Chapter 2

Preparation and evaluation of modified gluten flours
2.0 Introduction

In wheat, the prolamines comprise the major components of gluten. The amino acid sequences of individual prolamines fall into three groups namely sulphur–rich (S-rich), sulphur–poor (S-poor) and high molecular weight (HMG) prolamines (Tatham and Shewry, 1995). Apha-amylase inhibitor and gliadin are identified as allergens occurring in wheat. Researchers have found that glutenin was allergenic for most patients allergic to wheat and also have elucidated a Gln-Gln-Gln-Pro-Pro motif as the IgE binding epitope structure (Sturgess et al, 1994; Tanabe, et al 2000). Based on the above described epitope structure, Gln-Xaa-Xaa-Pro-Pro, it was assumed that wheat flour becomes hypoallergenic by hydrolysis of peptide bonds near the essential proline residues of the epitope (Tanabe, 2008). Watanabe in 2000 proposed a novel method for producing hypoallergenic wheat flour. Bromelain and actinase were selected for the purpose. The team also proposed methods for preparation of different wheat based products such as hypoallergenic bread, pasta like noodles. It is also reported that hypoallergenic flour could be prepared by washing the soluble allergenic fraction of the wheat flour (Tanabe and Watanabe, 1999). Leszczynska (2003) studied the effect of microwave heating on the immunoreactivity of gliadin and wheat flour and found that microwave heating of food could not be applied for the elimination of allergic properties of wheat gliadins. They have observed the increased immunoreactivity in samples exposed with lower energy and the immunoreactivity decreased and was comparable to untreated sample when exposed to higher energy. Other studies have shown that sourdough fermentation, use of prolyl endopeptidases from fungal source and proteases from germinated cereals are effective methods to detoxify gluten peptide (M’hir et al, 2012). However, modification of whole flour to reduce its immunogenicity without losing properties necessary for product preparation needs to be focused. Hence the first objective was preparation of modified gluten flour from Indian T.durum using different bio-processing methods and their functional and immunochemical characterization.
2.1 Materials and methodology

2.1.1 Raw Materials

*Triticum durum* semolina and other non-wheat flours such as maize, oats and sorghum flours were procured from local market. Gliadin, pepsin and pancreatin from porcine were procured from Sigma chemicals, USA. Molecular marker for SDS-PAGE, anti-rabbit IgG-ALP, BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetra-Zolium), para-Nitrophenylphosphate (pNPP), Freund’s complete adjuvant and Freund’s incomplete adjuvant were procured from Bangalore Genie, India. All other chemicals used were of analytical grade. Tomato paste (Splitz, Bangalore) and spinach were procured from local market. Spinach puree was prepared by finely grinding the fresh spinach leaves after blanching at 100°C for 2 min.

2.1.2 Methodology adapted for modification

The methodology used in development of modified gluten pasta products and its validation is schematically illustrated in the Fig 2.1. To modify the *T. durum* wheat flour in order to reduce its immunogenicity, different bioprocessing methods were adapted. Methodology is illustrated in Fig 2.2.

2.1.2.1 Enzyme treatment: *T. durum* semolina was treated with 0.5% protease (from *Aspergillus orizae*) according to the method of Tanabe et al (1996). Protease was dissolved in 30% water and incubated for 2, 4, 6, 8, and 24 h. The mixture was then freeze dried. Freeze dried samples were powdered and used for further analysis.

2.1.2.2 Thermal treatment: With the idea that microwave heating may produce conformational and chemical changes in the gliadin structure that have important consequences on immunoreactivity of the wheat flour. Semolina samples (each 500 g) were heated using microwave at 360W, 540W, 720W, and 900 W for 5 min. Cooled to room temperature and used for analysis.

2.1.2.3 Non-gluten blend: Non-gluten blend was prepared by replacing 40% of the durum flour by other non-wheat cereal flours such as maize, sorghum.
and oats flour. All the flours were mixed and sieved 3 times to make homogeneous mixture and stored at room temperature for further analysis.

2.1.2.4 Chemical modification by altering the pH of the wheat flour

_T. durum_ wheat flour with initial pH of 6.7 was adjusted to acidic pH 3.00 and 5.00 using concentrated lactic acid, neutral pH (7.00) and alkaline pH of 9.00 using 6N NaHCO₃ (CM- 3.00, CM-5.00, CM-7.00 and CM-9.00). Wheat flour and water proportion was 12 g in100ml. 15 ml lactic acid and 17.5 ml of 6N NaHCO₃ was used to adjust respective pH. After altering the pH the paste was heated in water bath at 80°C for 3 h. The resulted gel was dried at 60°C and powdered and analyzed for immunogenicity. Modification protocol is schematically presented in Fig 2.3. Natural sources like tomato paste and spinach puree were also used to adjust the pH. Tomato paste was used for acidic condition (pH 3.00) whereas spinach puree was used for alkaline condition (pH 9.00).

2.2 Analytical methods

2.2.1 Proximate analysis

Proximate compositions of all the flours used in the pasta preparation were evaluated using standard methods. Moisture, ash and protein content were estimated by standard AACC method (2010).

