stage only the TI and some nonspecific binding proteins were bound to the affinity gel. Seventy-five milliliters of $8M$ urea at pH 2.0 was then used as a dissociating agent to release all the trypsin inhibitors from the column. The effluent was collected fractionally. The column was regenerated by an immediate washing with eluting buffer until the eluent had an absorbance at 280 nm of less than 0.05. The inhibitor fractions were dialyzed in $0.5N$ acetate buffer, pH 5.3 (the dialyzing tubing had a molecular weight cut point at 3,000). The precipitate, if any, was removed by centrifugation. Fractions with high inhibitor activity were collected, pooled, and concentrated by rotary evaporation.

For cation-exchange chromatography, the concentrated inhibitor extract that had previously dialyzed against $0.05N$ acetate buffer (pH 5.3) was applied to the column. After the nonbinding proteins had been eluted, a linear salt gradient of up to $1.0N$ NaCl was used to dissociate the inhibitors from the column. The fractions with high inhibitor activity were collected, concentrated by rotary evaporation, and then dialyzed against $0.5N$ acetate buffer (pH 5.3).

For gel-filtration chromatography, 10 ml of the concentrated inhibitor solution was applied to the column. Blue dextran was used to indicate column void volume. Fractions with high TI activity were collected and used for further study.

Identification of the Number of TI
Resolved by Affinity Chromatography

After affinity chromatography, the dialyzed TI extract was applied on a Bio-Gel P-60 gel filtration column. The molecular weight of each peak was estimated from the standard curve constructed for proteins of known molecular weight.

Analyses

The assay for determining TI activity was described previously (Chang and Tsen 1979). An inhibitor unit was expressed as half inhibition of trypsin (HIT), as reported by Chang and Tsen (1979).

RESULTS AND DISCUSSION

Isolation of TI

Profiles of each isolation step for triticale TI are illustrated in Figs. 1-3. For affinity chromatography of triticale TI, an extract of 50,000 HIT was applied on the column and 41,650 HIT (83%) was recovered by the dissociating agent, $8M$ urea at pH 2.0. The fraction was collected at 11 ml per tube. For CM-ion exchange chromatography, a sample of 41,650 HIT was applied and 39,060 HIT (94%) was recovered under the peak area. The fraction was collected at 15 ml per tube. For the gel filtration of triticale TI, a sample of 13,000 HIT was applied; 130 HIT (1%) was recovered in fraction I and 12,300 HIT (95%) in fraction II. The fractions were collected at 4.2 ml per tube. Profiles for rye, durum wheat, and HRW wheat TI follow a very similar pattern. Tables
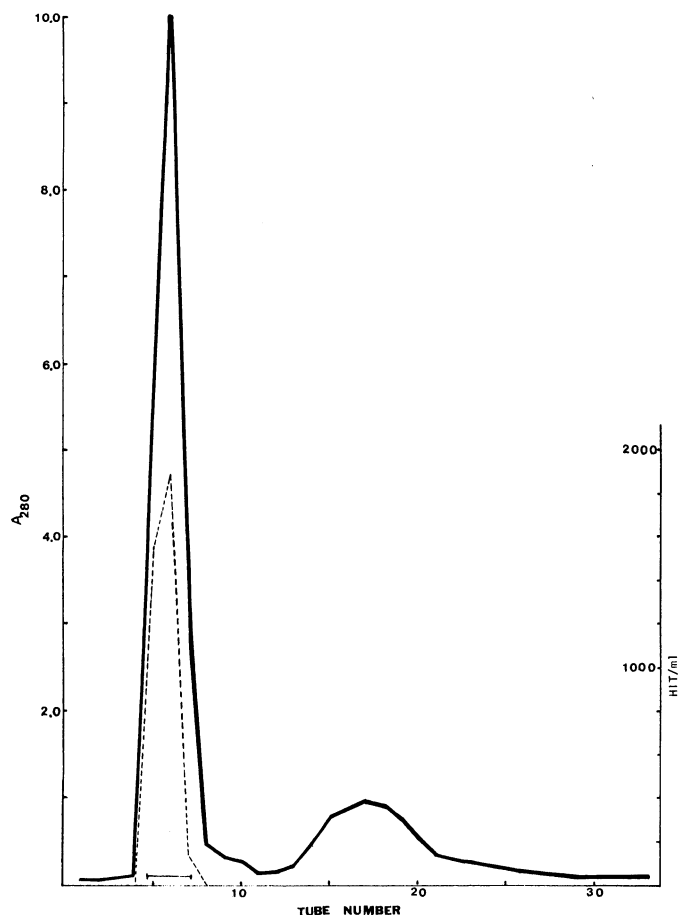

