NOTE

Trypsin Inhibitor Measurement: Effect of Order of Reagent Addition

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In the standard trypsin inhibitor (TI) assay method (AACC 1983; Kakade et al 1969, 1974), trypsin is dissolved in 0.001*N* HCl, trypsin inhibitor (soy meal extract) is diluted with water, and the substrate is dissolved in the assay buffer (0.05*M* Tris-HCl, pH 8.2; 0.02*M* $CaCl_2$). The enzyme is mixed with inhibitor (or with water, for the zero inhibition tube) and warmed to 37°C. Then the substrate solution, also at 37°C, is added to start the assay. After 10 min of incubation, the reaction is stopped by adding 30% acetic acid, and the absorbance value of the mixture is determined at 410 nm. One trypsin inhibitor unit (TIU) is defined as the amount of extract that causes a 0.01% decrease in the absorbance reading.

It has been reported that reversing the order of reagent addition increases the slope of the plot of absorbance versus amount of inhibitor extract (Viswanatha and Liener 1954; Liu and Markakis 1989a,b). In other words, there is a greater degree of inhibition when the substrate is preincubated with inhibitor (or water) and the assay is started by adding enzyme (E-last mode). Recommending that the standard TI assay use this order of reagent addition instead of the standard method of adding substrate to start the assay (S-last mode) is essentially arbitrary in the absence of an explanation for the discrepancy. There is no virtue in selecting a method simply because it yields somewhat larger numbers.

Liu and Markakis (1989b) offered one explanation for the difference based upon cleavage (by typsin) of a peptide bond in trypsin inhibitor, with the presumption that the inhibitor, modified during the preincubation period, was less effective. Another possible cause is that the alteration of trypsin during the preincubation occurs in the absence of calcium ion, a known stabilizer of trypsin. As reported here, the calcium ions were the main cause of the discrepancy between the two modes of running the TI assay.

MATERIALS AND METHODS

The materials used in this study were the same as those in an earlier report (Stauffer 1990). The stock enzyme solution contained 1.0 mg of porcine trypsin per milliliter in 0.001*N* HCl. The stock substrate was 40 mg of *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPA) dissolved in 1 ml of dimethylsulfoxide (DMSO). The standard buffer was 0.05*M* Tris-HCl, pH 8.2, 0.02*M* in $CaCl_2$.

The soy extract was prepared essentially as described by Liu and Markakis (1989a). To 50 mg of enzyme-active defatted soy flour was added 5 ml of distilled water. The suspension was incubated at 37°C for 30 min with occasional stirring. Then 5 ml of standard buffer was added. After about 10 min at room temperature, the solid material had flocculated and mostly settled out. The supernatant was filtered through a 0.45-μm membrane filter to get a clear solution; 25 μl of this extract gave about 50% inhibition of 10 μg of trypsin.

Two modes of reagent addition were used. In the S-last mode, enzyme, without or with inhibitor, was incubated at 37°C for 30 min. Substrate solution (warmed to 37°C) was then added to start the assay, which was terminated after exactly 10 min of reaction time by adding 30% (v/v) aqueous acetic acid. In the E-last mode, substrate was incubated in buffer (with or without inhibitor) at 37°C, and enzyme solution (at room temperature) was added to start the assay.

The solutions used were only half the volume of those described in the standard assay procedure (in either the S-last or E-last mode). The substrate solution contained 1 ml of the stock substrate added to 99 ml of standard buffer that had been previously warmed to 37°C; 2.5 ml of substrate solution was used per assay tube. The enzyme solution contained 1.2 mg of trypsin (1.2 ml of stock) diluted to 100 ml in 0.001*N* HCl; 1 ml of enzyme solution was used per assay tube. Each assay tube contained 440 pmol of active trypsin in 12 μg of trypsin, using values of 87% active enzyme and 23,560 molecular weight (Stauffer 1990). Each assay tube contained 1 ml of inhibitor solution comprised of 3 ml of extract diluted to 100 ml with water.

All assays were replicated fivefold. Absorbances were read at 385 nm, using water in the reference beam (Stauffer 1990). This wavelength is the peak of the difference spectrum (assay solution versus reagent blank), minimizing spectrophotometric error. Some of the readings were repeated at 410 nm, in order to determine the ratio of absorbances of hydrolyzed substrate at the two wavelengths. The blank-corrected A_{385} was $1.47 \pm 0.02 \times$ the blank-corrected A_{410}. (Blank absorbance, read versus water, was about 0.270 at 385 nm and 0.025 at 410 nm.) If one TIU is defined as a slope of −0.01 when the assay measurement is made at 410

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TABLE I
Trypsin Inhibitor Assays: Effect of Buffer and Ca^{++} During Preincubation for 30 Min at 37°C on Trypsin Inhibitor Unit (TU) Measurements