2.2.1.1 Estimation of Moisture content of flour

Moisture content of the flour was estimated according to AACC standard method 44-16.01. The moisture content is the weight after drying the flour for 1 h at 130°C expressed in percentage. Exactly 2 g of the flour was weighed in pre-weighed aluminium dish and placed in hot air oven maintained at 130°C for 1 h. Cooled in desiccators to room temperature and weighed. The percentage of moisture content was calculated as,

\[
\text{Moisture (\%) = } \frac{A-B}{A-C} \times 100
\]

Where, A- Weight of the flour + Aluminium dish before drying, B- Weight of the flour + Aluminium dish after drying, C- Weight of the Aluminium dish.
Fig 2.1 Overview of work plan adopted for the development of modified gluten pasta
**Fig 2.2 Methodology for modification of wheat flour**

1. **T. durum** wheat flour
2. **T. durum** wheat flour blending with non wheat flours
3. Enzymatic treatment
4. Thermal/microwave treatment
5. Modified gluten flour
6. Biochemical characterization
7. Immunochemical validation
8. Production of IgG antibodies against Gliadin in rabbits

**Fig 2.3 Methodology for chemical modification of wheat flour**

1. **T. durum** wheat flour
2. Acidic pH using Lactic acid (pH - 3.00 and pH - 5.00)
3. Alkaline pH using NaHCO₃ (pH - 7.00 and pH - 9.00)
4. Chemically modified flour
5. Rheological characterization
6. Biological characterization
7. Immunochemical validation
2.2.1.2 Estimation of Protein content of flour

Protein content of the flours was estimated using semi micro kjeldahl method (AACC-46-13.01). Approximately 1 g flour was weighed into kjeldahl flask. Digestion mixture (1.45% copper sulfate, 94.0% sodium sulfate, 1.5% Selenium content) of about 1-2 g was added along with 15-20 ml of concentrated sulphuric acid and 2-3 glass beads. This was digested until clear solution is obtained. After cooling the mixture was transferred into 100 ml volumetric flask and made up to the mark with distilled water and cooled to room temperature. Exactly 5 ml of the digested sample along with 10-15 ml of 40% sodium hydroxide (40 g of sodium hydroxide pellets dissolved in 100 ml distilled water and cooled to room temperature) is steam distilled into conical flask containing 2% of boric acid (2 g dissolved in 100 ml distilled water) with 2-3 drops of mixed indicator (5 ml of 0.1% bromocresol green and 1 ml of 0.1% methyl red was mixed to prepare mixed indicator). Ammonia collected in the conical flask in the form of ammonium borate was titrated against N/70 Hydrochloric acid (1.3 ml of Hydrochloric acid in 1000 ml distilled water) till pale pink colour. The amount of N/70 Hydrochloric acid required was noted down. Blank determination was carried out without the sample as above and the amount of N/70 HCl required was subtracted from the sample reading.

2.2.1.2.1 Standardization of HCl

Hydrochloric acid was standardized by distilling ammonium sulphate solution (0.942 g ammonium sulphate was dissolved in 1000 ml distilled water). 5 ml of this is equivalent to 1 mg of N\(_2\)) with 15 ml of 40% sodium hydroxide. The liberated ammonia was collected in the conical flask containing 5 ml of 2% boric acid and 4 drops of mixed indicator. This was titrated against N/70 HCl. The amount of N/70 HCl requires was taken for calculating acid factor.

The percentage of protein was calculated as,

\[
\% \text{ of protein} = \frac{\text{Volume of N/70 HCl required}}{\text{Volume of sample made after digestion}} \times \frac{\text{Volume of sample solution taken for distillation}}{\text{Weight of the sample taken}} \times \frac{100}{1000 \times \text{Acid factor}} \times 5.7
\]
2.2.1.3 Estimation of ash content of flour (AACC- 08-01.01)

Estimation of ash content of flour was carried out using AACC approved method (AACC- 08-01.01). Inorganic residual remained after incineration under atmosphere pressure represents the ash content. Ten grams of flour was weighed into pre-weighed silica dish and incinerated over a burner until smoke ceases then the dish was heated to 550-600°C in muffle furnace until light grey ash results. Cooled and weighed. Ash percentage is calculated as,

\[
\text{Ash (\%)} = \frac{(\text{Weight of the silica dish + ash}) - \text{Weight of the silica dish}}{(\text{Weight of the silica dish + Sample}) - \text{Weight of the silica dish}} \times 100
\]

2.2.2 Microstructure by using Scanning Electron Microscopy (SEM)

Flour samples after treatment along with control flour were scanned to understand microstructure of using scanning electron microscopy at both higher and lower magnification. The samples were mounted on the specimen holder and sputter-coated with gold then each sample was transferred to microscope (LEO 435 VP, USA) for observation where it was observed under different magnification. This procedure was applied to gain information about the arrangements of particles that correlates with the pasta characters. Scanning Electron Microscopy study was carried out for modified gluten flours as per procedure adopted by Prabhasankar et al (2009).

2.2.3 Determination of pasting characteristics of flour using Amylograph

The amylograph is a recording viscometer used to determine the effect of \(\alpha\)-amylase on the viscosity of flour. High viscosity of the starch present in flour is counter acted by action of \(\alpha\)-amylase which liquefies starch granules during heating of flour. Pasting characters of all the treated flour and control flour were studied using Brabender-visco-amylo-graph (Brabender OHG, Duisburg, Germany. Fig 2.4a), following the AACC method (AACC-22-10.01). The modified gluten flour samples were weighed based on the 14% moisture basis and mixed with water (100 ml) and made into paste without lumps. This slurry was transferred into Micro visco-amylograph bowl and allowed to heated up to
90°C and mixed with the speed of 250 (1/min). The pasting characteristics were measured with the measuring range of 300 cmg for the time of 27:50 min. Chemical modification i.e., altering pH to pH 3.00 and pH 9.00 was carried out just before the test run. Similarly enzyme was added just before the test run.

