Chapter 3

Material and Methods

3.1. Material

3.1.1. Pulse seeds

The certified seeds of three kidney bean cultivars (*Phaseolus vulgaris* L. cv. French Yellow, Contendor, Master Beans) were procured from Shere-Kashmir University of Agricultural Sciences and Technology, Shalimar, Srinagar, J&K, India whereas Local Red from 2006 harvest was procured from the local market of Srinagar, J&K, India. Seeds of two mash bean cultivars (*Phaseolus mungo* L. cv. PU-19 and T-9) were procured from National Seed Corporation, New Delhi whereas cultivar Mash 1-1 was procured from Department of Plant Breeding & Genetics, Punjab Agriculture University, Ludhiana, Punjab, India. Seeds were cleaned from the dirt, foreign material etc and stored until further use at 20 ºC. Moisture content of kidney beans was in the range 11.0 – 13.6% while mash beans had 9.6 – 10.0%.

3.1.2. Chemicals

Sulphuric acid, copper sulphate, potassium sulphate, sodium hydroxide, acetic anhydride and dimethyl sulphoxide were obtained from Quailagens Fine Chemicals, Mumbai, India. Boric acid, hydrochloric acid, phenolphthalin indicator, methyl red indicator, ethanol were obtained from Merk Specilities Private Limited, Mumbai, India. Hexane, toluene, sodium dodecyl sulphate, potassium hydroxide, papain enzyme were obtained from Sisco Research Laboratories, Mumbai, India. Amylose and amylopectin were obtained Central Drug House, Mumbai, India. Trinitro-benzene-sulphonic acid (TNBS) was procured from Hi Media Laboratories, Mumbai, India. All the reagents used in the study were of analytical grade.

3.2. Methods

3.2.1. Whole grain

3.2.1.1. Chemical composition

Moisture (925.10), protein (984.13), fat (920.85), ash (923.03) and crude fiber (962.09) contents were determined according to standard methods of *AOAC* (1990).
3.2.1.1.1. Moisture

Aluminium moisture dish (55 mm diameter and 15 mm height with inverted tightly fit cover) was dried at 130 ± 3 °C, cooled in air tight desiccator (having reignited CaO) and weighed soon after reaching to room temperature. Sample (2 g) was weighed in previously weighed moisture dish. Moisture dish was uncovered and placed in hot air oven (with an opening for ventilation) at 130 ± 3 °C for 1 h. After one hour the lid was placed on the moisture dish in the oven and transferred to air tight desiccators, allowed to cool to room temperature and weighed soon after reaching to room temperature. The loss in weight of sample was recorded as moisture content of sample on weight upon weight basis.

\[
\text{Moisture (\%) = \frac{\text{Loss in weight of sample upon drying (g)}}{\text{Weight of sample (g)}} \times 100}
\]

3.2.1.1.2. Protein

Sample (0.5 g db) was weighed into 500 mL Kjeldhal digestion flask. 1.5 g potassium sulphate (K₂SO₄), 0.04 g anhydrous copper sulphate (CuSO₄), 0.5 g alundum (8 - 14 mesh boiling stones) and 20 mL sulphuric acid (98% N free) were added to the flask. Digestion flask was heated on the heating mantle and allowed to heat at 5 min boil rate (burner preheated and adjusted to bring 250 mL water at 25 °C to boil in 5 min) until white fumes clear bulb of flask. Digestion flask was swirled gently and allowed to heat for additional 90 min. Digestion flask was cooled to room temperature; 250 mL double distilled water was added to it and allowed to cool again. 15 mL of standard HCl (0.5 N) and 3 – 4 drops of methyl red indicator (1% sodium salt of methyl red in methanol) were put in 250 mL Erlenmeyer flask. The flask was placed under the condenser with its tip immersed in standard HCl. The digested sample in Kjeldhal flask was transferred to distillation apparatus and 40 mL of NaOH (11.25 N) was added to it and allowed to mix. It was immediately distilled at 7.5 min boil rate until 150 mL distillate was collected in titration flask (Erlenmeyer flask containing standard acid). Blank (reagent) digestion and distillation was carried out simultaneously. Excess of standard acid (0.5N HCl) was titrated with standard NaOH (0.1N) in sample as well as in blank and nitrogen was calculated as:

\[
N(\%) = \frac{[(N_{\text{Acid}}) (V_{\text{Acid}}) - (N_{\text{Base}}) (V_{\text{Blank}}) - (N_{\text{Base}}) (V_{\text{Base}})][1400.67]}{\text{Weight of sample (g)}}
\]
Where $N_{\text{Acid}}$ is normality of standard acid (0.5 N HCl), $V_{\text{Acid}}$ is volume (mL) of standard acid (HCl) used for the sample, $N_{\text{Base}}$ is normality of standard NaOH, (0.1 N), $V_{\text{Blank}}$ is volume (mL) of standard base (0.1 N) needed to titre 1 mL standard acid (0.5 N) minus volume (mL) of standard base needed to titre reagent blank.

Crude protein was calculated by multiplying % N by the factor 6.25.

