Chapter 4

Validation of modified gluten pasta
4.1 Introduction

As explained earlier in the 1st chapter, gluten is the main structure forming protein in the flour which is responsible for the elastic characteristics of the dough (Gallagher et al, 2004) because of its extensive network of intermolecular disulfide bonds arising from cysteine residues of glutenins (Cornell and Hoveling 1998). It is well documented that on a dry matter basis, gluten contains 75-86% of protein, 10% starch and 5% fat (Gallagher et al, 2004; Cornell and Hoveling 1998) held strongly within the gluten protein matrix. Gluten comprises the protein fractions glutenins and gliadins. The glutenin is a rough rubbery mass when fully hydrated, while gliadin produces viscous, fluid mass on hydration. Hence gluten exhibit combined properties of these two components which results in unique property of viscoelasticity which is very essential for the most of the bakery products (Gallagher et al, 2004). Individuals with gluten sensitivity exhibit immune sensitivity to both soluble and insoluble fractions of the gluten proteins. Persons with CD are unable to consume many of the common wheat based products available in the market including breads, baked goods and other food products made with wheat flour (Moore et al, 2004). In symptomatic patients a strict gluten free diet is highly effective in relieving symptoms and may prevent long-term complication (Gallagher et al, 2004, Mustalahti et al, 2002). Foods that are not included in gluten free diets are: 1. Any bread, cereal or other food made with wheat, rye, barley, triticale, dinket, kamut and oat flour or ingredients. 2. Any processed foods that contain wheat and gluten derivatives as thickeners and fillers. 3. Medications that use gluten as pill or tablet binders (Gallagher et al 2004).

Now a day’s gluten free bakery products have become available in the market, prepared out of non-wheat flours such as rice maize, soya, guar and amaranth. However contamination of these food products by gluten containing cereals may take place at the stage of flour production or at the stage of production of the final product (Olexova et al, 2004). The report of the 20th session of Codex Alimentarius (1996) indicates that the consumption of prolamins by celiac patients should not exceed 10 mg per day. Hence
validation of any products that are made suitable for gluten sensitive
individuals or celiac disease patients is very essential. Many biochemical and
immunochemical assays helps in detecting the gluten contamination or
immunogenicity of the products. Recent research concentrates on the
development of immunological methods with high sensitivity and specificity
(Collin et al, 2004). In this regard many researchers have developed ELISA
tests to detect gluten content in gluten free foods. Chirdo et al, (2000) used a
polyclonal serum obtained against total gliadins, which recognize α, β, γ and
ω- gliadins and the HMW-GS of wheat as well as the prolamins of barley, rye
and triticale. Scientists have developed sandwich ELISA based on a cocktail
of antibodies using a combination of two monoclonal antibodies raised against
secalins and gliadins. Similarly, a more original protocol using a MAb
(monoclonal antibody) against a peptide derived from hydrolysis of α- gliadin
by pepsin and trypsin and corresponding to its N-terminal sequence is also
developed (Papini et al, 1999).

Hence, to confirm the changes during modification and effectiveness of
these modifications on immunogenicity of wheat flour, different variations of
optimized modified gluten pasta (explained in 3rd chapter) was validated using
biochemical immunochemical methods. This chapter deals with the
biochemical changes and immunogenicity of optimized modified gluten pasta
samples.

4.2 Materials and methodology

4.2.1 Materials

Gliadin, pepsin and pancreatin from porcine pancreas 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), Dithioerythritol (DTT), Freund’s
complete adjuvant and Freund’s incomplete adjuvant were procured from
Bangalore Genie, India. Nitro cellulose membrane of 0.2 µm was procured
from Sigma Chemicals, USA. Goat anti- rabbit IgG Alkaline phosphatase was
procures from Bangalore Genie, India. Anti human IgA (α-chain specific)
alkaline phosphatase and Anti-human IgE was procured from Sigma Chemicals, USA. All other chemicals used were of analytical grade.

4.2.2 Biochemical assays

4.2.2.1 Electrophoresis

All modified gluten pasta samples were analyzed for its protein profile by using Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Gel electrophoresis was carried out according to the method adopted by Prabhasankar, 2002. Detailed procedure of the SDS-PAGE is explained in the section 2.2.5.1 of 2nd chapter. Cooked freeze dried pasta samples were extracted using Lamelli sample buffer containing 6% 2M Tris-HCl, 50%glycerol, 20% SDS, 5% Mercaptothanol and 10% Bromophenol blue.

