# Characterization and Heat Stability of Trypsin Inhibitors from Rye, Triticale, and Wheat Samples[1]

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## ABSTRACT

Extinction coefficients were determined for the trypsin inhibitors (TI) isolated from rye, triticale, hard red winter wheat, and durum wheat. Except for TI from hard red winter wheat II, all the inhibitors contained carbohydrate. No free sulfhydryl group was detected in any of the inhibitors by the amperometric titration method. Amino acid composition studies on triticale and rye TI indicated the similarity of their amino acid constituents, ie, a high amount of glutamic acid, leucine, and glycine and very little methionine and tryptophan. All inhibitors were heat stable. Triticale TI was the most heat resistant among the inhibitors tested. Heating at 100° C for 1 hr inactivated only 7% of triticale TI. Heating up to 80° C caused minimal conformational perturbation of the inhibitors, and the perturbed molecules tended to go back to their original conformation upon cooling. However, heating at 125° C altered the conformation of the inhibitors and permanently inactivated all of them.


Trypsin inhibitors (TI) are extremely resistant to denaturants. Inhibitors from different sources show different degrees of resistance to high temperature, low pH, 8*M* urea, and 6*M* guanidine-HCl (Belew et al 1975, Madl and Tsen 1974, Ogiso et al 1976, Vincent et al 1971). Conformational changes by denaturants usually involve only minimal perturbation to the molecules, and these changes are reversed after the denaturants have been removed. The heat resistance of TI has drawn much concern because of their nutritional effect on food, and efforts have been made to inactivate the inhibitors during food processing.[3]

Because cereals provide a major food source, some information on the characterization and heat stability of cereal TI is of interest to cereal scientists and industry. This article presents such information based on our studies.

## MATERIALS AND METHODS

TI from triticale, rye, hard red winter (HRW) wheat, durum wheat, and soybeans, obtained from previous isolation procedures (Chang and Tsen 1981), were used.

### Determination of TI Activity

TI activity was assayed as described previously (Chang and Tsen 1979).

### Estimation of Carbohydrate Content

Carbohydrate was estimated according to Dubois et al (1956) by adding 1 ml of 5% phenol followed by 5 ml of concentrated $H_2SO_4$ to 1 ml of the sample solution. After 30 min, the absorbance of the mixture at 480 nm was measured. To quantitate carbohydrate content, a standard curve using D-mannose as the standard sugar was constructed. Because the carbohydrate content in each of the TI was very low, only its presence or absence was indicated.

### Free Sulfhydryl Group Determination

Free thiol groups were determined by amperometric titration (Tsen and Hlynka 1963, Madl 1973). Fisher Electropode model 65 was used as a galvanometer.

### Determination of Extinction Coefficient

The procedure for determination of the extinction coefficient was that of Babul and Stellwagen (1969). TI were first dialyzed thoroughly against 1m*M* HCl with 0.1*M* NaCl and then centrifuged at 10,000 rpm in a Beckman model E analytical ultracentrifuge. The concentration of the protein was determined from the number of fringes across the protein boundary. The absorbance of the same protein at 280 nm was measured. $E^{0.1\%}_{280\ nm}$ could be obtained according to Beer's law.

### Amino Acid Composition Analysis

Amino acid analysis was done according to Spackman et al (1958) and Moore et al (1959), using a Beckman automatic amino acid analyzer. Hydrolysis of the samples was done at 110° C for 22, 48, and 72 hr, respectively. Values of threonine and serine were obtained by extrapolating to zero hydrolysis time. Valine, isoleucine, and leucine were from 72-hr hydrolysis. Values for other amino acids were from 22-hr hydrolysis. The amount of tryptophan was determined by a spectrometric method (Edelhoch 1967).

### Heat Stability Studies

*Effects of Temperature on the Destruction of TI Activity.* The TI solution was divided into five portions and each portion was weighed in a test tube. One portion was used as a control and the other four were heated in an oil bath at 60, 80, 100, and 125° C, respectively, for 1 hr. A reflex device was used on top of the heating tubes to avoid excessive moisture loss. After the tubes were cooled to room temperature, the weight of each portion of solution was carefully adjusted back by adding water. Inhibitor activity of each portion was measured, and the percentage of inhibitor activity destruction was plotted against temperature.