3.2.1.1.3. Fat

5 g of ground dried sample (dried to constant weight at 100 °C under pressure of $\leq 100$ mm Hg) were weighed into paper extraction thimble (Whatman) and was covered with light layer of cotton. The thimble was placed in soxhlet extractor and connected with condenser from the top end and with pre weighed fat collection flask from the lower end. Extraction with petroleum ether was carried for 16 h at condensation rate of 2 – 3 drops/second. Petroleum ether was distilled off allowing only 10 – 15 mL of petroleum ether in the fat collection flask. Extract was dried in the flask at 100 °C for 30 min, cooled and weighed. Fat content was calculated as

$$\text{Fat} (\%) = \frac{\text{Weight of dried extract (g)}}{\text{Weight of sample (g)}} \times 100$$

3.2.1.1.4. Ash

Porcelain ashing dish was ignited, cooled in desiccators and weighed. Sample (5 g) was taken in pre-weighed ashing dish and ignited at 550 ± 10 °C for 6 h. It was then cooled in air tight desiccators (having reignited CaO) and weighed as soon as it reached room temperature. Ash content was calculated as

$$\text{Ash} (\%) = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \times 100$$

3.2.1.1.5. Crude fiber

Crude fiber is loss on ignition of dried residue remaining after digestion of sample with 1.25% H$_2$SO$_4$ and 1.25% NaOH solutions under specific conditions. Ground sample (2 g) was extracted with petroleum ether (boiling point 35 – 60 °C) to remove lipids for one hour. Extracted sample was transferred to 600 mL reflux beaker and 200 mL of near boiling 1.25% H$_2$SO$_4$ solution was added to it. Beaker was transferred to digestion apparatus and boiled under reflux conditions exactly for 30 min with intermittent rotation to keep solids
from adhering to sides. At the end of the H₂SO₄ digestion, sample was filtered under vacuum (25 mm Hg) through Buchner funnel fitted with 200 mesh screen. The sample residue remaining on filtration was washed with four 40 – 50 mL portions near boiling water and filtered after each washing. Residue from funnel was washed into reflux beaker with near boiling 1.25% NaOH solution. Beaker was placed on digestion apparatus and allowed to boil under reflux for 30 min. After NaOH digestion, sample was filtered under vacuum (25 mm Hg) through fritted glass crucible with coarse porosity. The sample residue remaining on filtration was washed with 25 mL near boiling 1.25% H₂SO₄ and then with two 25 – 30 mL portions near boiling water and filtered after each washing. Crucible with washed residue was dried at 130 ± 2 °C for 2 h for 3 h, cooled in a dessicator and weighed. Dried sample in crucible was asched for 2 h at 550 ± 2 °C, allowed to cool in muffle furnace to ≤ 250 °C. Crucible was taken from muffle furnace, cooled in dessicator and weighed. Crude fiber was calculated as:

\[
\text{Crude fiber (\%) = } \frac{\text{Loss in weight on ignition (g)}}{\text{Weight of sample (g)}} \times 100
\]

3.2.1.2. Colour of seeds

The surface colour was measured using Ultra Scan VIS Hunter Lab (Hunter Associates Laboratory Inc., Reston VA., USA). A glass cell containing pulse seeds was placed against the light source, covered with a black cover and ‘L’, ‘a’, and ‘b’ colour values were recorded. The instrument was calibrated with black and white tile before colour measurement. The ‘L’ value indicates the lightness, 0 – 100 representing dark to light. The ‘a’ value gives the degree of the red – green colour with a higher positive ‘a’ value indicating more red. The ‘b’ value indicates the degree of the yellow – blue colour, with a higher positive ‘b’ value indicating more yellow.

3.2.1.3. Physical properties of seeds

3.2.1.3.1. Length, width and thickness

Ten randomly selected seeds were used to measure length (L), width (W) and thickness (T), three principal dimensions which are in the three mutually perpendicular directions using a Vernier caliper reading to 0.01 mm. Average of ten determinations was reported.
3.2.1.3.2. Equivalent diameter

The geometric mean diameter, \( D_m \), was calculated using the following relationship (Mohsenin, 1970). Average of ten determinations was reported.

\[
D_m = (LWT)^{1/3}
\]

3.2.1.3.3. Sphericity

The sphericity (\( \Phi \)) was calculated as a function of the three principal dimensions as shown below (Mohsenin, 1970). Average of ten determinations was reported.

\[
\Phi = ([LWT]^{1/3}/L) \times 100
\]

3.2.1.3.4. Aspect ratio

The aspect ratio (\( R_a \)) of seeds was calculated as follows (Hauhouout-O’hara et al., 2000; Omobuwajo et al., 1999) and reported as average of ten determinations.

\[
R_a = W/L
\]

3.2.1.3.5. Seed volume

The volume, \( V \) (mm\(^3\)), of the seeds was calculated using the relationship (Mohsenin, 1970). Average of ten determinations was reported.

\[
V = \frac{\pi B^2 L^2}{6(2L - 3)}
\]

Where \( B = (WT)^{1/2} \)

3.2.1.3.6. Surface area

The surface area, \( A \) (mm\(^2\)), of the seeds was calculated using the relationship (Mohsenin, 1970) and reported as average of ten determinations.

\[
A = \frac{\pi BL^2}{2L - B}
\]

3.2.1.3.7. Bulk density

For the determination of bulk density, a 50 mL graduated cylinder previously tarred was gently filled up to the mark with seeds. The sample was then packed by gently tapping the cylinder on the bench top from a height of 5 cm until there was no further diminution of
the sample level after filling to the 50 mL mark. The weight of the filled cylinder was taken and the bulk density calculated as the weight of sample per unit volume of sample (g/mL).