4.2.2.2 Gel Filtration Chromotography

The glass column was packed with Sepharose 6B (Pharmacia fine chemicals, Sweden), pre-swollen granules at 25 ml per hour rate after equilibrating with 50 mM Phosphate buffer overnight. Phosphate buffer of 50 mM concentration was prepared by mixing 28 ml of 0.05 M disodium hydrogen orthophosphate dihydrate (0.8995 g in 100 ml) and 72.0 ml of 0.05 M sodium di-hydrogen orthophosphate dihydrate (0.7800 g in 100 ml) and pH was adjusted to 7.2. The cooked freeze dried pasta were extracted using 50 mM Phosphate buffer containing 0.5% SDS, pH 7.2 for 6 h and centrifuged twice. Extract were filtered through filter paper (Whatman No.1) followed by 0.45 μm membrane filter (Millipore). Samples were eluted with the same buffer using system connected to pump (Pharmacia Biotech) and fractions of 5-6 ml were collected in test tubes at fraction collector (Pharmacia Biotech). The OD was read at 280 nm using spectrophotometer (UV-1800 Spectrophotometer, Shimadzu, Japan).

4.2.2.3 High Performance Liquid Chromatography (HPLC)

Modified pasta samples were extracted using 70% ethanol with continuous agitation for 30 min. Centrifuged for 15 min at 10,000 rpm. The
obtained supernatant was filter through member filter (Millipore) of 0.45 µm porosity. HPLC was performed (Model LC 10 A, Shimadzu Corporation, Japan) using C18 columns (10µm of id) as described by Bietz et al, 1984. Samples 20 µl were analyzed using a linear gradient from 20 -55%, solvent B over 35 min. Solvent A was 15% Acetonytrile (ACN) + 0.1% Tri fluoro acetic acid (TCA) and solvent B was 80% ACN + 0.1% TCA. Components eluting from the column were detected at 210 nm using UV detector.

4.2.2.4 Two step extraction of gliadin

Gliadin was extracted by two step sequential extraction method according to van den Broeck et al, 2009 with slight modification. One gram of wheat flour and cooked, freeze-dried pasta was extracted with 10 ml of 50% aqueous isopropanol with continuous mixing at 250 rpm for 2 h using shaker and centrifuged thrice, at 8000 rpm for 15 min. Supernatant collected is marked as 1st gluten extract which is comprised mostly of gliadin. Residue was again mixed with 10 ml 50% aqueous isopropanol, 50 mM Tris-Hcl, pH 7.5 and 1% DTT (DTT was freshly added). This was vortexed for 5 min and sonicated (5X10% cycles) for 10 min (Bandelin Sonopuls, HD 2070, Berlin) with 45% power. After sonication this was heated at 60°C (water bath) for 30 min shaking at every 10 min and centrifuged at 8000 rpm. Supernatant was taken as 2nd extract (gluten extract). Gliadin extract was analyzed for immunogenicity.

4.2.3 Immunochemical assays

4.2.3.1 Fractionation of gliadin from durum wheat

Gliadin was fractionated from durum wheat flour as per the protocol mentioned in section 2.3.6.1

4.2.3.2 Raising antibodies against wheat gliadin

Production of antibodies against wheat gliadin was done according to the method followed by Prabhasankar et al 2002. Detailed procedure is described in section 2.2.5.2.
4.2.3.3 Collection of Celiac Disease Patients sera

Serum of the CD patients was a kind contribution of Dr Rakesh Khochhar, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Thirteen patients with CD were randomly selected between the age group of 5 to 48 years. Among them 11 were newly detected with CD, 6 patients were females and 3 patients were below the age of 15 years. Serum of all the patients were pooled and stored at -20°C. A pooled serum was used for immunoassay to detect the immunogenicity of the pasta product optimized.

4.2.3.4 Dot-Blot analysis

Dot-Blot analysis was carried out according to the method described by Prabhasankar (2002) as per the detailed procedure mentioned in section 2.2.6.3

4.2.3.5 Enzyme Linked Immunosorbant Assay (ELISA)

The allergenicity of the modified pasta extract was tested by ELISA as per the protocol given in section 2.2.6.5.