2.2.4 Determination of Farinograph characteristics of flour

Farinograph characteristics of flour was analysed using Brabender Farinograph-E (Brabender OHG, Duisburg, Germany. Fig 2.4a), following the AACC method (AACC-54-22.01). Farinograph records the resistance offered to the mixing blades during prolonged relatively gentle mixing at constant temperature. It also reveals the gluten characteristics during continuous mixing which is related to the behavior of gluten during fermentation. Farinograph is the most universally used dough testing instrument which determines water absorption and mixing profile of the dough.

About 50 g of flour on 14% moisture basis was weighed into 50 g of mixing bowl and mixed for 1 min. required amount of water was added through burette at 30°C so that the maximum consistency of 500 BU is attained. This test was carried out for 20 min to obtain water absorption, Dough Development Time, Dough stability and Mixing Tolerance Index. Chemical modification and enzyme treatment was carried out just before the test run.

2.2.5 Biochemical assays

2.2.5.1 Electrophoresis

Electrophoresis is a technique used to separate protein based on their electrophoretic mobility based on molecular weight. Protein to be analyzed is mixed with SDS an anionic detergent which denatures secondary and non disulphide linked tertiary structures and applies a negative charge to each protein in proportion to its mass. SDS-PAGE was carried out as per the method adopted by Prabhasankar, 2002.
Fig 2.4a Micro Visco Amylograph

Fig 2.4b Farinograph
Twelve percent acrylamide gel was used to separate the protein fractions of the sample. Separating gel was prepared by mixing 10 ml of 4x separating gel buffer with 12 ml 30% acrylamide, 8 ml water, 30 µl of N N N N Tetra methylene diamine (TEMED), 100 µl of freshly prepared 10% Ammonium per sulphate (APS) (0.1 g of APS dissolved in 1 ml). Separating gel buffer of 4x concentration was prepared by mixing 75 ml 2 M tris- HCl buffer (24.2 g dissolved in 100 ml distilled water and pH was adjusted to 8.8 with concentrated HCl) with 4 ml of 10% SDS and water. 30% acrylamide was prepared by dissolving 29.2 g acrylamide and 0.8 gm bis-acrylamide in 100 ml distilled water and filtered. Separating gel was mixed thoroughly and casted using 1 mm thick spacers placed between glass plates of 14x12 cm dimension. Stacking gel was prepared by mixing 2.5 ml of 4x stacking gel buffer with 2 ml 30% acrylamide, 5.5 ml water, 60 µl APS and 30 µl TEMED. Stacking gel buffer of 4x concentration was prepared by mixing 50 ml 1M tris- HCl buffer (12.1 gm dissolved in 100 ml distilled water and pH was adjusted to 6.8 with concentrated HCl) with 10% SDS and water. Flour samples were extracted using Lamelli sample buffer containing 6% 2M Tris-HCl, 50% glycerol, 20% SDS, 5% Mercaptoethanol and 10% Bromophenol blue. Gel was stained with coomassie brilliant blue R250 dissolved in methanol and acetic acid for 6 h at room temperature and de-stained using solution containing 20% methanol, 20% acetic acid and 60% water.

2.2.6 Immunochemical assays

2.2.6.1 Fractionation of gliadin from durum wheat

Fractionation of gliadin from durum wheat is graphically illustrated in Fig 2.5. Durum wheat flour was treated with Tris-Hcl buffer, pH- 8.8 for 1 h. Then this was centrifuged for 20 min. The supernatant was removed. This procedure was carried twice to remove the albumins and globulins. The pellet was treated with 75% ethanol to get gliadin fraction. Obtained gliadin fraction was dialyzed against water, freeze-dried and stored for further use.
2.2.6.2 Raising antibodies against wheat Gliadin

Production of antibodies against wheat gliadin was done according to the method followed by Prabhasankar, 2002. New Zealand white rabbit was immunized with 2 mg of gliadin (isolated from *T. durum* semolina- Fig 2.5) with Freund' complete adjuvant primarily. Rabbit was injected at 4 sites, one site subcutaneously and rest intradermally. This was followed by 3 booster doses of 1 mg of gliadin with Freund’s incomplete adjuvant with one week gap between each booster dose. Rabbit was test bled to collect the serum. And the presence of antibody was confirmed by Dot-Blot method as described below.

2.2.6.3 Dot-Blot analysis

Dot-Blot analysis was carried out according to the method described by Prabhasankar (2002). About 2.0 mg of flour was extracted with 70% ethanol and 4µl of extract (equivalent to 10µg of gliadin) was spotted on Nitrocellulose membrane (NCP- Sigma chemicals, USA) followed by blocking with 2% gelatin in PBS-T (Phosphate buffered saline, pH 7.4 containing 0.05% Tween). This was followed by treating with anti-gliadin antibodies raised in rabbit. The blot was then washed with PBS-T thrice and treated with goat anti-rabbit IgG- ALP conjugate. Finally the blot was treated with BCIP/NBT (5-bromo- 4-chloro- 3-indoyl phosphate / nitro blue tetra- Zolium) substrate in alkaline phosphatase buffer. After colour development reaction was stopped with water. Phosphate buffered saline was prepared by mixing 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium orthophosphate, 2 mM potassium di hydrogen orthophosphate. The pH was adjusted to 7.4 with HCl. Alkaline phosphatase buffer was prepared with the concentration of 100 mM sodium chloride, 100 mM tris- HCl (pH-9.5), 50 mM magnesium chloride and 1% Tween-20.