Fig. 1. Affinity chromatography profile of triticale trypsin inhibitors. — = Protein concentration, --- = trypsin inhibitor activity per milliliter. Inhibitor unit is expressed as half inhibition of trypsin (HIT).
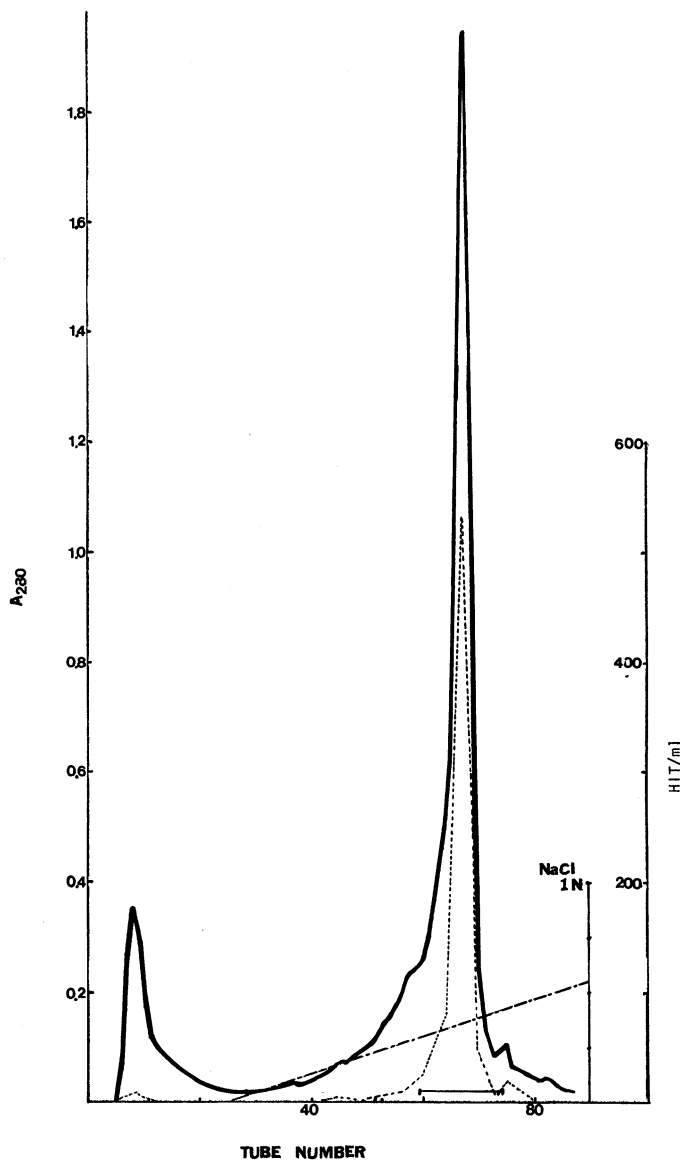

Fig. 2. CM-ion exchange chromatography profile of triticale trypsin inhibitors. — = Protein concentration, --- = trypsin inhibitor activity per milliliter. Inhibitor unit is expressed as half inhibition of trypsin (HIT).