4.2.3.6 Western blot

Immuno-blotting was carried out by transferring separated proteins on electrophorotic gel. Transferring was carried out using Biotech semi dry blot (BIOTECH, R & D Laboratories, India) with the power supply of 6 mA constant correct for 2h. Sandwich was prepared keeping 2 fiber pads (Whatman GB-003, 3 mm thickness), Nitrocellulose membrane, (sigma chemicals, USA) gel and 2 fiber pads one above the other. Nitrocellulose membrane was activated by soaking in water for 3 min and fiber pads were soaked in transfer buffer (0.25 M Tris, 1.92 M Glycine, pH 8.3) for 10 min. After transfer, the blot was washed with PBS buffer for 5 min then blocking was carried out with 0.5% gelatin in PBS for 1 h at room temperature. The blot was then washed thrice with PBS followed by incubation with primary antibody IgG raised in rabbit against gliadin overnight at 37°C. Blot was again incubated with Goat- anti
rabbit IgG - alkaline phosphatase conjugate for 1h at 37°C then was treated with BCIP/NBT substrate in alkaline phosphatase buffer for 15-20 min.

4.3 Results and discussion

4.3.1 Validation of chemically modified pasta

Effect of chemical modification by altering the pH on wheat proteins can be attributed to many conformational changes occurring through electrostatic repulsion or attraction as a result of changes in the ionizable groups of dough proteins. The influence of this chemical modification on pasta product quality is discussed in 3rd chapter. For further understanding of biochemical changes happening during the modification and influence on immunogenicity these chemically modified pasta variations (CM-3.00, CM/tp-3.00, CM-9.00 and CM/sp-9.00) were subjected to biochemical and immunochemical validation.

4.3.1.1 Electrophoresis

To confirm the changes during the alteration of pH of the wheat flour, electrophoresis was carried out. SDS-PAGE pattern of pH altered flour and pasta is shown in the Fig 4.1. The lane B shows the protein pattern of control flour and the lane C shows control pasta. Protein pattern of the control pasta were similar to control flour. The lane D shows CM-3.00 and E shows CM/tp-3.00. Both lanes showed distinct pattern of bands than the control. The bands of higher molecular weight (HMW) range between 205 kDa to 66 kDa were not visible and lower molecular weight bands between 66 kDa and 45 kDa and lesser were of very less intensity. Aminlari and Majzoobi (2002) also made similar observations where disappearance of bands were observed between 66-97 kDa and 55 kDa and lower but noticed reappearance of these bands as the pH increase above pH 5.00. Berti et al, 2007 states that conversion of protein amide groups to carboxylic groups with release of ammonia during mild acidic deamidation imparts negative charges that decrease the isoelectric point of protein and in addition leads to formation of lower molecular weight peptides. They also observed the decreased intensity
of bands of HMW proteins at molecular weight ranging 67 kDa and above as revealed by electrophoretic pattern of gluten suspension in acidic acid produced by heating at 90°C for 3 h. Shiau and Yeh, 2001 studied the effect of addition of lactic acid on characteristics of noodles. They reported that lactic acid increased the protein extractability because of positive charge imparted due to acidic condition. Acidic condition results in repulsion of protein and altered conformational changes. Visschers and de Jongh, (2005) reported that lowering of pH reduces the sulphhydril reactivity that decreases the electrostatic repulsion which causes protein aggregation and promotion of disulphide bond formation. Our findings are in agreement with Berti et al, 2007. The lane F shows CM-9.00 which had different protein profile than acidic modification and control showing very light bands at molecular weight nearing 97 kDa. The lane G shows CM/sp-9.00 had slightly different pattern than CM-9.00. Bands at higher molecular weight from 205 kDa to 97.4 kDa were not seen whereas bands at lower molecular range of 66 kDa were seen with very less intensity. Shiau and Yeh, 2001 also have observed that at alkaline pH, wheat protein undergo sulphhydril and disulphide interchange which contribute to aggregation and polymerization of wheat proteins. This is may be the reason for very less immunogenicity of alkaline modified pasta to IgG raised against Gliadin. This supports the present results and confirms the changes during the alteration of pH which may be the reason for very less immune response to anti-gliadin antibodies produced against gliadin in CD patients.

