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Changes in lipoxygenase isozymes and trypsin inhibitor activity in soybean during germination at different temperatures

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Abstract

Influence of germination temperature on lipoxygenase isozymes and trypsin inhibitor activity, the two undesirable components in soybean for human consumption, is not yet reported in soybean sprouts. Two Indian soybean genotypes were incubated for 144 h in a seed germinator at two different temperatures (25 and 35 °C) and the activities of lipoxygenase isozymes and trypsin inhibitor were monitored in the germinating seedlings every 24 h. Lipoxygenase-I as well as lipoxygenase-II + III were degraded continuously over the 144 h and the rate of degradation, of both the classes of lipoxygenase, was faster at the higher germinating temperature (35 °C) in both the genotypes. Trypsin inhibitor was also degraded continuously during germination upto 144 h and the degradation was faster at higher germination temperature. Protein extracts of seedlings of different periods, developed at different temperatures, and analyzed using polyacrylamide gel electrophoresis, indicated that the original Kunitz inhibitor band ($R_f = 0.75$) declined continuously in intensity during germination at both temperatures in both genotypes, and a new band ($R_f = 0.72$) possessing trypsin inhibitor activity appeared at 48 h at 35 °C, while it appeared at 72 h at 25 °C. Early appearance of a modified form of Kunitz inhibitor, a degraded product of native form, at 35 °C as compared to 25 °C, confirms that the faster quantitative reduction at higher temperature is due to faster degradation of the original Kunitz inhibitor form at higher temperature.
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Keywords: Lipoxygenase isozymes; Trypsin inhibitor; Germination; Temperature

1. Introduction

Soybean, being one of the most economical and nutritious food, can combat diseases stemming from malnutrition in developing countries and nutraceutical ingredients present in it can reduce the risk of major hazardous diseases, namely cancer, atherosclerosis, osteoporosis, diabetes, that afflict the developed world today. Apart from traditional soy preparations, namely tofu and soymilk, sprouts are one of the few other ways of availing health benefits of soybeans as young seedlings also contain nutraceutical ingredients, e.g. isoflavones (Park & Lee, 2004), that reduce the risk of the above-mentioned major killer diseases of the century. However, lipoxygenase and trypsin inhibitors, the two major undesirable components of soybean that limit its wider utilization, are also present in soybean seedlings. Lipoxygenase isozymes (EC1.13.11.12) are the major culprit responsible for the development of off-flavour in soy products as they catalyse the oxidation of polyunsaturated fatty acids containing the *cis*-*cis*-1,4 pentadiene moiety and thereby release off-flavour-producing aldehyde and ketone compounds (Rackis, Hinig, Sessa, & Steggerd, 1979). Trypsin inhibitor is an antinutritional factor that affects the protein digestibility (Liener & Kakade, 1980). Though it is heat-labile, the heat treatment insolubilizes the much-valued proteins (Anderson, 1992) and, more importantly, excessive heat treatment can cause loss of amino acids in soy proteins (Rios-Iriarte & Barnes, 1996). However, only a few reports of lipoxygenase isozymes

Abbreviations: Lx, lipoxygenase; TIA, trypsin inhibitor activity.

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(Bau, Villaume, & Mejean, 2000; Bordingnon, Ida, Oliveira, & Mandarino, 1995) and trypsin inhibitor activity (Bates, Knapp, & Avaujo, 1977; Collins & Sanders, 1976; Mcgrain, Chen, Wilson, & Tan-Wilson, 1989; Mostafa, Rahma, & Rady, 1987) in germinating seedlings are available in the literature. Contradictions in the results of these studies suggest that germination conditions, namely genotypes, humidity, temperature, can influence various biological components in young seedlings. Reports are lacking of lipoxygenase isozyme and trypsin inhibitor activities in germinating soybean seedlings developed at two different temperatures. The present investigation was undertaken to study the changes in lipoxygenase isozymes and trypsin inhibitor in germinating soybean seeds in the dark at two temperatures.

2. Materials and methods

2.1. Material

The seeds of two popular Indian genotypes viz. JS80-21 and JS71-05 were germinated from 0 to 144 h at 25 and 35 °C in a seed germinator with 100% relative humidity in the absence of light. After every 24 h, a few germinated seedlings were taken, in triplicate, and their fresh weight was recorded. Subsequently, they were allowed to dry in a seed drier at 37 °C. When completely dried, their weight was recorded again to calculate the moisture content. Freeze-dried samples were powdered and sieved to obtain a flour of 100 mesh size for analyses of trypsin inhibitor and lipoxygenase isozymes of seedlings.