2.2.6.4 Antigen and antibody dilution titer fixation

Antigen titre and antibody dilution titre fixation was carried out according to the method of Ragupathi et al, 1992. Gliadin fractionated from
T.durum semolina was coated on microtitre plates at different concentration level (156.25 ng-10 µg) to fix ELISA titration with different dilutions of anti-gliadin antibody (1:1000 µl, 1:2000 µl, 1:6000 µl and 1:8000 µl). Graph was constructed with different dilution of gliadin versus their reactivity with anti-gliadin antibodies. The concentration which showed maximum absorbency was considered as optimal antigen concentration and anti-gliadin antibody dilution for further ELISA experiments.

2.2.6.5 Enzyme Linked Immunosorbent Assay (ELISA)

Flour samples were extracted with buffer containing 6% 1 M Tris-HCl, 50% glycerol and 20% SDS. Immunogenicity of the modified flour extract was tested by ELISA. Microplates were coated with 200 µl (10 µg of protein /well) and incubated overnight at 4°C. Sites in the wells were saturated by incubating 200 µl of 0.5% gelatin in PBS buffer containing 0.05% Tween-20 at 37°C for 2 h. IgG, obtained from anti-sera of New Zealand white rabbit immunized with gliadin were used at the dilution of 1:1000 as primary antibody. This was incubated at 37°C for 2 h followed by the incubation of goat anti rabbit IgG ALP conjugate, at 37°C for 2 h. The wells were washed between each addition with PBS buffer containing 0.05% Tween-20. Colour development was done using substrate pNPP in Diethanolamine buffer (1 mg/ml). The ODs were read at 405 nm filter using an ELISA reader. Diethanolamine buffer was prepared with the composition of 0.97 ml diethanolamine, 20 mg sodium azide, 10 mg magnesium chloride and 80 ml of water. The pH was adjusted to 9.8 with 1 M HCl (1.16 ml in 100ml) and was made up to 100 ml.

2.2.6.6 Statistical analysis

Results are expressed as mean ± SD and analyzed by one way analysis of variance (ANOVA) and Tukey multiple comparisons test for significance at P< 0.05 using Graphpad statistical software- Graph pad Prism, version 5, 2007.
Fig 2.5 Protocol for fractionation of *T. durum* wheat flour

- **T. durum** Wheat semolina
  - Centrifuged at 3000 rpm for 20 min
  - Pellet
  - 75% ethanol
  - Gliadin
  - Supernatant (Albumins and globulins)
  - Tris-Hcl Buffer, pH 8.00

**Fig 2.6** Dot-Blot for the confirmation of production of antibodies

**Fig a** – Pre immune sera of rabbit, **b**- Sera after immunization

A- 20 µg/4µl of gliadin spotted, B-10 µg/4µl of gliadin spotted
2.3 Results and discussions

The preparation of modified gluten flours were carried out with three different methods along with one non-gluten blend preparation. In the first trial the flour was incubated with proteolytic enzyme such as protease (0.5%) for different time duration. These enzymes are used because of their low cost and effective proteolysing activity. The resulted mixture was freeze-dried and powdered. Colour of the enzyme treated flour was not affected than control flour. Moisture content of the flour was decreased to 7.70% whereas protein content was not affected.

In the second method, the flour was heated using the microwave oven at 540, 720 and 900 W for 5 min. This was cooled and packed immediately to avoid the moisture absorption. Hence the moisture content fell down to 4.28%. Protein content of this flour remained same as control flour.

In the third method, pH of the flour was adjusted to 3.00 and 5.00 using lactic acid and to pH 7.00 and 9.00 using 6 N NaHCO₃. The flour with acidic pH was whitish in colour than the unmodified and flour when adjusted to alkaline pH became yellow in colour.

Similarly to prepare non-gluten blends, 40% of wheat flour was replaced by non-gluten flours like sorghum, maize and oats. The blend was mixed thoroughly to get the uniform distribution. The moisture content of the blend was 8.5% whereas the control flour contained 9.32% of moisture. The protein content (10.03%) of the flour was not affected by the addition of non-gluten flours when compared with control (10.83%).

