# GRAIN QUALITY AND BIOCHEMISTRY

## Chickpea and Pigeonpea

## Antinutritional Factors

Prepared by:

Jmaid Singh

ICRISAT
International Crops Research Institute for the Semi-Arid Tropics
ICRISAT Patancheru P.O.
Andhra Pradesh 502 324, India

# GRAIN QUALITY AND BIOCHEMISTRY

## CHICKPEA AND PIGEONPEA

## 3. Antinutritional factors

Prepared by:

Umaid Singh

**International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)**

**Patancheru P.O., Andhra Pradesh 502 324, India.**

# FOREWORD

This is the third report of work of ICRISAT Grain Quality and Biochemistry Support Program. In this report, the work on antinutritional factors and *in vitro* protein digestibility of chickpea and pigeonpea has been described. Also, the effects of processing practices on the levels of these factors are reported. The work has been carried out during 1977-1982. In addition to this report, results on these aspects have appeared in the ICRISAT ANNUAL REPORTS. Our program has closely collaborated with Genetic Resources Unit, Pigeonpea Breeding, Chickpea Breeding and Pulse Physiology at ICRISAT and their contribution and assistance are gratefully acknowledged.

I sincerely thank Dr. R. Jambunathan for his comments on the earlier draft of this report.

This is not a formal publication of the Institute and should not be cited.

***

## Staff*

| | | |
|---|---|---|
| Dr. R. Jambunathan | .. | Principal Biochemist |
| Dr. Umaid Singh | .. | Biochemist |
| Mr. M.S. Kherdekar | .. | Research Associate II |
| Mr. N. Subramanyam | .. | Research Associate II |
| Mr. G.L. Waghray | .. | Research Associate I |
| Mr. G. Venkateswarlu | .. | Laboratory Assistant |
| Mr. B. Hanmanth Rao | .. | Laboratory Assistant |
| Mr. B.V.R. Sastry | .. | Stenographer |
| Mr. T.S. Noel Prashanth | .. | Clerk/Typist |

* Only those staff who were directly involved or contributed to the work reported in this report.

CONTENTS

# LIST OF TABLES

LIST OF FIGURES

## SUMMARY

Of the several antinutritional factors of food grain legumes, protease inhibitors (trypsin and chymotrypsin inhibitors), amylase inhibitors, flatulence causing sugars (oligosaccharides), polyphenols and phytic acid of chickpea and pigeonpea seeds have been studied in our laboratory and the results are summarised in this report.

The levels of trypsin inhibitor activity (TIA) were higher in both kabuli and desi seeds of chickpea than their chymotrypsin inhibitor activity (CIA). Mean values for the trypsin and chymotrypsin inhibitor units in dhal and seed samples of desi were higher as compared with kabuli cultivars. In case of pigeonpea, TIA values were generally higher in the wild species as compared with the cultivated species. But a clearcut difference in the CIA was observed between the wild and cultivated species. The highest TIA and CIA were observed in *Rhynchosia rothi*, only exception was *Atylosia cajanifolia* in which the inhibitor activities were similar to those in the cultivated-species. TIA was more in mature seed whereas green and mature seed differed little in CIA indicating that trypsin inhibitors accumulate with maturity.

The pressure-cooking at 15 lb for 15 min resulted in the reduction of TIA to the extent of about 80 and 90% in chickpea and pigeonpea, respectively. CIA was destroyed to a smaller extent than TIA as a result of heat treatment in both the crops. Open-vessel cooking was observed to be less effective in destroying TIA and CIA in comparison with the pressure-cooking in both chickpea and pigeonpea. Soaking of chickpea and pigeonpea in distilled water up to 12 hr brought about 20% reduction in TIA whereas as CIA changed very little as a result of soaking.

The *in-vitro* protein digestibility (IVPD) studies showed larger differences between desi seed and dhal samples when compared with kabuli seed and dhal samples. IVPD was negatively correlated, although low, with TIA and CIA in chickpea. Small variation in IVPD was observed in uncooked samples of several cultivars of chickpea and pigeonpea whereas cooked samples of the same cultivars differed greatly.

Amylase inhibitor activity (AIA) of chickpea extracts was investigated using pancreatic and salivary amylases. The extract showed higher inhibitor activity towards pancreatic amylase than salivary amylase. AIA of pigeonpea cultivars was determined by using pancreatic amylase and showed large variation among cultivars. Also, AIA of mature seed was higher than the green seeds of pigeonpea.

Flatulence causing oligosaccharide , stachyose was higher in desi cultivars as compared to kabuli cultivars. When considered together, raffinose and stachyose constituted about 40% of the total soluble sugars in chickpea seed whereas raffinose, stachyose and verbascose together constituted about 50% in pigeonpea. These oligosaccharides accumulated during later stages of seed development in both chickpea and pigeonpea.

The role of seed polyphenols of chickpea and pigeonpea in enzyme inhibitory activities of trypsin, chymotrypsin and amylase was examined by *in-vitro* methods. Chickpea polyphenols inhibited trypsin more than chymotrypsin whereas pigeonpea polyphenols did not show such a distinction. On the basis of the average percent enzyme inhibition in the various cultivars studied, pigeonpea polyphenols were found to

be more effective than those of chickpea. The addition of polyvinyl-pyrrolidone (PVP) remarkably reduced the enzyme inhibitory property of the polyphenols. The polyphenolic compounds of cultivars with dark testa colour showed more inhibitory activity than those with light testa color in both chickpea and pigeonpea. Both the crops contained very small amounts of tannins in comparison with total phenolic compounds and most of the phenolic compounds were located in seed coats. In case of both chickpea and pigeonpea no relationship between tannins and total polyphenolic compounds appeared to exist. Considerable amounts of phenolic compounds were removed by soaking and boiling of chickpea and pigeonpea seeds.

Most of the seed phosphorus was present in the form of phytic acid in both the crops. Phytic acid content of chickpea was slightly higher than pigeonpea. Cooking brought about a considerable reduction in the levels of phytic acid in pigeonpea whereas such a response was not observed in chickpea. Germination will have beneficial effects in terms of reducing the levels of phytic acid in both chickpea and pigeonpea.

# 1. INTRODUCTION

Basically, the food legumes are rich sources of protein and their importance has been very well recognized in human nutrition particularly in those countries where cereals and legumes are the staple diet of the people. Historically, the food legumes have been known to have the capacity to synthesize a wide variety of chemical substances that are known to exert a deleterious effect or interfere with the nutritive value when ingested by man or animals. Like other grain legumes, chickpea and pigeonpea have been reported to contain some antinutritional factors. The following antinutritional factors of these crops were studied.

1. Enzyme inhibitors
   - I. Protease inhibitors
     - . Trypsin inhibitor
     - . Chymotrypsin inhibitor
   - II. Amylase inhibitor
2. Flatulence causing oligosaccharides
   - . Raffinose
   - . Stachyose
   - . Verbascose
3. Polyphenols
4. Phytic acid

Also, our aim was to find out the genetic variability for these factors using many cultivars and some available wild relatives of these crops. Efforts were made to know if the concentration of these toxic constituents are reduced or eliminated as a result of processing

practices which are commonly followed in the utilization of these legumes. Therefore this report will primarily deal with the following aspects:

I. Genetic variability for antinutritional factors

II. Effect of processing practices on the levels of these factors.

2. Protease inhibitors:

The term 'Protease inhibitors' is used in its broadest sense to include inhibitors of trypsin and chymotrypsin, bearing in mind that individual inhibitors may differ in their specificity. These inhibitors which are proteinous in nature have attracted the attention of nutritionists because of the possible role they play in determining the nutritive value of legume proteins. Trypsin inhibitors and chymotrypsin inhibitors were studied separately in chickpea and pigeonpea.

2.1 Trypsin and chymotrypsin inhibitors:

Assay procedure: Trypsin inhibitor activity (TIA) was assayed according to Kakade et al. (1969). Trypsin inhibitor was extracted by shaking 200 mg of defatted material with 10 ml of 0.1 M phosphate buffer (pH 7.6) at room temperature for 1 hr. The extract was diluted four fold. The aliquots containing 0.2, 0.4, 0.6 and 0.8 ml were assayed for trypsin inhibitor activity. Protein content of the extract was determined according to Lowry et al. (1951) and percentage of protein extracted was calculated. Chymotrypsin inhibitor activity (CIA) was assayed according to Kakade et al. (1970). Inhibitor was extracted as described above except that 0.1 M borate buffer (pH 7.6) was used. Protein content of extract was determined as above.

2.1.1 Effect of seed coat on inhibitors extraction:

As trypsin inhibitors are soluble proteins, the activity of inhibitors to be assayed will depend on the extraction of these protein from the seed. Trypsin inhibitors were studied in dhal (decorticated) and seed samples of chickpea and pigeonpea. Total protein extraction as influenced by the presence of seed coat in seed samples was studied in order to find out its effect on the estimation of TIA and CIA. The effect of the seed coat of desi and kabuli chickpea varieties on protein extraction is shown in Table I and of red and white seeded pigeonpea varieties in Table 2.

Table 1: Effect of seed coat on protein extraction in chickpea[a]

| Cultivar | | Seed coat (%) | Phosphate buffer[b] | | Borate buffer[b] | |
|---|---|---|---|---|---|---|
| | | | Dhal | Seed | Dhal | Seed |
| | | | .....Protein extraction (%)..... | | | |
| Desi | USA-613 | 17.6 | 69.5 | 61.3 | 60.5 | 57.5 |
| | 850-3/27 | 12.8 | 63.9 | 51.9 | 59.4 | 53.0 |
| | Pant G-114 | 17.3 | 59.6 | 50.4 | 63.2 | 56.5 |
| | CPS-1 | 16.9 | 59.8 | 52.4 | 64.9 | 53.8 |
| | T-3 | 13.9 | 60.5 | 48.0 | 61.0 | 50.4 |
| | Annigeri | 16.2 | 68.3 | 54.5 | 62.5 | 56.3 |
| | G-130 | 16.8 | 70.2 | 57.4 | 64.3 | 54.0 |
| | P-5462 | 16.7 | 63.4 | 55.0 | 62.7 | 55.9 |
| Kabuli | K-4 | 8.3 | 70.8 | 61.7 | 65.2 | 64.3 |
| | G-104 | 6.0 | 71.1 | 63.4 | 69.1 | 68.4 |
| | Rabat | 6.7 | 67.5 | 61.8 | 63.9 | 60.6 |
| | L-550 | 5.7 | 68.0 | 63.5 | 64.8 | 63.4 |
| | GL-629 | 6.1 | 65.0 | 68.4 | 69.9 | 61.9 |
| | Giza | 8.2 | 65.4 | 62.0 | 67.8 | 65.0 |
| | No.501 | 8.8 | 66.7 | 64.5 | 64.0 | 64.4 |
| | Mean | 11.9 | 66.0 | 58.4 | 63.5 | 59.0 |
| | SE± | 4.7 | 3.90 | 5.8 | 3.0 | 5.2 |

[a]Extracted proteins were determined by Lowry's method (1951)

[b]0.1 M, pH 7.6.

The average seed coat content in desi seed was 16.0% while in kabuli it was 7.1%. Lower percentage of meal protein was extracted from desi seed samples (53.9) as compared to dhal (68.7) as shown in Table I. The differences in the extraction of proteins from the seed and dhal samples of kabuli cultivars were not as large. This could be due to the lower amount of seed coat in kabuli and may also be due to their chemical nature. The observed differences in protein extractions of desi and kabuli seeds influenced the trypsin inhibitor values and this is discussed in the following section. A similar effect of seed coat on protein extraction was observed in the case of pigeonpea (Table 2).

Table 2: Effect of seed coat on protein extraction in pigeonpea[a]

| Cultivar | Seed coat (%) | Phosphate buffer[b] | | Borate buffer[b] | |
|---|---|---|---|---|---|
| | | Dhal | Seed | Dhal | Seed |
| | | .....Protein extraction (%)..... | | | |
| C-11 | 15.7 | 67.5 | 58.0 | 64.7 | 53.9 |
| BDN-1 | 15.2 | 65.4 | 58.7 | 63.8 | 52.4 |
| No-148 | 14.8 | 70.3 | 60.3 | 68.7 | 61.5 |
| Hy-3C | 13.0 | 68.9 | 61.9 | 65.4 | 53.4 |
| NP(WR)-15 | 16.4 | 66.4 | 57.3 | 64.0 | 52.5 |
| Gwalior-3 | 15.3 | 63.9 | 54.0 | 67.5 | 58.4 |
| Mean | 15.1 | 67.1 | 58.4 | 65.7 | 55.4 |
| SE ± | 1.2 | 2.3 | 2.7 | 2.0 | 3.7 |

[a] Extracted proteins were determined by Lowry's method (1951);

[b] 0.1 M, pH 7.6.

## 2.2 Variation for trypsin and chymotrypsin inhibitors in chickpea:

The trypsin inhibitor activity TIA and chymotrypsin inhibitor activity (CIA) of dhal and seed samples of desi and kabuli cultivars were studied. These cultivars were grown at Hissar, India ($29^{o}$N) during the post rainy season of 1977-78. Material was obtained by pooling the seeds from single plots. The trypsin units inhibited (TUI/mg meal) in dhal samples of desi cultivars ranged between 9.3 and 14.6 with a mean value of 12.0 units (Table 3) and ranged from 6.7 to 12.3 with mean of 9.4 for kabuli (Table 4). In seed samples, the trypsin units inhibited (TUI/mg meal) varied from 9.9 to 15.7 with a mean of 12.7 units for desi (Table 3) and ranged between 8.1 and 12.1 with a mean of 10.3 for kabuli types (Table 4). This showed that trypsin inhibitor activity was higher in both the dhal and seed of desi when compared with dhal and seed samples of kabuli types. One might expect greater trypsin inhibitory differences between seed samples of desi and kabuli as compared to dhal samples, but this was not observed. This may be due to the observed lower protein extraction in the case of seed samples of desi cultivars. When the results were expressed as trypsin inhibitor units per mg of extracted protein, desi seed samples exhibited higher values (52.6 units) when compared with kabuli seed samples (31.9 units) and the observed differences for dhal samples of both types were small (Tables 3 & 4). The inhibition was about 65% higher in desi seed as compared to kabuli seed samples and only about 25% higher in desi dhal as compared with

kabuli dhal samples. The higher amount of trypsin inhibition in desi seed samples might have occurred due to the influence of polyphenolic compounds in the seed coat.

Table 3: Trypsin and chymotrypsin inhibition in dhal and seed samples of desi cultivars.

| Cultivar | Trypsin Inhibition | | | | Chymotrypsin Inhibition | | | |
|---|---|---|---|---|---|---|---|---|
| | Dhal | | Seed | | Dhal | | Seed | |
| | a | b | a | b | a | b | a | b |
| USA-613 | 14.6 | 40.6 | 15.7 | 58.2 | 7.5 | 22.0 | 7.8 | 37.8 |
| 850-3/27 | 13.6 | 36.6 | 14.4 | 56.6 | 8.3 | 25.2 | 8.7 | 39.2 |
| Pant G-114 | 9.3 | 26.0 | 9.9 | 37.4 | 7.2 | 20.6 | 7.7 | 29.8 |
| CPS-1 | 11.4 | 32.6 | 12.1 | 48.2 | 7.8 | 22.8 | 7.8 | 30.6 |
| T-3 | 13.6 | 41.2 | 14.5 | 60.4 | 7.3 | 24.4 | 8.0 | 35.8 |
| Annegiri | 10.0 | 33.0 | 10.4 | 44.0 | 7.1 | 23.2 | 7.6 | 34.6 |
| G-203 | 12.9 | 36.6 | 14.1 | 60.2 | 9.0 | 26.8 | 8.8 | 34.8 |
| P-5462 | 10.5 | 39.8 | 10.7 | 55.2 | 7.5 | 26.0 | 7.9 | 34.3 |
| Mean | 12.0 | 36.0 | 12.7 | 52.6 | 7.7 | 23.8 | 8.1 | 34.4 |
| SE ± | 1.8 | 4.8 | 2.1 | 7.9 | 0.6 | 2.0 | 0.4 | 3.0 |

[a]Inhibitor units/mg meal; [b]Inhibitor units/mg extracted protein.

The results of CIA of dhal and seed samples of desi and kabuli cultivars are shown in Tables 3 and 4. Less variability was observed in the CIA though the mean inhibitor activity was slightly higher for seed and dhal samples of desi as compared with kabuli cultivars. The mean chymotrypsin units inhibited (CUI/mg meal) was 7.7 units for desi and 6.5 units

for kabuli dhal while it was 8.1 units for desi and 7.3 units for kabuli seed samples. As observed for trypsin inhibitor, the chymotrypsin units inhibited (CUI/mg protein) were higher in the case of desi cultivars and the mean value was 34.4 units for desi seed and 23.2 units for kabuli seeds indicating the possible influence of seed coat constituents in these determinations.