3.2.1.3.8. True density

True density is the weight per unit volume of individual seed. True density of seeds was determined using the liquid displacement method. Toluene (C₇H₈) was used as it is absorbed by seeds to a lesser extent than water. Besides it has low surface tension so that it fills even shallow dips in a seed and its dissolution is low (Mohsenin, 1980).

3.2.1.3.9. Porosity

The porosity (ε) of the bulk is the ratio of spaces in the bulk to its bulk volume and was determined by the following equation

\[ \varepsilon = 100\left[1 - \left(\frac{P_b}{P_k}\right)\right] \]

where ε is the porosity in percentage; \( P_b \) is bulk density in g/mL and \( P_k \) is seed density in g/mL (Nimkar & Chattopadhyay, 200; Mohsenin, 1970).

3.2.1.3.10. Angle of repose

The angle of repose (θ) of seed was determined by a cylindrical tube (smallest diameter 45 mm, biggest diameter 80 mm and height 100 mm) having discharge gate at the bottom. After filling the tube with seed sample, the gate was quickly removed. The height (ht) of seed pile above the floor and the radius of the heap (r) were measured and used to determine the angle of response.

\[ \theta = \tan^{-1}\left(\frac{ht}{r}\right) \]

3.2.1.3.11. Static coefficient of friction

The static coefficient of friction (µ) was determined for three different structural materials, namely, mica ply, glass and polyethylene according to the method of Gezer et al. (2002). For this measurement one end of the friction surface was attached to an endless screw. The seed was placed on the surface and it was gradually raised by the screw. Vertical and horizontal height values were read from the ruler when the seed started sliding over the surface, then using the tangent value of that angle the coefficient of static friction was found.

\[ \mu = \tan \phi \]

Where µ is the static coefficient of friction and φ is the angle of tilt in degrees.
3.2.1.3.12. Husk content

Parameter was measured by manual husk removal. A sample (10 g) of seed was soaked in 50 mL water at room temperature (20 °C) overnight. Water was drained and the husk was removed manually. Husks and cotyledons were dried separately in an oven at 70 °C overnight and allowed to cool in dessicator for 1 h. Husk was weighed and calculated as percent of seed mass.

3.2.1.3.13. Hundred seed weight and volume

One hundred seeds were manually counted and then weighed on a digital weighing balance with accuracy upto 0.001 mg.

Seed volume was determined by counting one hundred seeds manually and putting them in 50 mL graduated cylinder. 20 mL of double distilled water was added to it. Seed volume (mL) was determined as:

\[
\text{Hundred seed volume} = \text{Total volume} - 20 \text{ mL}
\]

3.2.1.4. Cooking properties of pulse seeds

3.2.1.4.1. Swelling capacity and swelling index

The volume of 100 g of seeds was predetermined using a graduated cylinder and then soaked overnight in distilled water. The volume of the seeds after soaking was then measured. Swelling capacity (mL/seed) and the swelling index were determined (Adebowale et al., 2005) as follows:

\[
\text{Swelling capacity} = \frac{\text{Volume after soaking (mL)} - \text{Volume before soaking (mL)}}{\text{Number of seed}}
\]

\[
\text{Swelling index} = \frac{\text{Swelling capacity of seed (mL)}}{\text{Volume of one seed (mL)}}
\]

3.2.1.4.2. Hydration capacity and hydration index

Seeds (100 g) were counted and soaked in 100 mL of distilled water for 12 h at room temperature (20 ± 2 °C), drained and wiped with tissue paper. The weight of the swollen seeds was measured. Hydration capacity (g/seed) and hydration index were calculated (Adebowale et al., 2005) as follows:
3.2.1.4.3. Cooking time of seeds

A laboratory hotplate was used to maintain uniform and constant temperature during cooking time determination. 200 mL of distilled water was brought to boil in 500 mL spoutless beaker fitted with bulb condenser to prevent loss of water during cooking. 20 g of seeds from each cultivar were separately added to them. Boiling was continued, and samples (4–5 seeds) were withdrawn using a spatula at 5 min intervals up to 30 min and thereafter after every 2 min and tested for softness by pressing between finger and thumb as described by Singh et al. (1984). The time from addition of seeds till achievement of the desirable softness was recorded as the cooking time.

3.2.1.4.4. Gruel solid loss

20 g of seeds were cooked in 200 mL of double distilled water for minimum cooking time. The gruel was transferred to 500 mL beakers and then evaporated till completely dried in an oven at 110 °C. The solids were subsequently weighed and gruel solid loss was calculated as percentage.

3.2.1.4.5. Cooked length-breadth ratio

The cumulative length and breadth of 10 seeds was measured after cooking for minimum cooking time. The length-breadth ratio was determined by dividing the cumulative length with the cumulative breadth of cooked seeds.

