cystine to 0.30%. Since the supplemental residue supported optimal growth and PER, it appears that all of the methionine and cystine of this residue was utilized by the rat. However, neither water nor 0.1% sodium bicarbonate extraction in the sixth experiment removed all of the nonutilizable methionine and cystine from either raw or autoclaved beans. All extracted less protein than did 0.2% potassium hydroxide.

Autoclaved or boiled beans appeared to contain a water-soluble dialyzable substance which inhibited rat growth. This substance did not appear to be present in water extracts of raw beans, or in water from beans boiled in 0.1% sodium bicarbonate solution. The nature of the inhibitor is not known, but the fraction containing the inhibitor contained many peptides and/or free amino acids and substances which absorbed in the ultraviolet at 260 nm.

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Susceptibility of the Major Storage Protein of the Bean, *Phaseolus vulgaris* L., to in Vitro Enzymatic Hydrolysis

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The in vitro susceptibility to enzymatic hydrolysis of affinity-isolated G1, the major storage protein of the bean, has been examined. The extent of hydrolysis of G1 by a number of enzymes was less than that of native bovine serum albumin under similar conditions. Sequential treatments with different enzymes resulted in more complete hydrolysis. Discontinuous SDS gel electrophoresis of G1 after exposure to trypsin confirmed the susceptibility of the molecule to tryptic hydrolysis and indicated the presence of a number of extremely trypsin-labile peptide bonds. The existence of a number of relatively large trypsin-resistant peptides in G1 has also been observed. The effects of heat treatment on G1 suggest that there may exist some conformational constraints on hydrolysis of the native molecule. The concentration of tannins in different seed lines did not correlate with the in vitro susceptibility to hydrolysis of the affinity-isolated protein but added tannins readily decreased the in vitro susceptibility to hydrolysis.

Several factors are likely to limit the nutritional quality of legume seed proteins. Low content of sulfur-containing amino acids and the presence of a number of potentially antinutritive proteins are the most frequently discussed factors. However, sulfur content (cystine + methionine) does not correlate well with protein efficiency ratio for cooked legumes (Liener, 1976) which suggests that other factors may be dominant. In addition, the proteinase inhibitors and phytohemagglutinins which were once thought to be major determinants of nutritional quality in legumes may not play dominant roles (Kakade et al., 1973; Turner and Liener, 1975). As a result, the role that protein digestibility may play in determining nutritional quality has been referred to with increasing frequency in recent years (e.g., Kakade, 1974).

Susceptibility to in vitro enzymatic hydrolysis appears to correlate with in vivo measurements of nutritional quality even without any corrections for absorption or other complicating metabolic factors. Several procedures for in vitro hydrolysis with pepsin or with pepsin plus pancreatin were developed before 1965 and were shown to correlate better with biological value than did a number of chemical scores based only on amino acid analysis (Akeson and Stahman, 1964; Sheffner, 1967). Subsequent work with other enzymes or with simplified procedures has not substantially improved correlations with in vivo

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measurements of nutritional quality (Hsu et al., 1977; Maga et al., 1973; Saunders et al., 1973).

If a protein is resistant to enzymatic hydrolysis, simple amino acid analysis will give a deceptively high indication of nutritional quality. However, there are a number of factors which suggest that in vitro susceptibility to enzymatic hydrolysis of legume proteins should not be expected to correspond to the in vivo situation. Amylase treatment of soybean protein fractions has been shown to have substantial effects on the susceptibility of these fractions to hydrolysis by trypsin (Boonvisut and Whitaker, 1976). Starch complexed with protein is an important consideration for in vitro assays but is unlikely to be important for the in vivo situation. Product inhibition is also an important consideration for in vitro assays, but is not likely to play a role in vivo where absorption of amino acids and small peptides takes place. Galembeck et al. (1977) have shown that product inhibition can affect both the apparent rate and the apparent extent of in vitro hydrolysis of bovine serum albumin by chymotrypsin. Mihalyi (1972) has reviewed a number of other factors which can affect the susceptibility of native proteins to enzymatic hydrolysis and has indicated some of the complexities that should be expected. The binding of ligands to sites on a protein, for example, can have dramatic effects on susceptibility to hydrolysis. There are a number of cases in which binding of coenzymes can either dramatically decrease (Elodi and Szabolcsi, 1959; Trayser and Colowick, 1961) or dramatically increase (Nirenberg and Jakoby, 1960) the in vitro tryptic digestibility of a protein. Binding of two iron atoms to a molecule of ovotransferrin is reported to render the previously susceptible molecule completely resistant to proteolysis (Azari and Feeney, 1958). Protein associations, which are common in legume protein systems, are also known to affect susceptibility to hydrolysis (Kleczkowski and van Kammen, 1961). These factors make it unlikely that in vivo nutritional value of legume proteins can be predicted completely from in vitro digestibility studies.