Table 4: Trypsin and chymotrypsin inhibition in dhal and seed samples of kabuli cultivars.

| Cultivar | Trypsin Inhibition | | | | Chymotrypsin Inhibition | | | |
|---|---|---|---|---|---|---|---|---|
| | Dhal | | Seed | | Dhal | | Seed | |
| | a | b | a | b | a | b | a | b |
| K-4 | 11.4 | 36.6 | 11.0 | 32.0 | 5.7 | 19.4 | 7.1 | 23.0 |
| G-104 | 6.7 | 20.0 | 8.1 | 25.2 | 5.7 | 18.2 | 8.0 | 24.2 |
| Rabat | 8.0 | 25.0 | 9.7 | 30.2 | 6.3 | 19.6 | 6.1 | 20.1 |
| L-550 | 8.1 | 27.1 | 10.2 | 32.8 | 6.8 | 22.4 | 7.2 | 24.0 |
| GL-629 | 12.3 | 39.8 | 12.1 | 39.2 | 7.0 | 22.6 | 8.0 | 26.8 |
| Giza | 9.8 | 28.1 | 11.4 | 34.8 | 9.4 | 22.2 | 7.5 | 22.6 |
| No-501 | 9.6 | 28.4 | 9.7 | 30.6 | 5.9 | 23.4 | 7.3 | 21.8 |
| Mean | 9.4 | 29.2 | 10.3 | 31.9 | 6.5 | 21.2 | 7.3 | 23.2 |
| SE ± | 1.8 | 6.3 | 1.2 | 4.0 | 1.2 | 1.9 | 0.6 | 1.9 |

[a]Inhibitor units/mg meal; [b]Inhibitor units/mg extracted protein.

2.3 Trypsin and chymotrypsin inhibitors in pigeonpea and its wild relatives:

Seed samples of three pigeonpea cultivars - Baigani, Pant A-2 and UPS-120 and seven wild relatives were obtained from the Genetic

Resources Unit. Protein percent varied from 23.1 to 26.2 for pigeonpea cultivars and from 27.1 to 29.3 for wild relatives (Table 5). The mean protein content of the wild relatives was about 15% higher than those of pigeonpea cultivars. The variations in the trypsin and chymotrypsin inhibitor activities in pigeonpea cultivars were smaller than in wild relatives. The trypsin inhibitor activity (units inhibited/mg meal) ranged from 13.3 to 25.8 for the Atylosia species and from 12.5 to 15.1 for Cajanus. The trypsin units inhibited were the highest (82.4 units/mg meal) for Rhynchosia rothi. The mean chymotrypsin inhibitor activity in the wild species was more than three fold than the mean of the cultivated

Table 5. Protein contents, levels of trypsin and chymotrypsin inhibitors and in-vitro protein digestibilities (IVPD) in cultivars of pigeonpea and the wild relatives.

| Species | Protein (%) | Trypsin inhibition a | Trypsin inhibition b | Chymotrypsin inhibition a | Chymotrypsin inhibition b | IVPD (%) |
|---|---|---|---|---|---|---|
| 1. Cajanus cajan (L.) | | | | | | |
| Pant A-2 | 24.4 | 12.5 | 69.7 | 5.0 | 27.8 | 57.9 |
| UPAS-120 | 23.1 | 12.9 | 71.3 | 4.2 | 23.1 | 59.5 |
| Baigani | 26.2 | 15.1 | 67.1 | 3.5 | 15.3 | 64.1 |
| 2. Wild species | | | | | | |
| Atylosia scara baeoides (L.) Benth. | 27.8 | 14.2 | 60.4 | 14.2 | 60.9 | 67.8 |
| A. sericea Benth. Ex. Bak. | 28.4 | 17.9 | 76.4 | 20.1 | 85.3 | 68.1 |
| A. albicans W.& A. | 28.5 | 19.4 | 81.9 | 22.0 | 92.4 | 62.6 |
| A. volubilis (Blanco) Gamb. | 27.1 | 25.8 | 121.4 | 11.5 | 60.9 | 52.6 |
| A. platycarpa Benth. | 29.3 | 13.3 | 54.5 | 11.5 | 47.1 | 59.3 |
| A. cajanifolia Haines | 29.1 | 14.9 | 61.3 | 5.9 | 24.2 | 56.0 |
| Rhynchosia rothi Benth. Ex. Aitch. | 27.6 | 82.4 | 445.7 | 20.9 | 113.2 | 40.9 |
| Mean | 28.3 | 26.6 | 127.6 | 15.2 | 69.1 | 58.2 |
| SE ± | 0.3 | 0.5 | 2.0 | 0.2 | 1.3 | 1.6 |

[a] Units inhibited/mg meal; [b] Units inhibited/mg protein.

species. However, in the case of A. cajanifolia the level was similar to that of pigeonpea. CIA was the highest (20.9 units inhibited/mg meal) for Rhynchosia rothi and thus was similar to its TIA.

A similar trend was observed with respect to the differences between cultivated and wild species when the values of TIA and CIA were expressed as units inhibited per mg of extracted protein. TIA was several times higher in Rhynchosia rothi while A. volubilis exhibited the highest level among the Atylosia species. TIA of Rhynchosia rothi was comparable with the reported values for soybeans. When the chymotrypsin inhibitor activities were compared, large differences were observed between the cultivated and wild species, except in the case of Atylosia cajanifolia which had values similar to those of the pigeonpea cultivars (Singh and Jambunathan, 1981b).

Protein quality of pigeonpea is affected by the presence of protease inhibitors as in the case of other grain legumes. Due to the high levels of protein inhibitors in some of the wild species it is suggested that intergeneric lines obtained from crosses of Cajanus with wild species should be tested for the levels of protease inhibitors. However, the antimetabolic nature of such compounds may provide chemical resistance against some insect pests. Elevated levels of TIA in cowpea have been reported to confer resistance against the attach of the bruchid beetle. Clear differences in the levels of CIA between the wild species and pigeonpea have been observed in the present investigation. It would be worthwhile to find out if these compounds are associated with insect resistance mechanism in chickpea and pigeonpea. Detailed studies involving the bioassay of isolated inhibitors are required to

examine the role of such compounds in insect resistance mechanism.

As considerable amount of pigeonpea is consumed in the form of green seeds as vegetable, efforts were made to study the levels of protease inhibitors in green and mature seeds of pigeonpea. Eight lines of vegetable pigeonpeas and one of grain pigeonpea (C-11) were used for this study. These genotypes were grown on black soil at ICRISAT Center, during the 1980-81 post rainy season. A large variation was observed in the levels of these inhibitors both in green and mature seeds (Tables 6 and 7).

Table 6. Protease inhibitors and in-vitro protein digestibility (IVPD) of developing green seed of pigeonpea.

| Genotype | Protease inhibitors | | | | IVPD (%) |
|---|---|---|---|---|---|
| | Trypsin | | Chymotrypsin | | |
| | a | b | a | b | |
| ICPL-102 | 2.72 | 30.44 | 3.05 | 22.15 | 66.01 |
| ICPL-114 | 2.67 | 31.37 | 2.87 | 20.90 | 65.63 |
| ICPL-119 | 3.86 | 45.44 | 2.67 | 19.35 | 67.84 |
| ICPL-122 | 2.60 | 29.93 | 2.29 | 14.57 | 68.76 |
| ICPL-128 | 2.40 | 26.44 | 2.65 | 17.88 | 64.06 |
| ICPL-212 | 2.67 | 35.09 | 2.40 | 17.43 | 64.50 |
| ICP-6997 | 2.46 | 30.94 | 2.17 | 12.75 | 72.09 |
| ICP-7035 | 3.23 | 38.38 | 1.91 | 17.39 | 63.89 |
| C-11 | 2.53 | 28.76 | 2.90 | 21.05 | 68.40 |
| Mean | 2.80 | 32.97 | 2.55 | 18.16 | 66.80 |
| SE ± | 0.13 | 0.84 | 0.10 | 0.63 | 0.72 |

[a] Units inhibited/mg meal; [b] Units inhibited/mg protein.

While the differences in the chymotrypsin inhibitor activity between green and mature seed were small, trypsin inhibitor activity of mature seed was markedly higher than the green seeds. However, it was not possible to distinguish whether the increased trypsin inhibitor activity of mature seed was due to the polyphenolic compounds which also increased in mature seed or due to certain proteinous inhibitors which probably increased as the seed matured. Although additional studies may be necessary to understand the differences in trypsin inhibitor activity of green and mature seeds, the present finding indicate the green seed may be nutritionally better than the mature seed.

Table 7: Protease inhibitors and in-vitro protein digestibility (IVPD) of mature seed of pigeonpea.

| Genotype | Protease inhibitors | | | | IVPD (%) |
|---|---|---|---|---|---|
| | Trypsin | | Chymotrypsin | | |
| | a | b | a | b | |
| ICPL-102 | 9.25 | 76.76 | 3.06 | 18.83 | 61.45 |
| ICPL-114 | 11.75 | 103.52 | 3.47 | 23.31 | 59.72 |
| ICPL-119 | 9.46 | 82.96 | 2.99 | 20.47 | 52.51 |
| ICPL-122 | 11.17 | 86.56 | 3.17 | 19.53 | 63.14 |
| ICPL-128 | 8.90 | 95.66 | 3.07 | 21.74 | 62.00 |
| ICPL-212 | 12.08 | 102.40 | 3.63 | 26.71 | 57.05 |
| ICP-6997 | 10.05 | 89.75 | 2.60 | 17.50 | 58.43 |
| ICP-7035 | 8.19 | 67.95 | 2.60 | 17.41 | 56.81 |
| C-11 | 8.07 | 78.42 | 2.07 | 16.21 | 55.43 |
| Mean | 9.88 | 87.11 | 2.55 | 20.19 | 58.40 |
| SE ± | 0.50 | 1.45 | 0.09 | 0.27 | 0.82 |

[a]Units inhibited/mg meal; [b]Units inhibited/mg extracted protein.

3. In-vitro protein digestibility (IVPD) of chickpea and chickpea:

It is widely known that the amount of protein in a food does not necessarily represent the amount which is utilized when it is consumed. Although the amino acids in the protein of the food are required to be in the balanced proportion, the biological value of the protein depends on the release and availability of these amino acids. The factor which is most likely to affect the amino acid availability is the protein digestibility. The determination of protein digestibility by animal feeding is tedious, time consuming, and expensive as well. The in-vitro methods involving proteolytic enzymes have been studied and suggested by several workers as a useful method for evaluation of protein. In the laboratory we tried different procedures of in-vitro protein digestibility determination.

3.1 The use of different enzyme systems to determine protein digestibility:

In order to find out the optimum conditions and suitable enzyme system, different enzyme systems were studied to determine IVPD of chickpea and pigeonpea. A brief account of the method followed involving different enzyme systems is given below.

Pepsin/Pancreatin enzymes: An amount of sample containing 6.75 $\pm$ 0.1 mg N was placed into a 50 ml conical flask and 5 ml of HCl solution (pH 2.0) containing 2 mg of pepsin enzyme (Sigma Chem. Co., USA) was added. The flask was incubated in a water bath shaker at 37$^{o}$C for 16 hr. Then 2 ml of pancreatin enzyme (Sigma Chem. Co., USA) solution was added and the contents were further incubated for 24 hr. Pancreatin solution

was prepared by dissolving 50 mg pancreatin in 100 ml of 0.1 M borate buffer (pH 6.8) containing 0.025 M calcium chloride and the solution was filtered and used. After 24 hr of incubation the reaction was stopped by adding 7.0 ml of 10% (w/v) trichloroacetic acid (TCA) and the suspension was centrifuged at 10,000 x g for 15 min. The residue was washed twice with 5 ml of 5% TCA and the pooled supernatants made upto 25 ml with 5% TCA. An aliquot (5.0 ml) was taken and evaporated to dryness at low temperature (80-90$^{o}$C) and the nitrogen content was determined by the microKjeldahl procedure. The digestibility of sample was calculated by subtracting the enzyme blank from the nitrogen content in the supernatant and then expressed as percentage of total nitrogen in the sample. This procedure has been published earlier (Buchmann, 1979; Singh and Jambunathan, 1981a).

Pepsin/trypsin: This enzyme system was followed as described by Mauron (1970). Same amount of sample as above was taken in a 50 ml conical flask and incubated with 20 mg pepsin in 10 ml of HCl pH 2.0 for 24 hr at 37$^{o}$C. For trypsin treatment the pepsin digest was buffered with 1 g of $K_2HPO_4$ adjusted to pH 8.4 and incubated with 10 mg of trypsin at 37$^{o}$C for 24 hr. At the end of incubation, the reaction was stopped using 10% TCA and further processed as above.

Protease (Pronase): Digestibility determination using protease was carried out according to the method described earlier (Buchmann, 1979). An amount of sample containing 6.75 $\pm$ 0.1 mg N was placed into a 50 ml conical flask. After adding 2.0 mg protease (Sigma Chem. Co., USA), content was dispersed in 10 ml of 0.1 M borate buffer

and incubated for 18 hr. After the incubation period the enzyme reaction was stopped by using 10% TCA and further processed as in case of pepsin and pancreatin system.

Pepsin: The procedure followed for assay of pepsin digestibility was the same as in case of pepsin + pancreatin except for addition of pancreatin enzyme. After incubation with pepsin alone the enzyme activity was stopped by adding 10% TCA and processed as above.

Multienzyme: The multienzyme system consisted of trypsin, chymotrypsin and peptidase as described by Hsu et al. (1977). An amount of sample as above was suspended in 50 ml of distilled water and adjusted to pH 8.0 with 0.1 N HCl and/or NaOH while stirring in a water bath. The multienzyme solution 1.6 mg trypsin, 3.0 mg chymotrypsin and 1.3 mg peptidase (all from Sigma Chem. Co., USA) was maintained in an ice bath and adjusted to pH 8.0 with 0.1 N HCl/NaOH. Five ml of the enzyme solutions were then added to protein suspension which was being stirred at 37°C. The enzyme reaction was stopped at 2, 4 and 6 hr intervals by adding 5% TCA and processed as above.

Expectedly, different enzyme systems revealed large differences in IVPD values of chickpea and pigeonpea (Table 8). Using dhal samples of several cultivars of chickpea and pigeonpea it was observed that IVPD was highest in case of pepsin + pancreatin enzyme system and lowest in case of multienzyme system. It is desirable to obtain relationship between in-vitro and in-vivo methods before adopting any procedure in protein quality evaluation program. But the results on comparison of different enzyme systems suggest that pepsin + pancreatin system is

satisfactory. This enzyme system was followed to determine the IVPD of chickpea and pigeonpea cultivars.

Table 8: Different enzyme systems and in-vitro protein digestibility of chickpea and pigeonpea.

| Cultivar | Pepsin | Protease | Pepsin + Trypsin | Pepsin + Pancreatin | Multienzyme[a] 2 | 4 | 6 |
|---|---|---|---|---|---|---|---|
| | .....Protein digestibility (%)..... | | | | | | |
| Chickpea (dhal) | | | | | | | |
| Annigeri | 50.8 | 65.6 | 71.5 | 76.0 | 47.3 | 51.9 | 53.4 |
| G-130 | 48.9 | 64.5 | 68.3 | 72.6 | 41.3 | 50.4 | 55.8 |
| T-3 | 51.2 | 63.0 | 69.5 | 74.5 | 40.5 | 48.7 | 55.0 |
| G-104 | 47.5 | 62.5 | 67.0 | 72.9 | 38.9 | 47.5 | 51.4 |
| L-550 | 49.0 | 64.8 | 71.0 | 76.4 | 41.5 | 49.4 | 55.0 |
| Pigeonpea (dhal) | | | | | | | |
| C-11 | 45.6 | 54.5 | 60.1 | 62.4 | 35.6 | 40.4 | 43.2 |
| BDN-1 | 46.5 | 58.4 | 63.0 | 68.5 | 37.0 | 39.5 | 44.0 |
| No-148 | 43.0 | 54.3 | 62.8 | 69.5 | 39.7 | 41.3 | 46.5 |
| Hy-3C | 41.7 | 55.7 | 60.0 | 63.5 | 38.4 | 40.1 | 42.5 |
| NP(WR)-15 | 44.8 | 58.0 | 63.5 | 70.4 | 39.5 | 42.5 | 47.0 |

[a]Multienzyme solution contained trypsin, chymotrypsin and peptidase enzymes, and incubated for 2, 4 and 6 hr.

3.2 Variation for IVPD of chickpea and pigeonpea cultivars:

Results of in-vitro protein digestibility studies in desi and kabuli chickpea cultivars are shown in Table 9. The mean values for protein digestibility of desi seed and dhal were 63.9 and 71.0% respectively and

for kabuli seed and dhal were 73.6 and 75.3% respectively indicating a large variation between desi and kabuli types. Variation in the IVPD of pigeonpea and its wild relatives is shown in Table 5. IVPD of pigeonpea cultivars ranged between 57.9 and 64.1 percent and for wild relatives from 40.9 to 68.1 percent.