3.2.1.4.6. Water uptake ratio of cooked seeds

Pulse seeds (20 g) were cooked in 200 mL of double distilled water for minimum cooking time. The cooked seeds were then removed, drained and surface water on seeds was removed by filter paper. The samples were weighed and the water uptake ratio was calculated as the ratio of weight gained after cooking to weight before cooking.
3.2.1.5. Texture profile analysis of seeds

Texture of the soaked/cooked seeds was analyzed by using a Texture Analyzer (Model XT2i; Stable Micro Systems Ltd., Surrey, England) using 50 kg load cell. Seeds were placed at its natural rest position on the heavy duty platform of the texture analyzer and texture profile analysis (TPA) test was performed with a disc probe of 75 mm diameter for 70% of compression at a test speed of 2.0 mm min\(^{-1}\). Hardness, cohesiveness, gumminess, springiness, chewiness and adhesiveness were calculated from the TPA curve as described by Bourne (1978). At least 10 seeds were analyzed for each cultivar.

![Diagram of Texture Profile Analysis](image)

A typical texture profile analysis curve

3.2.2. Pulse flour

3.2.2.1. Flour preparation

To produce kidney and mash bean flour the whole seeds were milled in a laboratory mill (Newport Supermill-1500, Newport Scientific Pvt. Ltd, Warriewood, Australia). The resulting flour was then passed through 60-mesh screen and stored in airtight containers at refrigerated temperature until used.
3.2.2.2. Physicochemical properties of flour

3.2.2.2.1. Composition

Composition of legume flours was determined according to the methods described in section 3.2.1.1.

3.2.2.2.2. Bulk density

Bulk density was measured as a ratio of mass to volume using 10 mL graduated cylinder according to the method described in 3.2.1.3.7.

3.2.2.2.3. Swelling & solubility index

Swelling and solubility index of the flours were determined using 2% (w/v db) aqueous suspension of flour at 90 °C by the method of Leach et al. (1959). Flour (0.2 g db) was taken in pre-weighed centrifuge tubes with 10 mL of distilled water. The flour suspensions were then incubated in a water bath at 90 °C for 30 min with shaking after every 5 min. After cooling the samples to room temperature, the tubes were centrifuged at 3000 × g for 15 min. Supernatant containing soluble matter was decanted in pre-weighed moisture dishes. The gain in weight of centrifuge tubes was expressed as swelling index. Moisture dishes were dried at 110 °C to constant weight and then cooled in a desiccator to room temperature. The weight of residues in moisture dishes was expressed as solubility index.

\[
\text{Swelling index (g/g) } = \frac{\text{Increase in weight of empty centrifuge tube (g)}}{\text{Weight of sample (g)}}
\]

\[
\text{Solubility index (%) } = \frac{\text{Weight of residues in moisture dish upon drying (g)}}{\text{Weight of sample (g)}} \times 100
\]

3.2.2.2.4. Syneresis

Flour suspensions (2%, w/v db) were heated at 90 °C for 30 min in a water bath (TC-500, Brookfield Engineering Laboratories Inc., Massachusetts, USA.) with constant stirring. The flour sample was stored for 1, 2 and 5 days at 4 °C in separate centrifuge tubes for each day. Syneresis was measured as percentage amount of water released after centrifugation at 3000 × g for 10 min (C-24, Remi Laboratory Instruments, Mumbai, India).

3.2.2.2.5. Colour

Colour was determined according to the methods described in section 3.2.1.2.
3.2.2.6. **Scanning electron microscopy**

The flour was placed on an adhesive tape attached to a circular aluminium specimen stub and then coated vertically with gold-palladium. The samples were photographed at an accelerator potential of 10 kV using a scanning electron microscope (JSM-6100, JEOL Ltd., Tokyo, Japan).

3.2.2.3. **Pasting properties**

Pasting properties of the flours were measured using a Rapid Visco analyzer (RVA-4, Newport Scientific Pty Ltd., Warriewood, Australia). An aqueous dispersion of flour ~14% moisture basis (12.28%, w/w; 28.5 g total weight) was equilibrated at 50 °C for 1 min, heated at the rate of 12.2 °C/min to 95 °C, held for 2.5 min, cooled to 50 °C at the rate of 11.8 °C/min and again held at 50 °C for 2 min. A constant paddle rotational speed (160 rpm) was used throughout the entire analysis, except for rapid stirring at 960 rpm for the first 10 s to disperse the sample.

![A typical pasting curve](image)

A typical pasting curve
3.2.2.4. Flour gel texture

Textural properties of the flour gels were evaluated with slight modifications to the methods of Singh et al. (2006). The textural properties of RVA gels were evaluated by carrying out texture profile analysis (TPA) on a Texture Analyser (TA-XT plus; Stable Micro Systems, Surrey, UK). The flour pastes formed in the canister by RVA testing were poured into cylindrical plastic tubes (20 mm diameter, 40 mm deep). After cooling at room temperature (25 °C) for 1 h, the tubes were covered and then stored at 4 °C for 24 h. The gel formed in the tube was used directly for TPA. Each gel sample was penetrated (to a depth of 10 mm) with a cylindrical probe 6 mm in diameter. Force-time curves were obtained at a crosshead speed of 0.5 mm s\(^{-1}\) during two penetration cycles. From the texture profile curve, hardness, cohesiveness, adhesiveness, springiness, gumminess and chewiness were calculated. TPA was performed on gels prepared in triplicate for each sample.