*Effects of Temperature on the Conformational Change of TI.* A Cary-14 recording spectrophotometer with a water circulation device was used for determining temperature effects. The TI solutions were scanned from 350 to 260 nm vs 0.01*N* acetate buffer, pH 5.3, after each 10° C increase of temperature. A maximum temperature of 80° C was reached in this experiment. The inhibitor solution was then allowed to cool to room temperature, and the ultraviolet (UV) spectrum was remeasured. A graph was constructed by using the change of absorbance at 260, 278, and 290 nm plotted against different heating temperatures.

*Differential UV Spectrum of Native and Denatured TI.* TI solution (1 ml) was diluted with 1 ml of 0.05*N* acetate buffer solution, pH 5.3, and then divided equally into two portions. One portion was heated to 125° C for 1 hr to denature the inhibitor completely. The differential spectrum was scanned from 350 to 260 nm, using denatured inhibitor as sample and native inhibitor as reference.

## RESULTS AND DISCUSSION

Molecular weights, carbohydrate contents, and extinction coefficients of the TI are listed in Table I.

### Carbohydrate Estimation

All the tested TI except that for HRW wheat TI II contained a small amount of carbohydrate. Kassell and Williams (1976)

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[3]J. J. Rackis. 1975. Practical significance of soybean trypsin inhibitors. Presented at First Latin American Soy Protein Conference, Mexico City, Nov. 9-12.

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reported that all TI from egg white contained carbohydrate and most of the animal TI contained no carbohydrate. Whole wheat TI contained carbohydrate.

### Free Sulfhydryl Group Content

No free sulfhydryl groups (of either reactive or buried type) were detected in any cereal TI. This indicates that all the sulfhydryl groups in the molecules engage in disulfide linkage, which is partly responsible for the rigidity of the inhibitor molecules.

### Determination of Extinction Coefficients

Triticale and rye TI had lower extinction coefficients than did the wheat TI. The only reported value for plant TI was that from barley (Kassell and Williams 1976), which was 12.7.

### Amino Acid Composition

Amino acid analyses were done only for triticale and rye inhibitors. These have a similar amino acid composition (Table II). Glutamic acid, leucine, and glycine are the predominant amino acids in both TI. Methionine and tryptophan occur rarely. Like the other TI, they contain a higher amount of proline than do average proteins (Reeck and Fisher 1973). The amino acid composition of the rye TI in this study is different from that used by Hochstrasser and Werle (1969).

### Heat Stability Studies

*Effects of Temperatures on the Destruction of TI Activity.* Data on the destruction of TI by heat treatment are presented in Table III. All cereal TI tested in this study were heat stable, much more than soy TI was (Obara and Watanabe, 1971). Triticale TI was the most stable among the cereal TI tested, confirming the high heat stability of triticale TI found by Madl and Tsen (1974). Heating at 100° C for 1 hr inactivated different proportions of TI from different cereal samples: 7% from triticale, 35% from rye, 44% from HRW wheat I and durum wheat, and 71% from HRW wheat II.

*Effects of Temperature on the Conformational Change of TI.* The effects of various temperatures on conformational change were similar for all the TI. The effect is illustrated on triticale and rye TI in Fig. 1. The conformation of the inhibitor is not perturbed very much by the temperature up to 80° C. All the inhibitors tend to return to their original conformation after cooling.

*Differential UV Spectrum of Native and Denatured TI.* Triticale TI was used in the UV spectrum experiment (Fig. 2). Heating to 125° C could cause the conformational change of the inhibitor molecules to become permanent. Compared to the native triticale TI, the denatured TI had a higher absorbance at wavelengths greater than 290 nm and a lower absorbance at wavelengths between 250 and 290 nm. The other TI behaved similarly upon denaturation.

**TABLE 1**
**Some Physical Properties of Cereal Trypsin Inhibitors**

| | Molecular Weight[a] | Carbohydrate Content[b] | Extinction Coefficient[c] |
|---|---|---|---|
| Triticale | 19,500 | + | 0.97 |
| Rye | 17,000 | + | 1.00 |
| Hard red winter wheat | | | |
| I | 36,800 | + | 1.10 |
| II | 21,000 | − | 1.28 |
| Durum wheat | 38,500 | + | 1.43 |

[a] Data from Chung and Tsen 1981.
[b] + = Present, − = absent.
[c] $E^{0.1\%}_{280\ nm}$