Rhynchosia rothi had a substantially lower value (40.9 percent). The low protein digestibility of this species might be due to the presence of high levels of protease inhibitors. The green and mature seed of a number of genotypes of pigeonpeas were studied for IVPD. The IVPD of the green seed was more than the mature seed (Tables 6 & 7) while there were no large differences among the genotypes studied.

Table 9: Protein content, and in-vitro protein digestibility (IVPD) of chickpea [a]

| Cultivar | | Protein (%) | | IVPD (%) | |
|---|---|---|---|---|---|
| | | Dhal | Seed | Dhal | Seed |
| Desi | USA-613 | 26.8 | 22.0 | 63.7 | 58.8 |
| | 850-3/27 | 27.2 | 22.1 | 68.0 | 63.8 |
| | Pant G-114 | 29.3 | 23.5 | 65.4 | 52.4 |
| | CPS-1 | 26.4 | 23.2 | 73.6 | 68.5 |
| | T-3 | 23.5 | 20.8 | 74.5 | 63.2 |
| | Annigeri | 24.2 | 21.3 | 76.0 | 69.0 |
| | G-130 | 26.5 | 21.5 | 72.6 | 64.3 |
| | P-5462 | 24.4 | 19.8 | 74.5 | 64.8 |
| Kabuli | K-4 | 23.9 | 23.0 | 77.5 | 74.8 |
| | G-104 | 27.3 | 25.3 | 72.9 | 72.0 |
| | Rabat | 24.6 | 23.9 | 72.7 | 71.4 |
| | L-550 | 22.8 | 22.5 | 76.4 | 76.3 |
| | GL-629 | 23.6 | 23.5 | 79.1 | 77.6 |
| | Giza | 26.5 | 25.4 | 73.8 | 70.2 |
| | No.501 | 26.1 | 25.5 | 74.8 | 72.9 |
| | Mean | 25.5 | 22.9 | 73.0 | 68.5 |
| | SE ± | 1.8 | 1.6 | 4.1 | 6.6 |

[a] Pepsin + pancreatin enzyme system.

In addition, several cultivars of pigeonpea were examined for IVPD and results are presented in Table 10.

Table 10: Protein content and in-vitro protein digestibility (IVPD) of pigeonpea cultivars[a].

| Maturity group | Cultivar | Protein (%) | | IVPD (%) | |
|---|---|---|---|---|---|
| | | Dhal | Seed | Dhal | Seed |
| Early | UPAS-120 | 21.4 | 18.3 | 63.8 | 57.3 |
| | Pant-120 | 24.0 | 21.5 | 70.0 | 58.5 |
| | Prabhat | 20.1 | 17.6 | 64.5 | 54.9 |
| | T-21 | 20.2 | 17.8 | 63.0 | 51.0 |
| | DL-74-1 | 23.4 | 21.5 | 65.4 | 51.0 |
| Medium | C-11 | 23.7 | 21.0 | 62.4 | 50.2 |
| | No. 148 | 22.7 | 20.1 | 69.5 | 64.0 |
| | Hy-3C | 20.3 | 18.1 | 63.5 | 55.9 |
| | ICP-1 | 21.6 | 19.0 | 65.8 | 59.2 |
| | BDN-1 | 23.2 | 20.2 | 68.5 | 58.5 |
| | Mukta | 22.2 | 19.4 | 64.8 | 58.9 |
| | Hy-2 | 20.2 | 18.0 | 67.3 | 59.9 |
| | PM-1 | 19.7 | 17.2 | 59.7 | 46.8 |
| | AS-71-37 | 20.9 | 18.3 | 65.7 | 57.2 |
| | ST-1 | 21.8 | 19.2 | 62.9 | 54.3 |
| Late | NP(WR)-15 | 23.7 | 21.1 | 70.4 | 59.6 |
| | Gwalior-3 | 24.8 | 22.2 | 69.8 | 62.2 |
| | KWR-1 | 22.6 | 19.5 | 71.5 | 66.2 |
| | T-7 | 22.2 | 18.9 | 67.3 | 54.0 |
| | T-17 | 25.2 | 22.0 | 68.5 | 60.0 |
| | Mean | 22.2 | 19.5 | 66.2 | 57.0 |
| | SE + | 1.6 | 1.5 | 3.1 | 4.7 |

[a]Pepsin + pancreatin enzyme system.

The pigeonpea cultivars were grown at ICRISAT Center during the rainy season of 1976-77. Defatted dhal and whole seed samples of these cultivars were analysed for IVPD. The IVPD of pigeonpea cultivars ranged between 46.8 and 66.2 with a mean of 57.0 percent and these

values were considerably lower than those of the dhal samples (Table 10). It should be noted that percent mean value for IVPD was higher for chickpea than pigeonpea in case of both seed and dhal samples, indicating that protein digestibility of chickpea may be better than pigeonpea although clear cut differences did not exist among these cultivars. As these legumes are generally consumed after cooking, it would be desirable to study the variation in the IVPD in cooked samples of chickpea and pigeonpea cultivars.

Table 11: Relationship between protease inhibitors, polyphenol, seed coat percentage and in-vitro protein digestibility in 15 chickpea cultivars.

| | Protein (%) | Seed coat (%) | TIU[a] | CIU[a] | Polyphenols(%) |
|---|---|---|---|---|---|
| Seed coat (%) | -0.625** | - | | | |
| TIU[a] | -0.509* | 0.493* | - | | |
| CIU[a] | -0.331 | 0.457 | 0.530* | - | |
| Polyphenols (%) | -0.627** | 0.938** | 0.612* | 0.507* | |
| IVPD[b] | 0.134 | -0.731** | -0.439 | -0.339 | -0.832 |

TIU, trypsin inhibitor unit; and CIU, chymotrypsin inhibitor unit.
[a]Units inhibited/mg meal; ** and *Significant at 1% and 5% level respectively.

As protease inhibitors interfere with the protein digestibility, IVPD may be influenced by the levels of protease inhibitors. Using the results of 15 desi and kabuli cultivars of chickpea, interrelationship between the levels of protease inhibitors and IVPD was worked out (Table 11). A negative correlation, although of low magnitude was

observed between protease inhibitors and in-vitro protein digestibility.

4. Effect of traditional processing practices on protease inhibitors, and protein digestibility:

In the case of grain legumes, home processing practices are traditionally followed and this may possibly reduce or alter the levels of deleterious factors, and improve digestibility and palatability. Among the various processing practices, soaking and heating of chickpea and pigeonpea are the most common practices that are followed in India. Experiments were conducted to understand the effect of such processing treatments on the levels of protease inhibitors and protein digestibility.

4.1 Effect of soaking on protease inhibitors and digestibility:

Chickpea and pigeonpea dhal samples were soaked in water at room temperature for various periods of time. The amount of water was just sufficient to be absorbed by the material and the excess water was discarded after the soaking period. Dhal material was dried in the freeze drier and used for analysis. The protein content of chickpea and pigeonpea dhal decreased as the duration of soaking increased (Table 12). Reduction in the protein content as a result of soaking was found to be of more or less of the same magnitude in chickpea and pigeonpea. This might have been due to the slow leaching of water soluble proteins into the water. This was confirmed by analysing the protein content in soaking water which increased with the duration of soaking period.

Table 12. Effect of soaking on protein content in chickpea and pigeonpea

| Soaking duration (hr) | Chickpea | | Pigeonpea | |
|---|---|---|---|---|
| | G-130 | L-550 | C-11 | Hy-3C |
| | .....Protein (%)..... | | | |
| 0 | 24.5 | 20.4 | 22.9 | 22.9 |
| 6 | 23.8 | 20.2 | 22.5 | 22.9 |
| 12 | 23.6 | 20.0 | 22.4 | 22.3 |
| 24 | 23.4 | 19.5 | 21.6 | 21.4 |
| 36 | 23.0 | 19.1 | 21.4 | 21.0 |

[a]Dhal soaked in distilled water and freeze dried after discarding the soaking water.

Effect of soaking on protein solubility in chickpea and pigeonpea using dhal sample was studied and results are shown in Table 13. Results of soaking in distilled water and sodium chloride solution (0.5 M) were compared. Protein solubility in water and salt solution improved as a result of soaking and was more pronounced in chickpea than pigeonpea. Moreover, a considerable difference in protein solubility between chickpea and pigeonpea cultivars was observed as a result of prolonged soaking (Table 13). Protein solubility increased to a larger extent in chickpea as compared to pigeonpea as a result of soaking. This trend was observed in case of both water and salt soluble proteins (Table 13). In case of pigeonpea, soaking in salt solution was more effective. The improved protein solubility due to soaking might be useful from the digestibility point of view.

Table 13: Effect of soaking on protein solubility in chickpea and pigeonpea

| Soaking time (hr) | Chickpea (G-130) | | Pigeonpea (C-11) | |
|---|---|---|---|---|
| | Water | 0.5 M NaCl | Water | 0.5 M Nacl |
| | .....Protein solubility (%)..... | | | |
| 0 | 49.6 | 63.5 | 54.5 | 62.1 |
| 6 | 50.4 | 63.8 | 54.0 | 64.0 |
| 12 | 50.8 | 65.9 | 54.8 | 65.6 |
| 24 | 53.2 | 70.5 | 55.0 | 68.2 |
| 48 | 56.8 | 79.7 | 55.4 | 70.6 |

[a] Dhal soaked in distilled water and freeze dried after discarding the soaking water.

The levels of protease inhibitors and protein digestibility as influenced by soaking are presented in Table 14. A small reduction in trypsin inhibitory activity was observed whereas very little difference in chymotrypsin inhibitory activity was observed. Soaking up to 12 hr brought about 15 percent reduction in TIA in chickpea and about 25 percent in pigeonpea, while soaking beyond that period did not result in measurable differences in chickpea and pigeonpea.

Improvements in pepsin digestibility and IVPD were noticed when dhal was soaked for different durations. Protein digestibility increased considerably up to 24 hr of soaking in case of chickpea whereas increasing trend was noticed up to 36 hr of soaking in case of pigeonpea. Effects of soaking were more pronounced in chickpea as compared to pigeonpea and these differences might have existed because of differences in protein solubility as influenced by soaking.

Table 14: Effect of soaking on protease inhibitors, pepsin digestibility and *in vitro* protein digestibility (IVPD) in chickpea and pigeonpea[a]

| Constituent | Soaking time (hr) | | | | |
|---|---|---|---|---|---|
| | 0 | 6 | 12 | 24 | 36 |
| Chickpea (G-130): | | | | | |
| Trypsin inhibitor[b] | 12.2 | 11.5 | 10.2 | 10.2 | 10.0 |
| Chymotrypsin inhibitor[b] | 8.7 | 8.2 | 8.3 | 8.4 | 8.3 |
| Pepsin digestibility (%) | 49.8 | 50.7 | 55.4 | 63.9 | 64.5 |
| IVPD (%)[c] | 62.0 | 64.5 | 68.3 | 74.5 | 75.0 |
| Pigeonpea (C-11): | | | | | |
| Trypsin inhibitor[b] | 9.3 | 8.1 | 7.0 | 7.1 | 7.0 |
| Chymotrypsin inhibitor[b] | 6.0 | 5.8 | 5.5 | 5.3 | 5.3 |
| Pepsin digestibility (%)[c] | 48.4 | 49.7 | 53.6 | 56.3 | 60.9 |
| IVPD (%)[c] | 64.2 | 64.0 | 68.6 | 69.0 | 72.3 |

[a] Dhal soaked in distilled water and freeze dried after discarding the soaking water; [b] Enzymes units inhibited per mg of defatted dhal meal; [c] Percent digestible protein.

4.2 Effect of heating on protease inhibitors and digestibility:

Heat treatment is considered to inactivate the trypsin and chymotrypsin inhibitors to a certain extent. The procedures of open vessel cooking and pressure cooking were followed to study the effect of heat treatment on protease inhibitors and protein digestibilities. Effect of heat treatment on protein content and protein solubility in chickpea and pigeonpea are shown in Table 15. Protein percent value

was not affected by heat treatment. It is interesting to note that the solubility of proteins in water and salt solution (0.5 M NaCl) reduced remarkably as a result of heating (Table 15). One of the reasons for the reduction in protein solubility after heating may be due to the denaturation of proteins in pigeonpea and chickpea. Like in other grain legumes, chickpea and pigeonpea proteins are globular in nature. Such proteins are easily denatured as a result of heat treatment.

Table 15: Effect of heat treatment on protein content and its solubility in chickpea (G-130) and pigeonpea (C-11)[a].

| Heating time (min) | Chickpea | Pigeonpea | Chickpea | | Pigeonpea | |
|---|---|---|---|---|---|---|
| | | | Water | 0.5 M NaCl | Water | 0.5 M NaC |
| | .....Protein (%).... | | .....Protein solubility (%)..... | | | |
| 0 | 24.5 | 22.4 | 49.6 | 67.6 | 60.3 | 69.4 |
| 10 | 24.3 | 22.0 | 48.7 | 62.8 | 53.0 | 54.5 |
| 20 | 23.9 | 21.9 | 33.5 | 38.5 | 30.8 | 34.3 |
| 30 | 24.6 | 23.0 | 20.4 | 22.8 | 18.5 | 18.7 |
| 40 | 25.0 | 22.8 | 17.6 | 20.6 | 16.7 | 17.0 |
| 60 | 24.5 | 22.4 | 14.0 | 15.4 | 14.5 | 15.9 |

[a]Dhal boiled and freeze dried with broth.

The effect of heat treatment on protein solubility was also reflected in the protease inhibitor activities, pepsin digestibility and in-vitro protein digestibility as shown in Table 16. Although TIA and CIA are reduced considerably as a result of heat treatment, it was not clear whether this was due to the destruction of inhibitors activity or due to the poor extractability of inhibitors. In order to ascertain this, the extracted inhibitors were boiled for various

time period. Boiling chickpea extracts for 20 minutes resulted in about 50 and 40 percent reduction in trypsin and chymotrypsin inhibitors activity respectively (Table 17). Interestingly reduction in inhibitors activity of the extract was more in pigeonpea than chickpea.

Table 16: Effect of heat treatment on protease inhibitors, pepsin digestibility and in-vitro protein digestibility (IVPD) in chickpea and pigeonpea.

| Constituent | Heating time (min)[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 10 | 20 | 30 | 40 | 60 |
| Chickpea (G-130): | | | | | | |
| Trypsin inhibitor[b] | 12.2 | 8.8 | 8.5 | 7.3 | 4.6 | 2.4 |
| Chymotrypsin inhibitor[b] | 8.7 | 6.5 | 6.4 | 5.5 | 3.8 | 3.0 |
| Pepsin digestibility (%)[c] | 49.8 | 50.5 | 44.6 | 35.8 | 35.0 | 34.5 |
| IVPD (%)[c] | 62.0 | 68.5 | 63.6 | 50.8 | 40.4 | 40.3 |
| Pigeonpea (C-11): | | | | | | |
| Trypsin inhibitor[b] | 9.4 | 7.8 | 6.3 | 5.7 | 3.8 | 1.4 |
| Chymotrypsin inhibitor[b] | 6.0 | 4.4 | 3.9 | 3.8 | 3.1 | 2.6 |
| Pepsin digestibility (%)[c] | 48.4 | 46.0 | 40.5 | 39.6 | 38.8 | 37.4 |
| IVPD (%)[c] | 64.2 | 70.6 | 58.6 | 56.0 | 50.2 | 49.5 |

[a]Dhal boiled and freeze dried; [b]Enzyme units inhibited per mg of defatted dhal meal; [c]Percent digestible protein.

Comparison of pressure cooking and open vessel cooking showed noticeable differences in the activities of trypsin and chymotrypsin enzymes. Dhal samples were pressure cooked for 15 min in a pressure cooker whereas in case of open vessel cooking dhal sample was boiled

for 30 min in a glass beaker. The whole contents including the cooking water were freeze dried and analysed. Trypsin and chymotrypsin inhibitor activities were destroyed by pressure cooking to a larger extent than open-vessel cooking in both chickpea and pigeonpea cultivars shown in Table-18.

Table 17: Effect of heat treatment of chickpea and pigeonpea extracts on the protease inhibitor activities.

| Heating time (min)[a] | Chickpea (G-130)[b] | | Pigeonpea (C-11)[b] | |
|---|---|---|---|---|
| | Trypsin | Chymotrypsin | Trypsin | Chymotrypsin |
| 0 | 64.5 | 35.4 | 43.0 | 26.5 |
| 5 | 58.4 | 31.7 | 40.5 | 20.4 |
| 10 | 50.3 | 28.5 | 38.4 | 17.3 |
| 15 | 45.8 | 26.0 | 30.6 | 13.6 |
| 20 | 32.6 | 24.5 | 19.8 | 13.0 |
| 25 | 28.0 | 18.4 | 18.4 | 12.5 |
| 30 | 25.6 | 17.6 | 18.3 | 11.8 |

[a]Protein extract boiled in a test tube; [b]Units inhibited/ml of boiled extract.

