Journal of Cell and Tissue Research Vol. 8 (1) 1333-1338 (2008)
ISSN: 0973- 0028 *(Available online at www.tcrjournals.com)* **Original Article**

ASSESSMENT OF ANTINUTRITIONAL FACTORS AND PROTEIN CONTENT IN THE SEEDS OF CHICKPEA CULTIVARS AND THEIR DIVERGENCE

BHAGYAWANT, S. S.? AND SRIVASTAVA, N.

School of Studies in Biotechnology, Jiwaji University, Gwalior 474 011, India.
E. mail: bhagyawant123@rediffmail.com

Received: November 29, 2007; Revised: December 10, Accepted: December 13, 2007

Abstract: Legume grains constitute the primary source of diet protein for the population of many countries including India. However, these legume seeds are also known to contain factors like protease and amylase inhibitors as well as polyphenols etc. which are nutritionally antagonistic. These may reduce the availability of otherwise good proteins in the diet and cause diseases originating from malnutrition. However, presently protein antimetabolites are getting much attention due to their role in defense against insects or microbes. Biochemical characterization of these antinutritional factors in chickpea seed cultivars is attempted in the present investigation.

Key words: Protease inhibitors, Lectins, Chickpea seed

INTRODUCTION

Food legumes constitute an important part of human diet which source dietary proteins for populations in the Indian subcontinent and in many other developing countries. Most of the legume seeds are known to synthesize certain biologically active substances. Being considered as antinutritional substances, they devalue the nutritive importance of legumes when ingested by humans or animals [1]. Several such substances in grain legumes have been reported [2]. Antinutritional factors may be either heat-labile comprising lectins, protease inhibitors and cyanogens or heat stable including, among many others, antigenic proteins, condensed tannins and quinolizidine alkaloids etc. Most commonly observed antinutritional factors in grain legumes reported include phytolectins, polyphenols (tannins) and certain inhibiting proteins [3,4].

Protease inhibitors (PIs) in legumes such as winged bean, pigeonpea and cowpea have been extensively studied. These are prominently localized in storage tissues such as seeds and tubers. Both chickpea and pigeonpea contain considerably higher levels of PIs than other commonly consumed Indian edible legumes, however, much lower than those of soybeans. Protease inhibitors in chickpea seeds are considered as antinutritional factors. It is also known that chickpea seeds contain inhibitors of proteases [5-8].

In addition to PIs, lectins or phyto-hemagglutinins also accumulate in legumes. These are proteins, which agglutinate red blood cells by virtue of their specificity for glycoprotein receptor sites on the cell surface. Their antinutritional activity is because of either interference with the digestive enzymes and reducing the availability of nutrients or by disturbing the epithelial cells there by hampering nutrient transport into the cell. These being toxic for human consumption can be completely degraded by moist heat treatment [1]. Lectins however, are synthesized by plants for their defense and signal recognition for symbiotic nitrogen fixing bacteria [9].

Polyphenols, otherwise known as tannins and lignins are water-soluble phenolic compounds with a molecular weight greater than 500 daltons. They occur almost in all vascular plants. Hydrolysable

tannins and condensed tannins (proanto cyanidins) are two different groups of these compounds and differ in their nutritional and toxic effects. Generally tree and shrub leaves contain both types of these tannins. Condensed tannins have a more profound digestibility reducing effect than hydrolysable tannins and are reported to occur in some grain legumes that are already used as food and fodder [10].

Tannins are also known to inhibit enzymatic activity [11,12] thus may be possibly affecting protein digestion. Furthermore, tannins may react with amino acids thus decreasing their biological availability and also hinder mineral adsorption [13]. The heat-labile condensed tannins known to cause deleterious effects in humans and animals, can be removed by various domestic treatments like soaking, sprouting, cooking and roasting [14,15]. The present paper describes the screening and quantification of the antinutritional factors in the chickpea cultivars.

MATERIALS AND METHODS

Chemicals: Trypsin, BAPNA, phosphric acid was obtained from Sigma chemical company St. Loius, (USA). Human blood was graciously provided by the dispensary of National Chemical Laboratory, Pune. All other chemicals were of analytical grade.