Although considerable information is available on the interaction of proteolytic enzymes with low molecular weight substrates and substrate analogues, surprisingly little is known about their interaction with protein substrates. Even less is known about the molecular basis of resistance to enzymatic hydrolysis. It should be noted that, although nearly complete resistance to enzymatic hydrolysis has been observed with some proteins, even partial resistance can have large effects on the nutritional value of a protein. This could easily happen if a disproportionately large percentage of certain amino acids were contained in a resistant peptide or if the rate of release of a certain amino acid was sufficiently different from the rest that it was nutritionally unavailable at the same time. Several workers have reported the existence in legume proteins of either enzyme-resistant peptides or major fractions of the total legume protein which showed little susceptibility to proteolytic digestion (Fukushima, 1968; Seidl et al., 1969; Kakade et al., 1969; Boonvisut and Whitaker, 1976).

The purpose of the present study is to examine the in vitro susceptibility to enzymatic hydrolysis of G1, the major storage protein of the bean.

MATERIALS AND METHODS

Bovine serum albumin (fraction V powder), trypsin (type III), chymotrypsin (type II), and porcine pepsin were obtained from Sigma Chemical Corporation. Seeds of *Phaseolus vulgaris* L. var. Improved Tendergreen were obtained from Olds Seed Co., Madison, Wis. Seeds of PI 207227 and BBL 240 were obtained from F. Bliss, Department of Horticulture, University of Wisconsin, Madison.

Intact dry French bean seeds (*Phaseolus vulgaris* L. var. Improved Tendergreen) were ground in a Waring blender for 30 s and extracted in 0.5 M NaCl for 15 min. The slurry was centrifuged at 4 °C and 15000 rpm for 20 min in a Sorvall RC-5 centrifuge and the G1 fraction isolated from the supernatant using the affinity chromatography method of Stockman et al. (1976). The protein was not subjected to water precipitation but was obtained in 0.5 M NaCl and stored at 4 °C until used. G1 was prepared fresh weekly. The concentration of G1 was determined by absorbance at 276 nm ($A^{1\%}_{276} = 10$). Discontinuous SDS gel electrophoresis of the G1 fraction showed no apparent contamination with other proteins.

Substrate concentrations for enzymatic digestions varied from 1.0 to 2.8 mg/mL depending upon the concentration of G1 isolated by affinity chromatography. Bovine serum albumin was freshly prepared in 0.5 M NaCl at the same concentration as the G1. For trypsin and chymotrypsin digestions, 1.0 mL of substrate in 0.5 M NaCl was added to 100 μL of 0.08 M sodium barbital, pH 8.6, followed by the addition of 100 μL of enzyme in 100 mM $CaCl_2$ at a concentration equal to that of the substrate solution (1.0–2.8 mg/mL). The final ratio of enzyme to substrate in every case was therefore 1:10. Substrates and enzymes were adjusted to pH 8.6 before addition to digestion mixture. Pepsin digestions were unbuffered and substrates were adjusted to pH 1.4 by addition of substrate solution to an equal volume of 0.08 N HCl in 0.5 M NaCl. This procedure appeared to avoid precipitation of G1 at low pH. For each digestibility experiment three replicates of each substrate with enzyme, three replicates of each substrate without enzyme, and three replicates of an enzyme-only control were prepared. Digestions were carried out at 37 °C for 21 h in tubes sealed with parafilm. For sequential digestions, 100 μL of 0.08 M sodium barbital, pH 8.6, was added to each tube after 21 h digestion with pepsin. Freshly prepared trypsin or chymotrypsin was then added to give an enzyme–substrate ratio of 1:10, and the tubes were incubated another 21 h.