3.2.2.5. Thermal properties

Thermal properties of kidney and mash bean flours were analyzed using Differential Scanning Calorimeter-DSC (200 PC-Phox, Phoenix Netzsch, Burlington, Germany) equipped with a thermal analysis data station. A 10 mg sample was weighed into a 40 µl capacity aluminium pan and 20 µL distilled water was added with the help of Hamilton micro syringe. Pans were hermetically sealed and allowed to stand for 1 h at room temperature before heating in DSC. The DSC was calibrated using indium and an empty aluminum pan was used as reference. Sample pans were heated at a rate of 10 °C/min from 20 to 180 °C and thermal parameters viz. onset (T\(_o\)), peak (T\(_p\)), conclusion (T\(_c\)) temperature and enthalpy (\(\Delta H\)) were calculated from the DSC curves.

3.2.2.6. Functional properties of pulse flours

3.2.2.6.1. Protein solubility

Protein solubility was determined in the pH range 2 – 10 using 0.1% w/v flour aqueous dispersion. The suspensions were adjusted to the desired pH, solubilised by shaking at 210 rpm at 25 °C in a shaking incubator (LSI-3016R, Daihan Lab Tech Co., Ltd., Namyangju, Kyonggi, Korea) for 60 min and then centrifuged at 12000 × g for 10 min. The protein content of the supernatant was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard. Briefly 0.2 mL of protein solution was
taken and 2 mL of alkaline copper sulphate reagent (mixing 100 mL of alkaline reagent – 1:1 of 2% sodium carbonate and 0.1 N NaOH, with 2 mL copper reagent - 1:1 of 1.56% copper sulphate and 2.37% sodium potassium tartarate) was added and the solution was incubated at room temperature for 10 min). 0.2 mL of Folin Ciocalteau solution (0.1 N) was then added and sample was incubated for 30 min and the absorbance was measured at 540 nm.

3.2.2.6.2. Water and oil absorption capacity

To determine water and oil absorption capacity, 1g (db) of sample was weighed into 25 mL pre-weighed centrifuge tubes and then stirred into 10 mL of double distilled water or refined soyabean oil (Amrit Banaspati Co. Ltd., Rajpora, India) for one minute. The samples were allowed to stand for 30 min and then centrifuged at 2200 × g for 30 min. The water or oil released on centrifugation was drained. Water or oil absorption capacity was expressed as gram of water or oil held per gram of flour sample.

3.2.2.6.3. Foaming capacity and stability

Aqueous dispersions (2% w/v db) of the flour at pH 2, 4, 6, 8 and 10 were homogenized in 250 mL measuring cylinder with a high speed homogenizer (Remi Instruments Division, Vasai, India) at 10000 rpm for 1 min. Foaming capacity was calculated as the percent increase in volume of the flour dispersion upon mixing. The foam stability was determined by measuring the foam volume with time.

3.2.2.6.4. Emulsifying properties

Emulsifying properties were determined by the turbidimetric method of Pearce and Kinsella (1978). Refined soybean oil (2 mL) and 6 mL of 0.2% protein solution (pH 3, 5 and 7) were homogenized in a mechanical homogeniser at 10000 rpm for 1 min. 50 µL of emulsion were pipetted from the bottom of the container into 5 mL of SDS solution (0.1% w/v) at 0 and 10 min after homogenisation. Absorbance of the solution was read at 500 nm with spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan). The absorbance measured immediately ($A_0$) and 10 min ($A_{10}$) after emulsion formation were used to calculate the emulsifying activity index (EAI, m$^2$/g) and the emulsion stability index (ESI, min).

$$EAI = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \varphi \times \theta \times 10000}$$
\[
\text{ESI} = \frac{A_0}{A_0 - A_{10} \times 10}
\]

Where DF is the dilution factor (100), c is the initial concentration of protein (g/ mL), \( \phi \) is the optical path (0.01), \( \theta \) is the oil volume fraction of the emulsion (0.25) and \( A_0 \) and \( A_{10} \) the absorbance of the emulsion at 0 and 10 min, respectively.

3.2.3. Pulse starch

3.2.3.1. Starch isolation

One kilogram of the sample was soaked in 4 L of distilled water and kept at 4 °C for 12 h. Coats of the seeds were removed by manual abrasion. The cotyledons were pulverized along with water for 5 min in a mixer blender. The slurry obtained was then diluted to ten times (volume/volume) with distilled water and the pH was adjusted to 10 using 0.5M NaOH. The slurry was continuously mixed on magnetic stirrer for one hour, and then filtered through a 75 µm mesh sieve to separate the fiber. The filtered slurry was then centrifuged at 3000 \( \times \) g for 30 min at 10 °C (C-24 BL, Remi Industries Mumbai, India). The aqueous phase obtained on centrifugation was collected for the recovery of proteins, whereas the sediment obtained was scraped off from the surface and the lower white portion was washed with distilled water three times and allowed to sediment at refrigerated temperature (4 °C). This sediment was recovered as starch. The starch was dried at 40 °C in a hot air oven (NSW-143, Narang Scientific Works Pvt. Ltd., New Delhi, India).