Seed material: Total twenty-one chickpea (*Cicer arietinum* L.) cultivars (Table 1) were selected for analysis. The mature and dry seed material was obtained from Agricultural Research Station, Badnapur, Maharashtra.

Quantification of Trypsin Inhibitors: For protein extraction seeds of each cultivar were soaked in 1 ml of distilled water for overnight. Seed coats were removed and half cotyledon were weighed. To the tubes 2.5mM HCl was added in 10 multiple of cotyledonary weight. Seeds were macerated with metal rod and vertexed for 30 min. at 4 ^{0}C to remove any particulate matter. The clear supernatant was used as source for inhibitor studies.

Enzyme inhibitor assay: The inhibitor content was measured using BAPNA as a substrate [16]. For measuring trypsin inhibitory activity 10 µg of trypsin was mixed with suitable quantity of the sample (to get 50-60% inhibition) and incubated at 25 ^{0}C before measuring the residual trypsin activity. 10 µl of seed extract was mixed with 80 µl of 50 mM Tris-HCl buffer, pH 8.2, containing 20 mM $CaCl_2$, 10 µl of trypsin (in 1 mM HCl) and incubated at room temperature at 30 sec interval between two wells on a microtitre plate. The residual activity was measured by adding 125 µl of BAPNA (40 mg/ml dimethyl sulfoxide, freshly diluted 1:100 in 50 mM Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$ pre-warmed to 37 ^{0}C) and then incubated at room temperature for 30 mins. Reactions were stopped by the addition of 25µl of 3% (v/v) acetic acid. Liberated p-nitroanilide was measured at 410 nm. 100% trypsin activity was measured from the sample minus the inhibitor extract. One unit of trypsin activity was defined as the amount of enzyme which increases the optical density by one unit at 410 nm due to the release of p-nitroaniline. Further one TI unit was defined as the amount of inhibitor that inhibited 1 unit of trypsin activity [17].

Table 1: Details of *Cicer arietinum* L. germplasm used for ANFs – studies

Sr. No.	Cultivar / line	Type	Agronomic characters
1	BCP-15	Desi	Erect plant type, bold seeded, temperature tolerant
2	BCP-17	Desi	Angular bold seeded, wilt resistant
3	BCP-28	Kabuli	White flowered, smooth seeded, spreading type
4	BCP-48	Desi	Wrinkled seed, erect plant type
5	BCP-51	Desi	Small seeded
6	BCP-54	Desi	Medium seeded, erect plant type, low yield
7	BCP-73	Desi	White flowered, yellow seeded, semi erect
8	BCP-91	Desi	Early maturity, drought tolerant
9	BCP-201	Kabuli	Smooth seeded, high yield
10	BDN 9-3	Desi	Early maturity, twin podded, high yield
11	Vijay	Desi	Rain-fed as well irrigated, late sown
12	Vishal	Desi	Bold seeded, high yield
13	JG-62	Desi	Small seeded, twin podded
14	ICCV-2	Kabuli	Spreading plant type, early maturity
15	BDNG-787	Desi	Bold seeded, moderately wilt sensitive
16	BDNG 57-3	Desi	Spreading plant type, salt tolerant
17	KAK-2	Kabuli	Extra bold grain
18	Virat	Desi	White flowered, superior grain quality
19	Green Chafa	Desi	Green seed coat
20	Jaki – 9218	Kabuli	Semi spreading, rain-fed as well as irrigated, bold seeded
21	AKG 46	Desi	High yield, superior grain quality

Quantification of Tannins: Dry seeds were used as a source for tannin extraction. 400 mg of finely powdered defatted meal was mixed with 40 ml of water. The suspension was boiled for 30 mins. cooled, and subsequently centrifuged at 2,000 rpm for 10 mins. After extraction, 1 ml of the above clear supernatant was pipetted into duplicate test tubes. Five milliliter of Folin-Denis reagent, 10 ml of sodium carbonate solution, was diluted to 100 ml with water. Color thus developed was read at 700 nm after 30 mins with Schemadzu UV-Vis spectrophotometer. Tannins were estimated as tannic acid equivalents [18].

