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Triticale, a newly man-made cereal from a wheat-rye cross offers a unique food and feed potential because of its higher protein content with nutritionally balanced amino acid composition as compared to other cereal grains. However, some cereal proteins such as trypsin inhibitors, present in triticale and rye, can affect the digestion and utilization of dietary proteins in non-ruminant animals. Therefore, selections of triticale low in trypsin inhibition activity are desired.

This study was thus conducted: (1) To refine existing methods for assaying trypsin inhibitor activity (TIA), so that an accurate laboratory procedure for determining TIA in triticale seeds would be available; (2) To observe the influence of various storage conditions on changes in TIA of triticale seeds.

Eight triticale cultivars harvested in 1982 at the Hyslop Agronomy Farm near Corvallis, Oregon, were used in the study. It was

observed that seed meal of 0.45 mm and 0.75 mm in particle diameter did not significantly influence the assaying results. Either 100 or 125 µg/ml procine trypsin can be used to differentiate various levels of TIA among diverse triticale selections, but not 75 or 150 µg/ml. A larger sample weight of 40 mg resulted in less variation in TIA between duplicated samples than observed when 20 mg samples were used.

Experimental data indicated that TIA in seeds of eight triticale cultivars stored under desert (33°C, 65% RH), temperate (21°C, 65% RH) and arctic (1.7°C, 65% RH) conditions showed small but significant increases after 30 days of storage compared with that of the originals (seeds stored in the room conditions, 21 ± 4°C, 30-59% RH). After that, there was no further increase in TIA. For seeds stored under tropical (33°C, 87% RH) conditions, TIA increased significantly in some cultivars or stayed the same in others the first several days. then decreased significantly to an average decrease of 5.67% from the original TIA's. For artificially aged (33°C, 100% RH) seeds, TIA decreased even more significantly to an average decrease of 26.27% from the originals.

Assay of the Trypsin Inhibitor Activity In Seeds of Triticale

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INTRODUCTION

The ever-increasing world population mandates the development of new sources of food and feed superior in protein content and quality. Triticale, an amphiploid of wheat x rye, has been considered as a cereal crop for the future as it, in general, contains protein higher in the essential amino acids than other cereal grains. However, reduced feed efficiency was observed when triticale was fed to non-ruminant animals (Knoblauch et al., 1977). This has stimulated an interest in discerning the anti-nutritional compounds responsible for the reduced nutritional value of triticale. Trypsin inhibitors may be one of the factors responsible for the reduced utilization of dietary proteins from this source by monogastric animals (Knoblauch et al., 1977; Erickson et al., 1979). In other feeding trials, triticale selections low in trypsin inhibitor activity (TIA) were used (Erickson et al., 1980; Knoblauch et al., 1977). In their studies the deleterious effects associated with feeding triticale were not observed. This suggests the breeder should select triticale lines low in TIA as recommended by Knoblauch et al. (1977).

Development of triticale cultivars low in trypsin inhibitors is dependent on the availability of an inexpensive and rapid method for estimating trypsin inhibitor content of small seed lots. Thus, the first objective of this study was to develop a reliable laboratory procedure for determining the TIA in grains of individual triticale plants.

Known trypsin inhibitors are proteins which deteriorate over time as do other natural macromolecules. The extent of this deterioration, as influenced by temperature and humidity, was the second objective of this study.

Trypsin inhibitors are soluble proteins which may be released from insoluble storage proteins of the grains. The third objective of this study, therefore, was to determine the relationship between the soluble protein content and TIA of triticale grains stored under high relative humidity and temperature conditions.

LITERATURE REVIEW

Poor nutritional value of certain cereal grains is frequently attributed to a low quantity and quality of seed protein. However, low protein digestibility and amino acid availability in cereal diets may also adversely affect performance of monogastric animals (Munck, 1972). This low digestibility may be associated with the presence of trypsin inhibitors in cereal grains such as rye and triticale.

I. The Role of Trypsin Inhibitors

Since Osborin and Mendle (1917) demonstrated the nutrition value of soybeans was improved following a heat treatment, a great deal of effort has been directed to the identification of the so-called proteinase inhibitors. Much of the initial work in this field came from those interested in animal nutrition. They were concerned about the possible deleterious dietary effects exerted by the proteinase inhibitors found in important food plants and their products. The early work on the proteinase inhibitors, of plant origin, concentrated mainly on the inhibitors of trypsin, the important enzyme of the digestive tract of animals. In most feeding experiments with rats, raw soybeans caused enlargement of the pancreas, reduced growth and protein digestibility, impaired fat absorption, lowered energy utilization and reduced availability of amino acids, vitamins and minerals.

Trypsin inhibitor of protein nature in unheated soybean flours was first described by Read and Haas (1938). Since 1947, trypsin inhibitor in raw soybeans (SBTI) has been considered as one of the

factors affecting its nutritional value (Westfall et al., 1947; Borches et al., 1948). Investigations have indicated that SBTI caused pancreatic hypertrophy of chicks and rats (Chernick, 1949) and inhibited the animal's growth by upsetting the balance between methionine and cystine. This resulted from the increase in biosynthesis of cystine from methionine and the incorporation of excessive cystine into the pancreatic enzyme. The work conducted by Krogdahl et al., (1979); indicated that SBTI has a considerable inhibitory capacity towards trypsin and chymotrypsin in human pancreatic juice. Evidently protein digestion might be impaired by SBTI and it was concluded that the inhibition of human proteinases by soybean extract was also relevant in human nutrition.

Soybean trypsin inhibitors are a group of several proteins which make up 6% of the total proteins in soybean seeds (Rackis et al., 1962). Early data demonstrated that about 40% of the growth depression and pancreatic enlargement of rats was caused by the ingestion of trypsin inhibitors from soybean meal. Leiner (1953 and 1958) indicated that anti-tryptic activity could not account entirely for the detrimental effect on growth since it was found that SBTI could exert its growth depressing effect when fed with hydrolyzed protein.

The feeding of unheated soybean meal and trypsin inhibitor diets did not reduce the trypsin level in the intestinal tract of rats. Leiner further suggested that the growth depression reflected the combined effects of trypsin inhibitor factor and a feed intake reducing factor: soybean hemaglutinin. Rackis (1965) found that feeding highly purified SBTI to rats resulted in 100% pancreatic

hypertrophy and 30-60% growth inhibition and reduction of protein efficiency. It was estimated in another experiment (Kakade et al., 1973) that 40% of the pancreatic hypertrophy and growth inhibition observed in rats could be accounted for by the presence of trypsin inhibitors in raw soybeans.

Mitchell et al. (1976), assessed the nutritional effects of corn trypsin inhibitors on rat growth and found that neither average weight gains nor protein efficiency ratios were significantly effected by corn sources differing in TIA levels. Furthermore, no hypertrophy of the pancreas was observed. Thus, they concluded that the higher levels of trypsin inhibitors in opaque-2 corn were not deleterious to rats. Apparently, a threshold tolerance to TIA exists in rats below which a biologic effect cannot be demonstrated. Erickson (1979) in his unpublished data also showed that in practical swine diets, trypsin inhibitors from normal corn did not induce hypertrophy of the pancreas, and thereby significantly influence performance of pigs.

Knoblauch et al. (1977) reported significant correlations between trypsin inhibitor levels and the efficiency of protein utilization ($r = -.79$) of triticale diets fed to weanling voles. Erickson et al. (1979) evaluated nutritional value of triticale in swine starter (3-4 weeks) and grower (3 months) diets and reported the anti-tryptic activity of triticale was significantly correlated with average daily gain and gain per unit feed ($r = -.87$ and $-.84$, respectively) for young piglets. Erickson et al. (1978) demonstrated that triticale lines selected for low TIA could be incorporated into swine diets at high levels without detrimental effects upon pig performance.

II. Properties of Trypsin Inhibitors from Cereals

A. Distribution and specificity of trypsin inhibitors.

Trypsin inhibitors are a group of soluble proteins found in seeds that have the ability to inhibit the proteolytic activity of trypsins. Occurrence of proteinase inhibitors in plant organs such as seeds and tubers is widespread. They have been found in Leguminosae, Graminae, Solanaceae, and other families. Mikola and Kirsi (1972) estimated that the trypsin inhibitors present in both the embryos and endosperms of grains of barley, wheat, and rye, comprised 5-10% of the total soluble proteins. Various trypsin inhibitor levels were observed among different parts of grain. Kirsi and Mikola (1971) found that barley grains have six times more TIA in the embryo than in the endosperm. Halim et al. (1973), found that corn endosperm contains more trypsin inhibitors than the embryo. In the seeds of rye, anti-tryptic activity was observed in the endosperm (Mikola and Kirsi, 1972; Planowski, 1974) as well as in the embryo (Hochestrasser and Werle, 1969). Mikola and Kirsi (1972) compared the differences between endospermal and embroyal trypsin inhibitors in oats, barley, wheat and rye. They found TIA decreased in the order of rye, barley, wheat, and oat, and 3-7 times more inhibitor activities were detected in the embryo than their respective endosperms. They further noted that the total amount of trypsin inhibitors in the endosperm was always greater than the amount in the embryo of grains of all four cereals. Barber et al. (1973), claimed that in rice, TIA was concentrated mainly in the bran fractions. Distribution of inhibitor activity in flour, shorts

and the bran portion of triticale and wheat samples was estimated by Chang and Tsen (1979). Triticale shorts had 60-70% and bran had 42-60% of the TIA found in the flour. Wheat shorts had 87% of the TIA in flour and no detectable TIA was found in wheat bran.

Madle and Tsen (1974) reported that TIA in triticale is intermediate between its parental species with rye having a high TIA and wheat a low TIA rating. Boisen and Djurtoft (1981) also studied the specificity of trypsin inhibitors obtained from various cereals. The immunogenic properties of purified wheat endosperm inhibitors seemed to be different not only from those of wheat embryo inhibitors but also from the endosperm inhibitors of barley and rye. Wheat seeds contain at least four trypsin inhibitors in the embryo (Hochestrasser and Werle, 1969; Mistunaga, 1974 and 1979) and three in the endosperm (Mistunaga, 1974). These findings indicated that the structure of trypsin inhibitor is rather specific in each species and in different tissues within the species.

B. Heat stability of trypsin inhibitors.

The plant proteinase inhibitors are generally small proteins having molecular weights under 50,000 and more commonly under 20,000. Vogel et al. (1968) stated that the low molecular weight inhibitors were stable to acids and heat. Ogiso et al.(1975) purified trypsin inhibitors from Japanese barley. The inhibitors were found to be thermo-resistant and were stable over a broad pH range from 2 to 11. A possible mechanism for stability was given by the same group in 1976 based on study of the molecular structure. The inhibitor

contained a rigid disulfide loop and three disulfide bridges which contributed to structural stability. It was also shown that after treatment at 100°C for 10 minutes in 8M urea or 6M grannidine hydrochloride, conditions under which most proteins are completely denatured, the trypsin inhibitory activity was unaltered. McNaughton (1981) observed that trypsin inhibitor content in raw soybean meal decreased with increased heating time and decreased at a faster rate with increasing moisture levels in the meal. SBTI which have a higher molecular weight than those of cereals, were inactivated when soybean products were cooked.

Trypsin inhibitors isolated from the endosperm of rye seeds by Boisen and Djurtoft (1981) proved to be heat stable at 100°C. The pure preparation of trypsin inhibitors from wheat endosperm was reported to be very stable to pepsin at pH 2.0 and to heat at 100°C (Boisen et al., 1981). Mistunaga (1974) reported trypsin inhibitors from wheat grains retained 90% of their original activity after heating at 80°C for 2 hours. Earlier, Shamala and Lyman (1964) demonstrated that heating the crude wheat trypsin inhibitors, extracted from whole kernels, to boiling destroyed their activities. In view of the low level of TIA in wheat flour, which contains only about 0.3% of the TIA of soybean meal (Chang and Tsen, 1979 and 1981), and the inhibitor's lability, it is unlikely that trypsin inhibitors present in wheat could exert any influence on the nutritive value, even if it is consumed unheated.

A comparative study of the destruction of trypsin inhibitors by heat treatment was reported by Chang and Tsen (1981) in which

rye, triticale and wheat samples were heated in an oil bath at 60, 80, 100 and 125°C for 1 hour. All of the cereal trypsin inhibitors tested were more heat stable than those of SBTI (Obara and Watanabe, 1971). Trypsin inhibitors from triticale were the most stable among those tested, confirming the high heat stability of triticale trypsin inhibitors first reported by Madle and Tsen (1974). Heating at 100°C for 1 hour inactivated different proportions of the trypsin inhibitors from different cereal samples; 7% from triticale, 35% from rye, 44% from hard red winter and durum wheats. Conformation of the inhibitor was not perturbed very much by temperatures up to 80°C. Heating to 125°C caused the conformational changes of the inhibitor molecules to become permanent and reduced their activities. Unfortunately, such high temperature treatments also reduced nutritive value of the grains.

III. Genetic and Environmental Effect on Trypsin Inhibitors

Since the trypsin inhibitors of triticale are heat stable (Madle and Tsen, 1974) and the available triticale lines exhibit a range of anti-tryptic activity, genetic manipulation would appear to be the logical approach to reduce trypsin inhibitors and render the triticale useful for feed (Knoblauch et al., 1977). However, as suggested by Erickson (1979), if selection pressures were applied to increase total protein and lysin concentrations, a corresponding increase in TIA may be induced. Kirsi (1973) reported the quantity of TIA in cultivars was constant even though total protein content varied from 9 to 16%, suggesting the increase in protein was in the insoluble proteins and therefore did not increase TIA which is associated with the soluble proteins. Halim et al., (1973) investigated the distribution of trypsin inhibitors in several strains of corn and found the amount of TIA varied widely among the strains and between embryo and endosperm. The data suggested that lines containing the opaque-2 gene may be expected to produce higher levels of trypsin inhibitor in the endosperm than does normal corn. All of the above studies indicate that the level of TIA is inherited.

Effectiveness of selection against high TIA in triticale was demonstrated by Tanner's (1980) studies. A majority of the advanced generation lines were low in TIA. This indicates dominance effects were significant, approaching complete dominance in the direction of low trypsin inhibitor content in F_2 generation of ten hexaploid winter triticale crosses. The fact that the narrow sense heritability (h^2 = .46) of trypsin inhibitor content was significantly large,

should make selection against the trait more effective in early segregating generations (F_3 or F_4). Tanner and Reinbergs (1981), in the analysis of the 5 x 5 spring wheat diallel, revealed that TIA was controlled by both additive and dominance genetic effects and TIA in a 4 x 4 spring rye diallel was controlled mainly by additive genetic and reciprocal effects. It was concluded that selection for low TIA in wheat and rye should be effective in segregating lines since the narrow sense heritability of the trait was quite high in both species. In these studies, they also observed that 100 seed weight was positively related to TIA in wheat and rye. Although trypsin inhibitor is extracted as part of the soluble protein content of the seed, it may be independently inherited. No relationship was found between total or soluble protein content and TIA as trypsin inhibitor content in soluble protein may vary among genotypes.

Environmental conditions such as nitrogen fertilization may influence TIA. In each barley cultivar used in the experiment of Kirsi (1973), the levels of trypsin inhibitor remained constant when nitrogen fertilizer was applied even though total protein content of the grains increased. This result conflicts with the data given by Chang and Tsen (1979) who indicated that application of nitrogen fertilizer increased the TIA of triticale.

IV. Isolation and Purification of Trypsin Inhibitors

Among many purification techniques, affinity chromatography is the most versatile for isolating proteins and peptides. Many studies on SBTI have been reported, however, only a few deal with cereal trypsin inhibitors. Agarose affinity chromatography has been used to isolate trypsin inhibitors from various sources including cereals. Shyamala and Lyman (1964) reported a trypsin inhibitor that was isolated from whole wheat flour using carboxymethyl cellulose chromatography. Hochestrasser and Werle (1969) reported the use of a polyanionic trypsin resin in affinity chromatography to isolate trypsin inhibitors from many sources, including wheat and rye germs. Madle and Tsen (1974) isolated six protein fractions containing inhibitor activity from an acetate extract of triticale flour through gel filtration and electrophoresis. Recently, Chang and Tsen (1981) isolated trypsin inhibitors from rye, triticale, and hard red winter and durum wheats, by using four major steps: extraction, affinity chromatography, CM-cellulose ion-exchange chromatography and gel-filtration chromatography. After the second step, affinity chromatography, a relatively high yield of rye and triticale trypsin inhibitors (82 and 83%, respectively), was obtained and the specific activity of the concentrates was increased 100-200 times as compared to its activity in the crude extracts. The specific activity was not increased significantly following steps 3 and 4. Purified trypsin inhibitors, from different cereals, were heterogeneous as shown by disc gel electrophoresis (Madle and Tsen, 1974; Bruhn and Djurtoft, 1977). Using the isolation procedure, they

obtained the purified trypsin inhibitors and determined their molecular weights were: hard red winter wheat--36,800 and 21,000; durum wheat --38,000 and 22,500; triticale--19,500 and rye--17,000.