Picryl sulfonic acid (TNBS) was obtained from Sigma and recrystallized according to Fields (1972). The TNBS assay used was a modification of the procedure described by Fields (1971). After 21 or 42 h, each digest was diluted with 8.0 mL of distilled deionized water. A 0.5-mL aliquot of this diluted digest was added to 0.5 mL of 0.1 M $Na_2B_4O_7$, pH 9.5. To this was added 20 μL of a freshly prepared solution of TNBS (100 mg/0.2 mL) and the mixture thoroughly mixed. After 30 min the reaction was stopped by addition of 2.0 mL of 0.1 M NaH_2PO_4 containing 1.5 mM Na_2SO_3. A blank prepared at the same time as the digestions and containing only 0.5 M NaCl was assayed with TNBS following the same procedure. Absorbance of the samples at 420 nm was determined using a Beckman 25 spectrophotometer. Values of ΔOD for no-enzyme control and enzyme-only control were subtracted from ΔOD value of appropriate sample. Calculation of percent peptide bond hydrolysis from changes in absorbance involves determining the ratio of the number of new amino groups in the digestion to the total number of peptide bonds in the digestion mixture and expressing this ratio as a percentage. The number of new amino groups is calculated using 22000 as the molar extinction coefficient for TNP-α-amino groups (Fields, 1972), and the total number of peptide bonds is calculated by dividing the grams of substrate in the digestion mixture by 113

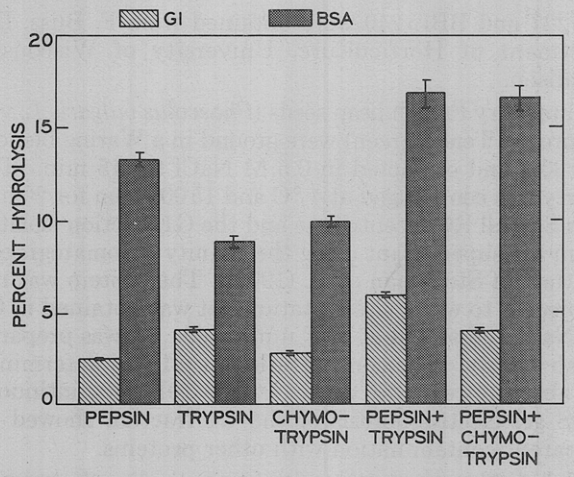

Figure 1. Percent total peptide bond hydrolysis of G1 or BSA with pepsin, trypsin, chymotrypsin, or combinations of these enzymes. Extent of hydrolysis is determined by TNBS assay of amino groups formed by hydrolysis. All digestions were done at 37 °C for 21 h with an enzyme to substrate ratio of 1:10.

g/mol, the average residue molecular weight of amino acids. Statistical comparisons were made using a one-tailed *t* test.

Discontinuous SDS gel electrophoresis was done as described by Laemmeli (1970) except that the running gel was 12% acrylamide with a ratio of acrylamide to bisacrylamide of 200:1. Electrophoresis was performed in a 0.75-mm slab gel in an apparatus obtained from Hoeffer Scientific Instruments, San Francisco, Calif. Samples of digestion mixtures for SDS gel electrophoresis were diluted with an equal volume of Tris–SDS–mercaptoethanol sample buffer, frozen immediately in acetone–dry ice, and stored frozen until all samples were ready for electrophoresis.

Tannins (Mallinckrodt Chemical Works, New York; oak nut gall tannins, crude extract, average molecular weight 1000–1500) were dissolved in 50% methanol and 50 μL was added to 1.0 mL of substrate buffered with 8 mM sodium barbital, pH 8.6. All concentrations of tannins used caused immediate precipitation of both G1 and BSA, except 0.11 mg of tannin/mg, which precipitated only the G1. Precipitates were vortexed into fine suspensions before the addition of trypsin and after dilution with water. The small standard errors obtained for this experiment show that error due to nonuniformity of the samples is not significant. Catechin equivalent values for Tendergreen, PI 207227, and BBL 240 were obtained using the vanillin–HCl assay described by Burns (1971), except that extractions were done with 100% rather than 50% methanol.