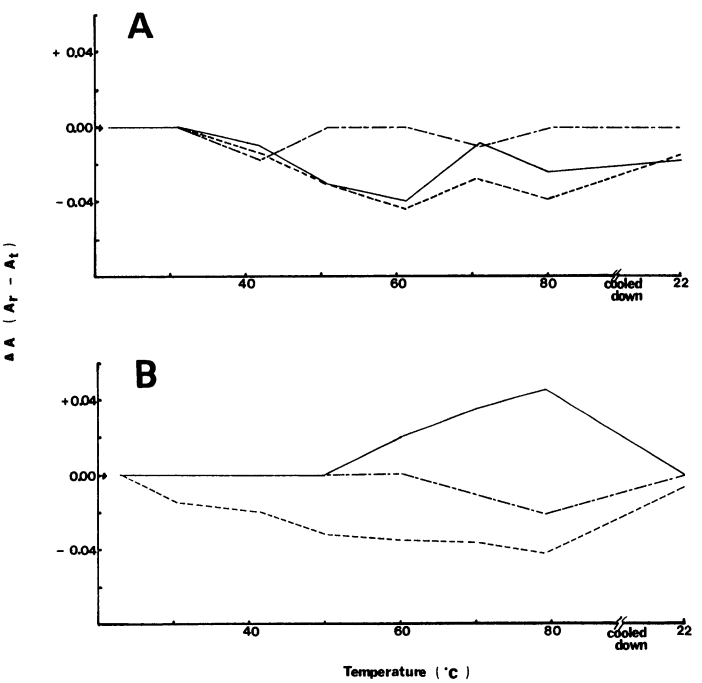


**Fig. 1.** Conformational change of rye **(A)** and triticale **(B)** trypsin inhibitor at various temperatures. $A_t$ and $A_r$ are the absorbance at various heat temperatures and at room temperature, respectively. — = 260 nm, - - - = 278 nm, - - - - = 290 nm.

**TABLE II**
**Amino Acid Composition (Molar Ratio) of Trypsin Inhibitors**

| | Triticale | Rye |
|---|---|---|
| Lys | 2.1 | 2.1 |
| His | 2.4 | 2.0 |
| Arg | 6.8 | 7.8 |
| Asp | 6.5 | 6.6 |
| Thr | 7.0 | 5.9 |
| Ser | 4.8 | 4.0 |
| Glu | 11.0 | 10.6 |
| Pro | 9.6 | 9.5 |
| Gly | 9.8 | 10.2 |
| Ala | 7.4 | 7.8 |
| Cys/2 | 4.9 | 5.7 |
| Val | 7.6 | 7.5 |
| Met | 0.3 | 0.3 |
| Ile | 3.6 | 3.6 |
| Leu | 10.5 | 10.2 |
| Tyr | 3.3 | 3.6 |
| Phe | 1.9 | 1.8 |
| Trp | 0.6 | 0.9 |

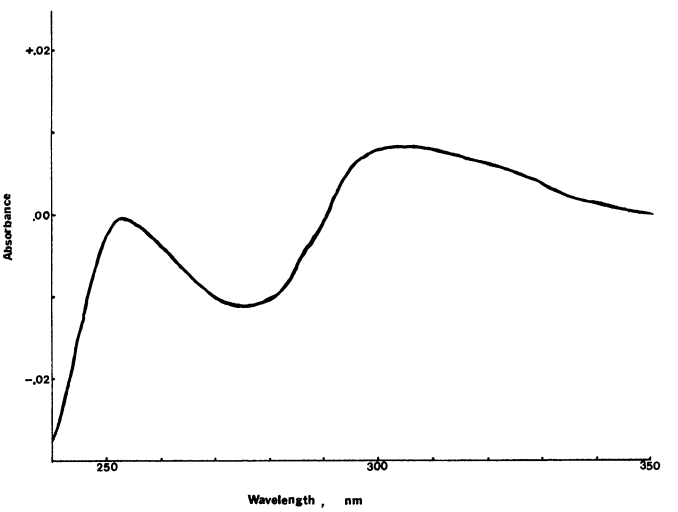


**Fig. 2.** Differential ultraviolet spectrum of denatured and native triticale trypsin inhibitors (denatured inhibitor as sample and native inhibitor as reference).

**TABLE III**
**Percentage of Trypsin Inhibitor Activity Destruction at Various Temperatures**

| Trypsin Inhibitor | Temperature (° C) | | | |
|---|---|---|---|---|
| | 60 | 80 | 100 | 125 |
| Hard red winter wheat | | | | |
| I | 29 | 36 | 44 | 100 |
| II | 28 | 32 | 71 | 100 |
| Triticale | 4 | 4 | 7 | 100 |
| Rye | 15 | 15 | 36 | 100 |
| Durum wheat | 0 | 14 | 38 | 100 |


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