I–IV list the yield of HIT for each isolation step for each of the TI.

One predominant TI was isolated from each of triticale, rye, and durum wheat and two from HRW wheat. Triticale and rye TI behaved very similarly throughout each of their respective purification steps. Both of the inhibitors had a higher final yield (~75%) than did the TI from durum wheat (~20%) or HRW wheat (~27% for the two inhibitors combined). As shown in Tables I–IV, all the yields were based on the total HIT of the extract. The final yield of the cereal TI through this purification procedure depends largely on the affinity chromatographic step. Triticale and rye TI yields were fairly high (83 and 82% of the extract yield, respectively), whereas those of HRW and durum wheats were low (37 and 29% of the extract yield, respectively) through this step. Many possibilities could account for the failure or ineffectiveness of the affinity matrix to bind TI. The most likely reason is steric hindrance exerted by the agarose polymeric back-bone against formation of the trypsin and inhibitor complex. This steric effect varies among different TI species. As shown in Table V, triticale and rye TI, which had relatively low molecular weights (19,500 for
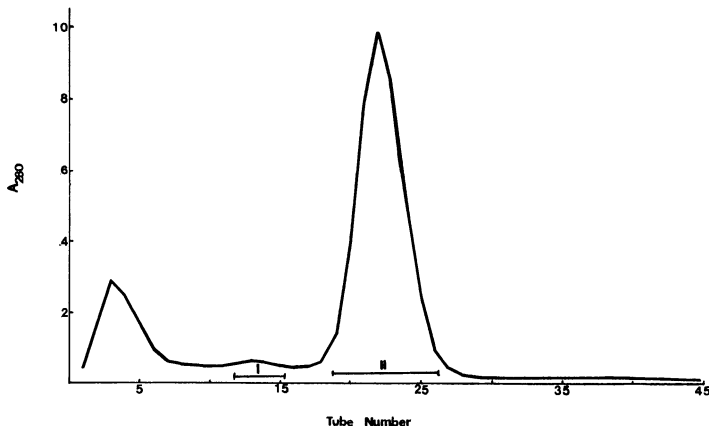

Fig. 3. Gel filtration profile of triticale trypsin inhibitor, showing recovery in fractions I and II.

TABLE I
Purification of Rye Trypsin Inhibitor

Purification Steps	HIT[a]/ml	Volume (ml)	Total HIT[a]	Protein (mg/ml)	Specific Activity (HIT[a]/mg)	Yield (%)
Extract at pH 7.5	8.0	3,000	24,000	3.74	2.1	100
Affinity column	700.0	28	19,600	2.50	280.0	82
Cation exchange column	80.0	230	18,400	0.24	333.0	77
Gel filtration column	480.0	38	18,240	1.31[b]	366.4	76

[a] Inhibitor unit expressed as half inhibition of trypsin.
[b] Protein concentration was based on $E_{280 nm}^{0.1\%} = 1.00$.

TABLE II
Purification of Triticale Trypsin Inhibitor

Purification Steps	HIT[a]/ml	Volume (ml)	Total HIT[a]	Protein (mg/ml)	Specific Activity (HIT[a]/mg)	Yield (%)
Extract at pH 7.5	10.0	5,000	50,000	4.56	2.2	100
Affinity column	1,190.0	35	41,650	4.88	243.9	83
Cation exchange column	180.0	217	39,060	0.62	290.3	78
Gel filtration column	970.0	38	36,860	3.10[a]	312.9	74

[a] Inhibitor unit expressed as half inhibition of trypsin.
[b] Protein concentration was based on $E_{280 nm}^{0.1\%} = 0.97$.