Lectin isolation: Lectin in the seeds was isolated using procedure as described by Gurjar et al. [19]. The dry matured seeds were finely ground in Warring blender. Seed meal (50 g), was added to 250 ml of Tris-HCl extraction buffer (20 mM Tris-HCl pH 7.2, containing 150 mM NaCl). The suspension was agitated for 12 h at 4 ^{0}C in cold and filtered through muslin cloth. The filtrate was subsequently centrifuged at 10,000 rpm for 20 min. at 4 ^{0}C. The clear supernatant was saved and used in hemagglutination assay.

The supernatant obtained in previous steps was maintained to the acidic as well as basic conditions to study the lectin behavior under different pH conditions. pH 4.5 of the filtrate was adjusted by gradual addition of 1M acetic acid to remove fats, re-centrifuged at 10,000 rpm for 15 mins in cold. The precipitate was discarded and pH of supernatant was adjusted to 8.0 with 1M NaOH (pH 7.0). The clear supernatant obtained after centrifugation was saved separately. The hemagglutination tests were thus performed using normal, acidic (pH 4.5) and basic (pH 8.0) conditions respectively.

Hemagglutination assay: The titre assay was initially performed using normal and trypsinised human erythrocytes (A, B, AB, and O) and thereafter with pronase treated rabbit erythrocytes. Fresh rabbit erythrocytes were separated from plasma by centrifugation at 3000 rpm for 4 minutes at 5-10 ºC and washed extensively with 10mM Tris-HCl buffer, pH 7.2, containing 150 mM NaCl (TBS). Finally, 3% suspension was prepared in TBS and used in hemagglutination assays. Hemagglutination tests were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocyte suspension was mixed with 50 µl of serially diluted lectin. Agglutination assay was examined visually after incubation for one hour at room temperature. Lectin free sample was used as a control. The unit of hemagglutination activity (U) termed as titre was expressed as the reciprocal of the highest dilution of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the titre of hemagglutination per mg of protein.

Preparation of pronase treated erythrocytes: Pronase treated erythrocytes for the hemagglutination assay were prepared by the method of Lis and Sharon [20]. Fresh rabbit erythrocytes were centrifuged at 2000 rpm for 10 minutes. The serum was removed and the erythrocytes were repeatedly washed with TBS. The RBC suspension (3%) was incubated with 0.05% (w/v) pronase at 37 ºC for 1 h. After incubation erythrocytes were repeatedly washed with TBS to remove pronase and finally suspended in TBS at a concentration of 3% and used for the hemagglutination assay.

Protein estimation: Protein content in the extracts was estimated by the Folin-phenol method [21] using BSA as a standard. Specific activities were expressed as Units/mg of protein.

RESULTS

Content and quality of protein are major concerned from the nutritional point of view in chickpea. Wide variations in concentration levels of various ANFs under consideration, for 21 cultivars of chickpea are seen when screened individually (Fig.1). The highest trypsin inhibitor activity was observed in BDN 9-3 (48.90 TIU/mg) while the lowest was in BCP-15 (7.10 TIU/mg) variety. A considerable difference was observed in the half seed weight of the cultivars when used as a source for measuring trypsin activity, thus showing no correlation between seed weight and TIU (Table 2). On an average, the half seed depicted a variation of 17.5%, the average optical density being 0.33 ± 0.11 accounting for inhibited trypsin units of 3.19 ± 1.08. The variation in half seed weight was

Table 2: Distribution of trypsin inhibitor content in the seeds of chickpea cultivars

Sr. No.	Cultivar/ line	Half seed wt. in mg	Mean O. D.	Units inhibited	TIU/mg
1	BCP-15	130	0.582	0.71	7.10
2	BCP-17	131	0.458	1.95	19.50
3	BCP-28	104	0.395	2.58	25.80
4	BCP-48	95	0.305	3.48	34.80
5	BCP-51	101	0.215	4.38	43.80
6	BCP-54	96	0.234	4.19	41.90
7	BCP-73	108	0.249	4.04	40.40
8	BCP-91	114	0.369	2.84	28.40
9	BCP-201	98	0.316	3.37	33.70
10	BDN 9-3	99	0.164	4.89	48.90
11	Vijay	98	0.230	4.23	42.30
12	Vishal	151	0.490	1.63	16.30
13	JG-62	91	0.445	2.08	20.80
14	ICCV-2	137	0.235	4.18	41.80
15	BDNG-787	145	0.303	3.5	35.00
16	BDNG 57-3	122	0.410	2.43	24.30
17	KAK-2	150	0.321	3.32	30.20
18	Virat	148	0.382	2.71	27.10
19	Green chafa	109	0.405	2.48	24.80
20	Jaki - 9218	142	0.275	3.78	37.80
21	AKG 46	130	0.215	4.38	43.80
	Mean	119	.033	3.19	31.83
	S.D.	20.87	0.10	1.07	10.78
	C.V.	17.54	32.37	33.72	33.87