4.3.1.2 Gel-Filtration chromatography

Gel Filtration chromatographic pattern of all pasta samples along with control flour (unmodified flour) and control pasta is presented in the Fig 4.2. According to Schofield et al, 1983 and Bombara et al, 1997, the elution pattern of wheat proteins can be divided into 4 regions, 1st region where higher molecular weight glutenin bands appear, 2nd represents lower molecular glutenin fractions and 3rd region comprises mostly of gliadins and between the molecular range of 135 to 35 kDa. The 4th region comprises of lower molecular weight proteins of 34 kDa and lesser. Pattern of control flour
showed higher amount of higher molecular weight proteins. This pattern had changed in the control pasta where the content of higher molecular proteins were reduced, correspondingly showing increase in the content of lower molecular proteins. This may be due to effect of temperature during pasta drying which may result in lower solubility and also polymerization of higher molecular weight glutenins to some extent. CM-3.00 showed distinct pattern than the control pasta where, higher molecular protein peak did not appear. It showed only the lower molecular protein peak. This may be due to deamidation. Mimouni et al, 1994 reported that acid treatment at 70°C for 2 h caused deamidation and limited peptide bond hydrolysis. Similarly CM/tp-3.00 showed distinct pattern than the control but showed the presence of higher molecular proteins. Pasta with alkaline pH (CM-9.00 and CM/sp-9.00) showed very distinct pattern than control pasta. The pattern showed only lower molecular weight protein peak indicating that almost all higher molecular proteins were reduced correspondingly increasing the lower molecular weight proteins whereas few bands of higher molecular weight proteins were visible in SDS-PAGE pattern. Similar pattern was observed in CM/sp-9.00. This may be due to sulphhydryl and disulphide bond interchange which results in aggregation and polymerization of wheat proteins. Shiau and Yeh, 2001 observed reduced protein extractability at alkaline condition because of polymerization of gluten proteins which could be related to disappearance of higher molecular proteins. These changes in the pattern might be the reason for reduced immunogenicity of both CM-9.00 and CM/sp-9.00. These altered patterns are supported by the SDS-PAGE pattern of the respective flours.

4.3.1.3 Reverse Phase-High performance liquid chromatograph

HPLC elution patterns of pastas treated with different enzymes are shown in the Fig 4.3. The elution pattern of Control flour which shows peak eluting at 1.6 min with the large area of 21% indicating the lower molecular weight proteins corresponding to gliadins followed by peaks eluting at 2.1, 2.6, and at 3.2 min representing the low molecular weight glutenins (Fig 4.3A). And at 28.4 min peak was shown with the similar large area of 21.4% representing the higher molecular weight glutenins. Fig 4.3 B shows control pasta.
elution pattern was changed in control pasta. An early elution of peak at 1.3 min was observed with the area percentage of 8.4 in control pasta. The area percentage of the peak was also reduced compared to control flour. A similar molecular distribution was observed in Gel filtration pattern also. A similar trend of elution pattern was observed in pasta with acidic pH (CM-3.00 and CM/tp-3.00). Area of 29.91% was observed with the peak eluted at 2.25 min. Peaks corresponding to higher molecular weight were not visible. This correlates with the data of Gel filtration pattern and electrophoretic pattern which were also in agreement with the findings of electrophoretic pattern of Berti et al, 2007. Similarly, CM/tp-3.00 also showed peak with the large area of 30.54% with the peak eluted at 1.6 min. Another peak corresponding to high molecular weight protein covering the area of 19.46% was shown in pasta pH altered by tomato paste was seen. This chromatographic pattern was entirely different in pasta with alkaline pH (CM-9.00 and CM/sp-9.00). Peaks eluted at 1.9 and 2.2 min had the larger area 17.9% and 23.72%. And few peaks corresponding to higher molecular weight were also seen. Similar pattern was observed in CM/sp-9.00 where peaks eluted at 2.0 min and 2.3 min had large area peak of 34.3% and 21.43% respectively. This clearly indicates polymerization of higher molecular weight glutenins and peptide hydrolysis to some extent due to alkaline condition. Mimouni et al, 1994 have reported similar observations. They observed increase in low molecular protein fractions and decrease in the high molecular weight protein fractions when gluten was hydrolyzed with alcalase and acid deamidation.
Fig 4.1 SDS-PAGE pattern of pH altered pasta

A-Molecular marker, B- Control flour, C- Control pasta, D- CM-3.00, E-CM/tp-3.00, F- CM-9.00, G- CM/sp-9.00, HMG- High Molecular weight Glutenins, LMG- Low Molecular weight Glutenins
Fig 4.2 Gel filtration chromatograms of chemically modified pasta
Fig 4.3 HPLC pattern of chemically modified pasta

A-Control flour, B-Control pasta, C- CM-3.00, D-CM/tp-3.00, E- CM-9.00, F-CM/sp-9.00
4.3.1.4 Immunological validation of chemically modified pasta.