3.2.3.2. Acetylation of starch

Acetylation of native starch was carried out following the method of Wang & Wang (2002) with slight modifications. Starch (100 g db) was dissolved in distilled water (185 mL) to make 35% slurry. pH of the slurry was adjusted with 1 M NaOH to 8.0 – 8.5 and then mechanically stirred for 30 min. Acetic anhydride (4 g for 4% acetylation and 8g for 8% acetylation ) was added drop wise to the slurry while maintaining at pH 8.0 – 8.5. The reaction was continued for 60 min before acidifying to pH 5.5 with 1M HCl. The slurry was then washed with three fold distilled water three times and dried at 40 °C.

3.2.3.3. Percent acetylation and degree of substitution

The percent acetylation (PAacet) and degree of substitution (DS) were determined titrimetrically following the method of Wurzburg (1978). Acetylated starch (1.0 g) was placed in a 250 mL flask and 50 mL of 75% ethanol solution was added. The loosely
stopped flask was agitated, warmed to 50 °C for 30 min, cooled and 40 mL of 0.5 M KOH was added. The excess alkali was back-titrated with 0.5M HCl using phenolphthalein as an indicator. The solution was stood for 2 h, and then any additional alkali, which may have leached from the sample, was titrated. A blank, using the original unmodified starch, was also used.

\[ \text{PAcet} = \frac{(V_{\text{blank}} - V_{\text{sample}}) \times \text{Molarity of HCl} \times 0.043 \times 100}{\text{Weight of sample (g)}} \]

\[ V_{\text{blank}} \] and \[ V_{\text{sample}} \] are volumes of HCl for blank and sample in mL.

DS is defined as the average number of sites per glucose unit that possess a substituent group (Whistler & Daniel, 1995) and calculated from PAc values.

\[ \text{DS} = \frac{162 \times \text{PAc}}{4300 - (42 \times \text{PAc})} \]

3.2.3.4. Physico-chemical properties of starch

3.2.3.4.1. Composition

Composition was determined according to the methods described in section 3.2.1.1.

3.2.3.4.2. Apparent amylose

The amylose content of starches cultivars was estimated using the method of Hoover and Ratnayake (2002). Starch (20 mg db) was dissolved in 90% dimethyl sulphoxide (8 mL) in 10 mL screw-cap reaction vials. The contents of the vials were vigorously agitated for 20 min and then heated in a water bath (with intermittent shaking) at 85 °C for 15 min. The vials were then cooled to ambient temperature, and the contents diluted with water to 25 mL in a volumetric flask. The diluted solution (1.0 mL) was mixed with water (40 mL) and 5 mL of iodine (I_2)/potassium iodide (KI) solution (0.0025 M I_2 and 0.0065 M KI) and the volume was adjusted to a final value of 50 mL. The solution was allowed to stand for 15 min at ambient temperature prior to absorbance measurements at 600 nm (UV Pharma Spec-1700, Shimadzu, Kayto, Japan). A standard curve plotted for mixtures of amylose and amyllopectin of potato was used to determine the amylose content.
3.2.3.4.3. Water absorption capacity

Starch (2.5g db) was mixed with 20 mL distilled water and then stirred manually for 30 min at 25 ºC, centrifuged at 3000 × g for 10 min (C-24, Remi, Industries Mumbai, India) and the supernatant was decanted. Gain in weight was expressed as percentage of water absorption capacity (Anderson et al., 1969).

3.2.3.4.4. Swelling and solubility index

Swelling and solubility index of starch was determined according to the method described in section 3.2.2.3.

3.2.3.4.5. Light transmittance

Starch gels were prepared by heating an aqueous starch suspension (1% db) at 90 ºC in water bath (TC 500, Brookfield Engineering Laboratories Inc., Massachusetts, USA) for 30 min with constant stirring. The suspension was cooled for one hour at 30 ºC. The samples were stored for five days at 4 ºC in a refrigerator and transmittance was determined every 24 h by measuring absorbance at 640 nm against a water blank with a UV-spectrophotometer (UV Pharma Spec-1700, Shimadzu, Kayto, Japan).

3.2.3.4.6. Syneresis

Syneresis of native and acetylated starches was determined according to the method described in 3.2.2.4.

3.2.3.4.7. Freeze thaw stability

Freeze thaw stability was determined by the method of Hoover and Ratnayake (2002). Aqueous starch slurry (6%, w/v db) was heated in a water bath (TC 500, Brookfield Engineering Laboratories Inc., Massachusetts, USA) at a temperature of 90 ºC, for 30 min. The gels were subjected to cold storage at 4 ºC for 16 h and then frozen at -16 ºC. To measure freeze thaw stability, the gels frozen at -16 ºC for 24h, were thawed at 25 ºC for 6 h and then refrozen at -16 ºC. Five cycles of freeze thaw were performed. The tubes were centrifuged (1000 × g for 20 min at 10 ºC) and the released water was measured as freeze thaw stability.

3.2.3.4.8. Colour

Colour values of starches were determined according to the methods described in section 3.2.1.2.
3.2.3.4.9. Scanning electron microscopy

Scanning electron microscopy of native and acetylated starches was determined according to the procedure described in section 3.2.2.2.6. The length and width of ten granules was measured with Vernier caliper and reported as average length and breadth.