2.2. Extraction and estimation of lipoxygenase isozymes activity

For determination of lipoxygenase isozymes, seedlings were ground. The sieved soy flour was defatted with petroleum ether at 0–4 °C, following Marczy, Simon, Mozsik, and Szani (1995).

The soybean extract was prepared by homogenizing sieved soy flour with 100 vol of phosphate buffer (0.2 M, pH 6.8) in a microtissue homogenizer for 20 min at 0–4 °C. The homogenized suspension so obtained was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant so obtained was used as the crude extract for assaying lipoxygense isozymes, following the standard method (Axelrod, Cheesebrough, & Laakso, 1981). Reaction mixture for lipoxygenase-I consisted of crude extract as enzyme source, boric acid borax buffer (0.2 M, pH 9.0) and 10 mM sodium linoleate as a substrate. Lipoxygenases II and III were analysed collectively with the reaction mixture consisting of crude extract as enzyme source, phosphate buffer (0.2 M, pH 6.8) and 10 mM sodium linoleate as a substrate. The change in absorbance was recorded in a Shimadzu UV-160 spectrophotometer at 234 nm as the enzyme assay was based upon the increase of absorbance at 234 nm due to appearance of the conjugated diene in fatty acid hydroperoxides. One unit of enzyme was taken as equivalent to the amount of enzyme that generated an increase in absorbance of 1.0 per min at 234 nm. Values given in Table 2 are means of observations in triplicate samples.

2.3. Estimation of trypsin inhibitor activity

Powdered flour (0.2 g) obtained from dried seedlings, was extracted with 10 ml of 0.01 N of NaOH for 3 h with constant stirring at 125 rpm in an orbital shaker so as to keep the samples in suspension. The suspension so obtained was appropriately diluted so that 2 ml of the sample extract inhibited 40–60% of the trypsin used as a standard in the analysis. Trypsin inhibitor (TI) activity was determined by standard procedure (Kakade, Rackis, Mc gee, & Pushki, 1974) as modified by Hammerstrand, Black, and Glover (1981). Of the five test tubes taken, 2 ml aliquots of the diluted sample were placed in the four test tubes. A fifth test tube was prepared for the trypsin standard by adding 2 ml of distilled water. To three of the four test tubes containing the sample extract, 2 ml of of trypsin solution (prepared by dissolving 4 mg of the trypsin in 200 ml of 1 mM HCl) were added and these tubes were maintained in a constant temperature water bath at 37 °C for 10 min. Five millilitres of benzoyl DL-arginine para nitroanilide hydrochloride (prepared by dissolving 80 mg of benzoyl DL-arginine paranitroanilide hydrochloride in 2 ml of dimethyl sulfoxide and diluted to 200 ml with 50 mM tris buffer of pH 8.2, containing 20 mM calcium chloride and the contents were warmed to 37 °C) were rapidly added to each tube. The contents were stirred immediately in a vortex mixer and the tubes were placed in a water bath at 37 °C. The reaction was terminated after exactly 10 min by the rapid addition of 1 ml of 30% acetic acid. The fourth tube, containing sample extract (sample blank), was prepared by the same procedure, except that the trypsin solution was added after the reaction was terminated by the addition of 30% glacial acetic acid. A fifth test tube was prepared for the trypsin standard by adding 2 ml of distilled water. The absorbance of each solution was determined at 410 nm against the sample blank. Values obtained from each of the two sample extracts were subtracted from the trypsin standard. These values were averaged and the trypsin content was determined as follows:

$$\text{TI mg/of defatted sample} = \frac{\text{Differential Absorbance} \times \text{dilution factor}}{0.019 \times 1000},$$

$$\text{Percent Inhibition} = \frac{100 \times \text{differential absorbance}}{\text{Absorbance of the standard}}.$$

Values given in Table 2 are means of observations in triplicate samples.

2.4. Qualitative analysis of Kunitz inhibitor

Kunitz inhibitor, from germinated seedlings, was extracted in Tris–Cl buffer (100 mM, pH 6.8) containing 0.23 M $CaCl_2$ and 5 mM phenyl methyl sulfonyl fluoride

(PMSF), following Kollipara, Domagalski, and Hymowitz (1991) and was resolved using a non-denaturing discontinuous polyacrylamide slab gel consisting of 5% stacking gel (pH 6.8) and 10% resolving gel (pH 8.8) in a BioRad Vertical Electrophoresis system, as reported by Laemmli (1970). A trypsin inhibitor–trypsin complexing method (Mies & Hymowitz, 1973) was used to determine the electrophoretic band with trypsin inhibitor activity. Images were captured in a Gene Genius Bio-imaging system, of Syngene.