None of these two procedures of cooking resulted in complete destruction of inhibitors activities. It appeared that trypsin inhibitors were more liable to heat treatment than chymotrypsin. Interestingly it was also observed that the destruction of the inhibitor activities was more in pigeonpea than chickpea as a result of heat treatment (Table-18). Differential responses of cultivars were noticed when the effect of pressure cooking on inhibitor activities was studied using several cultivars of pigeonpea (Table-19) and chickpea (Table-20).

However, it should be noted that when the results were expressed as enzyme units inhibited per mg extracted protein, these inhibitors increased in cooked samples and this appeared to be due to the reduced extractions of protein. This observation needs further explanation as it is not clear from the results of our experiment.

Table 18: Effect of methods of cooking on trypsin and chymotrypsin inhibitors activity in chickpea and pigeonpea.

| | Trypsin inhibitor[a] | | | Chymotrypsin inhibitor[a] | | |
|---|---|---|---|---|---|---|
| | Uncooked | Cooked | | Uncooked | Cooked | |
| | | b | c | | b | c |
| Pigeonpea: | | | | | | |
| C-11 | 9.4 | 1.1 (88.9) | 3.0 (68.1) | 6.4 | 2.0 (68.7) | 2.9 (54.7) |
| ICP-28 | 12.5 | 0.5 (96.0) | 3.9 (68.8) | 9.2 | 2.8 (77.6) | 3.0 (67.4) |
| Chickpea: | | | | | | |
| G-130 | 12.2 | 2.6 (78.7) | 4.6 (62.3) | 7.5 | 3.0 (60.0) | 3.5 (53.3) |
| L-550 | 13.4 | 3.1 (76.9) | 5.8 (56.7) | 8.0 | 3.5 (62.5) | 4.2 (47.5) |

Values within parenthesis indicate the percentage of destruction of inhibitor activity.

[a]Enzyme units inhibited/mg meal; [b]Pressure cooked for 10 min;

[c]Open-vessel cooked by boiling in a beaker for 30 minutes.

Also the IVPD of chickpea and pigeonpea was remarkably increased as a result of cooking (Table 21). Mean value for IVPD was higher in chickpea than pigeonpea in case of uncooked sample but reverse

was the trend when cooked samples were compared. It was interesting to note that IVPD of pigeonpea dhal was increased to a larger extent in comparison with chickpea. This observation should be confirmed by conducting animal feeding trials using chickpea and pigeonpea cultivars.

Table 19: Effect of cooking on protease inhibitor activity in pigeonpea[a]

| Cultivar/line | Trypsin inhibitor[b] | | Chymotrypsin inhibitor[b] | |
|---|---|---|---|---|
| | Uncooked | Cooked | Uncooked | Cooked |
| ICPH-6 | 7.4 | 1.0 | 2.7 | 0.3 |
| ICPL-234 | 9.2 | 1.0 | 2.8 | 0.1 |
| ICPL-270 | 7.6 | 1.7 | 2.6 | 0.1 |
| Hy-8 | 8.3 | 0.8 | 2.6 | 0.1 |
| GS-2 | 10.4 | 0.8 | 2.6 | 0.1 |
| No-148 | 8.9 | 1.0 | 2.6 | 0.2 |
| K-64 | 11.0 | 0.9 | 3.0 | 0.2 |
| PDA-5 | 8.8 | 1.1 | 2.7 | 0.1 |
| BDN-2 | 8.5 | 1.4 | 2.8 | 0.3 |
| MAUL-175 | 7.7 | 1.5 | 2.2 | 0.1 |
| ICPH-2 | 8.3 | 1.8 | 2.8 | 0.2 |
| ICPH-5 | 12.2 | 2.0 | 2.5 | 0.2 |
| AS71-37 | 6.2 | 0.6 | 2.4 | 0.2 |
| JA-5 | 10.5 | 1.5 | 2.7 | 0.3 |
| LRG-30 | 7.7 | 1.1 | 2.8 | 0.3 |
| LRG-36 | 9.1 | 0.6 | 2.6 | 0.1 |
| BDN-1 | 8.4 | 0.5 | 2.8 | 0.2 |
| C-11 | 5.4 | 0.5 | 1.7 | 0.1 |
| Mean | 8.6 | 1.1 | 2.6 | 0.2 |
| SE± | 1.54 | 0.4 | 0.3 | 0.1 |

[a]Dhal pressure cooked for 15 min and freeze dried with broth;

[b]Units inhibited per mg meal.

Table 20: Effect of cooking on protease inhibitor activity in chickpea[a]

| Cultivar/line | Trypsin inhibitor[b] | | Chymotrypsin inhibitor | |
|---|---|---|---|---|
| | Uncooked | Cooked | Uncooked | Cooked |
| L-550 | 10.3 | 0.2 | 3.1 | 0.2 |
| ICCC-24 | 11.1 | 0.3 | 3.3 | 0.5 |
| ICCC-25 | 10.8 | 0.2 | 3.3 | 0.2 |
| ICCC-26 | 9.6 | 0.3 | 3.2 | 0.4 |
| H-208 | 16.1 | 0.4 | 3.3 | 0.3 |
| Pant-G-114 | 17.3 | 0.3 | 3.3 | 0.4 |
| BDN-9-3 | 13.8 | 0.1 | 3.4 | 0.3 |
| P-326 | 20.2 | 0.5 | 3.2 | 0.4 |
| BG-212 | 15.7 | 0.4 | 3.2 | 0.3 |
| K-850 | 14.5 | 0.9 | 3.3 | 0.5 |
| G-130 | 15.9 | 0.4 | 3.4 | 0.6 |
| C-235 | 20.3 | 0.5 | 3.2 | 0.5 |
| G-543 | 21.0 | 0.4 | 3.4 | 0.5 |
| BG-203 | 19.2 | 0.2 | 3.3 | 0.5 |
| H-76-49 | 18.0 | 0.3 | 3.5 | 0.4 |
| Mean | 15.3 | 0.4 | 3.3 | 0.4 |
| SE ± | 3.5 | 0.18 | 0.11 | 0.11 |

[a]Dhal pressure cooked for 15 min and freeze dried with booth;

[b]Units inhibited per mg meal.

5. Amylase inhibitors and *in-vitro* starch digestibility:

Although briefly described, amylase inhibitors have not received much attention in the available literature on the nutritional quality of legumes. It has been reported that the growth inhibiting

property of raw beans is due to the presence of a heat labile factors which inhibited the in-vitro activity of pancreatic amylase. Also, a large variation in the inhibitor activity of pancreatic amylase among several species of food legumes has been reported. We are not aware of any detailed studies on amylase inhibitor of chickpea and pigeonpea. We have made an attempt to study the levels of these inhibitors in these crops.

Assay procedure:

The pancreatic amylase inhibitory activity was carried out according to the method of Jaffe et al. (1973). The salivary amylase inhibitor activity was determined according to the procedure of Granum (1978). Human saliva was collected and diluted about fivefold in 0.02 M phosphate buffer, pH 6.9. After standing overnight at 5°C, the mixture was centrifuged at 10,000 x g for 15 min. Amylase inhibitor was extracted by shaking a finely ground and defatted chickpea sample with 0.02 M phosphate buffer, pH 6.9 (1:10, w/v) for 2 hr at room temperature (25 ± 1°C). The suspension was then centrifuged at 10,000 x g for 15 min at room temperature. The supernatant was then heated for 10 min at 70°C, and centrifuged again, the supernatant so obtained was tested for amylase inhibitor activity.

The determination of in-vitro starch digestibility was carried out using pancreatic amylase (Sigma Chem. Co., USA). Starch digestibility was determined in meal samples and in isolated starch as well. Starch was isolated according to the procedure of Garwood et al. (1976). For the determination of in-vitro digestibility, a suitable amount of

defatted meal (50 mg) or the isolated starch (25 mg) was dispersed in 1.0 ml of 0.02 M phosphate buffer, pH 6.9. Pancreatic amylase (20 mg) was dissolved in 50 ml of the same buffer and 0.5 ml was added to the sample suspension and incubated at 37°C for 2 hr. After the incubation period, 2 ml of 3-5 dinitrosalicyclic acid reagent was quickly added and the mixture was heated for 5 min in a boiling water bath. After cooling, the solution was made to 25 ml with distilled water, and filtered prior to measurement of the absorbance at 550 nm. A blank was run simultaneously by incubating the sample first and 3-5, dinitrosalicyclic acid was added before the addition of the enzyme solution. Maltose was used as the standard and the values were expressed as mg of maltose released per g of sample.

5.1 Variation for amylase inhibitors and in-vitro starch digestibility (IVSD) in chickpea and pigeonpea:

Dhal samples of 8 desi and 7 kabuli cultivars grown at Hissar, India (29°N) during the post rainy season of 1977-78 were analysed. The amylase inhibitor activity of chickpea cultivars, when examined using pancreatic amylase (enzyme units inhibited/g meal), ranged between 7.8 and 10.5 in desi and from 5.6 to 10.0 in kabuli cultivars as shown in Table 22. This indicated considerable variations among these cultivars. A similar variation but of lower magnitude was observed with salivary amylase. A comparison under similar assay conditions indicated that the amylase inhibitor activity was more towards pancreatic amylase than salivary amylase and this was found to be the case in both desi and kabuli cultivars (Table 22).

Table 21: *In-vitro* protein digestibility (IVPD) of cooked and uncooked pigeonpea and chickpea[a]

| Cultivar | Protein (%) | | IVPD (%) | |
|---|---|---|---|---|
| | Uncooked | Cooked | Uncooked | Cooked |
| **Pigeonpea:** | | | | |
| ICPH-6 | 23.0 | 23.3 | 56.5 | 80.9 |
| ICPL-234 | 23.8 | 23.8 | 55.5 | 80.6 |
| ICPL-270 | 23.7 | 22.9 | 56.0 | 83.0 |
| Hy-8 | 21.5 | 21.3 | 56.3 | 83.4 |
| GS-2 | 23.4 | 23.7 | 56.2 | 81.1 |
| No. 148 | 24.4 | 24.2 | 53.5 | 79.8 |
| K-64 | 24.2 | 23.8 | 54.2 | 77.4 |
| PDA-5 | 23.6 | 23.5 | 55.9 | 77.1 |
| BDN-2 | 25.1 | 25.4 | 53.8 | 76.1 |
| MAUE-175 | 23.0 | 22.8 | 55.5 | 77.5 |
| ICPH-2 | 25.5 | 25.3 | 53.9 | 76.6 |
| ICPH-5 | 24.4 | 24.0 | 54.9 | 77.2 |
| AS.71-37 | 23.8 | 23.7 | 52.5 | 76.9 |
| JA-5 | 25.3 | 25.4 | 55.4 | 62.7 |
| LRG-30 | 23.7 | 23.3 | 56.3 | 59.4 |
| LRG-36 | 24.0 | 23.8 | 52.4 | 78.1 |
| BDN-1 | 24.7 | 24.6 | 54.8 | 78.7 |
| C-11 | 23.7 | 23.9 | 56.4 | 81.2 |
| Mean | 23.9 | 23.8 | 55.1 | 77.7 |
| SE ± | 0.94 | 1.03 | 1.61 | 4.90 |
| **Chickpea:** | | | | |
| Annigeri | 15.5 | 15.6 | 63.3 | 71.5 |
| L-550 | 16.8 | 16.8 | 59.8 | 73.2 |
| ICCC-24 | 17.2 | 16.9 | 60.4 | 71.1 |
| ICCC-25 | 16.5 | 16.7 | 60.7 | 68.3 |
| ICCC-26 | 16.5 | 16.0 | 65.0 | 69.8 |
| H-208 | 20.1 | 20.3 | 52.3 | 64.1 |
| Pant-G114 | 20.5 | 20.3 | 56.8 | 69.7 |
| BDN-9-3 | 19.2 | 19.0 | 58.2 | 69.6 |
| P-326 | 24.7 | 25.1 | 52.1 | 67.4 |
| BG-212 | 22.1 | 21.8 | 55.7 | 68.9 |
| K-850 | 17.9 | 17.6 | 61.5 | 75.8 |
| G-130 | 21.4 | 21.7 | 53.9 | 70.9 |
| C-235 | 23.8 | 23.3 | 54.0 | 72.2 |
| G-543 | 23.4 | 23.6 | 56.1 | 69.8 |
| BG-203 | 21.9 | 22.3 | 61.2 | 72.1 |
| H-76-49 | 21.9 | 21.5 | 58.0 | 75.4 |
| Mean | 20.0 | 19.9 | 58.2 | 70.6 |
| SE ± | 2.94 | 3.02 | 3.70 | 2.01 |

[a]Dhal pressure cooked for 15 min and freeze dried with broth.

Table 22: Amylase inhibitor activity (AIA) and starch digestibility in dhal samples of 15 chickpea cultivars

| Cultivar | Starch (%) | Amylase inhibitor activity[a] | | Starch digestibility | |
|---|---|---|---|---|---|
| | | Salivary amylase | Pancreatic amylase | b | c |
| Desi: | | | | | |
| USA-613 | 53.4 | 5.3 | 7.9 | 49.2 | 92.2 |
| 850-3/27 | 49.8 | 8.4 | 10.4 | 44.4 | 87.3 |
| Pant G-114 | 48.4 | 4.0 | 9.2 | 44.5 | 88.0 |
| CPS-1 | 50.1 | 7.8 | 8.6 | 39.8 | 85.4 |
| T-3 | 51.0 | 6.5 | 9.5 | 43.6 | 85.7 |
| Annigeri | 49.8 | 7.4 | 9.6 | 43.2 | 85.4 |
| BG-203 | 52.3 | 3.7 | 8.6 | 50.5 | 94.8 |
| P-5462 | 51.7 | 4.0 | 8.6 | 45.5 | 99.5 |
| Kabuli: | | | | | |
| K-4 | 51.6 | 3.1 | 5.7 | 46.1 | 89.3 |
| C-104 | 50.5 | 4.4 | 10.0 | 49.5 | 98.0 |
| Rabat | 52.1 | 4.4 | 7.3 | 46.8 | 90.0 |
| L-550 | 54.4 | 7.3 | 7.8 | 51.7 | 95.1 |
| GL-629 | 49.8 | 3.3 | 5.6 | 45.6 | 91.6 |
| Giza | 49.6 | 3.3 | 7.3 | 49.7 | 100.2 |
| No. 501 | 52.8 | 4.0 | 7.9 | 40.5 | 86.7 |
| Mean | 51.1 | 5.1 | 8.3 | 46.0 | 90.5 |
| SE ± | 2.4 | 0.2 | 0.3 | 3.0 | 6.5 |

[a] Units inhibited/g meal; [b] mg maltose released/g meal; [c] mg maltose released/g starch.

Reports are available to indicate that the partially purified kidney bean fractions inhibits the salivary amylase more than the pancreatic amylase. This shows that amylase inhibitors from different legume seeds may behave differently towards the enzyme. It has been

reported that inhibitors when supplied in large amounts, can overcome gastric digestion and inhibit α-amylase in man and other animals (Silano, 1977). However, when cooked starch is substituted for raw starch, α-amylase inhibitors are less effective in slowing down starch digestion.

Table 23: Correlation coefficients between amylase inhibitor and *in vitro* starch digestibility (IVSD) of 15 chickpea cultivars

| | | Correlation coefficients | | |
|---|---|---|---|---|
| | | IVSD (%) | | Starch content |
| | | b | c | (%) |
| IVSD : a | - | 0.642** | -0.016 | 0.203 |
| b | - | - | 0.435 | 0.154 |
| c | - | - | - | 0.182 |
| Amylase inhibitor activity[a] | -0.587* | -0.304 | 0.235 | -0.151 |

* and ** Significant at 5% and 1% level, respectively; [a] mg maltose released/g meal; [b] mg maltose released/g meal starch; [c] mg maltose released/g isolated starch; [d] pancreatic amylase.

Pancreatic amylase inhibitor is present in most of the legumes, but the highest inhibitor activity has been reported in kidney bean. The inhibitor activity in chickpea cultivars appeared to be considerably lower than those reported for other important food legumes. However, in well-cooked broad bean the inhibitor was reported to be completely inactivated at 100°C (Hernandex and Jaffe, 1968). We also observed the

complete inactivation of amylase inhibitors of a few chickpea cultivars when the extracts were boiled for 10 min. But the findings reported here suggest that in case of unheated chickpea meal, some inhibition of starch digestion by amylase inhibitors may be expected.