3.2.3.5. Thermal properties

Thermal properties of native starches were analyzed using DSC (200 PC-Phox Phoenix, Netzsch, Burlington, Germany) equipped with a thermal analysis data station. Starch (3.0 mg, db) was weighed into a 40 μL capacity aluminium pan and distilled water was added with the help of Hamilton micro syringe to achieve a starch–water suspension containing 70% water. Pans were hermetically sealed and allowed to stand for 1 h at room temperature before heating in DSC. The DSC was calibrated using indium and an empty aluminum pan was used as reference. Sample pans were heated at a rate of 10 ºC/min from 20 – 100 ºC and gelatinization parameters viz. onset (T_o), peak (T_p), conclusion (T_c) temperature and enthalpy (ΔH_{gel}) of gelatinization were calculated from the DSC curves.

3.2.3.6. Pasting properties

Pasting properties of the starches were measured using a Rapid Visco analyzer (RVA-4, Newport Scientific Pty Ltd., Warriewood, Australia). An aqueous dispersion of flour-14% moisture basis (10.7%, w/w; 28.0 g total weight) was equilibrated at 50 ºC for 1 min, heated at the rate of 12.2 ºC/min to 95 ºC, held for 2.5 min, cooled to 50 ºC at the rate of 11.8 ºC/min and again held at 50 ºC for 2 min. A constant paddle rotational speed (160 rpm) was used throughout the entire analysis, except for rapid stirring at 960 rpm for the first 10 s to disperse the sample.

3.2.3.7. Starch gel texture

Starch gel texture was determined according to the methods described in section 3.2.2.4.

3.2.4. Protein isolates form pulses

3.2.4.1. Preparation of pulse protein isolates

One kilogram of the sample was soaked in 4 L of distilled water and kept at 4 ºC for 12 h. Coats of the seeds were removed by manual abrasion. The cotyledons were pulverized
along with water for 5 min in a mixer blender. The slurry obtained was then diluted to ten times (volume/volume) with distilled water and the pH was adjusted to 10 using 0.5 M NaOH. The slurry was continuously mixed on magnetic stirrer for one hour, and then filtered through a 75 µm mesh sieve to separate the fiber. The filtered slurry was then centrifuged at 3000 × g for 30 min at 10 °C (C-24 BL, Remi Industries, Mumbai, India). The sediment obtained was recovered as starch. The aqueous phase (supernatant) obtained on centrifugation was adjusted to pH 4.5 with 0.1 N HCl to precipitate the protein. The proteins were recovered by centrifugation at 8000 × g for 10 min at 5 °C (C-24 BL, Remi Industries, Mumbai, India) followed by removal of the supernatant. Protein curd was washed with distilled water and the curd was re-dispersed in distilled water and adjusted to pH 7. It was then lyophilised (Heto, LL 3000, Allerod, Denmark) as protein isolate.

### 3.2.4.2. Preparation of protein hydrolysates

Protein hydrolysates were prepared according to the method of Bandyopadhyay and Ghosh (2002). Protein isolate (10 g db) were each dispersed in 200 mL of double distilled water, adjusted to pH 10 with 1 N NaOH and incubated at 50 °C for 1 h with shaking in a shaking incubator (LSI-3016R, Daihan Lab Tech Co., Ltd., Namyangju, Kyonggi, Korea). Each portion was then adjusted to pH 8 and hydrolysed with 0.01g of papain (1 Anosan units/gram) at 37 °C for 30 and 60 min with constant shaking. The resulting protein hydrolysates were adjusted to pH 8 and rapidly inactivated the enzymes by heating at 95 °C for 5 min. Each hydrolysate was then freeze dried and stored at 4 °C.

### 3.2.4.3. Degree of hydrolysis (DH)

Aliquots (0.25 mL) of test or standard solutions prepared in 1% w/v SDS were taken in test tubes containing 2.0 mL of phosphate buffer (0.2125 M, pH 8.2) (Adler-Nissen 1979a.). TNBS (w/v) reagent (0.1% w/v in water, 2.0 mL) was then added to each tube, followed by mixing and incubation at 50 °C for 60 min in a covered water bath to exclude light. After incubation, the reaction was stopped by the addition of HCl (0.1 M, 4.0 mL) to each tube. Samples were then allowed to cool at room temperature for 30 min, before absorbance values were measured at 340 nm using an UV-Visible spectrophotometer (1601, Shimadzu, Kyoto, Japan). L-Leucine (0 – 2.0 mM) was used to generate a standard curve. DH values were calculated using the following formula (Adler -Nissen, 1979b).

\[
\text{DH} (\%) = \frac{(\text{AN}_2 - \text{AN}_1)}{\text{N}_{\text{pb}}} \times 100
\]
Where, $AN_1$ - amino nitrogen content of protein before hydrolysis (mg/g protein)
$AN_2$ - amino nitrogen content of protein after hydrolysis (mg/g protein)
$N_{pb}$ - nitrogen content of the peptide bonds in the protein (mg/g protein)

3.2.4.4. Physico-chemical properties of protein isolates

3.2.4.4.1. Composition

Composition of native and hydrolysed protein isolates was determined according to the methods described in section 3.2.1.1.