Cooked freeze-dried and powdered pH altered pasta were subjected to ELISA and Dot-Blot analysis. Result of ELISA is presented in Fig 4.4. The results reveal the antigenic property of flour when modified using lactic acid and sodium bicarbonate. CM-3.00 and CM/tp-3.00 showed higher immunogenicity similar to control flour whereas CM-9.00 and CM/sp-9.00 showed very less immunogenicity with anti-gliadin antibodies raised against gliadin in rabbit than the other pH varied pasta. This data was further confirmed by Dot-Blot pattern of samples which also exhibited the similar immunogenicity as presented in Fig 4.5. CM-9.00 and CM/sp-9.00 showed very less antibody binding capacity. The spots of CM-9.00 and CM/sp-9.00 on blotting membrane were almost invisible but the spots of CM-3.00 and CM/tp-3.00 showed similar intense colour development as in control pasta and flour. This confirms that antigenic property of the wheat flour can be reduced by alkaline modification using sodium bicarbonate and also using natural ingredient such as spinach. From the SDS-PAGE pattern, it was evident that acidic condition results in the formation of lower molecular weight proteins which ranged from 44 kDa and lesser. This augmentation in lower molecular protein fractions formation may expose more amounts of these lower molecular weight proteins to anti-gliadin antibodies which could be related to increase in the immunogenicity as indicated by immunological assays. In the similar way CM-9.00 showed less immunogenicity. This may be attributed to aggregation and polymerization of glutenin fractions and disulphide formation due to alkaline condition which might inhibit the exposure of allergenic peptides responsible for the immunogenicity. Berti et al, 2007 reported that IgA reactivity of gluten (obtained from hard red spring wheat and winter wheat cultivars) was reduced from 1.6 to 4.3 fold by acid heat treatment. Berti et al, 2007 concluded that conversion of glutamine in glutamic acid resulted from chemical modification would modify epitopes that are recognizable by antibodies.
IgG reactivity

Fig 4.4 Immunogenicity of chemically modified pasta

Fig 4.5 Dot-Blot pattern of chemically modified pasta
A- Control flour, B- Control pasta, C- CM-3.00, D-CM/tp-3.00, E- CM-9.00, F- CM/sp-9.00
4.3.2 Validation of enzyme treated pasta

As explained in the 3rd chapter, Transglutaminase (TG) is an acyl transferase which catalyzes inter-or intramolecular cross linking through formation of peptide bonds between glutamine and lysine residues. The influence of this enzyme is discussed in 3rd chapter. To study protein profile further, these Transglutaminase treated pasta samples (TG 10, TG 50, TG 100 and TG 150) and other pasta variations prepared using enzymes such as protease, actinase and xylanase were subjected to SDS-PAGE.

4.3.2.1 SDS- PAGE pattern of transglutaminase treated pasta

Electrophoretic pattern is shown in the Fig 4.6. SDS-PAGE pattern indicated not much change in the profile compared to control. In all the variations of pasta, diffused bands were observed and this was increased as the level of TG increased. This may be due to cross linking and aggregation of gluten proteins by transglutaminase enzyme which makes the extraction of protein difficult. Lane A and B shows the pattern of control flour and control pasta respectively which showed clear and distinct bands whereas C, D, and F shows TG treated pasta TG 10, TG 50, TG 100 and TG 150 respectively. In these lanes more diffused bands were observed which may be because proteins were unable to separate. TG can enhance the cross linking of proteins which results in polymerization and aggregation of high molecular weight gluten proteins (Takacs et al 2008). Hence, there was not much change was observed in protein profile compared to control. Similar, observations were made by Truong et al, 2004. They reported the polymerized protein pattern of whey protein concentrate after transglutaminase treatment as indicated by SDS-PAGE. Larre et al, 2000 reported that polymerization is directly related to the quantity of enzymes used and also states that TG deamidates proteins apart from crosslinking of the proteins. More diffused bands in transglutaminase treated pasta samples may be due to this increased polymerization of gluten proteins. From the SDS-
PAGE pattern it is evident that this polymerization increased with the increase in the amount of transglutaminase.