The starch digestibility was studied using pancreatic amylase. An increase in digestibility was observed with increasing periods of incubation upto 2 hr and thereafter no measurable changes were noticed. Therefore, for comparing the digestibility of cultivars, an incubation period of 2 hr was followed. The results expressed as mg maltose released/g meal and mg maltose released/g meal starch are reported in Tables 22. No large variations in the starch digestibility of meal was observed among these cultivars and apparently no large differences in the digestibility of meal starches were noticed between desi and kabuli types. However, the mean value for digestibility (mg maltose released/g meal starch) was slightly higher for kabuli than for desi types (Table 22). On the other hand, digestibility of isolated starch from kabuli types was found to be higher than desi types. Moreover, the digestibility of isolated starch was apparently higher than those of meal starch.

There appeared to be no relationship between the digestibility of meal starch and isolated starch of chickpea (Table 23). Perhaps, some interfering substances are present in meal samples and in higher concentration in desi as compared to kabuli ones. In order to confirm this hypothesis, determination of in-vivo digestibility of starch of these cultivars is required.

A statistically significant negative correlation, although of low magnitude, was obtained between the amylase inhibitor activity and digestibility of meal (Table 23) indicating that the digestibility of starch is adversely affected by the levels of amylase inhibitor. But there was no significant correlation between amylase inhibitor activity and digestibility of isolated starch. It is known that oligosaccharides such as raffinose and verbascose are present in considerable amount in several grain legumes. However, due to the nonsignificant differences in these oligosaccharides among the legumes, the observed differences in the α-amylosis of different legumes could not be explained on the basis of the presence of these oligosaccharides. Our results also revealed no relationship between the in-vitro starch digestibility and the stachyose and raffinose contents of chickpea.

Determination of amylase inhibitor activity (AIA) and in-vitro starch digestibility (IVSD) was accomplished in 18 cultivars of pigeonpea in order to know the variation among cultivars. Determinations were carried out in similar way as reported in case of chickpea. These cultivars were grown during 1976-77 and obtained from our breeding programme. Some variation in AIA appeared to exist among these cultivars.(Table 24). AIA was estimated by using pancreatic amylase and salivary amylase. Unlike chickpea, no differences were observed when the two enzymes were used for measuring the inhibitor activity. Generally, AIA was remarkably higher in pigeonpea than chickpea. Further studies are required to know the biochemical differences if any in the amylase inhibitors of chickpea and pigeonpea. As mentioned

earlier, inhibitors are mostly inactivated by heat treatment. We also conducted an experiment and did not detect AIA in the dhal samples of two cultivars boiled for 20 minutes. In order to confirm this more number of cultivars need to be used for assay of AIA.

IVSD of pigeonpea cultivars studied is shown in Table 24. IVSD expressed as mg maltose liberated per g sample ranged between 31.9 and 46.7 with the mean being 37.4 . A similar variation was observed when IVSD was expressed as mg maltose released/g of starch in the samples. It should be mentioned here that IVSD might have been influenced by some factors other than amylase inhibitors as the relationship between AIA and IVSD was not strong. Efforts should be made to carry out these studies in cooked and uncooked samples of more cultivars in order to confirm this results. In view of a large variation for IVSD among the cultivars in-vivo starch digestibility would be useful to know whether such differences exist.

Since pigeonpea is consumed as vegetable, AIA and IVSD of developing green seed (vegetable pigeonpea) and mature seed were studied using 8 genotypes of vegetable pigeonpea and one grain pigeonpea (C-11) as shown in Table 25. AIA of green seeds was considerably lower than those of the mature seeds and this was found to be the case in both vegetable and grain types. From nutrition point of view, these inhibitors will be of considerable interest where fresh green seeds are consumed, while they may not be of importance in countries where cooked green seeds are consumed because they are reported to be inactivated when heated at 100$^{o}$C. IVSD expressed as mg maltose released per g meal ranged between 50.9 and 56.9 with the mean being 53.1 for

green seed of vegetable pigeonpea whereas that of grain type was 51.7 (Table 25). Considerably lower values for IVSD were obtained when mature seed samples of these cultivars were analysed. The lower values for IVSD in mature seed of pigeonpea could possibly be due to two reasons: 1) higher levels of amylase inhibitors in mature seeds; 2) a more complex nature of starch-protein matrix, as a result of seed maturation. There is a need to carry out detailed studies to investigate this aspect in pigeonpea.

Table 24: Variation in amylase inhibitor activity (AIA) and in-vitro starch digestibility (IVSD) of pigeonpea (dhal) cultivars.

| Cultivar | | Starch (%) | Amylase inhibitors[a] | IVSD[b] |
|---|---|---|---|---|
| Early | UPAS-120 | 54.5 | 21.8 | 32.8 |
| | Pant-A-2 | 51.4 | 18.4 | 35.6 |
| | Prabhat | 54.9 | 13.5 | 36.7 |
| | T-21 | 54.2 | 17.5 | 34.0 |
| | DL-74-1 | 54.2 | 12.8 | 37.5 |
| Medium | C-11 | 57.6 | 15.3 | 40.6 |
| | No-148 | 54.2 | 20.5 | 43.5 |
| | Hy-3C | 58.2 | 23.5 | 38.7 |
| | ICP-1 | 54.8 | 18.0 | 36.0 |
| | BDN-1 | 52.9 | 17.5 | 34.0 |
| | PM-1 | 58.2 | 19.2 | 32.5 |
| | AS-71-37 | 56.9 | 14.5 | 38.9 |
| | ST-1 | 55.7 | 16.7 | 31.9 |
| Late | NP(WR)-15 | 54.7 | 13.9 | 39.0 |
| | Gwalior | 51.5 | 18.5 | 35.8 |
| | KWR-1 | 55.0 | 24.7 | 34.6 |
| | T-7 | 53.7 | 19.0 | 44.5 |
| | BDN-2 | 54.5 | 21.9 | 46.7 |
| | Mean | 54.8 | 18.2 | 37.4 |
| | SE + | 2.0 | 3.4 | 4.2 |

[a]Units inhibited/g meal; (pancreatic amylase)

[b]mg maltose/g meal using pancreatic amylase.

Table 25: Amylase inhibitors and *in-vitro* starch digestibility (IVSD) of green and mature seed of pigeonpea.

| Genotype | Green | | | Mature | | |
|---|---|---|---|---|---|---|
| | Starch (%) | Amylase[a] inhibitor | IVSD[b] | Starch (%) | Amylase[a] inhibitor | IVSD[b] |
| ICPL-102 | 51.0 | 19.3 | 56.9 | 51.3 | 26.4 | 35.9 |
| ICPL-114 | 48.7 | 15.9 | 53.7 | 52.7 | 30.1 | 37.3 |
| ICPL-119 | 50.3 | 16.5 | 52.6 | 54.2 | 23.5 | 36.5 |
| ICPL-122 | 46.8 | 16.4 | 51.5 | 54.0 | 28.5 | 40.1 |
| ICPL-128 | 46.6 | 18.5 | 54.3 | 50.8 | 34.2 | 38.6 |
| ICPL-212 | 48.9 | 18.4 | 50.9 | 53.6 | 22.5 | 32.4 |
| ICP-6997 | 47.8 | 15.9 | 52.6 | 53.6 | 25.8 | 36.9 |
| ICP-7035 | 48.4 | 17.4 | 53.4 | 51.2 | 24.5 | 33.7 |
| C-11 | 47.0 | 17.4 | 51.7 | 55.2 | 26.3 | 34.6 |
| Mean | 48.4 | 17.3 | 53.1 | 53.0 | 26.9 | 36.2 |
| SE± | 1.4 | 1.2 | 1.7 | 1.5 | 3.4 | 2.3 |

[a]Units inhibited/g meal; (pancreatic amylase) [b]mg maltose released per g meal.

6. Flatulence causing oligosaccharides of chickpea and pigeonpea:

Three oligosaccharides, raffinose, stachyose, and verbascose structurally different are known to cause flatulence when grain legumes containing these sugars are ingested in large quantities. It has been fairly established that the enzymes responsible for hydrolysis of these oligosaccharides are absent in human digestive system. Consequently these sugars escape digestion and pass on to the lower intestine where these are reacted upon by the flora (bacterial) of the intestine. As a result of this reaction various gases are

produced (carbon dioxide, hydrogen etc.) leading to flatulence. These complex sugars can be determined by several techniques. But in the present investigation, the techniques of paper chromatography and thin layer chromatography were used.

6.1 Estimation of oligosaccharides by paper chromatography:

Paper chromatography was followed to estimate raffinose, stachyose and verbascose in chickpea and pigeonpea. The chromatography technique was carried out using the solvents butanol-pyridine-water (5:1:4, v/v). The chromatogram was run for about 72 hr. The paper was removed, dried with hot air and marginal strips were cut off and sprayed with a solution of ammonical silver nitrate (Leslie, 1968). The strips were heated in an oven at 110°C until the dark spots indicating the position of the sugars appeared. With the aid of lines ruled on the central unsprayed portion of the chromatogram and using the developed spots on the marginal strips as indicators, sections of paper corresponding to raffinose and stachyose positions were cut from the central portion. The sugars from the strips were eluted with water and their concentrations estimated colorimetrically by the phenol-sulphuric acid method as described earlier.

Using this technique raffinose and stachyose were determined in 15 cultivars of chickpea. Desi and kabuli cultivars were used to find out if any difference exist between these two groups. Data on the concentrations of total soluble sugars and oligosaccharides in chickpea desi and kabuli cultivars are given in Table 26. While the percentages of soluble sugars in these cultivars did not differ consi-

derably, fairly large variations in stachyose and raffinose contents were observed among these cultivars.

Table 26: Stachyose and raffinose contents in dhal samples of 15 chickpea cultivars.

| Cultivar | Soluble sugars (%) | Stachyose | | Raffinose | |
|---|---|---|---|---|---|
| | | a | b | a | b |
| Kabuli | | | | | |
| K-4 | 4.57 | 0.82 | 17.94 | 0.36 | 7.87 |
| C-104 | 4.78 | 1.38 | 28.87 | 0.55 | 11.50 |
| Rabat | 5.15 | 1.19 | 23.10 | 0.56 | 10.87 |
| L-550 | 5.67 | 1.12 | 20.36 | 0.37 | 7.72 |
| GL-629 | 5.24 | 1.30 | 24.80 | 0.38 | 7.25 |
| Giza | 4.77 | 1.36 | 28.51 | 0.62 | 12.99 |
| No.541 | 4.68 | 0.95 | 20.29 | 0.36 | 7.69 |
| Desi | | | | | |
| USA-613 | 4.24 | 1.06 | 25.01 | 0.39 | 9.20 |
| 850-3/27 | 4.73 | 1.49 | 31.05 | 0.51 | 10.78 |
| Pant G-114 | 4.9 | 1.27 | 25.92 | 0.46 | 9.39 |
| CPS-1 | 4.04 | 1.08 | 26.73 | 0.36 | 8.91 |
| T-3 | 4.15 | 1.13 | 27.72 | 0.47 | 11.32 |
| Annegiri | 5.08 | 1.74 | 34.25 | 0.66 | 13.02 |
| BG-203 | 4.32 | 1.85 | 42.82 | 0.59 | 13.65 |
| P-5462 | 4.30 | 1.13 | 26.28 | 0.53 | 12.32 |
| Mean | 4.70 | 1.26 | 26.91 | 0.48 | 10.52 |
| SE ± | 0.12 | 0.03 | 1.58 | 0.04 | 0.55 |

[a]g/100g sample; [b]g/100g soluble sugars.

The stachyose content (g/100g meal) ranged between 1.06 and 1.85 with a mean value of 1.34 in desi cultivars and varied from 0.82 to 1.38 with a mean value of 1.16 in kabuli cultivars (Table 26). When the results of desi and kabuli were considered together, it was noticed that on average, stachyose accounts for 26.9 percent and raffinose content accounts for 10.5 percent of the total soluble sugars. These results are comparable with those of earlier workers who reported that in chickpea, stachyose and raffinose account for 27.3 and 7.7 percent of total soluble sugars, respectively (Lineback and Ke, 1975).

In order to know if any relationship exists between the oligosaccharides and total soluble sugars, correlation coefficients among these variables were calculated and the results are presented in Table 27. Total soluble sugars were not significantly correlated with either of these two oligosaccharides expressed either as g/100g sample or as g/100g soluble sugars. The present study gave enough indication that the concentration of these oligosaccharides is independent of the levels of total soluble sugars in these cultivars.

Table 27: Correlation coefficients between total soluble sugars, and stachyose and raffinose in 15 chickpea cultivars.

| Oligosaccharides | | Raffinose | | Stachyose | | Soluble sugars (%) |
|---|---|---|---|---|---|---|
| | | a | b | a | b | |
| Raffinose | a | - | 0.923** | 0.765** | 0.696** | 0.091 |
| | b | - | -- | 0.692** | 0.781** | -0.289 |
| Stachyose | a | - | -- | -- | 0.917** | 0.154 |
| | b | - | -- | -- | -- | -0.244 |

[a]g/100g sample; [b]g/100g soluble sugars; **Significant at 1% level.

On the other hand, stachyose and raffinose were positively and significantly correlated with each other when the results were expressed either as g/100g sample or as g/100g soluble sugars.

As mentioned earlier, the ingestion of large quantities of legumes is known to cause flatulence in experimental animals and humans due to the presence of these oligosaccharides. Germination or cooking of chickpea or mungbean do not greatly alter their flatus inducing capacity as compared to the raw forms. In view of the observed variations in the levels of oligosaccharides among the chickpea cultivars and their implication in human nutrition, it would be worthwhile to screen and select cultivars having lower amounts of these oligosaccharides.

6.2 Accumulation of oligosaccharides at different stages of chickpea seed maturation:

Efforts were made to study the accumulation of these oligosaccharides with reference to the levels of precursor sugars, fructose and sucrose in seed at different stages. The technique of paper chromatography was not found suitable for this purpose primarily because of the inability of the procedure to separate different sugars on the same chromatogram. The requirement of a longer run for separation of stachyose and raffinose resulted in the elution of glucose, fructose and sucrose from the same chromatogram. In view of this difficulty, the separation and quantification of various sugars was achieved by thin layer chromatography.

Thin layer chromatography (TLC) was carried out on Silica gel G plates. The plates of 500 u thickness were prepared and activated before use by heating at 105°C for 30 min. The developing solvent was chloroform, acetic acid and water (30:35:5, v/v). The separated sugars were detected by spraying with aniline-diphenylamine solution which was prepared by mixing 5 volumes of 1% aniline and 5 volumes of 1% diphenylamine in acetone with 1 volume of 85% phosphoric acid. Equal concentration of sugars were applied for each stage of maturity. The sugars in the extracts were determined according to the procedure described earlier. The separated sugars were scanned in a densitometer and the area of the peaks and their concentrations were estimated by comparing with their respective standard sugars that were run under similar conditions. Glucose and fructose did not separate well and therefore were considered together.

Two chickpea cultivars (desi, G-130 and kabuli L-550) with considerable difference in sugar levels were selected to study the accumulation of oligosaccharides at different stages of maturation. These cultivars were grown during 1978-79 under normal cultural practices in the experimental plots of the pulse physiology program at ICRISAT Center. The plants that flowered on the same day were chosen at random and the flowers were tagged. The samples at 14, 21, 28, 35, 42 and 49 days after flowering were collected and chilled in ice. Seeds were removed from the pods and seed and pod wall samples were freeze dried. The freeze dried samples were ground to pass through a 100-mesh

sieve. The ground samples were defatted in a Soxhlet apparatus using hexane.

Using thin layer chromatography technique it was possible to demonstrate the relative changes in the concentrations of fructose and glucose, sucrose, raffinose, stachyose and other unidentified oligosaccharides (the unidentified oligosaccharides were not measured). The sugars of pod wall at different stages could not be measured quantitatively as these sugars failed to resolve satisfactority by both the thin layer and paper chromatography techniques.

Fructose and glucose, and sucrose were the predominant sugars of the seeds in the early stages of maturity (Tables 28 & 29).

Table 28: The levels of oligosaccharides and soluble sugars of seeds at different stages of maturation of kabuli chickpea (cv L-550)[a].

| Sugars | Days after flowering | | | | | |
|---|---|---|---|---|---|---|
| | 14 | 21 | 28 | 35 | 42 | 49 |
| | .....(g/100g soluble sugars)..... | | | | | |
| Glucose + Fructose | 35.4 (1.5) | 13.7 (1.1) | 9.3 (1.0) | 4.5 (0.6) | nd | nd |
| Sucrose | 49.7 (2.2) | 32.2 (2.6) | 25.7 (2.7) | 21.6 (2.8) | 19.2 (2.4) | 19.6 (2.5) |
| Raffinose | nd | 2.8 (0.2) | 4.7 (0.5) | 9.3 (1.2) | 11.5 (1.4) | 12.7 (1.6) |
| Stachyose + Unidentified | nd | 10.4 (0.8) | 18.3 (1.9) | 30.5 (3.9) | 29.7 (3.7) | 31.5 (4.0) |

nd = not determined; [a]Values within parenthesis are expressed as mg/seed.