Table 3: Relationship between seed coat color and tannin contents in chickpea

Sr. No.	Cultivar / line	Type	Seed coat color	Tannins (mg/g)
1	BCP-15	Desi	Light brown	3.0
2	BCP-17	Desi	Brown	0.75
3	BCP-28	Kabuli	Salmon white	2.25
4	BCP-48	Desi	Brown	2.25
5	BCP-51	Desi	Light brown	7.5
6	BCP-54	Desi	Light brown	1.5
7	BCP-73	Desi	Golden yellow	2.25
8	BCP-91	Desi	Dark brown	15.0
9	BCP-201	Kabuli	Salmon white	4.5
10	BDN 9-3	Desi	Brown	3.0
11	Vijay	Desi	Brown	4.5
12	Vishal	Desi	Brown	3.0
13	JG-62	Desi	Brown	4.5
14	ICCV-2	Kabuli	Salmon white	9.0
15	BDNG-787	Desi	Light brown	1.5
16	BDNG 57-3	Desi	Light brown	3.0
17	KAK-2	Kabuli	Cream white	4.5
18	Virat	Desi	Cream white	5.25
19	Green Chafa	Desi	Green	5.25
20	Jaki - 9218	Kabuli	Brown	6.0
21	AKG 46	Desi	Dark brown	20.25
	Mean			4.32
	S.D.			3.189
	C.V.			73.82

Table 4: Screening of chickpea cultivars for hemagglutination levels

Sr. No.	Cultivar / line	Protein conc.in mg/ml	Titre assay in wells	Units	Units/ml	Units/mg
1	BCP-15	9.2	4	32	25600	2782
2	BCP-17	14.2	6	128	102400	7111
3	BCP-28	8.4	5	64	51200	6095
4	BCP-48	6.4	5	64	51200	8000
5	BCP-51	3.4	4 1/2	48	38400	11294
6	BCP-54	7.2	4 1/2	48	38400	5333
7	BCP-73	7.2	4	32	25600	3555
8	BCP-91	2.8	3	16	12800	4571
9	BCP-201	2.7	3	16	12800	4740
10	BDN 9-3	6.8	5	64	51200	7529
11	Vijay	3.0	2 1/2	12	9600	3200
12	Vishal	3.2	3 1/2	24	19200	6000
13	JG-62	8.0	5	64	51200	6400
14	ICCV-2	11.2	4	32	25600	2285
15	BDNG-787	7.6	3	16	12800	1684
16	BDNG-57-3	4.8	3 1/2	24	19200	4000
17	KAK-2	9.2	4	32	25600	2782
18	Virat	10.8	6	128	102400	9481
19	Green chafa	12.0	5 1/2	96	76800	6400
20	Jaki - 9218	8.0	2	8	6400	800
21	AKG 46	14.4	3 1/2	24	19200	1333
	Mean	7.65	4.11	46.28	37028.57	5017.85
	S.D.	3.58	1.10	35.20	28163.11	2753.56
	C.V.	46.80	26.84	76.05	76.05	54.87