RESULTS AND DISCUSSION

Susceptibility of G1 and BSA to Enzymatic Hydrolysis. The extent of hydrolysis of G1 and BSA by trypsin, chymotrypsin, and pepsin or combinations thereof is shown in Figure 1. In every case the susceptibility to enzymatic hydrolysis of BSA is considerably greater than that of G1. Sequential treatments gave larger extents of hydrolysis than did treatments with single enzymes. The extent of hydrolysis of either substrate with any enzyme did not vary substantially within a given experiment. Values shown are means of three replicates and standard errors are shown. Variations in observed extent of hydrolysis from one day to another were larger. The differences in susceptibilities to hydrolysis of G1 and BSA may not be as large as it appears from this data. The percent of hydrolysis of bonds for which an enzyme has

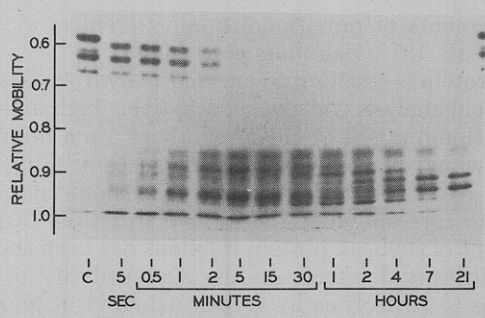

Figure 2. Discontinuous SDS gel electrophoresis of G1 exposed to trypsin for varying periods of time. Digestion was in 0.5 M NaCl, 8 mM Nabarbital, pH 8.6, with an enzyme to substrate ratio of 1:20. Aliquots of the reaction were taken at 5 and 30 s, 1, 2, 5, 15 and 30 min and at 1, 2, 4, 7, and 21 h. Aliquots were mixed with an equal volume of SDS–sample buffer and frozen immediately until ready for electrophoresis. C = no-enzyme control.

specificity would be a more appropriate comparison than the percent hydrolysis of total peptide bonds. For example, the lysine plus arginine content of BSA is 14.2% (King and Spencer, 1970), so cleavage by trypsin of 8.8% of the total peptide bonds corresponds to cleavage of 62% of the theoretically trypsin-susceptible bonds. The lysine plus arginine content of G1, however, is only 8.6% (Hall, 1977) so cleavage of 4% of the total peptide bonds corresponds to 46.5% cleavage of the trypsin-susceptible bonds. Calculations based on percent hydrolysis of normally trypsin-susceptible bonds suggest that there is a substantial difference in extent of hydrolysis between BSA and G1, but this difference is not as large as indicated by comparison of percent hydrolysis of total peptide bonds. In the case of trypsin, then, the low in vitro digestibility of G1 appears to be related both to structural constraints on hydrolysis and to a relatively low concentration of amino acid residues compatible with the substrate specificity of the enzyme. Unfortunately it is not possible to make similar comparisons for other enzymes whose specificity is not as restricted as that of trypsin.

The results of SDS gel electrophoresis of G1 after various periods of incubation with trypsin confirm that G1 is extensively hydrolyzed by trypsin (Figure 2). Controls without enzyme showed no changes in banding pattern during this time period. Banding patterns identical with those shown in Figure 2 were obtained on a number of different occasions from trypsin-digested G1. Several extremely rapid changes in G1 occurred on exposure to trypsin. The two bands with relative mobilities of 0.58 and 0.61 disappeared completely even at the earliest time point, and a band with relative mobility of 0.60, a number of faint bands with relatively mobility greater than 0.85, and a large amount of very low molecular weight material appeared. The four-banded pattern shown by the no-enzyme control has been invariably obtained in this laboratory from freshly prepared, affinity-isolated G1 on high-resolution SDS gels of the kind described in the Methods section. This four-banded pattern is also observed in samples of seeds extracted directly with SDS sample buffer and in rapidly isolated preparations of G1 (total time from seed to gel less than 10 min). The three-banded pattern seen after brief exposure to trypsin has been thought by ourselves (Stockman et al., 1976) and by others (Romero et al., 1975; Sun and Hall, 1975) to reflect the subunit size of the native molecule but this may not be the case. No similar rapid modifications are observed on exposure of G1 to either chymotrypsin or pepsin. The importance of preventing exposure to trypsin-like enzymes in crude seed extracts is clearly emphasized by this result. Further incubation with