The concentration of these sugars slowly declined during the later stages of development in the case of both desi (G-130) and kabuli (L-550) cultivars. The raffinose and stachyose with the unidentified oligosaccharides were found to be absent in the samples obtained at 14 days after flowering in case of cv.L-550 and in the samples of 14 and 21 days after flowering in the case of cv.G-130. These oligosaccharides appeared in samples of 21 days after flowering and their concentration increased as the seed matured. A rapid increase in the concentration of stachyose and raffinose was noticed between 21 and 35 days after flowering. The increase in the concentration of oligosaccharides was accompanied by a decrease in the concentration of glucose and fructose and sucrose during the early stages of maturation and possibly these mono and disaccharides are utilized for the synthesis of oligosaccharides during the course of development.

Table 29: The levels of oligosaccharides and soluble sugars of seeds at different stages of maturation of desi chickpea (cv.G-130)[a].

| Sugar | Days after flowering | | | | | |
|---|---|---|---|---|---|---|
| | 14 | 21 | 28 | 35 | 42 | 49 |
| | .....(g/100g soluble sugars)..... | | | | | |
| Glucose + Fructose | 29.7 (1.1) | 23.3 (1.0) | 8.9 (0.5) | 4.4 (0.3) | nd | nd |
| Sucrose | 43.5 (1.6) | 43.0 (1.8) | 31.9 (1.7) | 24.4 (1.2) | 20.9 (1.1) | 20.4 (1.1) |
| Raffinose | nd | nd | 9.3 (0.5) | 11.8 (0.6) | 13.7 (0.7) | 13.5 (0.7) |
| Stachyose | | | 14.1 (0.8) | 29.5 (1.5) | 40.8 (2.1) | 39.9 (2.1) |
| Unidentified | nd | nd | | | | |

nd = not determined; [a]Values within parenthesis are expressed as mg/seed.

For separation and quantification of oligosaccharides of pigeonpea, again the technique of paper and thin layer chromatography were followed. We have also used paper chromatography to estimate stachyose and raffinose in several cultivars (Table 26). Verbascose was not determined in these cultivars as it was not available initially. Later on this oligosaccharide was obtained and estimated in some pigeonpea lines. The levels of stachyose and raffinose concentration were similar in chickpea as compared to pigeonpea.

6.3 Oligosaccharides of green and mature seed of pigeonpea:

Green and mature seed of vegetable pigeonpea and grain type (C-11) were analysed for various sugars. Eight lines of vegetable pigeonpea and one of grain type were selected for this study. These lines were grown on black soil at ICRISAT Center during the post rainy season of 1980-81. Raffinose, stachyose and verbascose were estimated by using the thin layer chromatography (TLC) technique. Using this technique it was possible to demonstrate the relative concentrations of these oligosaccharides of green and mature seeds. These oligosaccharides were studied in comparison with other sugars, glucose, fructose and sucrose. Glucose and fructose were estimated together as these sugars could not be resolved completely by TLC. Glucose fructose and sucrose were the predominant sugars in green seed. The concentration of these sugars declined and consequently those of oligosaccharides increased as the seed matured. Raffinose and stachyose were present in very low amount in green seeds whereas these were present in considerable amount in the mature seed (Table 30). Verbascose was not detected in the green seed while this was the predominant sugar in the

mature seed. This clearly indicates that these oligosaccharides are accumulated in the seed during the later stages of maturation. From utilization point of view, the consumption of pigeonpea as vegetable seems to be better in view of the lower amount of flatulence causing oligosaccharides in green seeds. Furthermore, the observed variation in the levels of oligosaccharides among pigeonpea lines suggest that attempts should be made to screen and select cultivars having lower amounts of these oligosaccharides.

Table 30: Oligosaccharides of green and mature seed of pigeonpea[a].

| Genotype | Green | | | Mature | | | |
|---|---|---|---|---|---|---|---|
| | Soluble sugars(%) | Raffinose | Stachyose | Soluble sugars(%) | Raffinose | Stachyose | Verbascose |
| ICPL-102 | 5.3 | 9.0 | 11.9 | 3.8 | 13.7 | 19.0 | 26.7 |
| ICPL-114 | 4.9 | 9.5 | 6.2 | 3.2 | 11.7 | 12.3 | 21.0 |
| ICPL-119 | 4.7 | 9.4 | 3.1 | 3.5 | 12.0 | 13.7 | 22.1 |
| ICPL-122 | 4.9 | 6.1 | 3.2 | 2.5 | 13.4 | 19.4 | 21.7 |
| ICPL-128 | 5.5 | 6.2 | 2.5 | 4.1 | 10.3 | 19.0 | 27.5 |
| ICPL-212 | 5.4 | 7.7 | 2.5 | 3.0 | 12.4 | 15.4 | 25.0 |
| ICP-6997 | 5.2 | 5.0 | 2.8 | 2.3 | 17.3 | 15.6 | 25.1 |
| ICP-7035 | 5.0 | 1.5 | 2.5 | 3.0 | 11.7 | 12.8 | 24.8 |
| C-11 | 4.9 | 1.3 | 2.0 | 2.9 | 13.7 | 14.2 | 25.8 |
| Mean | 5.1 | 6.2 | 4.1 | 3.1 | 12.9 | 15.7 | 24.4 |
| SE ± | 0.3 | 3.2 | 3.2 | 0.6 | 2.0 | 2.8 | 2.3 |

[a]Expressed as g/100g soluble sugars; Verbascose was not detected in green seeds.

7. Polyphenols of chickpea and pigeonpea:

Polyphenols (loosely termed as tannins) have been the subject of several investigations from nutrition point of view in the past. Polyphenols of pigeonpea and chickpea have not received much attention of the nutri-

tionists. These compounds were studied and results are summarised under the following headings. Chickpea cultivars used for this work were grown at Hissar, during the post rainy season of 1977-78 excepting 67 genotypes the results of which are given in Table 34 were grown at ICRISAT Center during the same year by Genetic Resources Unit. All pigeonpea cultivars were grown at ICRISAT Center.

7.1 Extraction and estimation of polyphenols:

The polyphenolic compounds were extracted from defatted meal (500 mg) by refluxing with 50 ml of methanol containing 1% HCl for 2 hr. The extract was concentrated in a rotary flash evaporator and brought to a known volume with acidified methanol for quantitative estimation and with distilled water for enzyme inhibition study. The effect of duration of refluxing on extraction of polyphenolic compounds was also studied by refluxing for 1, 2, 3 and 4 hr as above. In order to study the effect of different solvents on extraction, the polyphenolic compounds were extracted using distilled water, acetone, methanol, and methanol-HCl by refluxing. Methanol-HCl extraction was also carried out at room temperature 25$^{o}$C for comparison. The amount of total polyphenolic compounds in the extracts obtained was estimated as tannic acid equivalent according to the Folin-Denis procedure (Swain and Hillis, 1959). Tannins were also extracted and estimated in chickpea and pigeonpea. Finely ground sample (500 mg) was taken in a 50 ml conical flask and dispersed in 25 ml methanol and stoppered. Flasks were gently shaken and allowed the extraction overnight (24 hr) at constant temperature (32$^{o}$C). After extraction period contents were filtered and

1 ml of the solution was pipetted into duplicate test tubes. Five milliliter of vanillin-HCl solution (equal volumes of 4% vanillin in methanol and 8% concentrated HCl in methanol) was added and the colour thus developed was read in the colorimeter at 500 mu. Tannins were estimated as catechin equivalents (Price et al. 1980).

The results illustrating the effect of different solvents on the extraction of total polyphenolic compounds are presented in Table-31. The boiling acidified methanol (methanol-HCl) had a remarkable effect on the extractability of these compounds in case of both chickpea and pigeonpea. Extraction of polyphenols was significantly higher in case of refluxing as compared to the extraction at room temperature in the same solvent.

Table 31: Effect of different solvents on the extraction of seed polyphenolic compounds of chickpea and pigeonpea

| Solvent[a] | Chickpea (G-130) | Pigeonpea (C-11) |
|---|---|---|
| | .....Polyphenols (mg/g)..... | |
| Acetone | 0.62 ± 0.05[c] | 0.87 ± 0.04[c] |
| Methanol | 0.28 ± 0.01 | 0.43 ± 0.02 |
| Water | 2.34 ± 0.08 | 3.06 ± 0.06 |
| Methanol-HCl[b] | 3.60 ± 0.07 | 5.14 ± 0.05 |
| Methanol-HCl | 6.18 ± 0.07 | 14.23 ± 0.07 |

[a]Extraction by refluxing for 2 hr; [b]Extraction at 25°C for 16 hr;

[c]Standard error of estimation based on six determinations.

Higher extraction of these compounds as a result of heat treatment in acidic conditions could be attributed to the polymeric nature of flavonoids which generate anthocyanidins as degradation product when they are heated in acid solution. The value for polyphenolic compounds was also considerably higher when water was used as a solvent in comparison with methanol and acetone solvents. But this might have been the result of extraction of some proteinous substances which also give Folin-Denis positive reaction. Table 32 shows the effect of durations of extraction using methanol-HCl by refluxing. The extraction of polyphenols increased up to 2 hr and thereafter no measureable differences were observed. Extraction of polyphenols using methanol-HCl by refluxing for 2 hr was observed to be satisfactory and therefore used in the present study.

7.2 Distribution of polyphenols in whole seed, dhal and seed coat components of pigeonpea and chickpea:

In order to know the relative contribution of different components of pigeonpea and chickpea seeds, the polyphenolic compounds were estimated in whole seed, dhal and seed coat samples. The mean value of polyphenolic compounds (mg/g) in desi seed was more than twice the amount that was present in desi dhal while a comparison of mean values between kabuli seed and dhal showed no such differences (Table 33). This observation could be related to the variability in the seed coat percentages and the colour of seed coat in desi and kabuli cultivars. This was confirmed by analysing the dhal, whole seed and seed coat samples for polyphenolic compounds which showed that seed coat contributed to about 75% of total polyphenolic compounds of

seed in desi cultivars. Similar results were obtained when the polyphenolic compounds of whole seed, dhal and seed coat samples of pigeonpea cultivars were compared (Table-33). The analysis of four pigeonpea cultivars with different seed coat colours showed that the seed coat contained the highest proportion of polyphenols and brown seed appears to have a higher concentration of polyphenols than white seed. These studies clearly indicated that polyphenolic compounds are mostly located in the seed coat and they are highly associated with the intensity of pigmentation in seed coat. This finding is particularly important in those areas where pigeonpea and chickpea are consumed as whole seeds and this will be more so where dark seeded cultivars of these crops are grown and consumed.

Table 32: Effect of different durations of extraction on polyphenolic compounds of chickpea and pigeonpea[a].

| Extraction (hr) | Chickpea (G-130) | Pigeonpea (C-11) |
|---|---|---|
| | .Polyphenols (mg/g) | |
| 1 | 4.80 ± 0.05[b] | 12.20 ± 0.06[b] |
| 2 | 6.18 ± 0.04 | 14.23 ± 0.04 |
| 3 | 5.86 ± 0.06 | 14.18 ± 0.07 |
| 4 | 6.05 + 0.05 | 14.25 ± 0.07 |

[a]Extraction by refluxing in Methanol-HCl;

[b]Standard error of estimation based on six determinations.

Table 33: Distribution of polyphenolic compounds in whole seed, dhal and seed coat of chickpea.

| Cultivar | Seed colour | Seed coat (%) | Whole seed | Dhal | Seed coat |
|---|---|---|---|---|---|
| | | | ..Polyphenols (mg/g).. | | |
| Chickpea: | | | | | |
| CPS-1 (D) | Brown | 16.9 | 4.30 | 2.10 | 14.34 |
| T-3 (D) | Light brown | 13.9 | 4.42 | 2.14 | 15.01 |
| BG-203 (D) | Light brown | 16.8 | 4.53 | 1.75 | 13.92 |
| Rabat (K) | Salmon white | 6.7 | 2.18 | 2.07 | 2.30 |
| L-550 | Salmon white | 5.7 | 1.74 | 1.70 | 1.98 |
| Giza (K) | Salmon white | 6.1 | 1.94 | 1.92 | 2.54 |
| Pigeonpea: | | | | | |
| BDN-1 | Brown | 15.2 | 15.10 | 1.89 | 106.87 |
| C-11 | Light brown | 15.7 | 14.23 | 1.70 | 92.28 |
| NP(WR)-15 | White | 16.4 | 6.04 | 1.45 | 37.19 |
| HY-3C | White | 13.0 | 3.74 | 1.60 | 27.04 |

D = Desi, K = Kabuli.

7.3 Relationship between tannins and total phenolic compounds:

Tannins are generally described as water-soluble high molecular weight polyphenols which precipitate proteins from solutions. These compounds are condensation products of flavan-3-ols and flavan-3, 4-diols, thus they give positive reaction with vanillin. Tannins were estimated by vanillin-HCl method in several cultivars of chickpea and pigeonpea and results were compared with those of the polyphenolic compounds. In case of chickpea, 67 cultivars representing different

group based on seed coat colour were analysed and results are summarised in Table 34 and detailed in Appendix 1. The results of tannins and phenolic compounds of pigeonpea cultivars are shown in Table 35. No relationship between tannins and total polyphenolic compounds was observed in both chickpea and pigeonpea. Unlike total polyphenolic compounds, tannin contents of chickpea and pigeonpea were not associated with the seed coat colour. Tannin contents of most of the chickpea and pigeonpea cultivars were very low whereas they were not detected in a number of cultivars. It may be mentioned here that vanillin-HCl method will not detect tannin in the samples which are low in tannins. This method is mostly used to distinguish between tannin and nontannin polyphenols. Hence, the present studies indicate that chickpea and pigeonpea seed contain large amounts of nontannin polyphenols where as tannin polyphenols are present in negligible amount.

Table 34: Relationship between seed colour, polyphenolic compounds and tannins in chickpea

| Seed colour | No. of cultivars | Tannins[b] | | Polyphenolic compounds | |
|---|---|---|---|---|---|
| | | Range | Mean | Range | Mean |
| | | | | (mg/g) | |
| Salmon white | 10 | 0.14-0.29 | 0.23 | 1.94-2.88 | 2.36 |
| Very light brown | 7 | 0.18-0.28 | 0.23 | 1.88-3.60 | 2.66 |
| Light brown | 14 | nd-0.15 | 0.08 | 4.22-5.28 | 4.67 |
| Brown | 11 | nd-0.14 | 0.09 | 3.29-5.58 | 4.43 |
| Dark brown | 11 | nd-0.14 | 0.07 | 4.05-5.63 | 5.15 |
| Black | 13 | 0.09-0.14 | 0.10 | 5.22-7.08 | 6.15 |
| Green | 1 | 0.07 | 0.07 | 5.69 | 5.69 |
| Total | 67 | nd-0.29 | | 1.88-7.08 | |

[a]Analysis of whole seed sample; nd = not detected.

Table 35: Relationship between seed coat colour, polyphenolic compounds and tannins in pigeonpea.

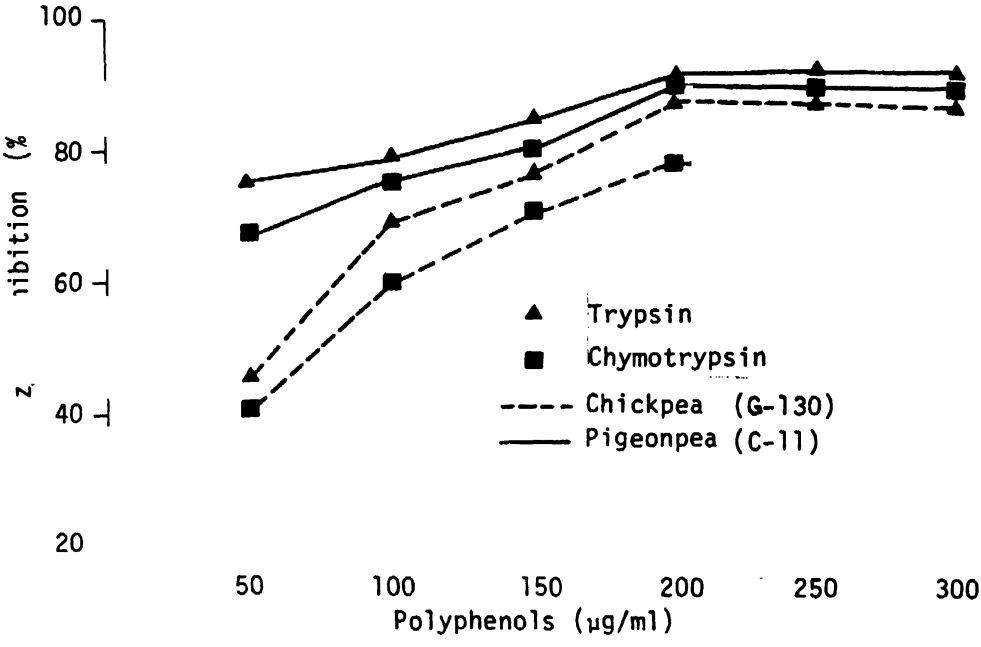
| Cultivar | Seed coat colour | Tannins[a] | Polyphenolic compounds |
|---|---|---|---|
| | | .....(mg/g)..... | |
| NP(WR)-15 | White | 0.19 | 6.04 |
| HY-3C | White | 0.54 | 3.74 |
| HY-2 | White | 0.12 | 4.85 |
| ST-1 | Light brown | 0.00 | 8.93 |
| KWR-1 | Light brown | 0.08 | 7.45 |
| Gwalior-3 | Light brown | 0.23 | 7.20 |
| T-7 | Light brown | 0.54 | 8.45 |
| T-17 | Light brown | 0.48 | 9.34 |
| UPAS-120 | Brown | 0.12 | 13.54 |
| Prabhat | Brown | 0.08 | 12.94 |
| T-21 | Brown | 0.06 | 14.04 |
| BDN-1 | Brown | 0.15 | 15.10 |
| Mukta | Brown | 0.00 | 13.45 |
| C-11 | Brown | 1.02 | 14.23 |
| No.-148 | Dark brown | 0.91 | 16.70 |
| ICP-1 | Dark brown | 0.09 | 15.90 |
| DL-74-1 | Dark brown | 0.23 | 16.84 |
| Mean | | 0.32 | 11.10 |
| SE$\pm$ | | 0.04 | 0.25 |

[a]Expressed as catechin equivalent

7.4 The inhibition of digestive enzymes by polyphenols:

The trypsin, chymotrypsin and amylase are the enzymes responsible for the digestion of proteins and carbohydrates which are the principal constituents of the diet.