V. Assay for Trypsin Inhibitor Activity

The level of TIA has been determined by a variety of methods (Kakade et al., 1969; Fritz et al., 1974). Among them, the one developed by Kakade et al. (1969) has been the most widely used. The official American Association of Cereal Chemists (AACC) methods 71-10 (1974) was established largely according to the method of Kakade et al. (1974) and Rackis et al. (1972). The AACC methods was designed primarily for determining the TIA of soy products. One gram of a ground sample was placed in 50 ml of 0.01N NaOH for 3 hours. A magnetic capsule was used to agitate the solutions. Portions of the suspension: 0, 0.6, 1.0, 1.4, and 1.8 ml, were pipeted into test tubes and adjusted to 2.0 ml with water. The pH of the suspension was maintained between 8.4 and 10.0 and the solution was diluted to the point where 1 ml produces trypsin inhibition of 40-60%. Two milliliters of trypsin solution (1 ml trypsin/50 ml 0.001M HCL) and 5 ml substrate solution, containing 40% benzoyl-DL-arginine-P-nitroanalide (BAPA) in Tris buffer (0.05M, pH 8.2) were added to each tube. After placing in a water bath for exactly 10 minutes, the reaction was stopped by adding 1 ml 30% acetic acid. The solution was then filtered and absorbance measured at 410 nm. One trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 ml of the reaction mixture. TIA was expressed in terms of trypsin inhibitor units (TIU). Kakade et al., (1969) recommended the use of BAPA as the substrate in the analysis of soybean extracts providing one allows for the competitive nature of the complex. The results of the kinetics studies conducted by Madle and Tsen (1974)

stated that the inhibition by triticale trypsin inhibitors is a non-competitive type, therefore, the complex should be formed prior to the addition of substrate. The AACC method appears to be inapplicable because of the non-competitive nature of the trypsin inhibitor in triticale. The TIA assay for triticale definitely needs to be improved.

Laskowski (1954) pointed out that the inhibitor activity of the soybean trypsin inhibitor was always higher on synthetic substrates (BAPA) than on natural protein substrates. Kakade et al. (1969) evaluated natural and synthetic substrates for measuring the anti-tryptic activity of soybean samples and proved the use of the synthetic substrates to be a convenient and reliable method for assaying for TIA. They also pointed out that when TIA deviates from linearity, the high level of inhibitor concentration could be attributed to the partial disassociation of the trypsin-inhibitor complex.

In Stewart's (1973) observation, slight variations in response to enzyme concentration was noted from day to day and between enzyme lots which necessitated running standard curves each day when quantitative assays were performed. It was also observed that no difference in inhibition was noted when trypsin was mixed with SBTI for either 15, 30, 60, 300, or 900 seconds prior to the addition of the substrate (BAPA).

Several modifications made the existing AACC method more suitable for determining the TIA of cereals. Chang and Tsen (1979) specified that a sodium acetated buffer, pH 3.8, was more effective than 0.01N NaOH as a solvent for extracting cereal inhibitors. They also defined one unit of TIA as the amount of inhibitor needed to inhibit 50% of the activity of 10 μg trypsin. They emphasized that the concentration

of inhibitor extract has to be adjusted so that 40-65% of inhibition of trypsin is obtained.

In order to obtain a reliable measure of the trypsin inhibitor content of foods, Smith et al. (1980) recommended the use of different dilutions of extracts of the foods which gave a 40-60% inhibition of bovine trypsin. Hamerstrand et al. (1981) modified the standard AACC analytical procedures for determining TIA in soy products; the TIA was determined from a single dilution of a sample extract that inhibited at least 40% but no more than 60% of the trypsin. Values obtained by the modified procedures although approximately 20% lower than those obtained by standard methods were considered a more accurate representation of the TIA in the sample and were more reproducible.

Hildebrand and Hymowitz (1980) developed a simple and rapid technique for determining the presence or absence of the Kunitz trypsin inhibitor in soybean seeds for use by breeders and crop testing people. They used Azocoll which is a general proteolytic substrate consisting of a dye bound to an insoluble protein similar to azoalbumin as a synthetic substrate for trypsin to react upon. When proteases such as trypsin react with Azocoll, the red dye is released into the solution. Trypsin inhibitors, however, prevent the tryptic digestion of Azocoll by complexing with the protease first and rendering the enzyme ineffective. According to the data obtained by Hidebrand and Hymowitz (1980), soybean cultivars identified as low TIA, as determined by electrophoresis, were also low in TIA when the new Azocoll procedure was used. However, the new procedure should facilitate screening for low TIA of soybean genotypes in breeding programs.

Erickson et al. (1979), published an essay specific for determining TIA in triticale. Two millimeters of acidified water (pH 4.9) were added to 20 mg of ground triticale seed meal in a test tube and the suspension was held for 2 hours at 4 to 5°C, then 22 mg of Azocoll were added to each tube. At the same time, 1 ml phosphate buffer (pH 7.0), 1 ml trypsin solution, and a marble were added to each tube. Vigorous shaking action was employed during a 10 minute incubation period at 37°C. The reaction was stopped by adding 1 ml of 30% acetic acid and the tubes were centrifuged for 10 minutes at 10,000 x g. The supernatant was read against a blank at 520 nm in a spectrophotometer.

Poyse (1981, personal communication) modified the procedures for assaying trypsin inhibitor levels among different cereal species by using 1 g sample flour with 13 ml acetate buffer solution (pH 3.8). After refrigeration for 2 hours, samples were centrifuged at 1075 x g for 15 minutes. The supernatant liquid was saved and diluted to give 40-60% trypsin inhibition. One millimeter of the diluted sample was put into a tube and was added to 1 ml of the trypsin solution. The tube was placed in a 37°C water bath for 3 minutes. After incubation, 7 ml of BAPA substrate solution was added and the tube was placed in the water bath for another 10 minutes. Then 1 ml of 30% acetic acid was added to stop the reaction. The absorbence of the fluid was read in a spectrophotometer against a blank at 410 nm.

MATERIALS AND METHODS

Seeds of eight cultivars of triticales (listed below) which exhibited high, medium or low trypsin inhibitor activity (TIA) in preliminary experiments, were planted at Hyslop Agronomy Farm in the fall of 1981. Seeds harvested in the summer of 1982 were used for all the studies conducted during these investigations:

I. The Eight Cultivars used

The eight cultivars used were:

Cultivar	seed source	Chromosome Number	Relative TIA Level
1. CT463/77	M82-267	42	high
2. 6TA876	M82-5001	42	high
3. LT176/73	M82-5006	42	high
4. CT525/78	M82-5028	42	medium
5. CT691/78	M82-6031	42	medium
6. LT487/77	M82-5008	42	low
7. LT582/77	M82-5016	42	low
8. TCMC100	M82-5056	56	low

II. The Reagents Used in Experiments 1 and 2

The following reagents were used in experiments 1 and 2:

1. Extracting solution (pH 4.9): One liter of distilled water was titrated to pH 4.9 with 1N HCL .
2. Reaction buffer (0.1M KPO_4, pH 7.0): 13.6 g of KH_2PO_4 was dissolved in 900 ml distilled water. The pH was adjusted to 7.0 with 1N NaOH and the final volume was brought to 1 liter.

3. 30% acetic acid: 99.9% glacial acetic acid was added to distilled water in 3:7 (v/v) ratio.
4. 0.001N HCL solution: 83 ml HCL solution (37.25%) was added to 917 ml distilled water to make 1N HCL solution than was diluted 1000 x.

   All above solutions, after being prepared, were stored in the cold room at 5°C.
5. Trypsin solution: Trypsin (porcine pancrease, Type II, twice crystallized, Sigma Chemical Co.) was weighed and dissolved in 0.001N HCL solution and was prepared fresh for each run. Concentration of the trypsin solution was varied for different experiments.
6. Azocoll: Ten milligrams of Azocoll, a general synthetic proteolytic substrate (Calbiochem-Behring Corp.) was placed in each test tube. Erickson (1979) used 22 mg Azocoll for each test tube. However, preliminary tests conducted by the writer showed that 10 mg of Azocoll provided sufficient substrate to measure maximum trypsin activity in the control within the designated incubation time.

Experiment 1. Improvement of the Assay of Trypsin Inhibitor Activity for Triticale

In preliminary tests, the assay procedure for TIA reported by Erickson (1979) failed to differentiate among cultivars high and medium in TIA. Experiment 1 was thus conducted to improve the existing method so one can distinguish between triticales high or medium in TIA. Concentration of trypsin solution, particle size of the seed meal and sample size were considered as the factors most likely to influence TIA assay results. In the first part of Experiment 1, two particle sizes: 0.75 mm and 0.45 mm (use 1.0 mm and 0.5 mm screen in the UDY Cyclone Sample Mill) and four trypsin concentrations: 75 ug/ml, 100 ug/ml, 125 ug/ml and 150 ug/ml were applied to a known quantity of seed meal extracts of eight cultivars as treatments. Four replications of each treatment were conducted in this part of this experiment.

In the second part of Experiment 1, four cultivars: cv.1, cv.3, cv.7 and cv.8, were chosen to study the effect of sample sizes (20 mg and 40 mg). Four replications of each treatment were assayed.

TIA assay: One to two grams of seeds were ground for each sample. One set of 20 mg seed meals was weighed and each was placed in a polyethylene tube to which 1 ml of extracting solution was added. After extracting at room temperature (22°C - 25°C) for 2 hours with occasional shaking, 10 mg of Azocoll, 1 ml of trypsin solution and 1 ml of reaction buffer were added to each tube. A small glass bead was also placed in each tube to facilitate the mixing as the reaction started in the tubes. Two control tubes (without

sample meals) and two blank tubes (with reagents only and without seed meals and substrate) were also prepared for each run. After all tubes were incubated at 37°C with vigorous shaking for 10 minutes, the reaction was stopped by adding 1 ml of 30% acetic acid. The tubes were centrifuged for 8 minutes at 10,000 x g. The absorbance of the supernatant was read against the blank at 520 nm in a Cary 219 spectrophotometer.

Expression of TIA was determined from the following calculation (Erickson, 1979):

$$\% \text{ inhibition} = \frac{\text{absorbence of control - absorbence of sample}}{\text{absorbence of control}} \times 100$$

Experiment 2. Effects of Storage Conditions on Trypsin Inhibitor Activity

In initial tests, differences were observed in TIA of seeds of different ages of the same cultivar. Since they had been stored under the same conditions, length of storage period, associated with temperature and humidity conditions were assumed to be factors which might have influenced the reduction in TIA. Experment 2 was therefore conducted to study effects of storage conditions on TIA.

Seeds of eight cultivars were wrapped in cheese cloth and stored in five conditions for different intervals of time.

Condition A: Hot and dry (33°C, 65% RH) for 30, 60, and 90 days, which simulated desert conditions.

Condition B: Medium (21°C, 65% RH) for 30, 60, and 90 days, which simulated temperate conditions.

Condition C: Cold (1.7°C) for 30, 60, and 90 days, which simulated arctic conditions.

Condition D: Hot and wet (33°C, 87% RH) for 7 to 42 days, which provided tropical conditions with relatively high humidity.

Condition E: Hot and saturated atmosphere (34°C, 100% RH) which provided an artificial aging environment. Seeds were placed on a wire-screen over a water layer (100% RH) in closed jars and the jars were stored in a temperature controlled chamber for 3 to 18 days.

Each seed sample consisted of 2 grams with two replications for each cultivar. The TIA of seeds was tested following termination of each storage period. Under conditions A, B, and C, TIA of the eight cultivars was determined at 30th, 60th, and 90th day after seeds were removed from storage. For seeds stored under condition D, TIA was tested after 7, 14, 21, 28, 35 and 42 days in storage. For the seeds stored under condition E, the TIA was tested after 3, 6, 9, 12, 16, and 18 days in storage. TIA of the eight cultivars stored under room conditions (21 ± 4°C, 30-59% RH) was also determined as originals to aid in comparing the effects of storage treatments. After the designated periods, seeds were removed from storage and ground immediately. TIA was assayed on the same day or the ground meals were stored in a refrigerator (3°C) and tested as soon as possible thereafter.

The assay procedure for TIA was modified further for Experiment 2 to minimize the variation between duplicated observations. TIA assay procedures reported by Erickson, 1979, and Smith et al., 1980, were modified as follows: (a) as suggested by Experiment 1, sample size was increased from 20 mg to 1 gram of seed meal. Each sample was

placed in a 50 ml centrifuge tube to which 10 ml of extracting solution was added. Extracting reactions were allowed to proceed at room temperature (21°C - 25°C) for 2 hours with occasional stirring. (b) After each sample was centrifuged at 17,000 x g for 10 minutes, 1 ml of the supernatant fluid of each sample was added to each of a pair of glass tubes and was diluted with extracting solution. The amount of extracting solution added depended on the level of TIA. Sufficient quantity of extracting solution was added so that each diluted sample fluid inhibited 40 to 60% of trypsin used in the assay. The remaining supernatant was kept in the freezer (-12°C) for later use. One milliliter of each diluted solution was transferred to a polyethylene tube into which 1 ml of trypsin solution and 1 ml of reaction buffer were added. (c) The tubes were placed in a water bath at 37°C and shaken rapidly for 3 minutes to allow the complex to be formed between trypsin and inhibitor. Then 10 mg of Azocoll and 1 glass bead were added to each tube. After incubation for another 10 minutes at 37°C with rapid shaking, 1 ml of 30% acetic acid was added to stop the reaction. The tubes were centrifuged again at 10,000 x g for 8 minutes and absorbence of the supernatant was read against the blank at 520 nm. (d) Inhibition was calculated as the relative inhibition units in reference to the trypsin used per unit of sample weight. The TIA, based on 1 g seed meal, was determined as follows:

$$\left(\frac{Ac - As}{Ac}\right) \times \text{dilution factor} \times 10 = \frac{\text{relative inhibition units}}{1 \text{ g seed meal}}$$

where Ac: absorbence of control

As: absorbence of sample

Experiment 3. Changes in Soluble Protein Content of Seeds Stored In Conditions D and E.

As shown by Mikola and Kirsi (1972), trypsin inhibitors are low molecular weight soluble proteins. High temperature and high relative humidity during storage may hydrolyze high molecular weight insoluble storage proteins in seeds during storage, thereby changing them into low molecular weight soluble protein that may become trypsin inhibitors. To determine if such changes occurred during storage conditions D and E, soluble protein contents of stored samples were measured in the extracted supernatant from Experiment 2. Coomassie blue G-250 stain method was used to measure the quantity of soluble protein (Bradford, 1976).

Procedures for measuring soluble protein contetn:

1. Reagent: 100 mg of Coomassie blue G-250 (Sigma Chemical Co.) was dissolved in 50 ml 95% ethanol to which 100 ml 85% phosphoric acid was added. After being filtered the volume was brought to 1 liter with distilled water.

2. Standard curve: 5 mg of bovin serum albumin (BSA) was dissolved in 5 ml of extracting solution (pH 4.9). Five concentrations of BSA solution were prepared from the stock and a standard curve was drawn for the color production. Absorbence of 0.0047 equals to 1 ug protein.