4.3.2.2 SDS-PAGE pattern of pasta treated with different enzymes

Fig 4.7 shows the SDS-PAGE pattern of different pasta samples treated with different enzymes. Lane A and B shows the protein profile of control flour and control pasta. The lanes showed clear molecular distribution of proteins of the wheat flour and pasta. The lane C is presented with protein profile of protease treated pasta. Distinct protein profile was observed, where most of the higher molecular bands were not visible and very few bands at lower molecular range were seen with less intensity. This indicated the process of hydrolysis of proteins by protease enzymes could digest almost all higher molecular weight gluten proteins. Transglutaminase, xylanase and actinase treated pasta showed similar protein profile compared to control pasta, where, in no change were observed in the protein profile of the pasta. Slight diffused bands were observed in these enzyme treated pasta compared to control flour and control pasta. This may be due to aggregation of proteins during the modification. Bombara et al, 1997 reported that added exogenous protease caused gradual decrease in the size corresponding to 25 kDa and also increased the smaller molecular mass fractions. Tanabe et al, 1996 studied the effect of bromelain enzyme in making hypoallergenic products. They reported that bromelain enzyme converted most of the wheat proteins to lower molecular weight proteins with molecular weight lesser than 40 kDa which were non-allergenic. Kong et al, 2007 have reported that commercially available protease such as alcalase, pancreatin, nutrase, protamex drastically reduced the molecular weight of the gluten proteins to 40 kDa and lesser. Watanabe et al, 1994(b) studied the effect of collagenase and transglutaminase enzyme in production of hypoallergenic wheat flour and concluded that collagenase treated flour and transgluaminase treated flour retained most of the high molecular weight proteins. Since xylanase enzyme is carbohydrate hydolysing enzyme, no change in the protein profile was observed.
4.3.2.3 Gel filtration

To study the protein profile of treated pasta, all modified pasta samples were subjected to gel filtration analysis. Results are graphically shown in the Fig 4.8. Pattern shows the distribution of gluten proteins based on the molecular size. Gel filtration pattern of wheat flour shows, higher amount of higher molecular proteins, whereas, this pattern had changed in control pasta which showed lesser amount of higher molecular protein and more amounts of lower molecular proteins. This may be due to aggregation of proteins during extrusion process and drying. As explained earlier, the elution pattern can be classified based on the molecular weight. First peak being higher molecular proteins and last being the lowest molecular proteins with molecular weight between (34 kDa to 10 kDa). Protease treated pasta showed very different pattern where increase in the content of lower molecular proteins were observed and simultaneously decrease in the higher molecular proteins. Transglutaminase treated pasta showed different pattern where, distinct peaks were not observed which may be due to aggregation of cross-linking of proteins catalyzing by transglutaminase enzyme. Xylanase and actinase treated pasta also showed different pattern of protein distribution but, was almost similar to pattern of control pasta. Bombara et al, 1997 studied the molecular distribution pattern after protease treatment and reported reduced molecular mass distribution. They concluded that higher molecular glutenin fractions were much affected by fungal proteases. Kong et al, 2007 reported that alcalase (commercial protease) hydrolyzed wheat gluten and showed smaller peptides when studied using size exclusion. Tanabe et al, 1996 developed hypoallergenic bread using protease enzyme. They reported that elution curve of this hypoallergenic wheat flour indicated the production of some low molecular weight proteins and some free aminoacids during the enzymatic reaction.