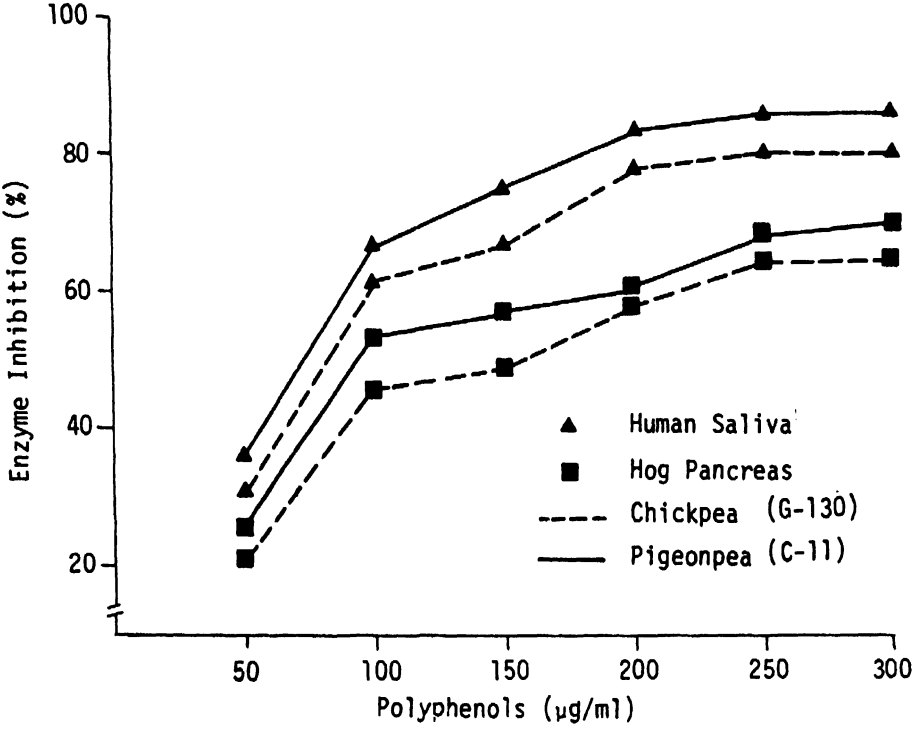
FIGURE 1: EFFECT OF DIFFERENT CONCENTRATIONS OF POLYPHENOLS ON TRYPSIN AND CHYMOTRYPSIN INHIBITION IN CHICKPEA AND PIGEONPEA.



The inhibition of these enzymes by polyphenols of pigeonpea and chickpea was assayed. The activities of these enzymes were assayed according to the procedures described earlier (Singh and Jambunathan 1981a and 1982). For salivary amylase the human saliva was collected and diluted about five fold in 0.02 calcium phosphate buffer, pH 6.8. After standing overnight at 5°C the mixture was centrifuged at 10,000 x g for 15 min and the supernatant was used for the assay. Chickpea and pigeonpea seed extracts containing polyphenolic compounds were added in the reaction mixture. Percent enzyme inhibition was determined by comparing the reduction in activity resulting from the addition of extract with that produced in the absence of any inhibitor. Experiments were conducted to study the effect of polyvinyl pyrrolidone (PVP), the tannin complexing agent on the enzyme inhibition. Seed extract containing phenolic compounds were treated with PVP (10% w/v) for 30 min at room temperature (25°C). PVP treated extracts were used for enzyme inhibition as described above.

The inhibition of trypsin, chymotrypsin and amylase enzymes (human saliva and hog pancreas) was studied using different concentrations of polyphenols of chickpea (cv.G-130) and pigeonpea (cv.C-11). Percent enzyme inhibition values for trypsin and chymotrypsin increased with increasing concentration of polyphenols up to 200 ug/ml of reaction mixture and thereafter remained constant (Figure I). In case of amylase, inhibition increased up to a concentration of 250 ug/ml of reaction mixture and additional amounts of polyphenols had no noticeable effect (Figure 2).

FIGURE 2: EFFECT OF DIFFERENT CONCENTRATIONS OF POLYPHENOLS ON AMYLASE INHIBITION IN CHICKPEA AND PIGEONPEA.



7.4.1 Effect of polyvinylpyrrolidone (PVP) on enzyme inhibition:

The use of PVP treated extract in the experiment indicated that presence of PVP increased the enzyme activity to a large extent (Table 36) However, the complete reversal of enzyme activity was not achieved even in presence of higher concentration of PVP in the extract indicating the presence of some other compounds which inhibit the enzyme activity but were not inactivated by PVP. The amount of such an inhibition for this enzyme differed considerably in the presence of PVP. The temperature of extraction of polyphenols had a considerable effect on enzyme inhibition. When the polyphenolic compounds extracted by refluxing were used enzyme inhibition was more than those extracted at room temperature (Table 36) implicating the qualitative differences in the polyphenolic compounds extracted by different procedures.

Table 36: Effect of polyvinylpyrrolidone (PVP) and methods of extraction of polyphenols on enzyme inhibitory activity of polyphenols of chickpea and pigeonpea[a]

| Enzyme | Methanol-HCl[b] | | | | Methanol-HCl[c] | |
|---|---|---|---|---|---|---|
| | Chickpea | | Pigeonpea | | Chickpea | Pigeonpea |
| | Control | +PVP | Control | +PVP | ..... Control ..... | |
| | .Enzyme Inhibition (%) | | | | | |
| Trypsin | 88.7 | 13.4 | 91.5 | 14.6 | 80.7 | 86.5 |
| Chymotrypsin | 79.0 | 12.3 | 90.3 | 11.0 | 70.6 | 81.4 |
| Amylase: | | | | | | |
| I. human saliva | 80.3 | 17.8 | 86.0 | 18.6 | 71.5 | 77.8 |
| II. hog pancreas | 64.5 | 12.5 | 80.9 | 15.3 | 60.7 | 62.3 |

[a]Results are averages of two independent assays; [b]Extraction by refluxing (boiling); [c]Extraction at room temperature (25°C).

7.4.2 Effect of seed coat colour on enzyme inhibition:

An experiment was conducted to study the effect of seed coat colour on enzyme inhibition using cultivars with different seed coat colours (Table 37). Polyphenols of white and brown chickpeas and pigeonpeas revealed striking differences in their enzyme inhibitory properties. Enzyme inhibition was highest in cultivar with brown seed coat colour, and lowest in cultivar with white seed coat colour. The larger differences were observed for amylase (hog pancreas) enzyme in comparison with trypsin and chymotrypsin enzymes in case of pigeonpea. Noticeable differences were also observed among the cultivars representing different testa colour (Table 37).

Table 37: Varietal differences in the enzyme inhibitory property of polyphenols of chickpea and pigeonpea.

| Cultivar | Seed colour | Polyphenols (mg/g) | Trypsin | Chymo-trypsin | Amylase | |
|---|---|---|---|---|---|---|
| | | | | | Human saliva | Hog pancreas |
| | | | .....Enzyme inhibition (%)..... | | | |
| Chickpea | | | | | | |
| Rabat | Salmon white | 1.9 | 33.6 | 26.3 | 29.8 | 17.5 |
| L-550 | Salmon white | 2.3 | 34.5 | 25.7 | 31.5 | 20.8 |
| Pant G-114 | Light brown | 5.3 | 86.4 | 72.5 | 73.4 | 56.9 |
| G-130 | Brown | 5.8 | 88.7 | 79.0 | 80.3 | 64.5 |
| USA-613 | Brown | 6.1 | 81.6 | 70.9 | 78.6 | 61.0 |
| Mean | - | 4.8 | 65.0 | 54.9 | 58.7 | 44.1 |
| SE ± | - | 0.1 | 1.8 | 1.6 | 1.7 | 1.5 |
| Pigeonpea | | | | | | |
| Hy-3c | White | 3.7 | 37.9 | 36.0 | 34.5 | 21.8 |
| NP(WR)-15 | White | 6.0 | 40.5 | 38.6 | 32.7 | 19.7 |
| C-11 | Light brown | 14.2 | 91.5 | 90.3 | 86.0 | 80.9 |
| BDN-1 | Brown | 15.2 | 90.3 | 91.6 | 79.4 | 69.3 |
| No.148 | Brown | 14.9 | 88.0 | 85.9 | 75.8 | 68.5 |
| Mean | - | 10.8 | 69.7 | 68.5 | 61.7 | 52.0 |
| SE + | - | 0.2 | 2.1 | 1.7 | 1.4 | 1.3 |

In conclusion, it may be mentioned that chickpea polyphenols showed higher inhibitory activity towards trypsin than chymotrypsin whereas pigeonpea polyphenols did not show such a difference. Further, the polyphenols of pigeonpea were found to be more effective than those of chickpea. Both chickpea and pigeonpea extracts showed higher inhibitor activities towards salivary amylase than pancreatic amylase. The addition of PVP to chickpea and pigeonpea extracts considerably reduced their inhibitor effects towards these enzymes.

## 7.5 Effect of processing practice on the polyphenols of chickpea and pigeonpea:

Like other antinutritional factors, the effect of some traditional processing practices on the polyphenols of chickpea and pigeonpea was studied. Most commonly followed practices of boiling and soaking in water and salt solutions were studied (Table 38). The boiling of chickpea and pigeonpea seeds for 20 min in distilled water removed a large amount of polyphenols in case of both chickpea and pigeonpea. More polyphenols were removed in case of cultivars having brown seed coat colour. The process of soaking was also found very effective in removing the polyphenols from seed. This indicate these polyphenols are water soluble and thus could be discarded in boiling and soaking water. Soaking in salt solutions removed more polyphenols than soaking in distilled water except in case of chickpea cultivars P-5462 where such a response was not observed. Soaking in sodium chloride and sodium bicarbonate solutions did not reveal large differences. These studies indicate that seed polyphenols of chickpea and pigeonpea could be eliminated to a large extent by following simple

procedure of soaking and boiling.

Table 38. Effect of soaking and boiling on the polyphenolic compounds of pigeonpea and chickpea

| Crop | Control | Soaking | | | Boiling[a] |
|---|---|---|---|---|---|
| | | Water | NaCl (1%) | $NaHCO_3$ (1%) | |
| | ..... Polyphenolic compounds (mg/g) ..... | | | | |
| Pigeonpea | | | | | |
| BDN-1 | 15.10 | 6.43 | 4.54 | 4.42 | 5.96 |
| C-11 | 14.23 | 6.90 | 4.03 | 4.20 | 6.40 |
| NP(WR)-15 | 6.04 | 2.84 | 2.10 | 2.14 | 2.13 |
| Chickpea | | | | | |
| USA-613 | 6.10 | 2.04 | 1.38 | 1.40 | 3.04 |
| P-5462 | 3.15 | 0.72 | 0.71 | 1.12 | 1.80 |
| L-550 | 1.43 | 0.88 | 0.39 | 0.58 | 1.21 |

[a] Boiled in distilled water for 20 min and water discarded;

[b] Soaked for 16 hr at room temperature (25°C) and water and salt solutions discarded.

8. Phytic acid content of chickpea and pigeonpea:

The ability of phytic acid to form a complex and reduce the availability of some important minerals and trace elements particularly calcium and zinc is a problem of general concern to nutritionists. Minerals from cereals, legumes and other plants in contrast to minerals from animal sources, are in general poorly utilized by man. This problem is more serious in diets containing plant proteins.

The phytic acid in chickpea and pigeonpea was determined according to the method described by Makower (1970) using 5% (w/v) trichloroacetic acid (TCA) for extraction. Total phosphorus was determined colorimetrically according to

the procedure described earlier using Technicon auto analyser (TAA, 1972). The studies on phytic acid content and phytase activity as a result of germination of chickpea and pigeonpea seeds were conducted.

Total phosphorus and phytic acid were determined in several cultivars of chickpea and pigeonpea (Table 39). Phytic acid phosphorus was calculated and results indicated that phytic acid represents 75.0-85.3 and 65.3-77.5 percent of total phosphorus in chickpea and pigeonpea, respectively. Phytic acid content of whole seed and dhal samples of chickpea and pigeonpea was estimated. More phytic acid was observed in case of whole seed samples of cultivars with brown seed colour as compared to those with white seed colour and this might have been due to seed coat interference (Table 40). The results indicated that most of the phytic acid is present in the dhal and this may be disadvantageous from consumption point of view.

Table 39. Relationship between phytic acid and total phosphorus in pigeonpea and chickpea[a]

| | Total Phosphorus | Phytate Phosphorus | Phytic acid | Phytate P as % of total P |
|---|---|---|---|---|
| | .....(mg/g)..... | | | |
| Pigeonpea: | | | | |
| UPAS-120 | 4.9 | 3.2 | 14.6 | 65.3 |
| Pant A-2 | 5.0 | 3.5 | 16.4 | 70.6 |
| BDN-1 | 4.8 | 3.7 | 15.9 | 77.5 |
| Mukta | 4.0 | 3.0 | 13.6 | 73.6 |
| NP(WR)-15 | 3.4 | 2.3 | 10.6 | 67.4 |
| Chickpea: | | | | |
| L-550 | 4.6 | 3.7 | 17.1 | 81.2 |
| ICC-4 | 4.5 | 3.8 | 17.8 | 85.3 |
| Annigeri | 4.0 | 3.0 | 14.1 | 75.0 |
| G-130 | 3.8 | 2.9 | 13.6 | 76.3 |
| BG-203 | 4.6 | 3.6 | 16.8 | 78.3 |

[a] Analysis of defatted dhal.

Table 40: Phytic acid content of whole seed and dhal samples of pigeonpea and chickpea cultivars with different testa colour.

| Cultivar/line | Seed colour | Phytic acid (mg/g) | |
|---|---|---|---|
| | | Whole seed | Dhal |
| Pigeonpea: | | | |
| C-11 | Brown | 15.75 | 12.42 |
| Hy-3c | White | 10.94 | 10.06 |
| Chickpea: | | | |
| G-130 | Brown | 15.75 | 13.50 |
| L-550 | White | 12.53 | 12.61 |

8.1 Effect of heating on phytic acid content:

Tables 41 and 42 show the effect of cooking on the phytic acid content. Also the results of analysis of several pigeonpea and chickpea cultivars are given in these tables. Phytic acid content of chickpea cultivars varied from 10.53 to 18.75 with mean being 15.0 mg/g (Table 41) and pigeonpea cultivars from 10.0 to 15.81 with mean being 13.57 mg/g (Table 42). This indicated that phytic acid of chickpea is slightly higher than pigeonpea. When pressure cooked, the phytic acid content of pigeonpea noticeably decreased whereas such differences were not observed in chickpea. Cooking process considerably decreased the phytic acid content of pigeonpea. The mean values for uncooked and cooked samples were 13.57 and 8.88 mg/g, respectively.

In case of chickpea, cooking process did not appear to play an important role in the levels of phytic acid. It seems phytic acid is degraded as a result of heat treatment in pigeonpea but not in chickpea. However, it is difficult to offer an explanation for such a difference. But the involvement of some chemical constituents cannot be ruled out in this context.