2.3.1 Rheological properties

2.3.1.1 Pasting characteristics of modified gluten flours

The Table 2.1a and Fig 2.7a shows the amylographic characteristics of the flours treated with different methods. The results indicated that the gelatinization temperature of the blends with the non-gluten flours decreased to 75.5°C than the control, which has 80.9°C. But the gelatinization
temperature of the microwave treated and enzyme treated sample were increased to 82.6°C and 85.2°C respectively. Non-gluten blend showed the increase in maximum viscosity 604 to 640 BU, hot paste viscosity from 497 to 633 BU and cold paste viscosity from 1047 to 1066 BU compared to control. Breakdown and set back values were higher than the control. All these results corroborates well with the findings of Indrani et al (2010). Their study reports, early the onset of initial viscosity of wheat starch in presence of other starch from different grains, they also reported that there was an increase in the viscosity of starch and multigrain system upon heating from 30 to 95°C which, was caused by the amyllose and low molecular weight amylopectin promoting the formation of polymer complexes. Increase in the cold paste viscosity, which represents the cooked paste after cooling indicates a strong tendency for retrogradation of starch molecules. Similarly, increase in breakdown values represents the resistance of starch granules to thermal treatment and mechanical shearing. The maximum peak viscosity of the microwave treated flour was decreased to 370 than the control flour indicating the considerable damage to starch with degradation of its macromolecules, due to microwave heating. Similarly, hot paste viscosity and cold paste viscosity, break down and set back values were decreased than the control. Song and Zheng (2007) reported in their review that thermal treatment at low temperature does not show changes in the mechanical properties of the gluten whereas, the higher temperature induces irreversible changes in rheological behavior of the gluten due to sulphydryl/disulphide exchange. The flour treated with enzyme also followed the similar trend of decrease in maximum viscosity than the control. Hot paste, cold paste setback and break down values also were decreased than the control indicating the damage of starch and protein molecules by enzymatic degradation or peptide hydrolysis by protease enzyme. Bombara et al (1997) reported the reduced viscosity and yield stress in gluten proteins hydrolyzed by protease and this coincided with the extent of hydrolysis of glutenins. This correlates well with our observations of electrophoretic pattern of protease treated flour explained under section 2.3.2.1. Amylographic results suggested that modifications occurred by enzymatic and microwave treatment greatly affected pasting characteristics of wheat flour, which is one
of the main rheological characteristics that influenced the product quality especially cooking quality of pasta. Increase in disulphide formation resulted in firm gluten network. High breakdown values are related to high peak viscosity and are related to the degree of swelling of starch granules during heating (Ragaee and Aal, 2006).

Amylograph correlation graph indicating the pasting characteristics of the chemically modified flour along with control flour is shown in the Fig 2.7b and Table 2.1b. Amylograph peak viscosity and gelatinization temperature were affected by the pH variations (Shiau and Yeh, 2001). Modification of flour with acidic pH, CM-3.00 and CM/tp-3.00 showed early onset of the gelatinization temperature. It showed 61.8°C and 63.5°C respectively than control (80.9°C). The flour with starch molecules which rapidly gets gelatinized will give softer products like noodles (Shiau and Yeh, 2001). They reported that addition of lactic acid results in weakening of the dough with more liquid like behavior resulting in less shear thinning. This was supported by the less shear values of CM-3.00 and CM/tp-3.00 indicating the decreased firmness of pasta. Maximum peak viscosity of 710 AU and 684 AU respectively were observed which were higher than the control sample. Song and Zheng, 2007 reported that increasing pH induces a decrease in Tangent delta or loss angle (tan δ) with an increase in G' (Storage modulus) and apparent viscosity. In the case of alkaline modification the onset of gelatinization was found to be slightly delayed than the acidic pH modification but was early than the control. CM-9.00 showed 67.4°C and CM/sp-9.00 showed 66.4°C. Similar pattern of change in the maximum peak viscosity was also observed. CM-9.00 had peak viscosity of 639 AU and CM/sp-9.00 showed 620 AU. Shiau and Yeh, 2001 reports that alkaline condition increases the peak viscosity and also delays the onset of gelatinization temperature. This may be the reason for increased firmness of CM-9.00 and CM/sp-9.00. Shiau and Yeh, 2001 have studied the effect of alkali and acid on rheology and properties of noodles. Lactic acid was used to decrease the pH of the dough and Kansui (alkaline salt) which gives pH 9.00 was used to modify the protein. They reported that, addition of Kansui slightly increased S-
H and S-S (sulphydryl and disulphide) content. They reported that alkaline conditions imparted by Kansui salt induced the sulphydryl group and disulphide bond interchange which resulted in aggregation of proteins through inter-chain disulphide bonds. Increase in disulphide formation resulted in firm gluten network. High breakdown values are related to high peak viscosity and are related to the degree of swelling of starch granules during heating (Ragaee and Aal, 2006). Except control flour all other chemically modified pasta showed increase breakdown values with a concomitant increase in peak viscosity values indicating a high degree of swelling of starch granules. Lower setback values indicate the low rate of starch retrogradation and syneresis. Due to this starch and gluten interaction CM-9.00 and CM/sp-9.00 showed better firmness and lower cooking loss. Supporting these data microgram showed compact arrangement and formation of gluten matrix.