Assay procedure: 0.1 ml of sample supernatant (saved from Experiment 2) was placed in a test tube and 5 ml of Coomassie reagent was added and mixed well with a shaker. Two minutes later, the absorbence of each sample was read against a blank (containing distilled water only) at 595 nm in a Cary 219 spectrophotometer. A blank containing 0.1 ml extracting solution plus 5 ml Coomassie reagent was used to correct the sample absorbence. The soluble protein content of each sample was calculated as follows:

$$\left(\frac{As - Ab}{0.0047}\right) \times 100 = \mu g \text{ soluble protein/1 g seed meal}$$

where As: absorbence of sample

Ab: absorbence of blank

RESULTS

Experiment 1: Improvement of the Assay of TIA for Triticale

Data obtained from the methodological study were analyzed using a factorial with completely randomized block design. The analysis of variance is shown in Table 1. Replications were performed on different days, which resulted in significant replications differences in TIA. Although assay procedures were strictly adhered to, significant replication differences suggested some unknown factors were not adequately controlled. A larger sample size might have helped minimize the variation associated with replications. Assay results were not affected significantly by particle size. Neither the interaction of particle size x trypsin concentration nor the interaction of particle size x trypsin concentration x cultivar was significant. Apparently, trypsin inhibitor activity was not adversely influenced by either of the particle sizes of seed meal analyzed.

The treatment of four trypsin concentrations and the interaction of trypsin concentration x cultivar was significant. When the trypsin concentration was varied, the eight cultivars differed signficantly in TIA (Table 2 and Figure 1). However, when 100 µg/ml or 125 µg/ml of trypsin concentration was used, differences among cultivars with high, medium or low TIA were readily discernible (Table 3). The standard for differentiating among high, medium and low TIA level was established by results obtained from vole feeding trials (Elliot, F.C., 1983; personal communication). In this case, TIA levels below

Table 1. Analysis of Variance Test for Trypsin Inhibitor Activity of Eight Triticale Cultivars with Treatments Consisting of Two Particle Sizes and Four Trypsin Concentrations

Source of variation	d.f.	Mean of square
Replication	3	1624.13 **
Particle size (P.S.)	1	54.49 NS
Trypsin concentration (T.C.)	3	2305.70 **
P.S. X T.C.	3	10.44 NS
Cultivar (CV.)	7	12852.20 **
P.S. X CV.	7	31.61 NS
T.C. X CV.	21	351.58 **
P.S. X T.C. X CV.	21	13.24 NS
Error	189	30.92
Total	255	
C.V.		11.94%

NS: Not significant at the 5% probability level

**: Significant at the 1% probability level

Table 2. Analysis of Variance Test for Trypsin Inhibitor Activity of Eight Triticale Cultivars within Each Trypsin Concentration Treatment

Source of variation	d.f.	Mean of square
Cultivars withen each trypsin concentration		
75 g/ml	7	2046.852 **
100 g/ml	7	4241.563 **
125 g/ml	7	4434.584 **
150 g/ml	7	3183.356 **
Error	189	30.920

** : Significnat at the 1% probability level

Table 3. Least Significant Difference Test for Trypsin Inhibitor Activity of Eight Triticale Cultivars withen Each Trypsin Concentration Treatment

Cultivar	Trypsin concentration			
	75 g/ml	100 g/ml	125 g/ml	150 g/ml
3	a	a	a	a
1	ab	b	b	c
2	ab	b	c	c
6	bc	b	bc	b
5	cd	c	d	d
4	d	c	d	d
7	e	d	e	e
8	e	d	e	e

Significant different at the 5% probability level

Table 4. Analysis of Variance Test for Trypsin Inhibitor Activity of Four Triticale Cultivars with Two Sample Sizes

Source of variation	d.f.	Mean of square
Replication	3	192.623 **
Sample size	1	4411.892 **
Cultivar	3	4425.018 **
Sample size x Cultivar	3	18.141 NS
Error	21	37.131
Total	31	
C.V.		11.889%

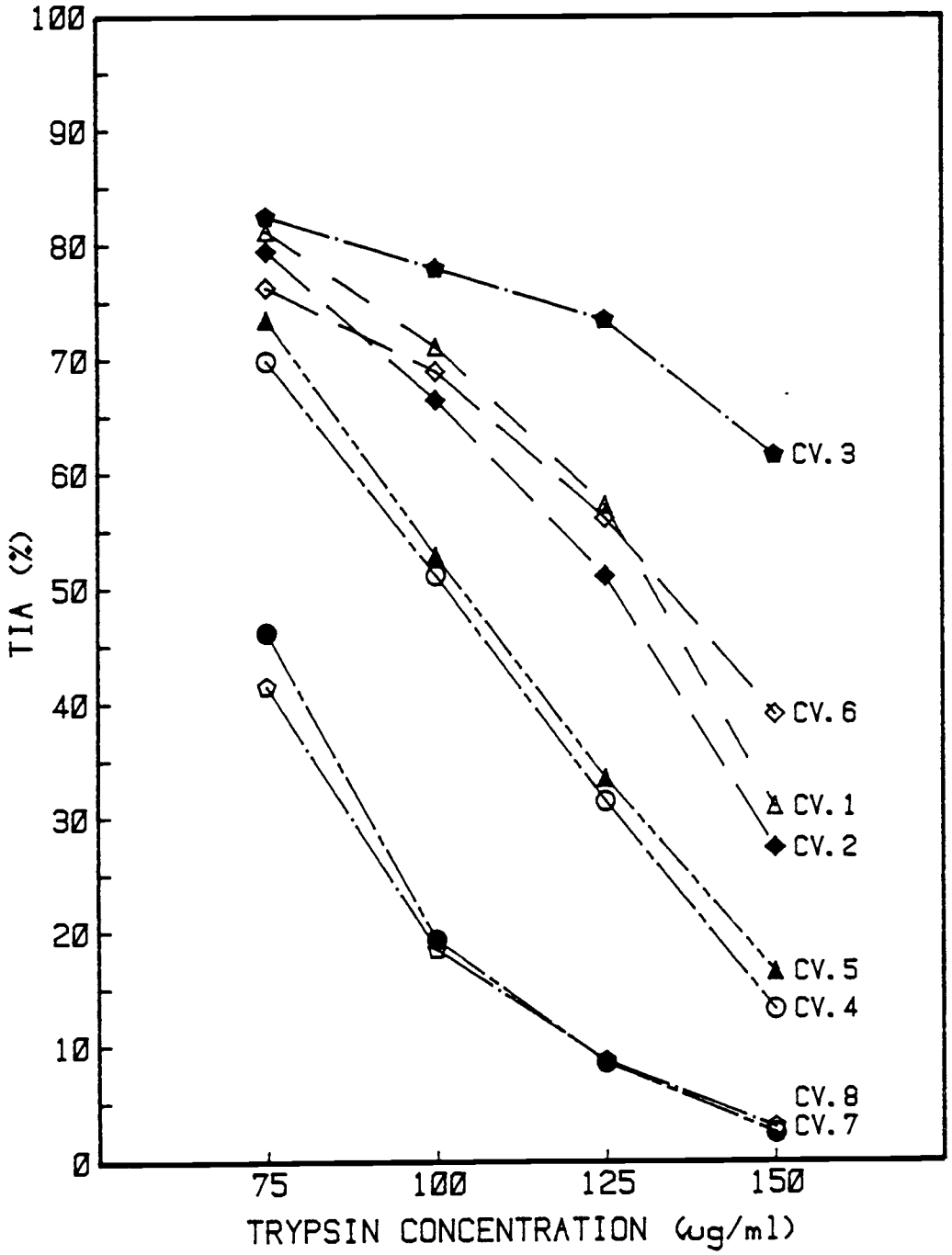
NS : Not significant at the 5% probability level

** : Significant at the 1% probability level

Table 5. Least Significant Difference Test of Standard Deviation for Trypsin Inhibitor Activity between Duplicated Samples in Four Crultivars with Two Sample Sizes

Sample size	Standard deviation of TIA	L.S.D. 0.05
20 mg	9.23%	a
40 mg	4.35%	b

Figure 1. Distribution of Trypsin Inhibitor Activity of Eight Triticale Cultivars under Four Trypsin Concentration Treatments



40% were considered acceptable. Differences of TIA among the eight cultivars were not clearly defined when concentrations of 75 and 150 µg/ml of trypsin were used.

From the analysis of variance presented in Table 4, it appears differences in TIA associated with sample sizes of 20 mg and 40 mg were significant. The larger sample size used in testing for TIA, the smaller the standard deviation of duplicated observations, as shown in Table 5. It is suggested that the weight of sample should be increased in order to reduce the variation between duplications.

Experiment 2: Effects of Storage Treatments on Trypsin Inhibitor Activity

Currently, triticales are consumed by animals without any pre-treatment. Seed processing procedures and storage conditions which could affect TIA in the grains have never been addressed. This study was instigated to determine the effect of aging on seeds of the same cultivar stored under different conditions on TIA.

Split-plot analysis of variance test for TIA of eight triticale cultivars stored in desert, temperate and arctic conditions for 30, 60, and 90 days is summarized in Table 6. Signficant differences in TIA were found due to storage conditions and cultivars. Interactions of cultivar x storage conditions x length of storage period and storage treatment x original were significant.

TIA of all eight cultivars increased signficantly during storage. Data, analyzed by the student-t test and the least significant difference test, are presented in Table 7. This increase in

TIA after storage may be associated with the following factors: (a) the conversion of some storage proteins into soluble forms that are trypsin inhibitors, (b) more trypsin inhibitors can be extracted from aged seeds, or (c) less interfering compound, e.g., phenolics, exist in aged seeds.

Information given in Table 7 and Figure 1 demonstrated that ranking of TIA among eight cultivars remained consistent as in Experiment 1 even though procedures for measuring TIA were modified further in Experiment 2 as stated in materials and methods section. This suggests that the method for assaying TIA used in Experiment 1 is reliable for differentiating TIA levels among triticale cultivars.

Changes of TIA in each cultivar under three storage conditions: desert, temperate, and arctic, for three storage periods; 30, 60, and 90 days; are shown in Figure 2 and the analysis of variance test for the effect of each storage treatment on individual cultivars is presented in Table 8. The data obtained by averaging TIA across three storage periods indicated that TIA of cv. 2 (6TA876) and cv. 4 (CT525/78) was significantly higher under the desert conditions. The data obtained by averaging TIA over three storage conditions indicated that TIA of cv. 1 (CT463/77) and cv. 4 (CT525/78) increased significantly after 30 days of storage. Also, the TIA of cv. 4 (CT525/78 and cv. 6 (LT525/78) was significantly increased after 90 days of storage regardless of storage conditions.

The TIA of remaining cultivars did not change significantly for most of the storage treatments. Longer storage periods probably would be required in order to detect other significant changes

Table 6. Analysis of Variance Test for Trypsin Inhibitor Activity of Eight Triticale Cultivars Stored in Desert, Temperate and Arctic Conditions for 30, 60 and 90 Days

Source of variation	d.f.	Mean of square
Treatment	9	134.31 **
Storage condition (S.C.)	2	299.40 **
Storage period (S.P.)	2	6.47 NS
S.C. X S.P.	4	37.65 NS
Treatment vs original	1	446.47 **
Error (a)	10	14.27
Cultivar (CV.)	7	1715.69 **
CV. X Treatment	63	17.04 **
CV. X S.C.	14	29.72 **
CV. X S.P.	14	12.86 **
CV. X S.C. X S.P.	28	15.29 **
CV. X (Treatment vs original)	7	7.05 *
Error (b)	70	3.24
Total	159	
C.V. (a)		13.56%
C.V.. (b)		6.46%

NS : Not significant at 5% probability level

** : Significant at the 1% Probability level

* : Significant at the 5% probability level

Table 7. Student-t Test to Compare Trypsin Inhibitor Activity of Individual Cultivars under Three Storage Conditions in Original and Stored Samples and Least Significant Difference Test of Trypsin Inhibitor Activity in Original and Stored Samples of Eight Triticale Cultivars under Three Storage Conditions.

Cultivar	TIA (Trypsin inhibition units / g seed meal)		Student-t test	L.S.D.$_{0.05}$
	Original	Average across three storage conditions		
3	36.859	46.012	**	a
1	28.034	33.823	**	b
6	26.525	31.734	**	b
2	23.316	30.148	**	c
4	20.693	24.856	**	d
5	18.531	24.404	**	d
8	15.601	18.869	**	e
7	12.936	15.781	**	f

** : Significant different at the 1% probability level

Taboe 8. Mean of Square of Trypsin Inhibitor Activity of Individual Cultivars under Each Storage Condition and Each Storage Period

Source of variation	d.f.	Mean of square							
		cv. 1	cv. 2	cv. 3	cv. 4	cv. 5	cv. 6	cv. 7	cv. 8
S.P. within Con. A	2	0.94	12.35 **	1.10	27.37 **	3.06	4.53	1.47	2.84
S.P. witnin Con. B	2	1.81	0.92	6.17	1.47	1.27	1.92	3.43	2.64
S.P. within Con. C	2	1.39	0.19	3.50	6.74	2.85	0.39	0.62	7.53
S.C. within S.P.-30	2	10.82 **	3.99	5.56	67.44 **	6.28	9.14	1.32	4.41
S.C. within S.P.-60	2	8.55	8.55	9.48	0.81	2.20	5.43	0.06	3.51
S.C. within S.P.-90	2	4.75	0.95	7.39	16.77 **	0.06	16.18 **	2.93	2.64

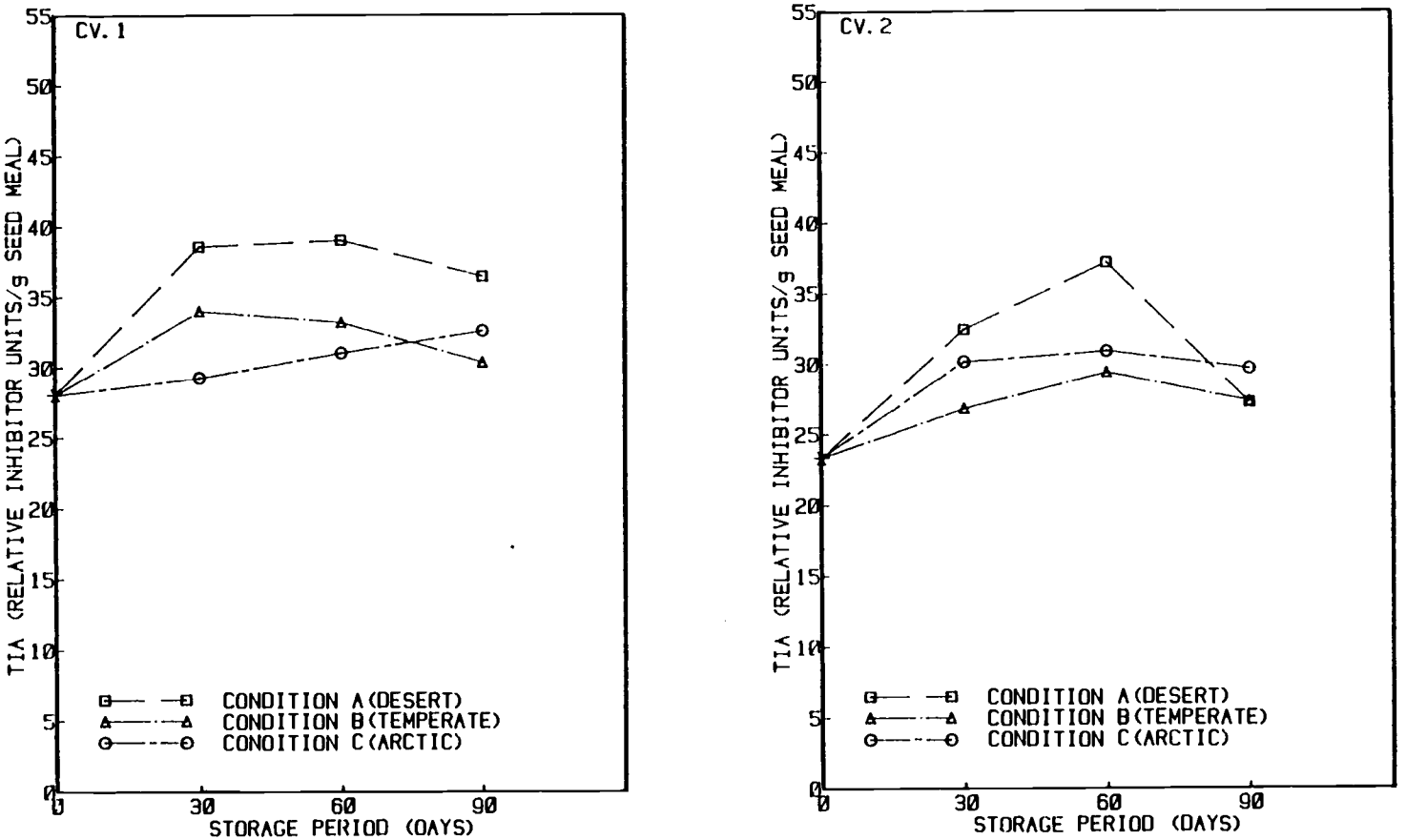
** : Significant at the 1% probability level