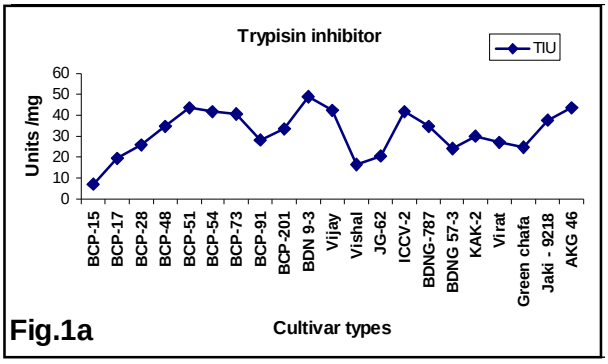

Fig.1a

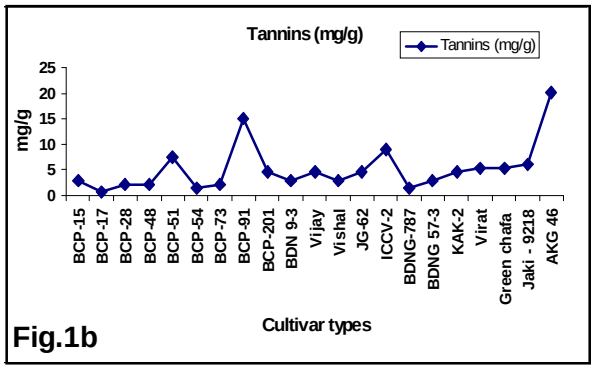

Fig.1b

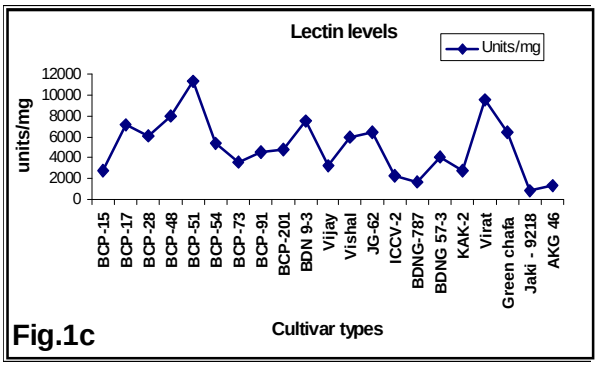

Fig.1c

Figs. 1a,b,c represent trypsin inhibitor (Fig. 1a), Tannins (Fig.1b) and lectin levels (Fig. 1c) in chickpea (*Cicer arietinum* L.) cultvars

less (C.V. = 17.54 %) while that for other parameters like units inhibited and trypsin inhibitor units per mg, the C.V. observed was more than 33% (Table 2).

A large variation in tannin content between different cultivars was observed (Table 3). The lowest tannic acid was observed in BCP-17 cultivar whereas the highest tannin concentration was estimated in AKG-46 variety. On an average, tannin content was 4.3 ± 3.1 with 73.8% variation. The cultivars with dark brown testas showed highest tannin content (Table 3). The results obtained are summarized in Table 4. Neither human erythrocytes of the blood group A,B, AB and O nor rabbit erythrocytes, but for the pronase treated rabbit erythrocytes were agglutinated by the lectin. Considerable variation was shown in hemagglutination activity by different cultivars of chickpea. Protein content varied lowest of 2.7 mg/ml in BCP-201 to highest of 14.2 mg/ml in BCP-17 variety. On an average, the protein concentration in crude extract was 7.6 ± 3.5 mg/ml with a 46.8 %

variation. A significant variation was found in the units of hemagglutination wherein the minimum of 8 units were detected in Jaki-9218 cultivar which gave 2 well titre and the maximum 128 units were observed in BCP-17 and Virat cultivar which gave 6 well titres. An average of 46 ± 3.5 units of lectin was observed in chickpea (Table 4).

Of the 21-desi and kabuli type cultivars screened, the kabuli type exhibited lower lectin levels in comparison with those of the desi types. The highest 11294 µ/mg level of lectin was found in BCP-51, whiles the lowest 800 µ/mg hemagglutination activity was depicted in JAKI-9218. However, the mean lectin values were 5017.85 µ/mg with a deviation of 2753.56 µ/mg for all the 21 cultivars studied showing 54.87 % of the variation.