3. Results and discussion

Table 1 indicates the changes in biomass and percent moisture content during germination from 0 to 144 h in two genotypes at two temperatures. Fresh weight of seedlings increased continuously with germination period in both genotypes at both the germination temperatures. When compared to 0 h samples, maximum fresh weight observed in young seedlings during germination from 0 to144 h at 25 °C was 3.28 times for genotype JS80-21 and 3.53 times for genotype JS71-05. However, these values were observed to be higher for both the genotypes when germination was carried out at 35 °C (4.18 times for genotype JS 80–21 and 4.21 times for JS71-05). Moisture percentage increased in both genotypes at both the temperatures. It is evident from Table 1 that the higher fresh weight for seedling at 35 °C was mainly due to the higher moisture retention rather than to the dry matter.

Lipoxygenase (EC1.13.11.12) in soybean seeds is present in the form of three isozymes, namely Lx-I, Lx-II and Lx-III (Axelrod et al., 1981) which have been categorized into two classes. Class I is characterized by a high pH optimum of around 9.0 and produces large amounts of 13-hydroperoxides, such as Lx-I, while class II has a pH optimum of around 7.0 and gives equal amounts of 9 and 13-hydroperoxides, such as Lx-II and III. Table 2 lists the changes in the lipoxygenase isozymes in two genotypes during germination from 0 to 144 h at two temperatures. Lx-I, as well as Lx-II + III activities, decreased during germination from 0 to 144 h in both genotypes at both temperatures. Soaking for 24 h (when the mature seeds had imbibed completely) at 25 °C depressed Lx-I activity by about 13.6% for genotype JS80-21 and 14.2% for genotype JS71-05 while soaking for the same period at 35 °C resulted in higher percent decline in Lx-I activity (about 30.7% for genotype JS80-21 and 41.7% for genotype JS71-05). Lx-I activity in six day old seedlings developed at 25 °C declined by 83.1% for JS80-21 and 89.4% for JS71-05 when compared to the 0 h samples. Seedlings of the same age developed at 35 °C exhibited slightly higher percent declines in both genotypes (93.8% for genotype JS80-21 and 92.6% for genotype JS71-05) of Lx-I activity. With regard to changes in Lx-II + III activity, soaking for 24 h at 25 °C depressed the activity by about 28.7% for JS 80-21 and 9.5% for JS71-05. Soaking for 24 h at 35 °C did not result in a statistically higher percent decline for genotype JS80-21 when compared to soaking at 25 °C. However, soaking for a similar period at 35 °C resulted in a greater value (16.8%) decline for genotype JS71-05. Six day old seedlings, developed at 25 °C, exhibited a percent decline of 86.6% for variety JS80-21 and 71.2% for variety JS71-05. No significant differences for percent decline in Lx-II + III activity were observed in six day seedlings of JS 80-21 developed at 35 °C when compared to seedlings of the same period developed at 25 °C while a higher percent decline was observed for genotype JS71-05.

Table 1
Changes in seed/seedlings' biomass and percent moisture content during germination at different temperatures

Genotype	Stage of germination (h)	Germination temperature					
		25 °C			35 °C		
		FW[a] of 100 seeds/seedlings (g)	% Increase in FW of 100 seeds/seedlings	% Moisture	FW of 100 seeds/seedlings (g)	% Increase in FW of 100 seeds/seedlings	% Moisture
JS80-21							
	0	11.6	–		11.6	–	
	24[b]	21.46	84.6	45.14	21.1	82.1	52.50
	48	23.66	104.2	55.05	22.0	90.4	57.62
	72	29.23	152.4	66.76	31.6	172.0	68.61
	96	33.64	190.9	72.15	41.9	262.0	77.24
	120	35.03	202.4	76.80	44.4	282.5	82.03
	144	38.04	228.0	78.02	48.5	318.0	83.31
JS71-05							
	0	13.2			12.4		
	24[b]	21.31	61.5	42.6	24.42	85.6	55.91
	48	22.20	68.0	51.8	30.09	128.4	62.26
	72	32.20	144.3	67.5	32.60	146.8	67.63
	96	36.43	175.8	72.2	48.00	264.7	78.78
	120	43.56	230.7	76.8	52.66	298.5	81.64
	144	46.60	253.7	78.0	58.34	342.7	83.40

[a] FW-fresh weight.
[b] When the mature seeds had imbibed completely.