2.3.1.2 Farinograph characteristics

Table 2.2 and Fig 2.8 shows the Farinograph results of the different flour samples. Fig 2.8 d shows the graph of enzyme treated flour. It showed very low water absorption of 45.2% but the dough development time of 19.9 min. This clearly shows the degradation of gluten proteins due to hydrolysis of peptide bonds by enzymes, that made flour very week and unable to form complete dough even after 19 min. Disulfide bonds and glutenins contributes to proper formation of gluten network imparting visco-elastic properties thereby by giving stability to the dough (Aminlari and Majzoobi, 2002). Since gluten network formation was affected, water absorption rate was decreased due to its inability to hold water between gluten matrixes. Pedersen et al (2005) investigated the influence of chemical and enzymatic modification on biscuit dough rheology and reported that hydrolysis of peptide bonds by protease enzymes reduce the molecular weight of gluten proteins resulting in weaker gluten network. Bombara et al (1997) also studied the influence of protease enzyme on flour functional properties. They reported that Farinograph water absorption increased at lower degree of hydrolysis and then decreased to lower values than unmodified flour. This initial increase in water absorption may be due to increased number of polar sites such as
carboxyl and amino groups and of hydrophobic groups as a result of hydrolysis. Decrease in water absorption capacity may be related to disruption of protein network responsible for imbibitions and holding of water leading to decrease water absorption capacity (Bombara et al, 1997). Farinogram of microwave treated sample is presented in the Fig 2.8 b that indicates the very high water absorption of 71.9% and the dough development time of 20 min. This could be due to the irreversible changes of gluten proteins by microwave heating that affects the gluten network formation even at higher water absorption rate. Fig 2.8 c shows the graph of non-gluten blends. Results indicated the increased percentage of water absorption from 61.1-62.6% compared with that of control flour (Fig 2.8 a). Similarly the dough development time was increased from 4.5 to 8.0 min. This could be due to delay in hydration and gluten network formation due to the presence of starches and fiber particles coming from the non-gluten flours added in the blend. Indrani et al (2010) also reported the increase in water absorption and dough development time with the use of multigrain in the preparation of bread. Bahnassey and Khan (1986) also found the higher water absorption when supplemented semolina with legume flours or their protein concentrates. They also observed higher dough development time and stability for blends containing bean flours. It is evident from the results that, the water absorption rate dough development was much affected by microwave heating and enzymatic treatment when compared with blend of non-gluten flours.

Farinograph characteristic of the chemically modified flour is shown in the Fig 2.8 and Table 2.2. Since chemical modification by altering pH affects the gluten forming proteins, water absorption percentage, dough development time (DDT) and stability values varied among the modified samples. Control flour showed water absorption percentage of 61.1% and this was increased to 74.8% when lactic acid was added to bring the pH to 3.00. Water absorption was decreased to 66.0% when 20% of Tomato paste was added along with the lactic acid to bring the pH to 3.00. These two modifications with acidic pH showed increased DDT, stability, mixing tolerance index (MTI) than the control. Gennadios et al, 1993 studied the effect of acidic and alkaline
condition on wheat gluten formation and reported that at pH 1-3 less intermolecular protein cross linking occurs under acidic condition. In the case of alkaline modification where pH was adjusted to 9.00 using NaHCO$_3$ water absorption was increased to 77%. Shiau and Yeh, 2001 reported that alkaline salt Kansui increased the Farinograph water absorption capacity. And DDT and stability were decreased to 3.2 and 3.8 min respectively. It showed higher mixing tolerance of 74 BU. CM/sp-9.00 showed water absorption of 76.6%. DDT was further decreased to 2.7 min than CM-9.00, whereas it showed the stability of 5.1 min and MTI of 29 BU. This supports the results cooking quality where alkaline pasta showed higher cooked weight and acidic pasta showed lower cooked weight. Aminlari and Majzoobi (2002) studied the effect of pH on rheological properties of hard red autumn variety wheat and reported that alkaline pH increase the dough development time and stability. They reported that more insoluble higher molecular weight proteins increase the dough development and stability. But they observed sharp decrease in dough weakening as the pH increased.

2.3.2 Validation of modified gluten flours

2.3.2.1 Biochemical characterization

Fig 2.9 shows the SDS-PAGE pattern of the flours with different treatment. The lane C and D shows the flour treated with microwave heating at 900 W and 720 W respectively. The protein profile was not much distinct when compared to control showing the less efficiency of microwave heating in reducing the immunogenicity. The lane E and F shows the protein profile of protease treated flour for 8h and 24h respectively. In this pattern band corresponding to 43 kDa and less were present when compared to control flour as shown in the lane B and other high molecular bands were not visible. This could be due to hydrolysis of high molecular weight proteins that were converted to lower molecular weight peptides. This is in agreement with the findings of Bombara et al (1997). They observed the reduction in the molecular mass distribution of glutenin fraction of wheat proteins affected by exogenous fungal protease and concomitant increase in the smaller molecular
<table>
<thead>
<tr>
<th>Sample</th>
<th>Gelatinization temperature (°C)</th>
<th>Maximum viscosity (BU)</th>
<th>Hot paste viscosity (BU)</th>
<th>Cold paste viscosity (BU)</th>
<th>Breakdown (BU)</th>
<th>Set back (BU)</th>
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<td>604</td>
<td>497</td>
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<td>1</td>
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<tr>
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<td>633</td>
<td>1066</td>
<td>110</td>
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<th>Maximum viscosity (BU)</th>
<th>Hot paste viscosity (BU)</th>
<th>Cold paste viscosity (BU)</th>
<th>Breakdown (BU)</th>
<th>Set back (BU)</th>
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<th>Stability (min)</th>
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<th>Mixing tolerance index (FU)</th>
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Fig 2.7a Pasting characteristics of flours treated with different methods

*NGB* - Low gluten blends, *ET* - enzyme treated and *MT* - Microwave treated.