DISCUSSION

Chickpea is consumed in various forms and chickpea containing lower levels of ANFs are nutritionally preferred. Quantification of these factors is one of the requirements for breeding programmes to aim at the development of cultivars suitable for food industry. This information therefore, can be useful for germplasm selection. The objective of quality evaluation programmes is to identify the germplasms with superior quality for inclusion and also for the exclusion of germplasms of inferior quality. In the present study seeds of chickpea cultivars contain considerable amount of trypsin inhibitor, tannins and lectin. These may contribute functionally as insect repellents or defense against predators but are harmful for human consumptions in the raw state. Heat treatments can completely destroy these factors [22]. Smirnoff et al. [23] studied the trypsin inhibitor and hemagglutination activities in chickpea (*Cicer arietinum* L.) and the effects of heat on germination. They found a complete elimination of lectin and trypsin inhibitors by heating chickpea seeds in boiling water for 10 min. and also on germination of seeds for 8 days. They further noted that the overnight soaking of seeds in water could remove as much as 23 % trypsin inhibitor and 50 % lectin levels.

Variations in the content of trypsin inhibitor in pulses and of different chickpea cultivars as seen in the present study have also been reported [24] and being attributed to the lower concentration of an inhibitor fraction or to the lower affinity (weaker inhibition) towards bovine trypsin [25]. Roy and Bhat [26] correlated the variation in quantity of trypsin inhibitor to variation in extractability of proteins having trypsin inhibitor activity. ELISA screening revealed considerable variations in the Bowman-Birk inhibitor (BBI) content of accessions of the genus *Glycine*. However, under identical assay conditions, trypsin inhibitor activity was observed to be in decreasing order for soybean, field bean, pea, lentil, pigeonpea and chickpea. This however, needs confirmation in chickpea through further studies.

Many workers have assessed the qualitative variations in protenase inhibitors based on electrophoresis. Kollipara and Hymowitz [25] noticed variation among the wild perennial species in the electrophoretic profiles of trypsin and chymotrypsin inhibitors. They found that the variation in trypsin and chymotrypsin inhibitor banding pattern could be useful in genetic studies of the protease inhibitor and the biosystematic studies of the genus *Glycine*. McNiven et al. [27] characterized a low trypsin inhibitor soybean cultivar by electrophoresis and enzyme activity measurement and demonstrated that shorter heating times were required to inactivate both trypsin and chymotrypsin inhibitor activity in low trypsin inhibitor cultivar compared to control.

Chavan et al. [28] studied the tannin content in seeds of chickpea (whole seed) and cotyledons. As revealed in the present study, the marked variations in the tannin content of chickpea has also been reported in other studies. Singh [29] studied the inhibition of digestive enzymes by polyphenols of chickpea (*Cicer arietinum* L.) and pigeonpea (*Cajanus cajan* L.). They observed that chickpea seeds (whole seed) contained 78 to 272 mg per 100 g tannins, while the cotyledons have only 16 to 38 mg per 100 g of the seed which were located mainly in the seed coat. The polyphenols in cultivars containing darker testa color inhibited the digestive enzyme activity more than cultivars with lighter color testa [30]. These compound also impart astringent flavors, which are not always desirable, as such chickpea seeds with light color of chickpea are preferred for whole seed consumption. In the present investigation hemagglutination activities were observed using pronase treated rabbit erythrocytes. Reports of chickpea producing a certain amount of agglutinating activity with cow erythrocytes are reported. This contradicted earlier observation that no hemagglutination factor was present in chickpea. Our report, therefore, also contradics the study of

Liener [31]. Hemagglutinins are highly sensitive to heat treatment, and the greatest reduction of agglutination can be obtained with moist heat at 100 ^{0}C [1].

Though lectins cause adverse physiological effects in humans, they play an important role against insect pests of agricultural crops [32]. In similar studies Gurjar et al. [33] reported the growth inhibition and total loss of reproduction potential in *Tribolium castaneum* by *Artocarpus hirsuta* lectin. They also found that, the post embryonic development was affected to a significant level when freshly hatched larvae of *T. castaneum* were fed with the lectin treated diet at 0.5 % concentration.

From overall data it is concluded that chickpea seed contains trypsin inhibitor, tannin and lectin at significant levels and can have similar activities as discussed above. It is hoped that the present study of quantification of antinutritional factors can extend guidelines in developing controlled breeding program to develop the cultivars with high seed protein contents as well as selecting and release of such varieties which are appropriate for human consumption.

ACKNOLEDGEMENTS

The authors are thankful to Dr. M.I. Khan, Scientist-F and Dr. Sushma M. Gaikwad, Scientist, Biochemical Sciences Division, National Chemical Laboratory, Pune for providing research facilities and support throug out the work. The authors are also grateful to Prof. K.K. Koul, Head, School of studies in Botany, Jiwaji University, Gwalior for critically evaluating the manuscript.