3.2.4.4.2. Colour

Colour values of native and hydrolysed protein isolates were determined according to the method described in section 3.2.1.2.

3.2.4.4.3. Bulk density

Bulk density was of native and hydrolysed protein isolates were determined according to the method described in section 3.2.2.2.

3.2.4.5. Functional properties of protein isolates

3.2.4.5.1. Protein solubility

Protein solubility of native and hydrolysed protein isolates was determined according to the method described in section 3.2.2.6.1.

3.2.4.5.2. Water and oil absorption capacity

One gram (db) of sample was weighed into 25 mL pre-weighed centrifuge tubes. For each sample, deionized water or refined soyabean oil (Amrit Banaspati Co. Ltd., Rajpora, India) was added in small increments and mixed with stainless steel wire (3 mm diameter) until the sample was saturated with water or oil. The samples were allowed to stand for 30 min at room temperature (20 °C) and then centrifuged at $2200 \times g$ for 30 min. The water or oil released on centrifugation was drained. Water or oil absorption capacity was expressed as gram of water or held per gram of sample.

3.2.4.5.3. Foaming capacity (FC) and foaming stability (FS)

FC and FS of native and hydrolysed protein isolates were determined according to the method described in section 3.2.2.6.3.
3.2.4.5.4. Emulsifying properties

Emulsifying properties of native and hydrolysed protein isolates were determined according to the method described in section 3.2.2.6.4.

3.2.5. Flat bread (unleavened) from pulses

Kidney bean cultivar French Yellow and mash bean cultivar T-9 were selected for flat bread making because they are widely grown cultivars of our region. The commonly cultivated wheat cultivar PBW-343 was selected for blending with pulse flour.

Wheat was milled in a stone mill (Amar Industries, Amritsar, India) to produce whole wheat flour with 100% extraction rate. To produce pulse flour, whole seeds of kidney bean and mash bean were milled in a laboratory mill (Newport Supermill-1500, Newport Scientific Pvt. Ltd., Warriewood, Australia). The resulting flour was then passed through 60-mesh screen to get flour to be blended with wheat flour for flat bread making.

3.2.5.1. Preparation of wheat-pulse (WP) flour and wheat-pulse protein isolate (WPPI) composite flours

In order to improve the quality and quantity of protein in wheat flour flat bread it was supplemented with pulse flours and pulse protein isolates. Wheat flour was replaced with pulse flour from 5 – 20% and with pulse protein isolate from 1 – 4%.

3.2.5.2. Flat bread (chapatti) from pulses

Preliminary trials were carried out to determine the amount of water to be added to the flour (200g) to develop non sticky viscoelastic dough that could be easily rolled and sheeted to make a flat bread (Gujral and Pathak, 2002). The flour was mixed with optimum water for three minutes in a laboratory pin mixer (National Manufacturing Company, Lincoln, NE). The dough was left to rest for half an hour. Dough ball (50 g) was rounded and then placed on a rolling board and sheeted with a rolling pin. The dough was rolled in one direction, inverted, and then rolled in a perpendicular direction.

The raw flat bread was immediately placed on an electric hot plate at 280 ± 3.0 °C and baked on one side and then inverted and baked on the other side followed by final baking on the first side. The baking time varied from 70 to 94 s due to differences in dough water absorption. The flat bread was allowed to cool for 10 min at 25 °C (80% relative humidity) and weight was recorded to determine the amount of water lost upon baking and this was
reported as bake loss. Puffing of the flat bread during baking is a desirable characteristic and it was reported as percentage of the flat bread upper layer that puffed during baking. The change in the flat bread diameter before and after baking was also noted and the reduction in diameter was reported as percentage shrinkage.

3.2.5.3. Sensory evaluation of flat bread

A semi-trained panel of fifteen members comprising of staff and students from the department, evaluated the sensory properties of the flat breads. Sensory evaluation was conducted at ambient conditions under natural light. The samples were coded with random code numbers to eliminate bias. Panelists were instructed to evaluate colour, taste, aroma, breakability, chewability, stickiness and overall acceptability. A nine-point hedonic scale with 1- dislike extremely; 5- neither like nor dislike and 9- like extremely was used (Yadav et al., 2009). Water was provided to rinse the mouth between evaluations. All the samples were analysed under similar conditions in different sittings.

3.2.5.4. Drying of flat bread and production of flat bread flour

Flat breads were freeze dried at -40 ºC and then ground in laboratory mill (Newport Supermill-1500, Newport Scientific Pvt. Ltd., Warriewood, Australia) to produce flour passing through 60 mesh sieve. The resulting flour was then packed in air tight containers and stored at refrigerated temperature until used.

3.2.5.5. Pasting properties of composite flours and flat breads

Pasting properties of flat breads and flour blends were determined according to the method described in section 3.2.2.3.

3.2.6. Statistical analysis

Mean values, standard deviation, analysis of variance (ANOVA) and correlation coefficients were computed using a commercial statistical package SPSS 16.0 (SPSS Inc, Chicago, USA). These data were then compared using Duncan’s multiple range tests at 5% significance level.