	Standard Assay[a]		Buffered Assay[b]	
	S-Last Mode[c]	E-Last Mode[d]	S-Last Mode	E-Last Mode
A_{385}, no inhibitor	0.808 ± 0.025	0.904 ± 0.012	0.942 ± 0.005	0.939 ± 0.018
A_{385}, 1 ml inhibitor	0.552 ± 0.022	0.588 ± 0.006	0.649 ± 0.006	0.631 ± 0.006
TIU per ml	17.5 ± 1.0[e]	21.5 ± 0.4	19.9 ± 0.2[f]	20.9 ± 0.6

[a] Inhibitor diluted with water and enzyme diluted with 0.001 *N* HCl.
[b] Both reactants diluted with standard buffer (0.05 *M* Tris-HCl, pH 82, 0.02 *M* $CaCl_2$.)
[c] Substrate added to initiate assay.
[d] Enzyme added to initiate assay.
[e] LSD ($P > 0.05$) = 2.0.
[f] LSD ($P > 0.05$) = 0.8.

nm, then one TIU gives a slope of −0.0147 when the measurement is made at 385 nm. This factor was used in calculating TIUs in Table I.

RESULTS

Standard Method Assay

This assay was run using standard method solutions, in both the S-last (standard) and E-last (proposed) modes (Table I). The data from this experiment confirm the observations of Liu and Markakis (1989a,b). The value for TIU (slope of absorbance versus inhibitor) is significantly lower (19%) in the S-last mode, compared to the E-last mode.

Buffered Standard Assay

In this assay, both reactants were diluted with standard buffer, rather than with 0.001*N* HCl for enzyme and water for inhibitor. Thus, the preincubation buffer, pH 8.2, contained Ca^{2+} as in the first experiment. The results are shown in Table I. The noninhibited rates are identical for both modes of addition, and in the E-last mode, the amount of inhibition is 5% greater.

Enzyme Stability

These results indicate that, in 0.001*N* HCl and the absence of Ca^{2+}, porcine trypsin is less stable than at pH 8.2 in the presence of 0.02*M* Ca^{2+}. In routine TI assay, a dilute solution of trypsin (100 ml) is prepared for use over several hours. Should this dilution be in dilute acid or in the standard assay buffer? Stock trypsin was diluted to 12 μg/ml in 0.001*N* HCl and in standard buffer. Both were assayed immediately, and again after being held at 15°C for 6 hr. At this temperature the activity loss was minimal in both diluents: 6% in acid, and 4.5% in standard buffer. For practical purposes, either medium is acceptable. The use of standard buffer is preferable, however, from the standpoint that there is less perturbation of the assay pH when the enzyme solution is added.

DISCUSSION

The data given in Table I were obtained as part of a study undertaken to find the reason for the difference in TIU measurement (the slope of the plot of absorbance versus amount of inhibitor extract) when the assay reaction was initiated by adding enzyme (E-last) as compared to adding substrate (S-last). Although that work continues, these data are of immediate interest because a collaborative study is presently underway, seeking to improve the standard method for measuring trypsin inhibitors in various soy-derived food products. The results reported here, plus the earlier work of Liu and Markakis (1989a,b), support the following recommendations for changing the standard assay procedure: 1) initiate the assay reaction by adding enzyme solution (E-last mode); 2) make dilutions of enzyme and inhibitor in standard buffer (0.05*M* Tris-HCl containing 0.02*M* $CaCl_2$).

Other improvements are being pursued to make the TI assay method more convenient and to reduce interlaboratory variation.

LITERATURE CITED

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1983. Approved Methods of the AACC. Method 71-10, approved November 1973. The Association: St. Paul, MN.

KAKADE, M. L., SIMONS, N., and LIENER, I. E. 1969. An evaluation of natural versus synthetic substrates for measuring the antitryptic activity of soybean samples. Cereal Chem. 46:518.

KAKADE, M. L., RACKIS, J. J., McGHEE, J. E., and PUSKI, G. 1974 Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chem. 51:376.

LIU, K., and MARKAKIS, P. 1989a. An improved colorimetric method for determining antitryptic activity in soybean products. Cereal Chem. 66:415.

LIU, K., and MARKAKIS, P. 1989b. Trypsin inhibition assay as related to limited hydrolysis of inhibitors. Anal. Biochem. 178:159.

STAUFFER, C. E. 1990. Measuring trypsin inhibitor in soy meal: Suggested improvements in the standard method. Cereal Chem. 67:296.

VISWANATHA, T., and LIENER, I. E. 1954. Inhibition of trypsin: I. Sequence of mixing the reactants. J. Biol. Chem. 210:97.

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