REFERENCES

[1] Liener, I.E.: J. Am. Oil Chemists Society., 56: 121-128 (1979a).
[2] Chrispeels, M.L.: Phil. Trans. R. Soc. Lond., B304:309-322 (1984).
[3] Singleton, V.L.: Adv. Food Res., 27: 149-242 (1981).
[4] Ryan. C.A.: Annu. Rev. Phytopathol., 28: 425-449 (1990).
[5] Liener, I.E.: J. Food Sci., 41: 1076-1081 (1976).
[6] Kadam, S.S., Smithard, R.R., Eyre, M.D and Armstrong, D.G.: J. Sci. Food Agric., 39: 267-275 (1987).
[7] Sing, U. and Jambunathan, R.: J. Food Sci., 46: 1364-1367 (1981).
[8] Price, M.L., Hagerman, A.E. and Butler, L.G.: J. Agric. Food Chem., 5: 947-952 (1980).
[9] Sharon, N.: Trends Biochem. Sci., 18: 221-226 (1993).
[10] Kumar, R. and Vaithiyanathan, S.: Animal Feed Science and Technology., 30: 21-38 (1990).
[11] Griffiths, D.W.: J. Sci. Food Agric.,30: 458-462 (1979).
[12] Singh, U.: Nutr. Rep. Int., 29: 745-753 (1984).
[13] Roy, S.N.: Ind. J. Biochem. Biophy., 16: 151-159 (1979).
[14] Ologhobo, A.D. and Fetuga, B.L.: J.Food Sci., 49: 199-201 (1984).
[15] Khokar, S. and Chauhan, B.M.: J. Food Sci., 51: 591-594 (1986).
[16] Erlanger, B.F., Kokowsky, N. and Cohen, W.: Arch. Biochem. Biophys., 95: 271-278 (1961).
[17] Kakade, M.L., Rackis, J.J., McGhee, J.E. and Puski, G.: Cereal Chem., 51: 376-382 (1974).
[18] Swain, U. and Hillis, W.E.: J. Sci. Food Agric., 10: 63-68 (1959).
[19] Gurjar, M. M., Khan. M.I. and Gaikwad., S. M.: Biochem. Biophys. Acta, 1381: 256-264 (1998).
[20] Lis, H. and Sharon, N.: Methods in Enzymol., 138:544-551 (1987).
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: J. Biol. Chem., 193: 265-275 (1951).
[22] Shemer, M. and Perkins, E.G.: J. Agric. Food Chem., 23: 201-205 (1975).
[23] Smirnoff, P., Khalef, S., Birk, Y and Applebaum, S.: Biochem. J., 157: 745-751 (1996).
[24] Harsulkar, A., Giri, A., Patankar, A., Gupta, V., Sainani, M., Ranjekar, P. . and Deshpande, V.: Plant Physilogy., 121: 497-506 (1999).
[25] Kollipara, K.P. and Hymowitz, T. : J. Agri. and Food Chem., 40: 2356-2363 (1992).
[26] Roy, D.N. and Bhat, R.V.: J. Sci. Food Agric., 25: 765-769 (1974).
[27] McNiven, M.M., Grimmelt, B., MacLeod, J.A. and Voldeng, H. : J. Food Sci. 57: 1375-1377 (1992).
[28] Chavan, J.K., Kadam, S.S. and Salunkhe, D.K.: Critical Rev. Food Sci. Nutri., 25: 107-158 (1986).
[29] Singh, U.: Nutrition Reports Intern. 29(3): 745-753 (1984).
[30] Price, M.L., Hagerman, A.L. and Butler, L.G.: J. Agric. Food. Chem. 28: 459-461 (1980).
[31] Liener, I.E: J. Food Sci., 41: 1076-1081 (1976).
[32] Ignacimuthu, S: Indian. J. Exp .Biol.,37: 215-222 (1999).
[33] Gurjar, M.M., Gaikwad, S.M., Salokhe, S.G., Mukherjee, S. and Khan, M.I: Mol. Biol. Int., 38: 95-98 (2000).