S.P. : Storage period

S.C. : Storage conditions

Con. : Condition

Figure 2. Trypsin Inhibitor Acitvity of Eight Triticale Cultivars Stored under Desert, Temperate and Arctic Conditions for 30, 60 and 90 days



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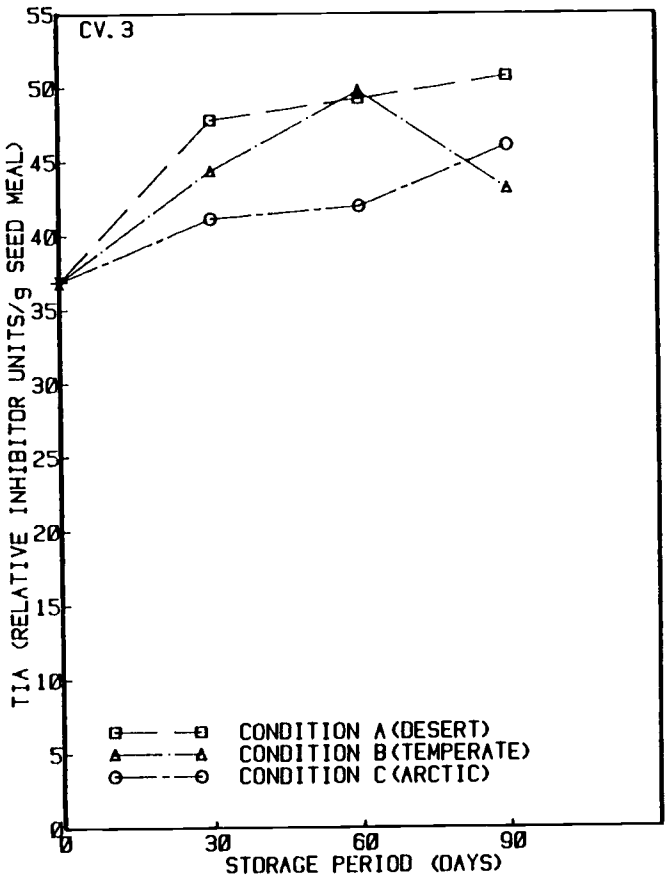

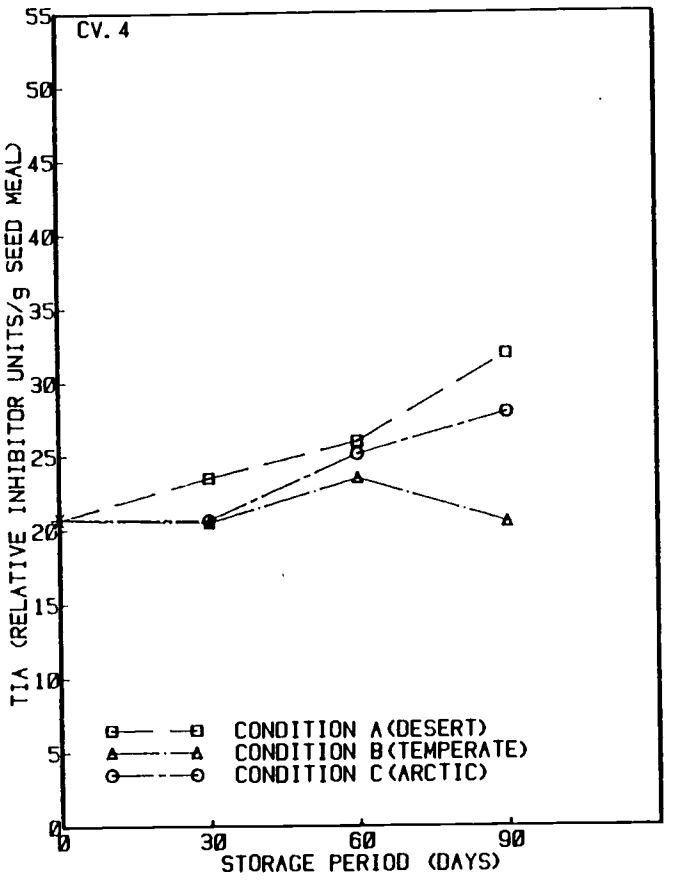

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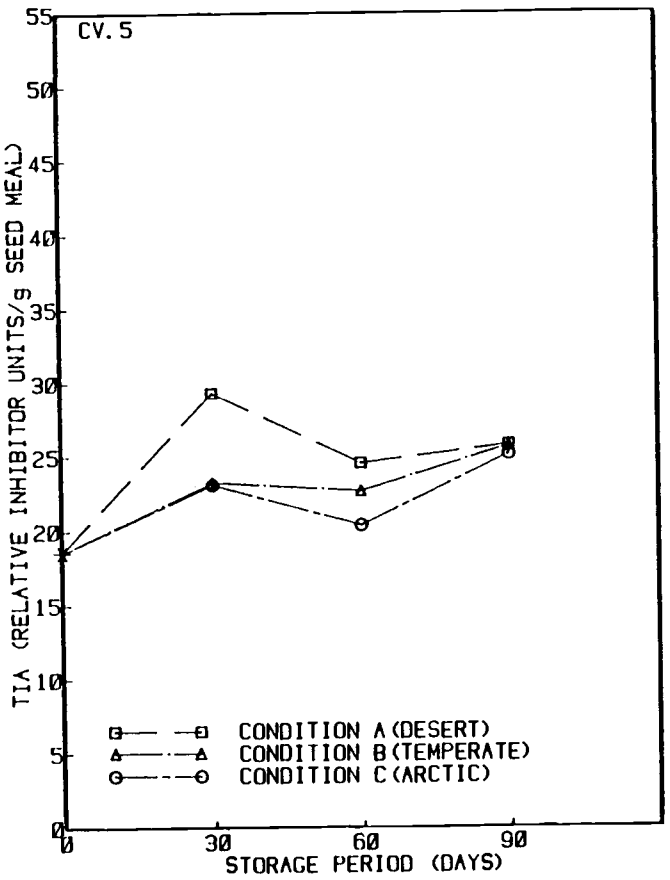

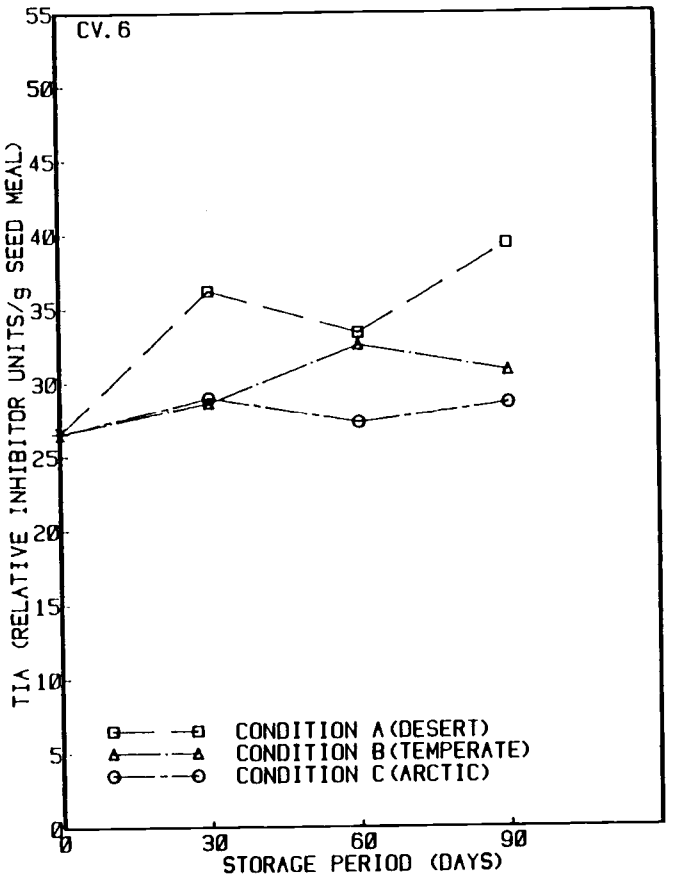

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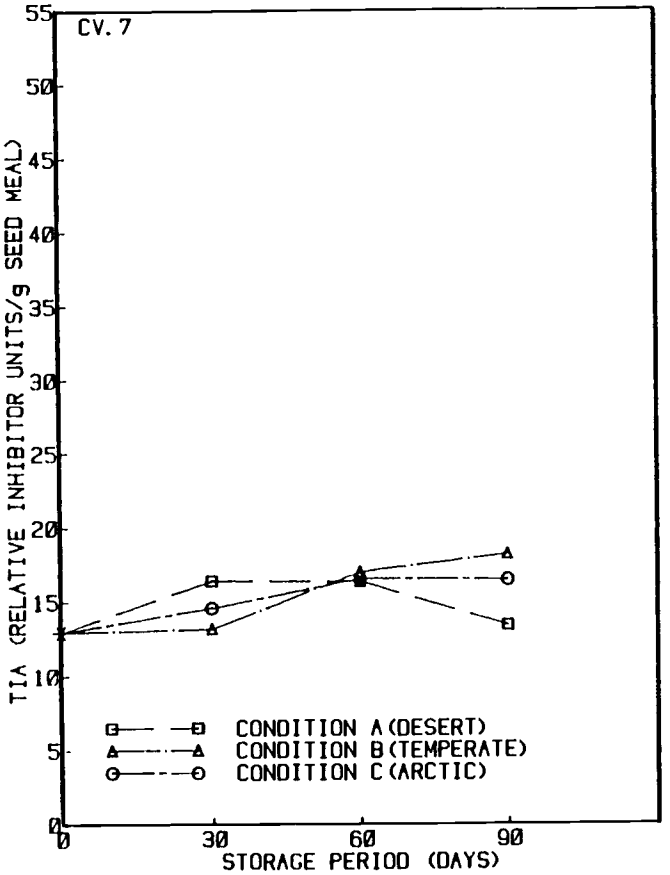
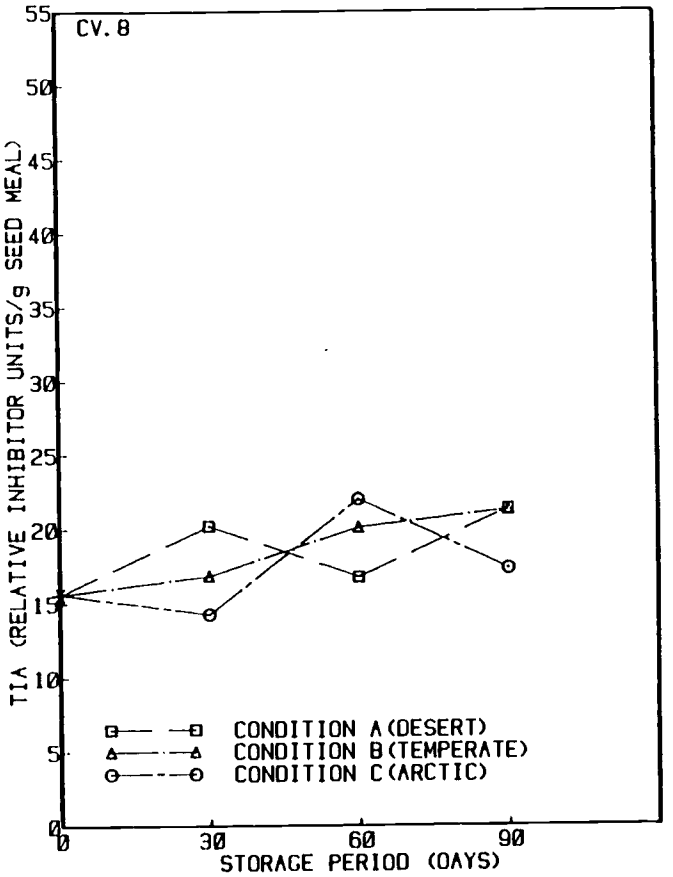




in TIA among treatments. Although the storage results failed to display dramatic changes in TIA during the 30 to 90 day storage periods under desert, temperate, and arctic storage conditions, TIA did increase significantly during storage under different temperature and humidity treatments in all cultivars as compared to the original TIA (Table 7). However, the increase was significantly greater in certain cultivars under given sets of conditions as compared to other cultivars (Figure 2 and Table 8) . Furthermore, the TIA ranking after storage treatments was similar to the ranking found when 20 mg sample size of each cultivar was assayed using 100 µg/ml of trypsin per assay (Figures 1 and 2).

Experiment 3: Changes of Trypsin Inhibitor Activity and Soluble Protein Content in Seeds Stored under Tropical and Artificial Aging Conditions

Two storage conditions, tropical (D) and artificial aging (E) were simulated by increasing both temperature and relative humidity as described in materials and methods section. TIA and soluble protein content were determined every 7 days for seeds stored at 87% humidity and every 3 days for those stored in 100% relative humidity. The high relative humidity under both conditions may change TIA and soluble protein content within a short period, thus the frequent sampling was conducted. Changes of insoluble storage proteins to soluble ones may result from the action of proteinases present in the moist seeds under these two conditions.

The analysis of variance tests for TIA and soluble protein content, presented in Tables 9 and 12 show the interactions of

"cultivar x storage period" were significant. This suggests TIA and soluble protein levels were not influenced by the same degree in each of the cultivar during a given period of storage. As shown in Figure 3, TIA of each individual cultivar stored under tropical conditions (D) was gradually decreased; in cultivars 1, 2, 4, 6 and 8, TIA reached the lowest level at the 21st day; at the 28th day for cultivars 3 and 4; and at the 14th day for cultivar 7. Across the storage periods, the TIA of eight cultivars was significantly lower in the samples stored under tropical conditions than the original samples. The decrease in soluble protein content under tropical storage conditions paralleled the TIA pattern (Figure 3). The range of the lower levels occurred between 21 to 28 days of storage. The increase of soluble protein content after 28 days might be attributed to fungi infection since the seeds were stored in high humidity conditions. Even though the mycelium and other contaminants were removed from the surface of all seeds prior to chemical analysis, internal fungi growth may have increased soluble protein content. In general, the correlation between the changes in TIA level and soluble protein content was poor as evidenced by an average R^2 value of 0.282 (ranged from 0.02 to 0.629, as shown in Figure 3). Only cultivar 6 (LT525/78) showed a higher degree of correlation with a $R^2 = 0.629$.

Although the level of TIA was significantly decreased in seeds stored in the tropical room with a humidity of 87%, another artificial aging condition (E) with 100% humidity, was provided to determine the effect a much higher humidity might have during

storage at high temperatures on TIA. A gradual decrease in TIA was observed in every individual cultivar stored under condition E (Figure 4). Soluble protein content was increased after first declining. Again the increase was probably associated with presence of fungi grown both on the surface and inside the seeds. Changes in soluble protein content and TIA in seeds during storage were poorly correlated as evidenced by the pattern in Figure 4 and the average $R^2$was 0.318 (ranging from 0.003 to 0.797). Only cultivar 2 (6TA876) exhibited a higher degree of correlation between soluble protein content and TIA (R^2= .797).

Seeds of cultivar 8 (TCMC100) which had the lowest TIA but highest soluble protein content before storage exhibited the poorest correlations: R^2 = 0.02 for condition D and R^2 = 0.003 for condition E, between TIA and soluble protein content. Generally speaking, cultivars with higher original TIA showed the greater decrease in TIA under the poor storage conditions when compared to cultivars with lower initial TIA (Table 13). It was demonstrated clearly that TIA in all cultivars was decreased under two storage conditions (D and E) with high relative humidity and temperature. In general, soluble protein content decreased as its storage period was lengthened, but an increase was evident during the late phase probably because of a build-up of microorganisms.