Table 2
Lipoxygenase isozymes[a] and trypsin inhibitor activity[b] in germinating soybeans at different temperatures

Variety	Stage of germination (h)	Lx-1		Lx-II + III		Trypsin inhibitor	
		25 °C	35 °C	25 °C	35 °C	25 °C	35 °C
JS80-21							
	0	1525		486		85.2	
	24[c]	1317 (13.6)	1057 (30.7)	347 (28.7)	359 (26.1)	80.7 (6.28)	74.0 (13.1)
	48	1193 (21.8)	700 (54.1)	293 (39.9)	285 (41.4)	73.6 (13.6)	62.8 (26.3)
	72	788 (48.3)	506 (66.8)	201 (58.6)	140 (71.3)	67.5 (20.8)	50.2 (41.1)
	96	583 (61.8)	300 (80.3)	154 (68.3)	109 (77.6)	61.4 (27.9)	49.8 (41.6)
	120	376.7 (75.3)	177 (88.4)	87.0 (82.1)	56.7 (85.3)	59.6 (30.1)	40.2 (52.1)
	144	258 (83.1)	94.6 (93.8)	65.1 (86.6)	53.7 (89.0)	46.3 (45.7)	32.3 (62.1)
Lsd ($p = 0.05$) JS71-05		35.4		13.9		6.4	
JS71-05							
	0	1666		285		90.6	
	24[c]	1429 (14.2)	976 (41.4)	258 (9.5)	237 (16.8)	83.4 (7.95)	79.1 (12.7)
	48	1236 (25.8)	655 (60.7)	225 (14.0)	186 (34.7)	71.2 (21.4)	59.3 (34.6)
	72	655 (60.7)	568 (65.9)	190 (33.3)	114 (60.0)	66.7 (26.4)	54.4 (39.7)
	96	373.2 (77.6)	362 (78.3)	122 (57.2)	108 (62.1)	60.8 (32.3)	46.0 (49.2)
	120	227 (86.4)	267 (84.0)	110 (61.4)	82.0 (71.2)	56.5 (37.6)	38.0 (58.1)
	144	177 (89.4)	123 (92.6)	82.0 (71.2)	54.0 (81.1)	48.7 (48.7)	28.6 (68.4)
Lsd ($p = 0.05$)		37.6		14.2		6.6	

[a] Units per gram of defatted ground seedlings as mean of triplicate samples, values given in parentheses are percent decline in activity.
[b] mg per gramme of defatted ground seedlings, values given in parentheses are percent declines in activity.
[c] When the mature seeds had imbibed completely.

About 80% of soybean TI activity is thought to be due to the protein SBTI-A_2 which is commonly known as Kunitz inhibitor. The changes in the electrophoretic banding patterns and specifically the Kunitz inhibitor (SBTI-A_2), protein bands as germination proceeded from 0 to 144 h at the two temperatures are depicted in Figs. 1 and 2. At both germination temperatures, band (R_f 0.75), corresponding to Kunitz inhibitor (the protein which constitutes about 80% of the trypsin inhibitor activity) marker protein appeared in both the genotypes from 24 h and were visible upto 144 h; though this band progressively decreased in intensity as germination progressed. A new band appeared at R_f 0.72 after 72 h at 25 °C and after 48 h at a germination temperature of 35 °C in both the genotypes. The new band progressively increased in intensity. The new band formed during germination was shown to possess a trypsin inhibiting ability, as confirmed by the trypsin inhibitor–trypsin complexing method (Lane No. 14 in Figs. 1 and 2) as the original as well as the new band disappeared when trypsin was added in the extract. The new band may be the degraded product of the Kunitz inhibitor during germination, which is supported by the early study indicating that protease K1 initiates the degradation of Kunitz soybean inhibitor during germination (Papastoitisis & Wilson, 1991) and the activity of enzyme has been reported to increase greatly during germination (Wilson, 1988). An early appearance of the new band at higher germination temperature may be because of the early expression of protease K1.