TABLE III
Purification of Hard Red Winter Wheat Trypsin Inhibitors (TI)

Purification Steps	HIT[a]/ml	Volume (ml)	Total HIT[a]	Protein (mg/ml)	Specific Activity (HIT[a]/mg)	Yield (%)
Extract at pH 7.5	3.0	7,000	21,000	2.66	1.1	100
Affinity column	59.0	130	7,670	0.62	95.2	37
Cation exchange column	16.0	360	5,760	0.12	133.3	27
Gel filtration column						
TI I	100.0	35	3,500	0.16[b]	625.0	17
TI II	44.0	50	2,200	0.16[b]	275.0	10

[a] Inhibitor unit expressed as half inhibition of trypsin.
[b] Protein concentration were based on $E_{280 nm}^{0.1\%} = 1.10$ for TI I and $E_{280 nm}^{0.1\%}$ for TI II.

TABLE IV
Purification of Durum Wheat Trypsin Inhibitor

Purification Steps	HIT[a]/ml	Volume (ml)	Total HIT[a]	Protein (mg/ml)	Specific Activity (HIT[a]/mg)	Yield (%)
Extract of pH 7.5	3.0	6,500	19,500	3.54	0.8	100
Affinity column	81.0	70	5,670	0.52	155.8	29
Cation exchange column	16.0	320	5,120	N.A.[b]	N.A.[b]	26
Gel filtration column	55.0	70	3,850	0.09[c]	611.1	20

[a] Inhibitor unit expressed as half inhibition of trypsin.
[b] Not available.
[c] Protein concentration was based on $E_{280 nm}^{0.1\%} = 1.43$.

TABLE V
Yields and Molecular Weights of Trypsin Inhibitors Isolated by Affinity Chromatography

Inhibitors	Yield (%)	Molecular weight
Rye		
I	9	44,000
II	84	17,000
III	5	5,300
Triticale		
I	95	19,500
II	1	5,400
Hard red winter wheat		
I	60	36,800
II	39	21,000
III	2	5,000
Durum wheat		
I	67	38,500
II	20	22,500
III	7	...

triticale inhibitor I and 17,000 for rye inhibitor II), could be absorbed to the trypsin ligand effectively. TI from HRW and durum wheats could be absorbed to the trypsin ligand poorly because of their higher molecular weights (36,800 and 21,000 for the former and 38,500 and 22,500 for the latter). At least 25 and 12% of escaped TI activity could be detected for HRW wheat and durum wheat TI, respectively, after the extract had been passed through the the affinity matrix. None of the escaped TI activity could be found for triticale and rye TI extracts. Other possible reasons for the failure of TI to interact with the immobilized trypsin are 1) electrostatic repulsion between the two components, 2) irreversible denaturation of TI by urea of pH 2, 3) incapability of the dissociating agent to dissociate the TI from the affinity matrix, and/or 4) precipitating out of the TI upon the removal of urea by dialysis.

In spite of the limitation of steric hindrance, the affinity chromatographic step concentrated the cereal TI effectively enough to increase their specific activities (HIT/mg of protein) up to 70–200 times. The CM-cellulose cationic exchange and the gel filtration chromatographic steps purified the TI further to increase their specific activities.

Identification of the Number of TI
Resolved by Affinity Chromatography

The trypsin inhibitors and their estimated molecular weights are listed in Table V. Each cereal sample with the exception of triticale has three different TI. Affinity chromatography separated predominantly one rye TI (mol wt 17,000), one triticale TI (mol wt 19,500), two hard red winter wheat TI (mol wt 36,800 and 21,000), and two durum wheat TI (mol wt 38,500 and 22,500). Disc gel electrophoresis on these cereal TI also showed them to be heterogenous.

A number of investigators have found several TI in soybeans (Laskowski and Sealock 1971), lima beans (Haynes and Feeney 1967), *Lathyrus sativus* (Roy 1972), wheat and rye germs (Hochstrasser and Werle 1969), and triticale flour (Madl and Tsen 1974). The results of this study not only substantiate the findings on the heterogeneity of TI, but also provide new information on the isolation, identification, and molecular weights of TI from whole grain samples of rye, triticale, HRW wheat, and durum wheat.

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