Data presented in Table 7 illustrate how TIA increased significantly when seeds were stored at relatively low humidity regardless of temperature. However, under both tropical and artificial aging conditions with relatively high humidity and temperature, TIA and

soluble protein content decreased significantly (Figures 3 and 4), but the changes were not highly correlated.

The average reduction of TIA under tropical conditions was 5.67% in 42 days while a substantial reduction of 26.27% resulted under artificial aging conditions in 18 days (Table 13). The reduction of TIA of all cultivars stored under condition D for 42 days and under condition E for 18 days was significant except for cultivar 2 (6TA876) assayed on the 42nd day under tropical conditions (Table 14 and Figure 3). Comparison was also made between the reduction of TIA of seeds stored under condition D for 21 days and under condition E for 18 days (Table 14) which showed that reduction of TIA of most cultivars under tropical conditions occurred mostly before 21 days. This indicated the reduction of TIA of triticale seeds could be decreased to a significant level within a short storage period (before 21 days) when seeds were stored under high temperature and humidity environments.

Table 9. Analysis of Variance Test for Trypsin Inhibitor Activity of Eight Triticale Cultivars Stored in Tropical Conditions for 7, 14, 21, 28, 35 and 42 Days

Source of variation	d.f.	Mean of square
Treatment	6	145.32 **
Storage period (S.P.)	5	169.33 **
S.P. vs original	1	25.25 NS
Error (a)	7	8.03
Cultivar (CV.)	7	573.13 **
Cultivar X Treatment	42	10.22 **
CV. X S.P.	35	10.85 **
CV. X (S.P. vs original)	7	7.09 *
Error (b)	49	2.46
Total	111	
C.V. (a)		12.80%
C.V. (b)		7.09%

NS : Not significant

** : Significant at the 1% probability level

* : Significant at the 5% probability level

Table 10. Analysis of Variance Test of Soluble Protein Content of Eight Triticale Cultivars Stored in Tropical Conditions for 7, 14, 21, 28, 35 and 42 Days

Source of variation	d.f.	Mean of square
Treatment	6	659.03 **
Storage period (S.P.)	5	754.50 **
S.P. vs original	1	181.69 **
Error (a)	7	31.71
Cultivar (CV.)	7	652.86 **
CV. X Treatment	42	80.60 **
CV. X S.P.	35	82.69 **
CV. X (S.P. vs original)	7	70.16 **
Error (b)	49	15.78
Total	111	
C.V. (a)		8.50%
C.V. (b)		5.99%

** : Significant at 1% probability level

* : Significant at 5% probability level

Figure 3. Trypsin Inhibitor Activity and Soluble Protein Content of Eight Triticale Cultivars Stored under Tropical Conditions for 7, 14, 21, 28, 35 and 42 Days

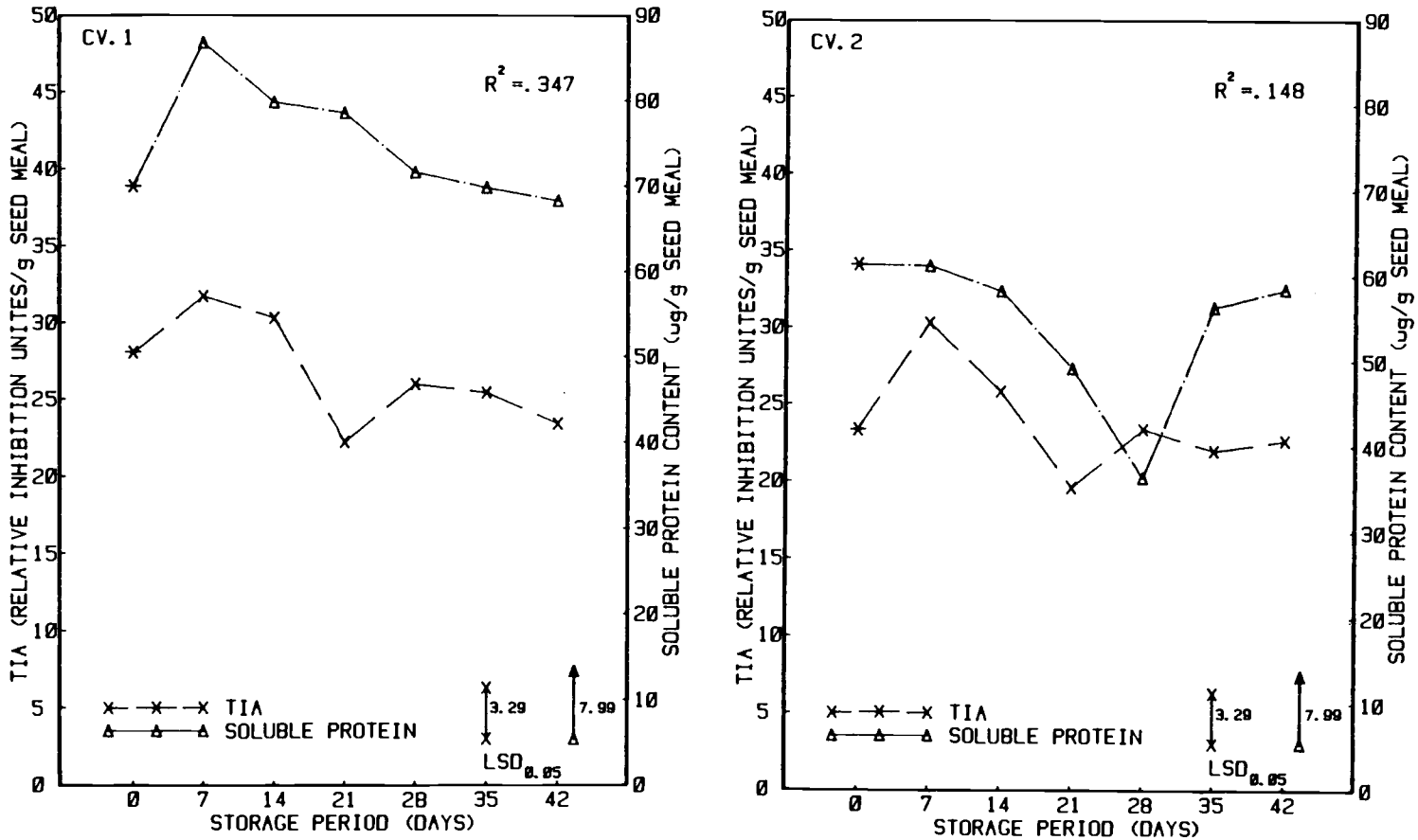


Figure 3. (continued)

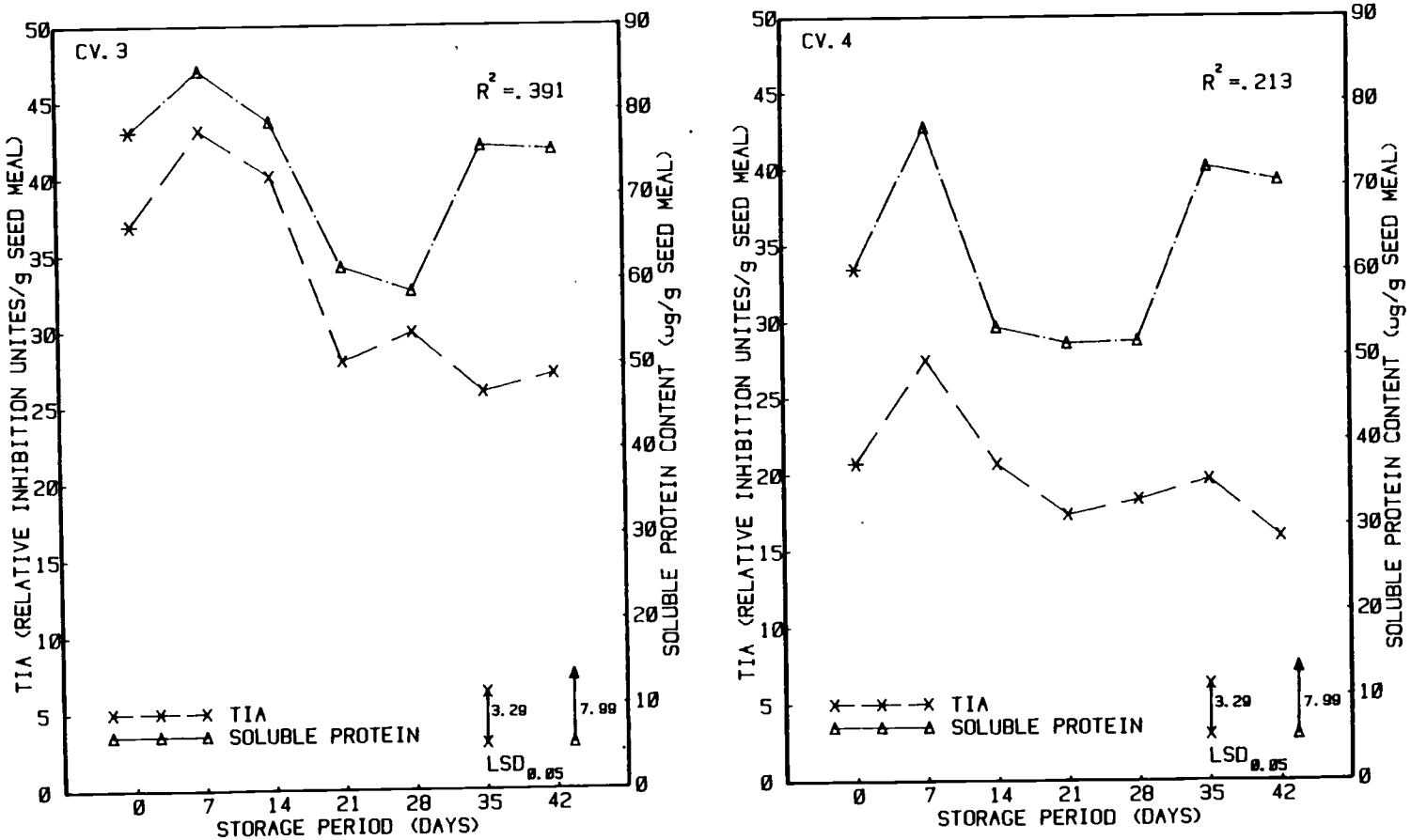
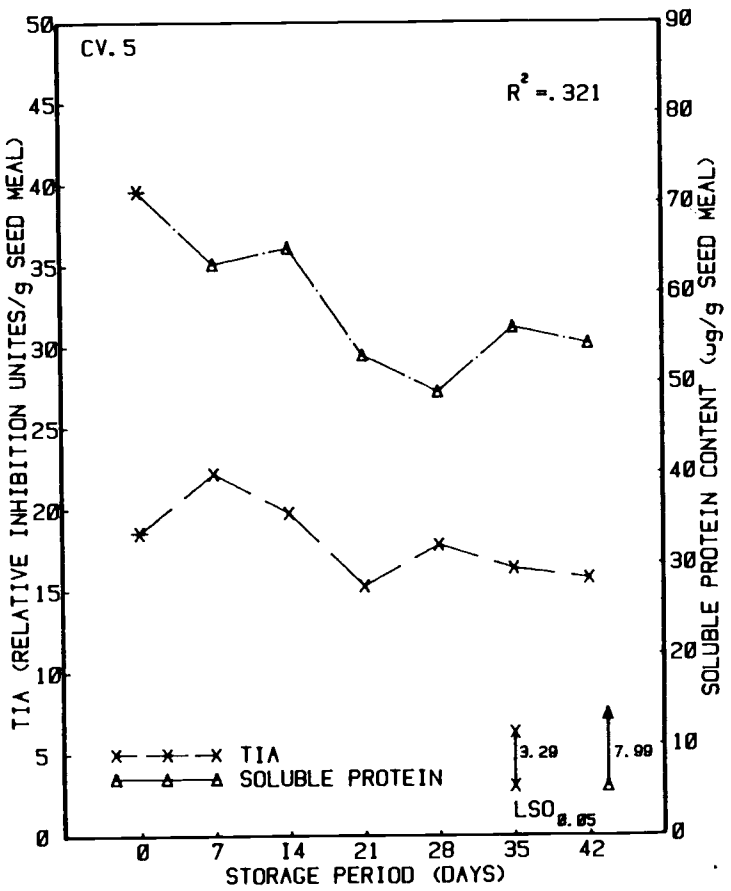


Figure 3. (continued)



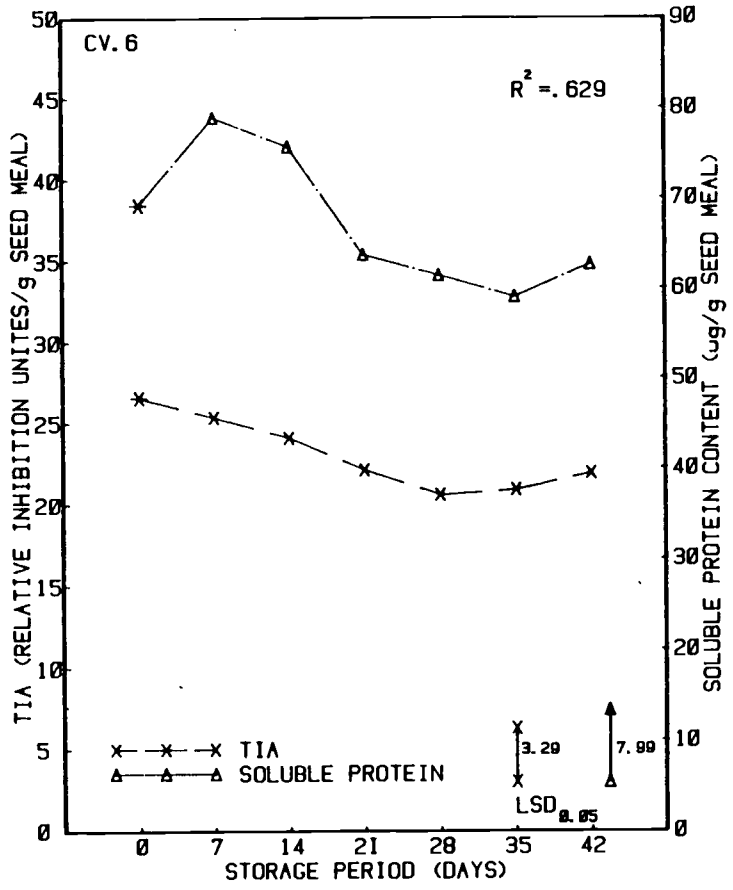
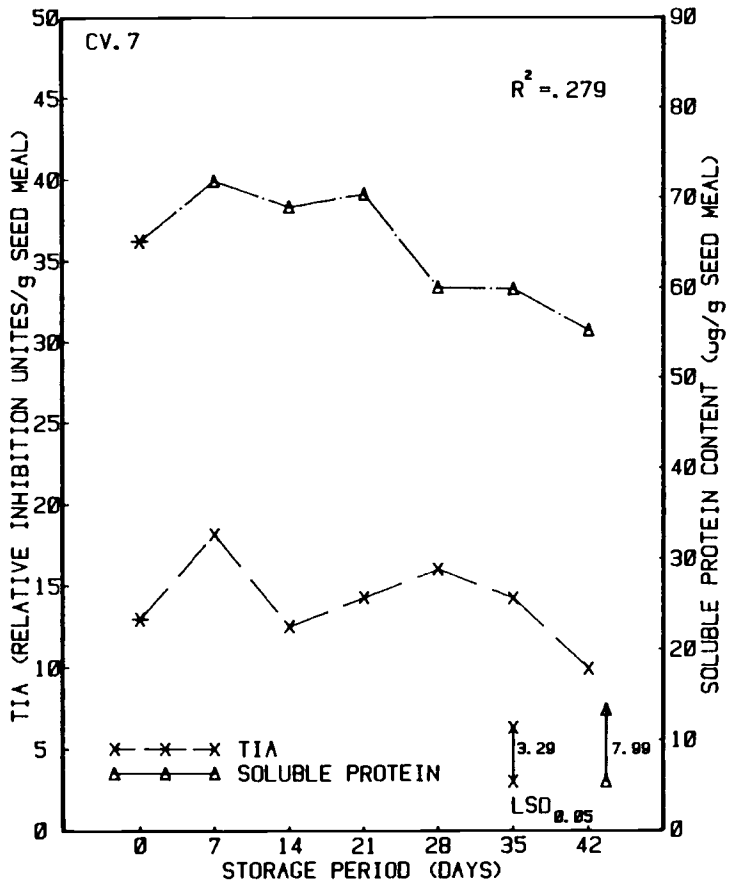