Fig 2.7b Pasting characteristics of chemically modified flour
Fig 2.8 Farinograms of modified wheat flours

a- Control flour, b- Microwave treated flour, c- Low gluten blends, d- Enzyme treated flour, e- CM-3.00, f-CM/tp-3.00, g- CM-9.00, and h- CM/sp-9.00
weight fractions. This change contributed to less viscosity as indicated by the rheological studies. The present study observation was also corroborated with the results of Tanabe et al (1996), where they have reported that by treating wheat flour with bromelain, most of the wheat proteins converts to low molecular peptides of nearly 20-40 kDa, which were considered to be non-allergenic peptides. Watanabe et al (1994a) also reported that actinase treated product contained low molecular weight proteinacious components but collagenase and transglutaminase treated products retained high molecular weight proteins. Pasini et al (2001) also observed similar kind of results during *in-vitro* digestion of bread dough, crumb and crust. They observed that during pepsin treatment of bread dough, the (high molecular weight) HMW prolamins were rapidly converted into a smaller number of bands with Mr Values between 66 and 31 kDa, which tended to disappear after the addition of pancreatin. The lane G shows protein profile of flour with acidic pH (pH 3.00) where bands at molecular weight range 66 kDa-43 kDa were not visible and bands at 97.4 kDa were shown with less intensity. The lane H showed flour with pH 9.00 wherein, similar protein profile was observed but bands at all molecular range were with less intense. Aminlari and Majzoobi (2002) also observed the disappearance of bands between 66-97kda and 55kDa and lower, but, noticed the appearance of these bands as the pH increases above 5. Lane I shows the low gluten flour prepared by blending 40% of non-gluten cereal flours. The protein profile showed less intensity bands corresponding to the allergen profile than the control flour.

### 2.3.2.2 Immunochemical characterization

#### 2.3.2.2.1 Dot Blot analysis of treated flour

The Fig 2.10 shows the Dot-Blot pattern of all flours with different treatment. The spot B shows control flour. The spot E shows the protease treated flour. The colour developed in these was less intense showing less immunoreactivity against IgG. The spot shows the Low gluten blend with less intensity of colour developed but the spot D which is microwave treated flour showed similar kind of colour development compared to control flour as
shown in the spot B. This supports the data of SDS-PAGE pattern confirming the less immunogenicity of enzyme treated flours. Zorzi et al (2007) have studied the allergenicity of durum wheat proteins after in vitro digestion using pepsin and pancreatin as influenced by pasta drying temperature. In this study, immunoblotting analysis with polyclonal antibody reveals that antibody recognized the same bands in all the undigested pasta samples confirming the heat resistance of some components of the wheat prolamines. The same protein fractions were immune-detected after pepsin digestion, but after pancreatin digestion the binding of the antibody was no longer detectable. Hence they reported that, prolamin that are recognized by the antibody although showing pepsin resistance were degraded by pancreatin to fragments with the molecular weight so low as to allow them to run off the electrophoresis gel. Similar results were observed for the bread after baking. Leszczynska et al (2003) studied the effect of microwave treatment on the immunoreactivity of gliadin by immunoblotting method. They reported that immune response decreased in comparison with the level at the maximum energy and for the energy of 90 kJ the immuno-reactivity of microwave treated gliadins fell down to the level comparable with that of the untreated.

Dot–blot analysis of chemically modified flour by altering pH is shown in Fig 2.10 which clearly shows the reactivity of modified flours with anti-gliadin antibody raised against gliadin rabbit. Acidic modified flour with pH 3.00 and 5.00 showed similar reactivity like control. Even though there were changes in the functional properties of the modified flour, no changes in the immunogenicity was observed. The neutral pH (CM-7.00) also showed similar intensity of colour development. Alkaline pH of 9.00 showed marked reduction in the intensity of the colour development confirming the less immunogenicity. This may be due to deamidation that brings the conformational changes which is not recognized by the anti-gliadin antibody. Berti et al (2007) also reported the lower IgA-AGA (IgA class anti-gliadin antibody) immunoreactivity of gluten proteins when deaminated chemically.
2.3.2.2 Antigen and antibody dilution titer fixation

Optimal dilution of anti-gliadin antibody and antigen concentration was determined by constructing the graph from the absorbance values. The dilution and antigen concentration that gave the maximum absorbency was considered as optimal. From the graph as presented in the Fig 2.11 it was clearly indicated that antigen concentration of 10 µg and anti-gliadin antibody dilution at 1:1000 µl was considered as optimum as maximum OD value was observed in this titer.

2.3.2.2.3 Enzyme linked immunosorbent assay of treated flour

The data also supported by ELISA pattern, the Fig 2.12 shows the ELISA pattern of allergens extracted from flours with different treatment. The graph reveals that flour treated with enzyme (protease) showed less immunoreactivity against IgG which was negligible where as the antigenic property of flour treated with microwave heating showed increased immunoreactivity and flour with Low gluten blends showed not much significantly reduced compared to control flour. Watanabe et al (1994a) also reported less ELISA values in flour treated with collagenase and transglutamases. And they confirmed that wheat flour can be made hypoallergenic when treated with enzymes like actinase, collagenase and transglutaminase. Leszczynska et al (2003) have also studied the immunoreactivity of microwave treated gliadins and wheat flour by ELISA. They observed immunoreactivity at lower doses of applied energy. But at the level of 500 W there was drop in reactivity of gliadins for 2 min and reactivity reached the level of untreated sample after 3 min exposition. The graph clearly indicates that among the chemically modified flours, flour with alkaline pH showed very less antigenic property than other samples. This was supported by Dot-Blot analysis also. The flour with acidic pH (3.00) and pH - 7.00 showed similar antigenic properties supporting the data of dot blot where they had similar intense coloured spots. The pH 5.00 also showed similar decrease in the reactivity but only CM-9.00 was not recognized by the anti-gliadin antibodies. This confirms that by adjusting the pH of the flour to
alkaline condition it is possible to reduce the immunogenicity of the wheat flour.