Table 41: Effect of heating on the phytic acid content of chickpea dhal[a]

| Cultivar/line | Phytic acid (mg/g) | |
|---|---|---|
| | Cooked | Uncooked |
| Annigeri | 13.75 | 13.93 |
| L-550 | 13.06 | 13.00 |
| ICC-24 | 17.81 | 18.13 |
| ICC-25 | 13.75 | 14.38 |
| ICC-26 | 10.53 | 10.31 |
| Annigeri | 14.06 | 14.69 |
| H-208 | 17.50 | 17.69 |
| Pant G-114 | 15.94 | 15.39 |
| ICC-4 | 15.81 | 15.94 |
| BDN-93 | 10.50 | 10.31 |
| PT-26 | 18.75 | 18.75 |
| BG-21 | 16.44 | 17.50 |
| K-850 | 15.31 | 14.69 |
| G-130 | 13.63 | 13.31 |
| C-235 | 17.06 | 17.06 |
| G-543 | 15.94 | 17.19 |
| BG-203 | 16.75 | 16.48 |
| H-76-49 | 14.06 | 14.38 |
| Mean | 15.00 | 15.13 |
| SE ± | 2.30 | 2.43 |

[a]Pressure cooked for 15 min and whole content used.

Table 42: Effect of cooking on phytic acid content of pigeonpea dhal[a].

| Cultivar/line | Phytic acid (mg/g) | |
|---|---|---|
| | Uncooked | Cooked |
| ICPH-6 | 12.50 | 9.06 |
| ICPL-234 | 14.88 | 8.75 |
| ICPL-270 | 14.69 | 9.06 |
| HY-8 | 15.81 | 8.75 |
| GS-2 | 13.14 | 8.75 |
| No.148 | 14.08 | 9.06 |
| K-64 | 15.00 | 8.75 |
| PDA-12 | 10.31 | 8.75 |
| BDN-2 | 14.38 | 9.06 |
| MAUL-175 | 13.13 | 8.91 |
| ICPH-2 | 13.63 | 9.06 |
| ICPH-5 | 10.00 | 8.91 |
| AS-71-37 | 13.31 | 8.13 |
| JA-5 | 13.63 | 8.91 |
| LRG-30 | 14.30 | 9.06 |
| LRG-36 | 14.06 | 8.91 |
| BDN-1 | 14.88 | 9.06 |
| C-11 | 12.50 | 8.75 |
| Mean | 13.57 | 8.88 |
| SE ± | 1.53 | 0.23 |

[a]Pressure cooked for 15 min and whole content used.

8.2 Phytase activity and phytic acid as influenced by germination:

The soaking followed by sprouting is a common process followed in case of several grain legumes. Although these processes are variably followed for chickpea and pigeonpea in India, efforts were made to study

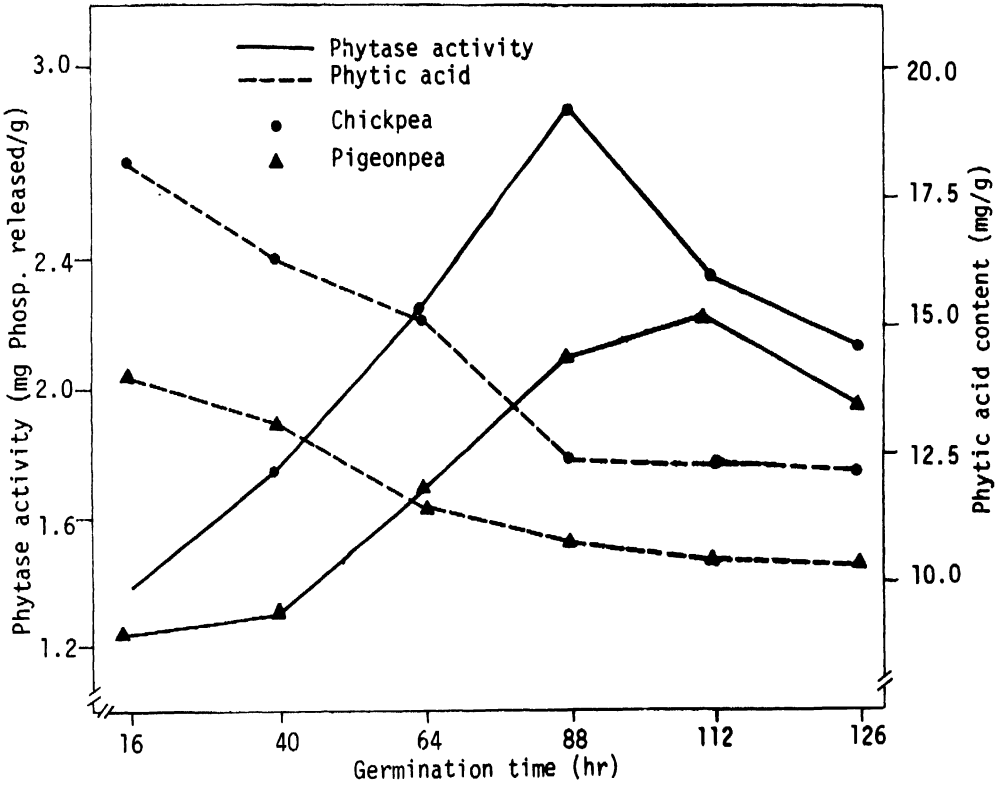
the effect of sprouting on phytic acid content and phytase activity.

Chickpea and pigeonpea seeds were first soaked in distilled water for 4 hr at room temperature. Then the seeds were placed on sheets of filter paper and allowed to germinate for different durations as given in Figure 3. After the germination period, seed coat was removed manually and germinated cotyledons along with radicles were taken for analysis of phytic acid and phytase activity. For enzyme extraction, cotyledons (1 g) were ground with mortar and pestle in 20 ml of 0.01 M maleate buffer pH 6.5 and phytase activity was measured by the release of O-phosphate from phytic acid according to the procedure described by Walker (1974). The reaction was carried out at 37°C for 2 hr. Phosphorus thus liberated was measured on the basis of reduction of the ammonium molybdiphosphate complex by ascorbic acid in the presence of antimony (Watanabe and Olsen, 1965). All the results of phytase activity and phytic acid were expressed on fresh weight basis. Phytase activity was defined as the amount of enzyme that liberated 1 mg of phosphorus under the assay conditions described.

The results of such studies are given in Figure 3 for chickpea and pigeonpea. The germination process was very slow in case of pigeonpea and this could be due to the nature of seed coat. Like other grain legumes, phytase activity increased with increasing duration of germination and this was more pronounced in case of chickpea (Figure 3). As expected the phytase activity was closely associated with a decreasing trend in the levels of phytic acid.

To summarise the results on phytic acid, it may be mentioned that most of the seed phosphorus is present in the form of phytic acid.

FIGURE 3. EFFECT OF GERMINATION ON PHYTIC ACID AND PHYTASE ACTIVITY IN CHICKPEA AND PIGEONPEA.



Phytic acid content of chickpea is slightly higher than pigeonpea. Cooking brought about a considerable reduction in the levels of phytic acid of pigeonpea whereas such a response was not observed in chickpea. Germination will also have beneficial effects in terms of reducing the levels of phytic acid in both chickpea and pigeonpea.

## REFERENCES


1. Buchmann, N.B. 1979. *In vitro* digestibility of protein from barley and other cereals. Journal of the Science, Food and Agriculture 30:583-589.

2. Garwood, D.L., Shannon, J.C. and Green, R.G. 1976. Starches of endosperms possessing different alleles at the amylose extender of locus in Zea Mays L. Cereal Chemistry 53:355-364.

3. Granum, P.E. 1978. Purifucation and characterization of an α-amylase inhibitor from rye (*Secale cereale*) flour.

4. Hernandex, A. and Jaffe, W.G. 1968. Inhibitor de la amilasa pancreatica en caraotas (*Phaseolus vulgaris*). Acta Cient Venez 19: 183-189.

5. Hsu, H.W., Vavak, D.L. and Satterlee, L.D. 1977 . A multienzyme-technique for estimating protein digestibility. Journal of Food Science, 42:1269-1273.

6. Jaffe, W.G., Raquol Moreno and Valentilla Wallis, 1973. Amylase inhibitors in legume seeds. Nutrition Reports International 7:169-173.

7. Kakade, M.L., Swenson, D.H. and Liener, I.E. 1969. An evaluation of natural vs synthetic substrates for measuring antitryptic activity of soybean samples. Cereal Chemistry 46:518-526.

8. Kakade, M.L., Swenson, D.H. and Liener, I.E. 1970. A note on the determination of chymotrypsin and chymotrypsin inhibitor activity using casein. Analytical Biochemistry 33:255-258.

9. Leslie, H. 1968. Analysis of mixtures of sugars by paper and cellulose column chromatography. Methods of Biochemical Analysis 1:205-240.

10. Lineback, D.R. and Ke, C.H. 1975. Starches and low molecular weight carbohydrates from chickpea and horse bean flours. Cereal Chemistry 52:334-342.

11. Lowry, O.H., Rosebrough, N., Farr, A.L. and Randall, R.J. 1951. Protein measurement with Folin-phenol reagent. Journal of Biological Chemistry 193:265-270.

12. Makower, R.U. 1970. Extraction and purification of phytic acid in beans (*Phaseolus vulgaris*). Cereal Chemistry 47:288-295.

13. Mauron, J. 1970. Nutritional evaluation of proteins by enzymatic methods. In - Evaluation of novel protein product, ed. A.E. Bander, Pergamon Press, New York. p. 211.

14. Price, M.L., Hagerman, A.E. and Butler, L.G. 1980. Tannin content of cowpeas, chickpeas, pigeonpeas, and mungbeans. Journal of Agriculture and Food Chemistry 28:459-461.

15. Silano, V. 1977. Biochemical and nutritional significance of wheat albumin inhibitors of α-amylase. Cereal Chemistry 55:722-731.

16. Singh, U. and Jambunathan, R. 1981a. Studies on desi and kabuli chickpea (*Cicer arietinum* L.) cultivars. The levels of protease inhibitors, levels of polyphenol compounds and *in vitro* protein digestibility. Journal of Food Science 46:1364-1367.

17. Singh, U. and Jambunathan, R. 1981b. Protease inhibitors and *in vitro* protein digestibility of pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives. Journal of Food Science and Technology 18:246-247.

18. Singh, U. and Jambunathan, R. 1982. Studies on desi and kabuli chickpea (*Cicer arietinum* L.) cultivars. The levels of amylase inhibitors, levels of oligosaccharides and *in vitro* starch digestibility. Journal of Food Science 47:510-512.

19. Singh, U. and Jambunathan, R. 1983. Changes in starch, oligosaccharides and soluble sugars in developing pod wall and seed of chickpea. Phytochemistry 21:297-299.

20. Swain, T. and Hillis, W.E. 1959. The phenolic constituents of *Prumus domestica*. 1. The quantitative analysis of phenolic constituents. Journal of the Science, Food and Agriculture 10:63-68.

21. Technicon Auto Analyser. Industrial Method No. 144.71A. Phosphorus in food products, 1972.

22. Walker, K.A. 1974. Changes in phytic acid and phytase during early development of *Phaseolus vulgaris* L. Planta 116:91-98.

23. Watanabe, F.S. and Olsen, S.R. 1965. Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil. Soil Science Society Proceedings 96:677-678.

## Appendix I

The levels of total phenolic compounds and tannins in chickpea

| S.No. | ICC # | Pedigree | Color | Phenolic compounds | Tannins (catechin eq. |
|---|---|---|---|---|---|
| | | | | .....(mg/g)..... | |
| 1. | 2400 | P-2173-1 | Salmon white | 2.28 | 0.23 |
| 2. | 2584 | P-2591 | " | 2.60 | 0.14 |
| 3. | 2593 | P-2602 | " | 2.31 | 0.25 |
| 4. | 2767 | P-2940 | " | 2.88 | 0.25 |
| 5. | 4973 | L-550 | " | 2.28 | 0.29 |
| 6. | 4985 | NP-34 | " | 2.28 | 0.18 |
| 7. | 8923 | K-1189 | " | 2.34 | 0.19 |
| 8. | 8924 | K-1258 | " | 2.56 | 0.25 |
| 9. | 8284 | HYB-16.31 | " | 1.94 | 0.29 |
| 10. | 10035 | - | " | 2.13 | 0.23 |
| 11. | 1164 | P-1081-1 | Very light brown | 2.22 | 0.26 |
| 12. | 2524 | P-2422 | " | 2.82 | 0.26 |
| 13. | 2526 | P-2422-2 | " | 3.60 | 0.28 |
| 14. | 2828 | P-3010 | " | 2.56 | 0.18 |
| 15. | 5316 | M-1 | " | 1.88 | 0.20 |
| 16. | 5320 | M-2XM-3 | " | 2.13 | 0.18 |
| 17. | 8358 | HY-13-4 | " | 3.43 | 0.26 |
| 18. | 596 | P-472 | Light brown | 5.16 | 0.15 |
| 19. | 788 | P-623 | " | 5.28 | 0.01 |
| 20. | 1990 | P-1610 | " | 4.80 | 0.15 |
| 21. | 2021 | P-1630 | " | 4.25 | 0.08 |
| 22. | 2204 | P-1774 | " | 4.46 | 0.07 |
| 23. | 3718 | P-4341-2 | " | 4.22 | nd |
| 24. | 4934 | Chafa | " | 4.54 | 0.07 |
| 25. | 4951 | JG-62 | " | 4.94 | 0.09 |
| 26. | 7745 | NEC-44 | " | 4.88 | 0.09 |
| 27. | 8316 | Chafa 8-16 | " | 4.29 | 0.13 |
| 28. | 8319 | ChikodiNu | " | 4.68 | 0.07 |

| S.No. | ICC # | Pedigree | Colour | Phenolic compounds | Tannins (catachin eq.) |
|---|---|---|---|---|---|
| 29. | 10131 | CPS-2 | Light brown | 4.66 | 0.07 |
| 30. | 10956 | RPSP-344 | " | 4.96 | nd |
| 31. | 10969 | RPSP-355 | " | 4.22 | 0.07 |
| 32. | 1669 | P-1387 | Brown | 3.57 | nd |
| 33. | 1810 | P.1469-2 | | 3.29 | 0.09 |
| 34. | 4700 | P-6292 | " | 4.42 | 0.06 |
| 35. | 5002 | WF WG III x 816-140-164 | " | 3.89 | 0.13 |
| 36. | 5003 | 850-3/27 | " | 4.79 | 0.10 |
| 37. | 5434 | Ponaflar-2 | " | 4.73 | 0.09 |
| 38. | 7688 | 1-81-19 | " | 3.29 | 0.07 |
| 39. | 7689 | 1-209-15 | " | 5.58 | 0.07 |
| 40. | 10070 | Coll-120 | " | 5.03 | 0.14 |
| 41. | 10071 | Coll-120-1 | " | 5.28 | 0.09 |
| 42. | 10966 | RPSP-352 | " | 5.07 | 0.11 |
| 43. | 431 | P-317 | Dark brown | 4.73 | nd |
| 44. | 535 | P-416 | " | 5.11 | nd |
| 45. | 1030 | P-861 | " | 4.98 | 0.09 |
| 46. | 1127 | P-1022 | " | 5.31 | 0.09 |
| 47. | 2042 | P-1642-1 | " | 4.63 | nd |
| 48. | 5780 | F-3 Parmar 4-14 | " | 4.60 | nd |
| 49. | 6118 | JG-109 | " | 5.34 | 0.14 |
| 50. | 6119 | JG-110 | " | 4.05 | nd |
| 51. | 10955 | RPSP-343-1 | " | 6.60 | nd |
| 52. | 10961 | RPSP-348 | " | 5.63 | 0.07 |
| 53. | 10965 | RPSP-351-1 | " | 5.63 | nd |
| 54. | 2396 | P-2170 | Black | 6.23 | 0.09 |
| 55. | 3594 | P-4265 | " | 7.08 | 0.09 |
| 56. | 3616 | P-4278-2 | " | 6.66 | 0.09 |
| 57. | 3792 | P-4412-1 | " | 6.73 | 0.10 |
| 58. | 3820 | P-4446-1 | " | 5.89 | 0.11 |
| 59. | 3822 | P-4449-1 | " | 6.37 | 0.09 |

| S.No. | ICC # | Pedigree | Color | Phenolic compounds | Tannins (catachin eq.) |
|---|---|---|---|---|---|
| 60. | 3832 | P-4459 | Black | 5.62 | 0.12 |
| 61. | 3850 | P-4500 | " | 5.66 | 0.09 |
| 62. | 3859 | P-4515 | " | 5.48 | 0.10 |
| 63. | 3866 | P-4528 | " | 7.06 | 0.14 |
| 64. | 4004 | P-4706 | " | 6.31 | nd |
| 65. | 4404 | P-5384 | " | 5.66 | 0.13 |
| 66. | 5810 | Harigantas | " | 5.22 | 0.09 |
| 67. | 4957 | Hema | Green | 5.69 | 0.07 |

nd = not detected.