Table 2 also details the quantitative changes in trypsin inhibitor activity (TIA) in soybean seedlings during germination. Trypsin inhibitor declined continuously as germination proceeded upto 144 h in both genotypes at both germination temperatures. Soaking for 24 h at 25 °C decreased TIA by 6.28% for JS 80-21 and by 7.95% for JS71-05. It was notable that a much greater drop in TIA was observed on soaking for the same period at 35 °C (13.1% for JS80-21 and 12.7% for JS71-05). The germination period of 48 h at 25 °C depressed TI activity by 13.6% for JS 80-21 and 21.4% for variety JS71-05 while seedlings developed at 35 °C exhibited a drop of 26.3% for JS80-21 and 34.6% for JS71-05. This may be attributed to an early appearance of a dense band that was confirmed to be a modified product of Kunitz inhibitor possessing trypsin inhibitor activity in both genotypes at 35 °C. At a germination temperature of 25 °C, six day old seedlings exhibited a decline of 45.7% for genotype JS80-21 and 48.7% for JS71-05 when compared to 0 h samples; however, six day old seedlings, developed at 35 °C, exhibited greater declines in TIA in both genotypes (62.1% for JS80-21 and 62.5% for JS71-05) when compared to 0 h samples.

With regard to lipoxygenase isozyme activity, both genotypes exhibited a decrease for Lx-I as well as Lx-II + III during germination at both temperatures when samples at 0 and 144 h were compared. As germination proceeded from 0 to 144 h, declines of Lx-I and Lx-II + III activities were faster at higher temperature throughout except in the case of JS71-05 wherein the rate of decline in Lx-I activity was faster upto 72 h at 35 °C. These results could not be compared, as such, with earlier works because of the absence of any similar report. Bordingnon et al. (1995) reported a decrease in Lx-I activity during germination, though authors did not report changes in Lx II + III activity. Bau et al. (2000) also reported reduction in lipoxy-

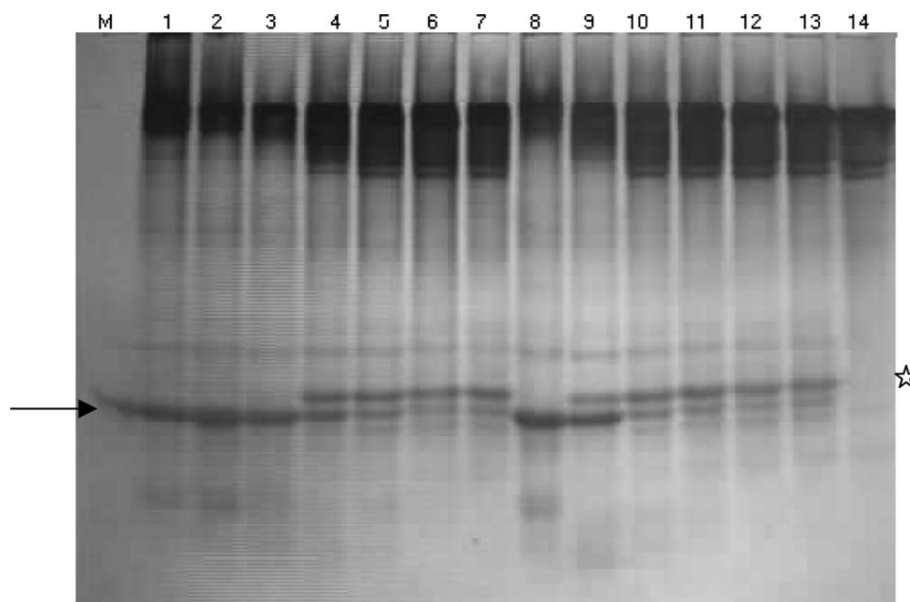

Fig. 1. Polyacrylamide gel of seedlings of genotype JS 80-21 showing Kunitz inhibitor (SBTI-A_2). Lane M depicts marker protein for Kunitz inhibitor. Lanes 1–7 (from left to right) depict mature seed, 24, 48, 72, 96, 120 and 144 h' seedlings germinated at 25 °C while lanes 8–13 depict protein for 24, 48, 72, 96, 120 and 144 h' seedlings developed at 35 °C. Lane 14 depicts disappearance of original ($R_f = 0.75$) and new band ($R_f = 0.72$) by trypsin inhibitor + trypsin complexing method. Arrow points to original SBTI-A_2 ($R_f = 0.75$) while star indicates the new SBTI-A_2 band ($R_f = 0.72$).