Figure 3. (continued)



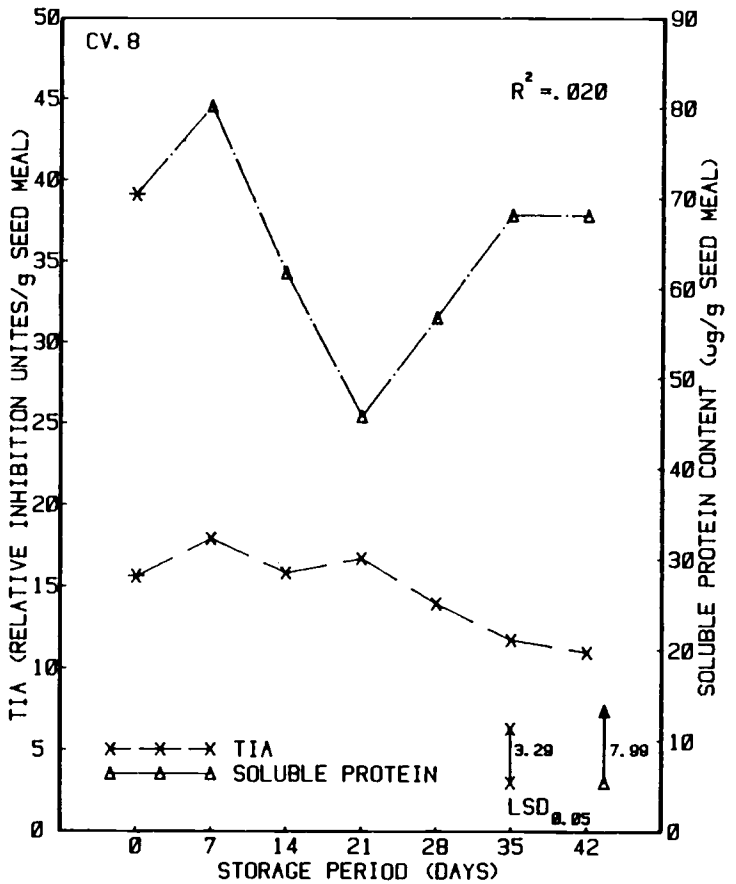


Table 11. Analysis of Variance Test of Trypsin Inhibitor Activity of Eight Triticale Cultivars Stored under Artificial Aging Conditions for 3, 6, 9, 12, 15 and 18 Days

Source of variation	d.f.	Mean of square
Treatment	6	352.81 **
Storage period (S.P.)	5	336.94 **
S.P. vs original	1	432.15 **
Error (a)	7	5.33
Cultivar (CV.)	7	552.12 **
CV. X Treatment	42	42.64 **
CV. X S.P.	35	49.97 **
CV. X (S.P. vs original)	7	305.20 **
Error (b)	49	2.61
Total	111	
C.V. (a)		11.54%
C.V. (b)		8.07%

** : Significant at the 1% probability level

Table 12. Analysis of Variance Test of Soluble Protein Content of Eight Triticale Cultivars Stored under Artificial Aging Condiitons for 3, 6, 9, 12, 15 and 18 Days

Source of variation	d.f.	Mean of square
Treatment	6	439.37 **
Storage period (S.P.)	5	402,88 **
S.P. vs original	1	621.78 **
Error (a)	7	47.62
Cultivar (CV.)	7	689.11 **
CV. X Treatment	42	53.29 **
CV. X S.P.	35	55.54 **
CV. X (S.P. vs original)	7	42.07 **
Error (b)	49	16.88
Total	111	
C.V. (a)		10.66%
C.V. (b)		6.35%

** : Significant at the 1% probability level

Table 13. Student-t Test to Compare Average Trypsin Inhibitor Activity of Individual Cultivars Stored under Tropical and Artificial Aging Conditions with Originals

Cultivar	TIA (Trypsin Inhibition Units / g Seed Meal)				
	Originals	Tropical Condition[a]	% reduction or increase	Artificial Aging[b]	% reduction or increase
1	28.034	26.473 *	- 5.57%	20.849 **	-25.63%
2	23.316	23.934 NS	+ 1.03%	16.295 **	-30.11%
3	36.859	32.342 **	-12.25%	28.268 **	-23.31%
6	25.525	22.488 **	-15.22%	20.858 **	-21.36%
7	12.936	14.169 *	+ 1.10%	9.820 **	-24.09%
4	20.693	19.881 NS	- 3.92%	16.710 **	-19.25%
8	15.601	14.333 *	- 8.13%	8.726 **	-44.07%
5	18.531	18.088 NS	- 2.39%	14.398 **	-22.30%
			- 5.67%		-26.27%

NS : Not significant at the 5% probability level

** : Significant at the 1% probability level

* : Significant at the 5% probability level

a = The average of trypsin inhibitor activity level obtained after 7, 14, 21, 28, 35, and 42 days of storage.

b = The average of trypsin inhibitor activity level obtained after 3, 6, 9, 12, 15, and 18 days of storage.

Table 14. Least Significant Difference Test of Trypsin Inhibitor Activity of Eight Triticale Cultivars between Originals and 21 Days and 42 Days under Tropical Condition and between Originals and 18 Days under Artificial Aging Condition

Cultivar	TIA (Trypsin Inhibition Units / g Seed Meals)			
	Original	Tropical Condition for 42 Days 33°C/87% RH	Tropical Condition for 21 days 33°C/87% RH	Artificial Aging Condition for 18 Days 34°C/100% RH
1	28.034	23.415 *	22.220 *	15.790 *
2	23.316	22.625 NS	19.580 *	12.700 *
3	36.859	27.095 *	27.965 *	11.300 *
4	20.693	15.925 *	17.310 *	10.435 *
5	18.531	15.765 *	16.690 NS	11.165 *
6	26.525	21.920 *	22.105 *	18.380 *
7	12.936	9.915 *	14.255 NS	3.640 *
8	15.601	10.945 *	15.280 NS	7.305 *

* : Significant at the 5% probability level

Figure 4. Trypsin Inhibitor Activity and Soluble Protein Content of Eight Triticale Cultivars Stored under Artificial Aging Conditions for 3, 6, 9, 12, 15 and 18 Days

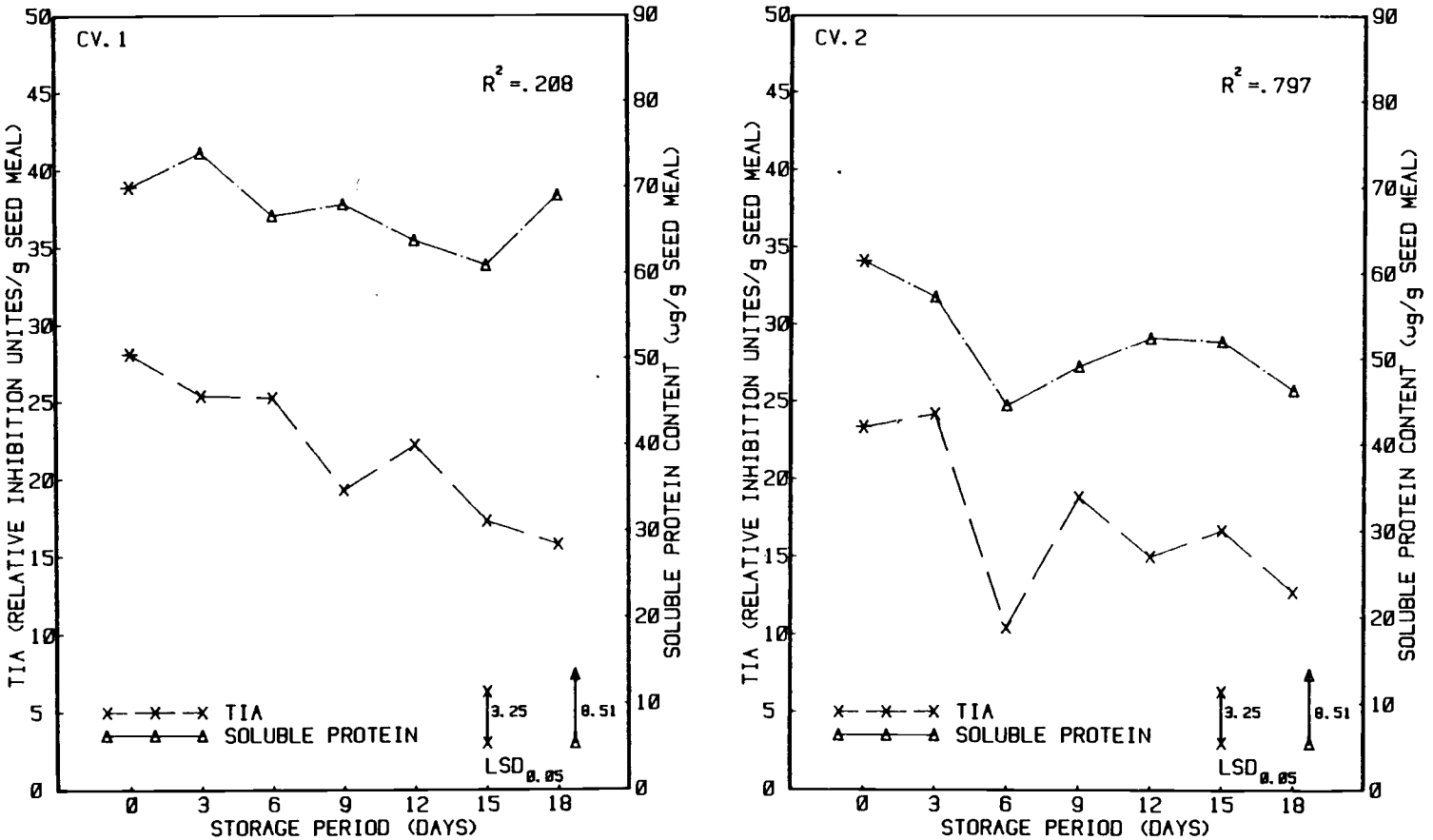
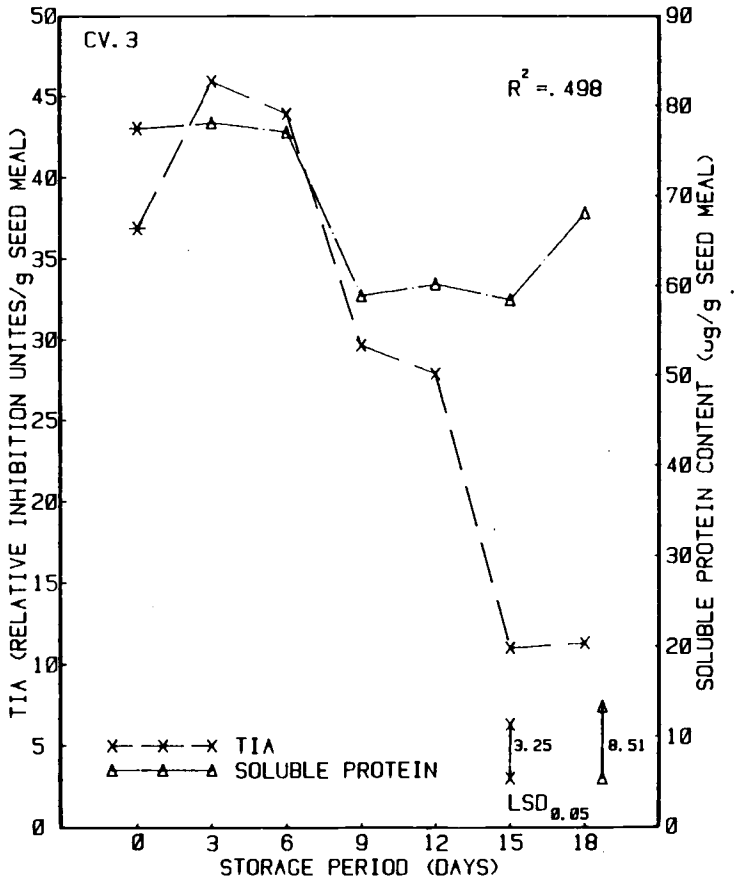


Figure 4. (continued)



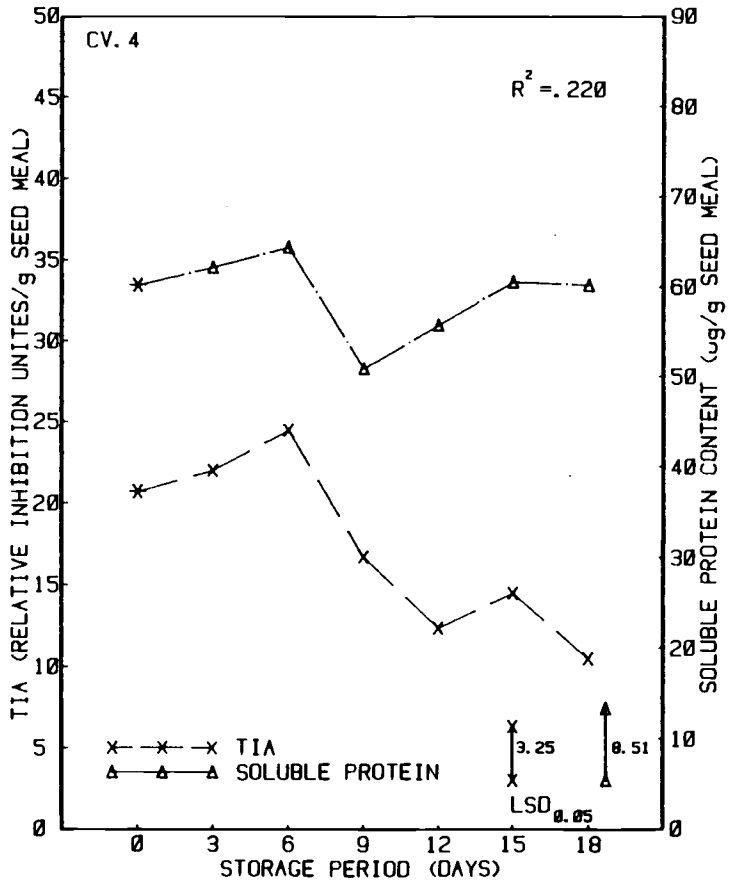


Figure 4. (continued)