Table I. Effect of Heating on the Susceptibility of G1 and BSA to Enzymatic Hydrolysis

Enzyme	Substrate	Percent peptide bond hydrolysis		Increase on heating (percent of unheated)
		Unheated	Heated	
Pepsin	G1	1.7 ± 0.3	4.3 ± 0.2	152
	BSA	13.7 ± 1.4	13.7 ± 1.4	0
Trypsin	G1	1.8 ± 0.5	5.8 ± 0.5	222
	BSA	11.8 ± 0.5	16.3 ± 1.4	38
Chymotrypsin	G1	1.5 ± 0.5	5.6 ± 0.3	273
	BSA	10.5 ± 0.2	15.4 ± 1.0	47

Table II. Effect of Tannins on in Vitro Tryptic Hydrolysis of BSA and G1

Mg of tannin/mg of protein	% hydrolysis	
	BSA	G1
0	12.8 ± 0.5	2.2 ± 0.2
0.11	13.1 ± 0.3	0.9 ± 0.2
0.27	10.2 ± 0.7	0.2 ± 0.2
2.75	0.3 ± 0.3	0
11.0	0	0
22.0	0	0

trypsin resulted in a gradual decrease in intensity of the three major bands and increased intensity of the lower molecular weight bands with relative mobilities between 0.85 and 1.0. The absence of any major hydrolysis products with relative mobilities between 0.66 and 0.85 is somewhat surprising and suggests that one of the most trypsin-labile bonds in each peptide occurs near the middle of the sequence of that peptide. Cleavage of these labile peptide bonds would result in peptides of roughly half the molecular weight of the original peptide. Two relatively large and apparently trypsin-resistant peptides are present in the hydrolysate after 21 h when all other hydrolysis products have been reduced in molecular weight to the point where they diffuse readily from the gel and therefore do not appear after staining.

Effect of Heat Treatment on Susceptibility to Hydrolysis. The effects of heat treatment (15 min, 100 °C, 2.1 mg/mL of substrate) on the susceptibility to enzymatic hydrolysis of both G1 and BSA are shown in Table I. In all cases except the hydrolysis of BSA by pepsin, heat treatment increased significantly ($P < 0.05$) the extent of hydrolysis. However, the increases in hydrolysis caused by heat treatment of G1 were substantially larger in every case than the increases seen after heat treatment of BSA. This may suggest that conformational or structural constraints on hydrolysis of G1 are larger than in the case of BSA. Thermal denaturation of BSA is not thought to involve extensive disruption of the secondary and tertiary structure of the molecule (Oakes, 1976); however, increases in the rate of tryptic hydrolysis after heat treatment have been reported (Gorini and Audrain, 1952). Nothing is known about thermally induced changes in the structure of G1.

Effect of Tannins on Susceptibility to Enzymatic Hydrolysis. Exposure to tannins significantly decreased the susceptibility to tryptic hydrolysis of both G1 and BSA (Table II). The effect of tannin content of seeds on in vitro digestibility of affinity-isolated G1 is not pronounced (Table III). Digestibility with trypsin appears to be lowest in the high tannin seed line but no clear relationship exists. Differences in digestibility with chymotrypsin or pepsin are not statistically significant. The length of time during which the seed protein is present as a crude extract containing tannins may be as important a factor as the tannin content. With affinity isolation procedures, this is typically quite short. It may be easier, then, to demonstrate an effect of tannins on digestibility of isolated protein if classical isolation procedures are used. It is also possible that examination of a larger number of seed lines would show a clearer effect of tannin content on digestibility as has been observed with other seeds (Ramachandra et al., 1977).