2.3.3 Microstructure of modified gluten wheat flours

The micrographs highlight the importance of continuous protein network in the entrapment of starch and good cooking quality. Fig 2.13A and B shows the images of control flour at different magnifications. The image showed compact arrangement of lens shaped wheat starch molecules which were covered with protein fractions. Similar observations were reported by Dexter et al (1979). In Fig 2.13G and 2.13H image of with non-gluten blend at different magnification are presented. Indrani et al 2010 reported that increase in the level of incorporation of multigrain mixture in preparation of bread interrupt the continuity of the matrix. They also observed thin protein matrix owing to the disruption of continuity because of higher level of incorporation of the multigrain mixture. Ryu (1999) studied the influence of additives on preparation of waxy barley and wheat flour bread. SEM images of bread demonstrated non-continuous, loose protein–starch matrix where in starch granules were dispersed. But more continuous structure was observed in the dough made with barley flour along with additives like hydroxypropylmethyl cellulose (HPMC). Findings of Prabhasankar et al (2009) supports the data by reporting that incorporation of seaweed beyond 2.5% level disturbs the microstructure of pasta. The Fig 2.13C shows the images of microwave treated flour. The image clearly indicates the denaturation of gluten proteins by microwave heating when compared to control that is supported by rheological studies. Similarly, the microstructure of enzyme treated flour (Fig 2.13B) shows the hydrolyzed protein fragments resulted in increased number of smaller protein fractions compared to control flour that affected the formation of gluten network, during dough development. This is supported by the pattern of SDS-PAGE and rheological studies of present study.

Modification of the wheat protein is in practice in food industry for various purposes. Enzymatic and chemical modification of the wheat gluten to achieve the improved functional properties of wheat dough is the corner stone
of the research in baking technology. Exogenous enzymes, chemicals, pH variations are used to achieve the purpose. In this study these different techniques were used to reduce the immunogenicity of the wheat flour. pH variation of wheat gluten influences the modification of structure of wheat proteins through electrostatic repulsion or attraction that results from changes in the degree of ionization of ionizable groups on proteins (Aminlari and Majzoobi, 2002). Protease enzyme hydrolyses the gluten proteins resulting in increased number of lower molecular weight protein fractions that are not recognized by the antibody against the gliadin thereby decreasing the immunogenicity. Microwave treatment failed to induce changes in the molecular mass distribution thereby failed to reduce the immunogenicity of the flour.

2.4 Conclusion

Different bio-processing methods were adapted in this section to reduce the immunogenicity of the wheat flour. As described in the methodology, non-gluten blends, microwave treatment, enzyme treatment and chemical modification by altering the pH of the flour to acidic and alkaline condition were the different methods adapted. Results of the analytical, biochemical and immunochemical assays confirm that, among these different methods, enzyme treatment and altering the pH to alkaline condition is more effective in reducing the immunogenicity. Non-gluten blend also showed reduced immunogenicity whereas microwave treatment did not show any satisfactory results. Since gluten is the main structure protein in most of the wheat based bakery and pasta products and is also a main culprit for CD, development of the products devoid of gluten is a challenging job. These above described modifications also affect the conformation of gluten proteins which influences the final product quality when applied in food processing. To study the application of these modified flours in food processing and to assess the final product quality these modified flours were taken for the pasta preparation which is discussed in the next chapter.
Fig 2.9 SDS-PAGE pattern of modified flours

A-Molecular Marker, B- Control flour, C- Microwave Treated-900 W and D-900W, E-flour treated with protease for 8hr, F- flour treated with protease for 24hr and G- pH modified to 3.00, H- pH modified to 9.00 and I- Low gluten blend.

Fig 2.10 Dot-Blot pattern of the different flours

A- Gliadin, B- Control flour, C-Low gluten blends, D-Microwave Treated, E- protease treated flour, F-CM-3.00, G-CM-5.00, H-CM-7.00, I-CM-9.00
Fig 2.11 Calibration curve for fixation of Titer value of antibody and antigen
Antigen concentration: 1-10 µg, 2- 5 µg, 3- 2.5 µg, 4- 1.25 µg, 5- 0.625 ng, 6- 312.5 ng, 7- 156.25 ng

Fig 2.12 ELISA pattern of modified gluten flour
NGB- Low gluten blends, ET- Enzyme treated and MT- Microwave treated, CM-3.00- Chemically modified flour with pH 3.00, CM-5.00- Chemically modified flour with pH 5.00, CM-7.00- Chemically modified flour with pH 7.00, CM-9.00- Chemically modified flour with pH 9.00.
Fig-2.13 Microstructure of the flours

A and B- Control flour at different magnification, C and D- Enzyme treated flour, E and F- Microwave treated flour, G and H- Low gluten blend. S- Starch molecules, P- Protein film