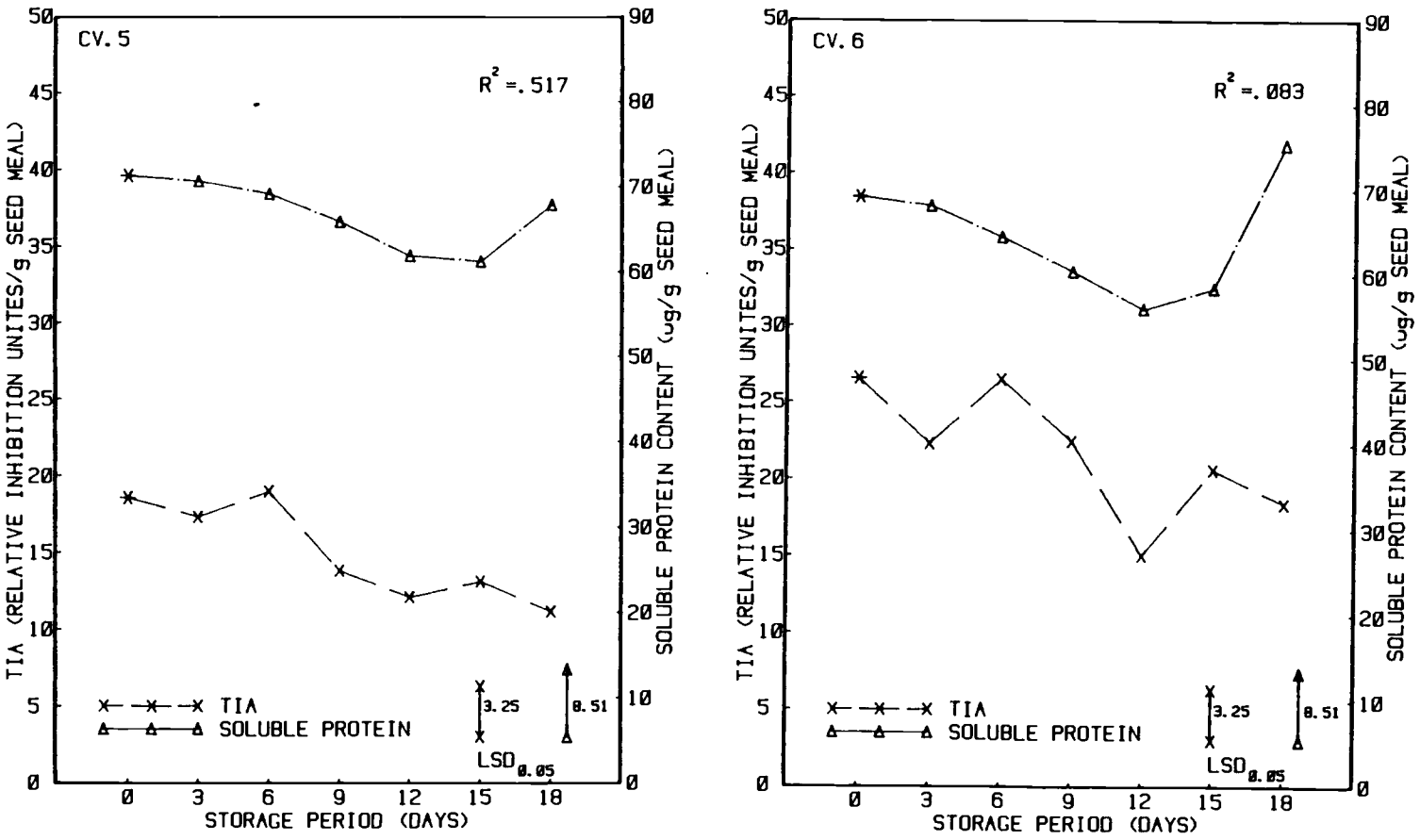
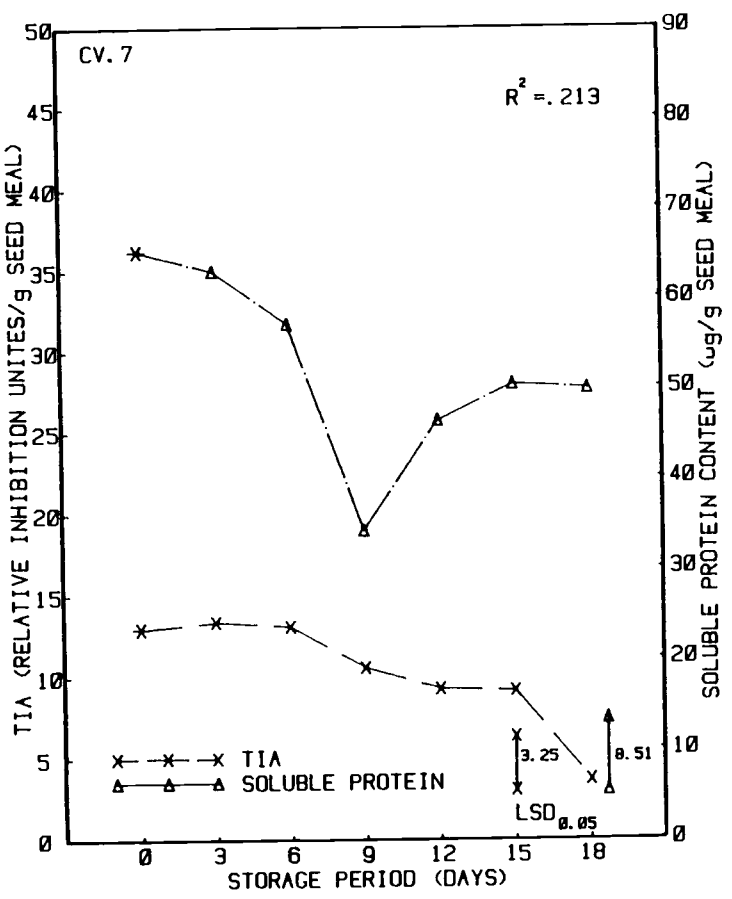
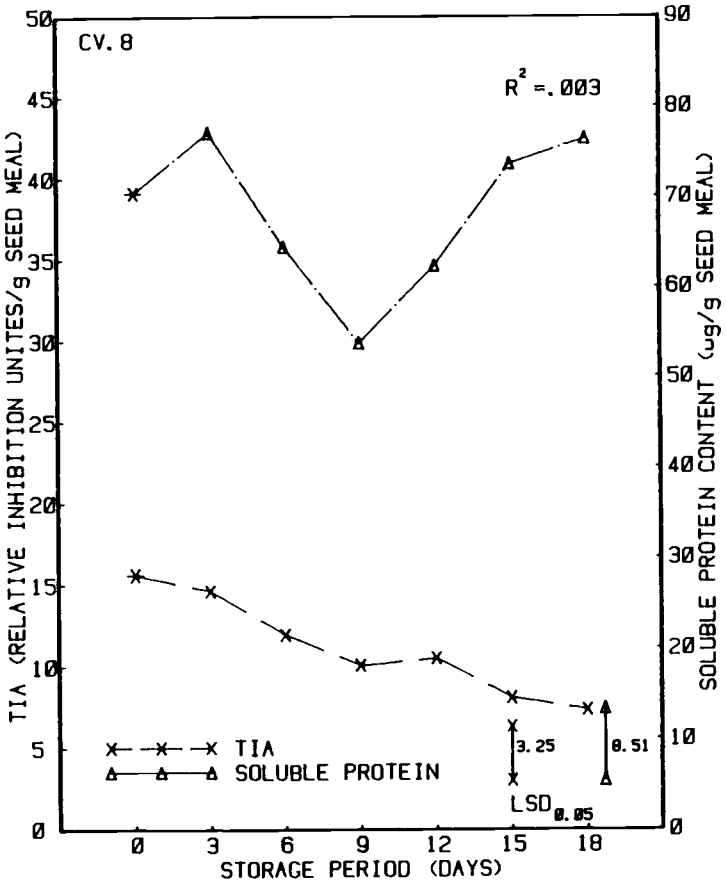


Figure 4. (contiuned)





DISCUSSION

Seeds of many plants are known to contain proteins capable of binding trypsin, an enzyme secreted from the digestive tract of animals, to form inactive complexes that result in reduced digestibility of feed protein in animals. Seeds of leguminous plants are especially rich in this kind of protein known as trypsin inhibitor. Among the cereals, trypsin inhibitor activity (TIA) has been detected in many species such as rye, barley, wheat, sorghum, oat, rice, and triticale. Triticale (X Triticosecale Wittmack), a polyploid cereal developed from a cross between Wheat (Triticum spp.) and rye (Secale spp.) has been found to surpass parental values for lysine and protein content, and in certain cases, for yield and agronomic versatility. Unfortunately, triticale selections currently available have been beseiged by a number of problems. One such problem is the inherent high TIA apparently inherited from the rye parent (Madle and Tsen, 1974). Shimada et al. (1974) have reported that poor palatability and elevated trypsin inhibition may have affected dietary intake and protein utilization when triticale was fed at the 80 and 100% levels in swine diets. If true, a benefit of the superior amino acid profile of triticale might be lost unless triticale cultivars low in TIA can be developed.

The discovery of trypsin inhibitors and their possible involvement limiting the nutritive value of feeds processed from triticale resulted in extensive work to investigate the chemical, biochemical and nutritional properties of these factors. The aim

of the present work is a) to improve the assaying procedure for TIA in aqueous extracts from triticale seeds, b) to obtain some indication of stability of TIA under different storage conditions, and c) to determine the changes in the relationship that may occur between TIA and soluble protein content under two storage conditions.

Experiment 1: Improvement of the Assay of Trypsin Inhibitor Activity for Triticale.

Methods presently available for determining TIA of plant origin partially fulfill the requirement for screening extensive numbers of samples. The standard AACC method for measuring the TIA of soy products (AACC 71-10) is based on use of a synthetic substrate, benzoyl-DL-arginine-P-nitroanalide hydrochloride (BAPA). The method evolved, primarily, from the work of Kakade et al. (1969 and 1974), who concluded the synthetic substrate could be utilized in assaying TIA of soy products. In cereal grains, TIA can readily be determined by several modified methods (Stewart, 1973; Smith et al., 1980; Hildebrand et al., 1980) by following the inhibition of trypsin hydrolysis of specific substrates. The assaying procedure described by Erickson (1979) is easily utilized for processing large numbers of samples and is specific for measuring TIA in triticale. However, some improvements can be made to increase differentiation among cultivars, to reduce variations between replicated assays, and to decrease expenses of the assay. Experiment 1 was thus conducted.

Results obtained from Experiment 1 indicated a) the level of

TIA was not influenced by particle size, b) the level of TIA was measured more accurately when 40 mg samples were used in the assay rather than the 20 mg samples recommended by Erickson, and c) a higher level of porcine trypsin concentrations was required in the study to differentiate among the level of TIA in the triticale and rye cultivars assayed (Appendix I) than originally recommended by Erickson (1979). Significant replication differences suggest some other factors were not recognized and adequately controlled. Perhaps thorough mixing of a large quantity of seed meal is essential to reduce the variation among replicated assay. Based on the experimental results, the recommended procedure for assaying trypsin inhibitor activity for a large population of triticale selection program is described in Appendix II.

Once the technique for testing TIA is optimized, the TIA assay could become a rapid and inexpensive method for screening large numbers of breeding lines for quantity of protein and minimum TIA in grains. Further, isolation of trypsin inhibitors and determination of their biological activities in animal feeding trials will ultimately have to be conducted, not only to establish their nutritional importance, but also to set a standard of TIA level for accepting or rejecting the unknown lines for feeding purposes. However, it is difficult to observe the combined and possible synergistic effects of all the anti-nutritional proteolytic factors in an animal feeding trial since such synergistic effects could be detrimental to the animals which would make it difficult to determine the effect of individual proteolytic factors.

Experiment 2: Effects of Storage Treatment on Trypsin Inhibitor Activity

Storage proteins also known as aleurone grains and protein bodies are stored in the aleurone layer and sub-aleurone endosperm tissue of the cereal grains, respectively. Proteinases, a number of enzymes located in storage organs, which could be activated from preformed or zymogen state, or synthesized de novo from messages of unknown "vintage," hydrolyze storage proteins and may result in new types of soluble proteins such as trypsin inhibitors. Trypsin inhibitors are small polypeptides synthesized according to genetic information during seed formation. Under unfavorable environmental conditions, trypsin inhibitors might deteriorate or degrade as do other natural macromolecules.

The effect of storage conditions on TIA in triticale seeds has never been studied. This experiment was conducted to determine the influence of temperature and humidity on the stability of TIA in stored grains. Seeds of eight triticale cultivars were stored for 90 days under three storage conditions, TIA of all eight cultivars was increased significantly for the first 30 days of storage. Although the TIA after 90 days storage under all three conditions was signficantly greater than that of the original seed lots, most of, not all of the increase in activity occurred during the first 30 days of storage in all eight cultivars. The increases in TIA could result from: a) conversion of some zymogen or complexed trypsin inhibitors into an active form, b) less interference from compounds such as phenolics in aged and/or non-dormant seeds and,

c) easier extractibility of trypsin inhibitors in aged and/or non-dormant seeds.

Under temperate conditions (21°C, 65% RH) which was the one most similar to the condition of the originals (25°C, 59% RH), the TIA of eight cultivars was still increased significantly for the first 30 days by comparing with that of the originals. It seemd the elevation of TIA could only be attributed to one factor which was the length of storage. Until the heterogeneity and the properties of each specific trypsin inhibitor is fully recognized and understood, the observation obtained here remained speculative.

The further modified procedures for assaying TIA are not only based on the adjustments from Experiment 1, but also on information obtained from several references (Smith et al., 1980; Hamerstrand et al., 1981; and Poyse, 1981). The major modifications included a) increase of sample size from 20 mg used in Experiment 1 to 1 g in Experiment 2. Even larger sample size (13 g) was applied in Poyse's method. b) The assaying of TIA was further improved by diluting the extracted trypsin inhibitors to the range of 1 ml of trypsin inhibitor inhibits 40-60% of 1 ml trypsin solution. Although the dilution procedure was more time consuming, more accurate data was obtained.

Information obtained from Experiment 1 and Experiment 2, as presented in Table 7 and Figure 1, demonstrated that ranking of TIA among eight cultivars remained consistent even though further modification was applied in measuring TIA in Experiment 2 as specified above. This indicated both procedures used in Experiment 1 and

Experiment 2 serve the same discerning purpose. However, sample size definitely needed to be increased in methods used in Experiment 1 in order to obtain more consistent and reliable data. Also, the additional dilution procedure applied in Experiment 2 might prevent under-estimating the TIA in some materials and give more reliable results.

Experiment 3: Changes of Trypsin Inhibitor Activity and Soluble Protein Content in Seeds Stored under Tropical and Artificial Aging Conditions.

Aging is a universal physiological phenomenon occurring in living organisms. Loss of seed vitality and vigor due to artificial or natural aging has been well documented. Chang et al. (1968) indicated that the insoluble protein content was not influenced by moisture content and storage temperature in crimson seeds and ryegrass seeds stored at lower temperature. But, at a high storage temperature, protein content of ryegrass seeds showed a definite reduction. The activity of proteases might be associated with aging in degrading vital structural and soluble proteins of organellar membranes, nuclear proteins, ribosomes, and enzymes. In 1972, Ching demonstrated that seeds of some materials stored at a high temperature (38°C) the soluble protein content showed a 68.55% breakdown compared to those stored at lower temperatures (3°C). Apparently, the proteins deteriorated under aging conditions of high temperature and humidity treatments. Therefore, in Experiment 3, both TIA and soluble protein content were measured.

On the average, TIA of eight triticale cultivars stored at 33°C/87% RH and 34°C/100% RH conditions deteriorated 5.67% and 26.27% as compared to the originals, respectively (Table 13). These data suggest that TIA of triticale seeds could be decreased to a great extent by increasing both temperature and relative humiditiy during storage.

The problem of purifying native proteins from plants that have phenolic compound content is well-recognized. However, according to Robinson (1979) when polyphenolic compounds are present, the most reliable method for measuring protein content is the Bradford procedure using Coomassie Brilliant Blue G-250 which ensured the true changes of soluble protein content obtained in this experiment. The results of Experiment 3 demonstrated that both TIA and soluble protein content were deteriorating under both tropical and artificial aging conditions (Figures 3 and 4).

Although the quantity of trypsin inhibitors comprises 5 to 10% of water soluble proteins both in the embryos and endosperms of grains of barley, wheat and rye (Mikola and Kirsi, 1972), Tanner et al. (1981) claimed that total and soluble protein contents in seeds showed no relationship with TIA within the range of several wheat and rye cultivars studied. This conclusion was also supported by the results of the experiment. As shown in Figures 3 and 4, the changes of TIA were poorly correlated with that of soluble protein content in most cultivars tested under both tropical and aging conditions. Poor correlation between TIA and soluble protein content further suggested that trypsin inhibitors and soluble

proteins are inherited independently. The possibility of hydrolyzed storage protein becoming trypsin inhibitors was not supported by experimental data.

However, the increase of soluble protein content caused by the infection of fungi in later storage periods might confuse the results so the correlation might not be truly indicative of genetic relationship of these two traits. For example, cv. TCMC100 which had the poorest correlation value for TIA and soluble protein content among eight cultivars was the one with the most visible contamination.

SUMMARY AND CONCLUSIONS

Knowledge of the nutrients and improvement in their availability in cereal grains should help men solve the world food supply problems. The content of anti-nutritional factors appears to influence the availability of nutrients in some cereal grains. Trypsin inhibitors have been identified in several cereal grains which adversely influence protein digestion in non-ruminant animals. Similarly, trypsin inhibitors in triticale seeds have been found to be the main contributors to the total anti-tryptic activity associated with incomplete utilization of proteins in monogastric animal diets (Shimada, 1974; Erickson et al., 1979).