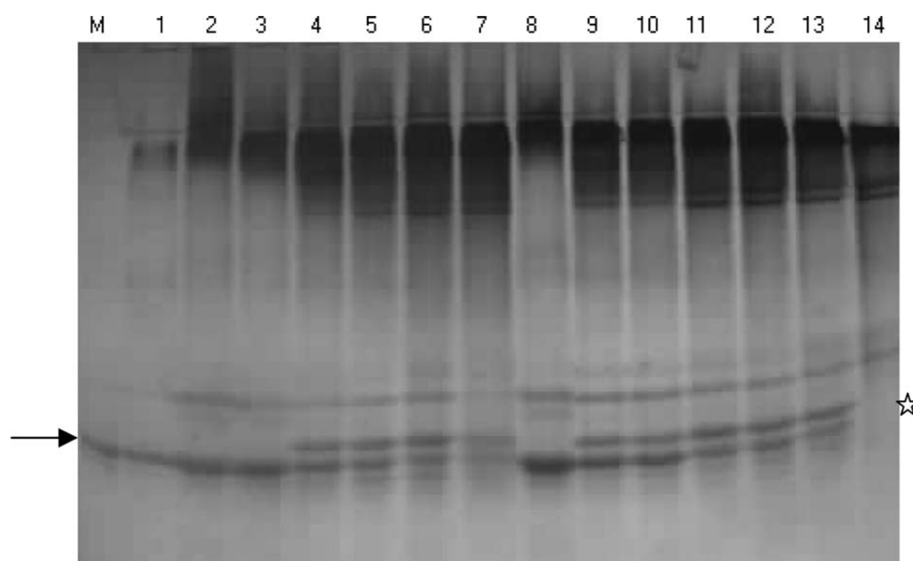

Fig. 2. Polyacrylamide gel of seedlings of variety JS71-05 showing SBTI-A_2. Lane M depicts marker protein for Kunitz inhibitor (SBTI-A_2). Lanes 1–7 (from left to right) depict mature seed, 24, 48, 72, 96, 120 and 144 h' seedlings germinated at 25 °C while lanes 8–13 depict protein for 24, 48,72, 96, 120 and 144 h' seedlings developed at 35 °C. Lane 14 depicts disappearance of original ($R_f = 0.75$) and new band ($R_f = 0.72$) by trypsin inhibitor + trypsin complexing method. Arrow points to original Kunitz inhibitor ($R_f = 0.75$) while star indicates the new Kunitz inhibitor band ($R_f = 0.72$).

genase activity after a short period of germination (72 h), though activities for both classes of lipoxygenases were not studied. Decline in lipoxygenase isozyme activities during germination at both temperatures may be attributed to their utilization in lipid mobilization during germination. A decline in TIA observed in both genotypes as germination proceeded from 0 to 144 h at 25 °C is in consonance and contradiction to some earlier works. Collins and Sanders (1976) reported varietal differences for changes in trypsin inhibitor activity during germination. They reported that, after 3 days of germination, trypsin inhibitor activity decreased in soybean (12.3% for variety Kanrich or 8% for variety Dare) whereas a third variety did not show any apparent changes. Mostafa et al. (1987) reported a decline (by 32%) in TIA in six day-germinated soybean seedlings when the germination was carried out at room temperature (23–25 °C) in the absence of light while Bates et al. (1977) reported a decline in one third of the original value after 4 days of germination carried out at room temperature. In view of the unavailability of reports in which germina-

tion was carried out at 35 °C, the results could not be compared. Furthermore, declines in TI activity observed in our study during germination are in contrast to the investigations carried out in other legumes. Recently, trypsin inhibitor content has been reported to increase during germination in two varieties of *Vicia faba* while it remained unchanged in *Cicer arietinum* (Muzquiz et al., 2004). Weder and Link (1993) observed that sprouting for 72 h did not alter the TIA in lentils while Vider-Valvarde et al. (1994) reported a decrease in TIA in six day-old seedlings. It is evident from our results that both the genotypes exhibited a faster and higher decline of trypsin inhibitor during germination at 35 °C as compared to germination carried out at 25 °C. This is also confirmed by the early appearance of degraded product of (Kunitz inhibitor) in both genotypes at higher temperature (Figs. 1 and 2).

Conclusively, our studies suggest that lipoxygenase isozymes and Kunitz inhibitor are degraded continuously from 0 to 144 h at both germination temperatures; and degradation is faster at higher germination temperature (35 °C). It is suggested that the sprouts developed at higher temperature (35 °C) may possess better nutritional quality and improved flavour; this will be true for other soy products prepared from soybean seedlings developed at higher temperatures.

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