Table III. Effect of Tannin Content of Seeds on in Vitro Digestibility of G1 Isolated by Affinity Chromatography

Variety	Tannin content (catechin equiv) (mg/g of flour)	Percent peptide bond hydrolysis		
		Pepsin	Trypsin	Chymotrypsin
PI 207227	195.0	2.4 ± 0.2	1.6 ± 0.7	1.8 ± 0.5
Improved Tendergreen	28.4	2.0 ± 0.3	4.5 ± 0.5	3.2 ± 1.0
BBL 240	3.8	2.2 ± 0.3	2.5 ± 0.2	3.6 ± 1.0

CONCLUSIONS

The major storage protein of *Phaseolus vulgaris* seeds has been studied in a number of laboratories (Barker et al., 1976; Racusen and Foote, 1971; Sun and Hall, 1975; Stockman, et al., 1976), but little agreement has been obtained on the subunit structure of the molecule. A number of complicating factors are likely to be responsible for this. The resolution obtained in banding patterns on SDS gel electrophoresis is highly dependent on small variations in experimental technique. In addition, the SDS gel electrophoresis banding pattern shown by the major protein of *P. vulgaris* has been shown to vary genetically (Romero et al., 1975) and this probably accounts for some of the variability which has been reported. The results presented in this paper may suggest a third possible reason for variability. The extreme lability of a number of peptide bonds to hydrolysis in vitro by trypsin suggests that caution should be exercised to minimize exposure to seed proteinases.

The extent of susceptibility of legume seed proteins to enzymatic hydrolysis may play a role in determining the overall nutritional value of the seed. Our results suggest that the major seed protein of *P. vulgaris* is less susceptible to hydrolysis by a number of proteinases than is bovine serum albumin, and it is certainly possible that this difference between the two substrates is sufficiently large to account for differences in nutritional quality. The existence of proteinase-resistant peptides may be a more significant factor although we do not have evidence to suggest that trypsin-resistant peptides are also resistant to hydrolysis by other proteinases. To represent a major determinant of nutritional quality, the resistant peptides would probably need to be resistant to a number of proteinases and to contain a percentage of certain amino acid high enough to affect the amino acid balance of the protein.

Tannins have been shown by a number of other workers to affect the *in vitro* digestibility of proteins (Feeny, 1969; Basaraba and Starkey, 1966; Ramachandra et al., 1977) as well as a number of in vivo measures of digestibility (Nelson et al., 1975; Marquardt et al., 1977; Ward et al., 1977). It is certainly not surprising that we observe decreases in in vitro susceptibility to enzymatic hydrolysis in the presence of tannins. It seems likely that the extent of interaction of tannins with seed proteins during isolation will depend on details of the isolation procedure. The potential importance of tannin content for nutritional value of bean seeds is suggested by the decreases in in vitro digestibility which we have observed.

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Comparative Nutritive Value and Amino Acid Content of Triticale, Wheat, and Rye

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A study was conducted to compare the nutritive value, i.e., PER (protein efficiency ratio) and NPR (net protein retention), and amino acid content of triticale (a cross between wheat and rye) with that of wheat and rye. The protein quality index based on PER and NPR at a 10% protein level was found to be highest in rye, followed by triticale and wheat. A chemical score based on the essential amino acid content of egg protein and an FAO provisional pattern of milk protein indicates the level of amino acids which are limiting in rye, triticale, and wheat. The EAAI (essential amino acid index) and BV (biological value) were also calculated.

Triticale, a relatively new cereal, was developed by crossing two species of cereal grains, wheat and rye. The name was derived from the generic classification of these grains (*Triticum*, wheat and *Secale*, rye). Plant breeders have since developed some tetraploid and hexaploid triticales that combine the characteristics of both wheat and rye (Briggle, 1969). These new selections have shown improvement in plant fertility, vigor, and yield. They are drought resistant and yield about 50% more than wheat under poor moisture conditions.

The dietary value of triticale in comparison with other cereals like wheat, sorghum, barley, corn, and bran in different species has been reported by Bragg and Sharly (1970), Mertz et al. (1975), Stringham (1971), Lofgreen (1971), McCloy et al. (1971), and Palta and Arora (1973). Research data are very limited concerning the use of tetraploid or hexaploid triticales as food grain by rats. No biological test data based on PER, NPR, and amino acid content have been reported so far in parent, offspring, and newly developed dwarf variety wheats. The present paper describes results of such investigation on amber-colored dwarf mutant variety Kalyan Sona (normal protein wheat variety), HD4502 (a high protein wheat variety), rye, and triticale (tetraploid) developed at the Indian Agricultural Research Institute, New Delhi.

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MATERIALS AND METHODS

Crops Samples. Seeds of Kalyan Sona, HD4502, rye, and triticale were collected from a field experiment conducted by the Division of Genetics of the Indian

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