Rapid and inexpensive laboratory tests must be developed to identify breeding lines low in trypsin inhibitor activity. In the first part of the study, two particle sizes of seed meals: 0.45 mm and 0.75 mm; two sample sizes of seed meals: 20 mg and 40 mg; and four trypsin concentrations: 75 ug/ml, 100 ug/ml, 125 ug/ml and 150 ug/ml were used as treatments in various combinations to determine the best procedure for measuring TIA. These factors were considered to be the variables most likely to affect the assay results.

It is known that most grains are used in animal feeding without any processing or pretreatment; storage conditions might change TIA in grains with time. In addition to that, the differences in TIA level were observed in seed samples of different ages of same cultivar in a preliminary experiment. The study on the effects of the storage conditions on TIA was

thus conducted in the second experiment. Five storage conditions - (A) desert (33°C, 65% RH); (B) temperate (21°C, 65% RH); (C) arctic (1.7°C); and (D) tropical (33°C, 87% RH) and (E) artificial aging (35°C, 100% RH) which simulated to the world's climates, were applied to the storage of triticale seeds. The seeds were tested for TIA at designated times; every 30, 60, and 90 days under conditions (A), (B), and (C); every 7 days for 42 days under condition (D) and every 3 days for 18 days under condition (E).

In Experiment 3 of the study, the soluble protein contents of seeds stored in tropical and artificial aging conditions (D and E) were also determined. The relationship of TIA with soluble protein content was inspected in these two conditions since more dramatic changes were expected to occur under high temperature and high humidity conditions.

Based on the results of the above three experiments, the following conclusions were drawn:

1. Particle size of seed meals, 0.45 mm and 0.75 mm which were commonly used for chemical analysis did not influence the result of TIA assay.

2. Differences between duplicated sample tubes were significantly decreased when sample size was increased from 20 mg to 40 mg per tube.

3. Cultivars were separated into more distinguishable TIA classes when two trypsing concentrations: 100 μg/ml or 125 μg/ml was added to each sample tube.

4. Under desert, temperate, and arctic storage conditions, the TIA in triticale seeds was a small but significant increase for the first 30 days of storage and then changed non-significantly until reaching 90 days of storage. Overall, the changes of TIA were significantly increased by comparing with the originals.

5. In general, under two high temperature and relative humidity storage conditions (D and E), TIA in most cultivars increased 10 to 20% then decreased signficiantly as storage period lengthened when compared with that of original samples. The general decrease of TIA and soluble protein content during storage under tropical and artificial aging conditions was probably due to the degradation of proteins in seeds. The increase of soluble protein content in later storage periods might be caused by fungi which thrived in the high temperature and humidity environments.

6. The conversion of insoluble storage protein to soluble form and trypsin inhibitors appears to be unlikely as the increase of soluble protein in seeds under prolonged tropical storage conditions did not correlate with changes in TIA.

7. Additional studies are needed to determine the factors responsible for the increase in TIA observed during the initial peirod of each of the five storage conditions.

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APPENDIX

APPENDIX 1. Trypsin Inhibition Level In Selected Triticale and Rye Cultivars Determined by Using Experiment 1 Assay Procedure

	Cultivar	Source of Seed	Ploidy Level	% of Trypsin Inhibition
1.	LT176/73	M80-6003	6x	85.1
2.	Daws/Snoopy Rye 6TA876	M80-6018	8x/6x	71.6
3.	6TA876/6TB163// 6TA876	M80-6019	6x	70.0
4.	EMS 6TA313A	M80-6227	6x	64.0
5.	"	M80-6230	6x	84.4
6.	"	M80-6237	6x	75.1
7.	EMS 6TA876	M80-7052	6x	71.5
8.	TCMC100, Nyon	M80-7053-1	8x	1.5
9.	EMS 6TA876	M80-7059	6x	60.7
10.	Druchamp/Kodiak Rye	M80-7465	8x	77.1
11.	6TA876/6TB164, F_4	M80-7141	6x	78.3
12.	6TB219/6TA876, F_5	M80-7143	6x	82.6
13.	Daws/Short Rye// EMS 6TA313A	M80-7160	8x/6x	59.4
14.	Daws/Antelope Rye// 6TA876, F_3	M80-7171	8x/6x	65.1
15.	Daws/Kodiak Rye	M80-7323	8x	86.4
16.	Daws/Kodiak Rye	M80-7330	8x	86.0
17.	Druchamp/Kodiak Rye	M80-7333	8x	41.6
18.	Crane/Snoopy Rye	M80-7450	6x	79.9
19.	61-1523/Druchamp//Rye	M80-7463	8x	35.2
20.	Nugaines/Dakold Rye	M80-7476	8x	82.9
21.	Pullman Sel. 101/ Secale montanum	M80-7478	8x	82.4
22.	Daws/Kodiak Rye	M80-8030	8x	83.0
23.	"	M80-8031	8x	75.6
24.	Pitic 62/Kodiak Rye	M80-8040	8x	77.1
25.	IAC-5/Rye	M80-8053	8x	72.9

26.	T. carthlicum-9 Kodiak Rye	M80-8056	6x	41.4
27.	Saragolla/Kodiak Rye	M80-8062	6x	85.3
28.	Daws/R1443 Rye	M80-8067	8x	84.1
29.	Daws/Rye	M80-8079	8x	81.7
30.	Daws/Rye	M80-8080	8x	83.4
31.	VT75229	M80-8864	6x	82.4
32.	6TA3i3A	M80-8871	6x	87.8
33.	Daws/Rye-1	M80-8898	8x	85.3
34.	Daws/Rye-2	M80-8899	8x	84.1
35.	N91,	TA76-163A	6x	78.8
36.	VT75229, selection	B804736	6x	74.0
37.	MC100, Kasarda No. 63, Triticale		8x	21.8
38.	Petkus Kasarda No. 63 Rye		2x	73.7
39.	Secalotriticum			65.8
40.	Kyoto 17, Rye		2x	55.5
41.	MC27, Triticale	Switzerland	8x	9.8
42.	MC41, Triticale	"	8x	80.8
43.	SV 6970, Winter Rye	Poland	2x	69.7
44.	DUSSI, Winter Rye	"	2x	81.6
45.	L Alfa, "	"	2x	81.8
46.	Carokurz, "	"	2x	79.4
47.	Hallo, "	"	2x	72.4
48.	Woschod 2, "	"	2x	73.3
49.	CHodar (M8), "	"	2x	75.8
50.	L 5/78, "	"	2x	79.8
51.	D 14/79, "	"	2x	88.0
52.	Otello	"	2x	78.1
53.	L 04	"	2x	85.5
54.	Char Kouska 60	"	2x	85.7
55.	L 6/78	"	2x	85.5
56.	L 13/79	"	2x	89.7

57.	L 254	Winter Rye	Poland	2x	84.0
58.	M 12A	"	"	2x	83.8
59.	L 11/79	"	"	2x	86.9
60.	Dankowskie, Selekeyene	"	"	2x	85.9
61.	Secale cereale Secale montanum Hybrid, wild rye	"	Hermiston Oregon	2x	88.1
62.	Dankowskie Nowe	"	Poland	2x	82.7
63.	D6644/T. urartu	Facultative	M80-8007	2x	7.6
64.	LT582/77	Winter Triticale	M81-6140	6x	7.4
65.	LT582/77	"	M81-6142	6x	18.3
66.	Elliott	Spring Triticale	Y-80, Plot 29	6x	64.3
67.	"	"	"	6x	65.0
68.	Daws/Snoopy /2/274-320		M80-7749-1		60.9
69.	1978h, Tcl 1023 1980 Nur., Tcl 243	Spring Triticale		6x	73.4
70.	Elliot,	"	Y-80, Plot 60	6x	66.8
71.	LT1317/77	Winter Triticale	M81-6144	6x	63.7
72.	CT93/76	"	M81-6148	6x	55.3
73.	CT525/78	"	M81-6158	6x	32.6
74.	LT487/77	"	M81-6138	6x	5.5
75.	CT463/77	"	M81-6154	6x	60.8
76.	CT317/78	"	M81-6152	6x	69.0
77.	CT138/77	"	M81-6150	6x	48.6
78.	CT513/78	"	M81-6156	6x	43.4
79.	CT691/79	"	M81-6160	6x	43.4

80.	LT176/73	Winter Triticale	M81-6136-11	6x	78.4
81.	LT176/73	"	M81-6136-12	6x	79.8
82.	LT176/73	"	M81-6136-13	6x	79.9
83.	"	"	M81-6136-14	6x	84.3
84.	CT525/78	"	M81-6158-1	6x	69.3
85.	"	"	M81-6158-2	6x	52.2
86.	"	"	M81-6158-3	6x	23.6
87.	"	"	M81-6158-4	6x	84.4
88.	LT894/77	"	M81-6142-11	6x	21.9
89.	"	"	M81-6142-16	6x	24.6
90.	"	"	M81-6142-18	6x	57.0
91.	"	"	M81-6142-19	6x	61.0
92.	"	"	M81-6142-20	6x	66.4
93.	LT582/77	"	M81-6140-11	6x	14.8
94.	"	"	M81-6140-12	6x	21.6
95.	"	"	M81-6140-14	6x	19.4
96.	"	"	M81-6140-15	6x	31.5
97.	LT487/77	"	M81-6138-14	6x	27.7
98.	"	"	M81-6138-15	6x	16.8
99.	"	"	M81-6138-16	6x	28.0
100.	"	"	M81-6138-17	6x	35.8
101.	CT513/78	"	M81-6157-2	6x	58.4
102.	"	"	M81-6157-3	6x	63.4

103.	61-1523/Druchamp//Rye-1	M81-8375-11	8x	33.0
104.	"	M81-8375-12	8x	42.3
105.	"	M81-8375-13	8x	43.1
106.	"	M81-8376-11	8x	38.8
107.	"	M81-8376-12	8x	32.6
108.	61-1523/Druchamps//Rye	M81-8376-13	8x	33.1
109.	"	M81-8377-1	8x	26.5
110.	"	M81-8377-2	8x	52.0
111.	"	M81-8377-3	8x	47.4
112.	"	M81-8377-4	8x	55.5
113.	"	M81-8378-1	8x	61.5
114.	"	M81-8378-2	8x	76.9
115.	"	M81-8379-1	8x	74.5
116.	"	M81-8379-2	8x	64.5
117.	"	M81-8380-1	8x	78.4
118.	Daws/Frontier	M81-8384-1	8x	82.1
119,	61-1523/Druchamps//Rye-1	M81-8459-1	8x	50.4
120.	"	M81-8459-2	8x	66.7
121.	"	M81-8459-3	8x	62.0
122.	"	M81-8459-4	8x	30.0
123.	"	M81-8460-1	8x	53.5
124.	"	M81-8460-2	8x	57.9
125.	"	M81-8461-1	8x	37.9
126.	"	M81-8461-2	8x	48.0
127.	"	M81-8462-1	8x	59.9
128.	"	M81-8462-2	8x	71.9
129.	"	M81-8462-3	8x	57.1

130.	Daws/Antelope//6TA876	M81-8043	6x	41.6
131.	"	M81-8044	6x	43.3
132.	"	M81-8047	6x	24.4
133.	BH1146/Pierre Rye	M81-8346	8x	84.6
134.	Bezostaya/Caribou Rye	M81-8347	8x	78.0
135.	Penjamo/R1003 Rye	M81-8353	8x	89.0
136.	Pitic62/73102 Rye	M81-8354	8x	93.1
137.	Pitic62/Kodiak Rye	M81-8357	8x	85.0
138.	Pitic62/Prolific Rye	M81-8361	8x	87.0
139.	6TA876	M81-8688-1	6x	67.5
140.	Salinas CA81	B 648	6x	84.1
141.	Salinas CA61	B 650	6x	57.0
142.	Salinas CA81	B 652	6x	85.4
143.	Burt/Dankowskie Zlote	M81-7138	8x	81.0
144.	E. Blackhull/Dankowskie Zlote	M81-7165	8x	61.3
145.	" , "	M81-7196	8x	72.2
146.	Daws/Secale montanum// VT75229 , F_3	M81-7749-1	8x/6x	71.6
147.	EMS 6TA 876, Sel. M79-8409-16	M81-8652-1	6x	70.9
148.	" , "	M81-8652-2	6x	75.4
149.	" , "	M81-8652-3	6x	62.6
150.	" , "	M81-8652-4	6x	75.9
151.	" , "	M81-8652-5	6x	62.3
152.	Carman	Canada	6x	64.1
153.	Welsh	"	6x	79.6
154.	75 L021		6x	70.4
155.	Triwell		6x	72.0
156.	79 P439		6x	65.6

157.	MN 81,82, Bulk	605-14	6x	73.0
158.	MN 81,83, Bulk	605-14-1	6x	61.7
159.	MN 81	61	6x	67.1
160.	Blenlea	Canada	6x	49.3
161.	Welsh	"	6x	82.7
162.	Vaca " S "	"	6x	83.2
163.	PND "R", Ye 75	Canada	6x	84.2
164.	Satu	"	6x	81.2
165.	PFT 765	"	6x	86.0
166.	Delfin 205	"	6x	77.9
167.	79 P058	"	6x	76.1
168.	75 L005	"	6x	74.7
169.	79 P056	"	6x	75.1
170.	75 L008	"	6x	80.7
171.	79 P057	"	6x	80.0
172.	79 P038	"	6x	77.0
173.	79 P186	"	6x	78.5
174.	79 P158	"	6x	76.0
175.	Chuu "S"	"	6x	78.9
176.	Wintri	"	6x	80.9
177.	Kodiak Rye	"	2x	86.3
178.	780018001	"	6x	71.4
179.	780021002	"	6x	19.8
180.	780005001	"	6x	76.4
181.	780014001	"	6x	80.7
182.	780015001	"	6x	80.8
183.	798033	"	6x	29.0

184.	6TA876	M76-6007-1	6x	80.6
185.	"	M76-6007-2	6x	80.2
186.	"	H77-201-5	6x	76.6
187.	"	M79-8749	6x	79.9
188.	"	M79-8749	6x	79.8
189.	"	M80-7280	6x	78.3
190.	"	M81-8753-1	6x	73.9
191.	6TA876	M81-8753-2	6x	76.4
192.	"	M82-6301	6x	76.6
193.	"	M82-6302	6x	76.9
194.	B858,	Jenkins	6x	73.3
195.	79B125001	Hhm82-317	6x	79.3
196.	78001801	Hhm82-299	6x	47.0
197.	780014001	Hhm82-295	6x	40.5
198.	780017001	Hhm82-301	6x	41.6
199.	780021002	Hhm82-313	6x	14.5
200.	780015001	Hhm82-297	6x	36.2
201.	79304001	Hhm82-315	6x	72.8
202.	6TA876/M76-6269 F_6	M826101	6x	38.4

APPENDIX 2. Recommended Procedure for Assaying Trypsin Inhibitor Activity in Triticale Selections.

1. Grind two grams of well mixed seeds for each sample.

2. Weigh one gram of seed meal and place each in a polyethylene tube to which 10 ml of sodium acetate buffer solution (0.02N, pH 3.8) is added.

3. After extracting at room temperature for two hours with occasional shaking, centrifuge the suspension at 3,000 x g for 15 minutes. Prepare one set of two tubes containing 0.5 ml sample plus 0.5 ml acetate buffer, to which 1 ml of trypsin solution (100 µg porcine trypsin dissolved in 1 ml of 0.001N HCL) and 1 ml of reaction buffer (pH 7.0) are added to each tube.

4. Prepare two control tubes (without sample extracts but with 1 ml trypsin, 1 ml acetate buffer and 1 ml reaction buffer) and two blank tubes (with reagents only) for each run.

5. Add 10 mg of Azocoll and a small glass bead to each tube. Mix well.

6. After incubating at 37°C for 10 minutes, stop the by adding 1 ml of 30% acetic acid.

7. Centrifuge tubes at 10,000 x g for 8 minutes and read absorbence of the supernatant against the blank at 520 nm in a spectrophotometer.

8. Trypsin inhibitor activity (TIA) as expressed is:

$$\% \text{ inhibition} = \frac{\text{Absorbence of control (Ac) - Absorbence of sample (As)}}{\text{Absorbence of control}} \times 100$$

9. If the % of inhibition is <40% or >60% then use original supernatant or higher dilution, respectively to assay again.

10. Calculate relative inhibition unit per gram of seed meal =

$$\frac{Ac-As}{Ac} \times \text{dilution factor} \times 10.$$