4.3.2.4 HPLC

HPLC elution profiles of pasta samples treated with different enzymes are shown in the Fig 4.9. Fig 4.9A shows the elution pattern of control flour
and B shows control pasta. Control flour showed peak eluting at 1.6 min with the large area of 21% followed by peaks eluting at 2.1, 2.6, and at 3.2 min representing the lower molecular weight proteins. At 28.4 min peak was observed with the similar large area of 21.4%. This pattern had changed in control pasta. There was an early elution of peak at 1.3 min was observed with the area percentage of 8.4. The size of the peak was also reduced compared to control flour. In protease treated pasta, different protein profile was observed. There was an elution of peak at 1.59 min and at 2.2 min with the large area of 40.53% indicating the increase in the content of low molecular weight proteins and no peak representing high molecular weight were appeared. This was supported by results of gel filtration where increase in the area of the peak representing low molecular weight proteins was observed. Fig 4.9D showed transglutaminase treated pasta where profile was similar to control pasta. Higher molecular weight protein peak was observed with larger area percentage. Fig 4.9 E is presented with HPLC pattern of xylanase treated pasta. It was observed that the enzymes did not affect protein profile of the pasta as it was supported by the SDS-PAGE and gel filtration pattern. Actinase treated pasta is presented in Fig 4.9 F which shows similar protein elution like control pasta. There was not much difference were observed in retention time and area percentage. Whereas this pattern had changed in pasta treated with combination of protease and other enzymes. Fig 4.9 G, Fig 4.9 H and Fig 4.9 I are presented with chromatographic pattern of protease+actinase treated, protease+xylanase treated and microwave and protease treated pasta respectively. The protease and actinase treated pasta showed higher amount of lower molecular weight protein fractions similar to protease treated pasta but some peaks of higher molecular weight peaks were also visible. This had changed when treated in combination with xylanase enzyme. Higher molecular weight protein fractions were peaks were reduced. Similar pattern was observed in pasta treated with both microwave and protease. Based on the biochemical analysis explained in 2nd chapter, microwave treatment had no effective in reducing the immunogenicity. But when protease was used along with microwave treatment enzyme protein profile was altered than control, because of hydrolysis by protease.
**Fig 4.6 SDS-PAGE pattern of Tansglutaminase treated pasta**

*M-Molecular marker, A-Control Flour, B-Control pasta, C-Tg 10, D-Tg 50, E-Tg 100, F-Tg 150.*

**Fig 4.7 SDS-PAGE pattern of pasta treated with different enzymes**

*M-Molecular Marker, A-Control flour, B-Control Pasta, C-P1-protease treated pasta, D-Tg2-transglutaminase treated pasta, E-Xy3-Xylanase treated pasta, F-Act4-Actinase treated pasta*
Fig 4.8 Gel filtration pattern of enzyme treated pasta
Fig 4.9 HPLC pattern of enzyme-modified pasta: A - Control flour, B - Control pasta, C - P1-Protease treated pasta, D - Tg2-Transglutaminase treated pasta, E - Xy3- Xylanase treated pasta, F - Act4- Actinase treated pasta, G - Protease + actinase treated, H - Protease +xylanase treated, I - Microwave and protease treated.
4.3.2.5 Immunochemical validation of enzyme treated pasta

Enzyme treated pasta variations were subjected to ELISA for immunological validation of the products. Results are graphically shown in the Fig 4.10. As indicated by the graph protease enzyme treated pasta showed very less reactivity with anti gliadin antibody ALP conjugate. Transglutaminase, xylanase and actinase treated pasta samples did not show any reduction in the immunogenicity. Similar reactivity compared to control was observed in these samples. Pasta with combination of protease, xylanase and actinase also showed very less immunogenicity whereas, these xylanase and actinase were ineffective when used alone. This low immunogenicity of protease treated pasta may be attributed to hydrolysis of gluten proteins by protease enzyme. SDS-PAGE, HPLC and gel filtration pattern of protease treated pasta clearly indicated the change in the protein profile of protease treated pasta where hydrolysis of higher molecular protein fractions were reduced to lower molecular weight fractions which were not recognizable by anti-gliadin antibodies. As explained earlier concomitant increase in the content of lower weight proteins were observed. ELISA pattern also supports these changes during modification and confirms that these lower molecular weight proteins were not recognized by anti gliadin antibodies thereby indicating the reduced immunogenicity of protease treated pasta. When protease was used in combination with other enzymes such as xylanase and actinase, similar reduced immunogenicity was seen as evident by the ELISA graph. But xylanase, transglutaminase and actinase were not effective in reducing the immunogenicity when used alone. Supporting this results HPLC, gel filtration and electrophoretic pattern also showed no change in the allergen profile of pasta with these enzymes. Watanabe, et al 1994(b), reported that treatments with actinase, transgluataminse and collagenase decrease the allergenicity of both salt soluble and insoluble protein fractions of the wheat flour. Watanabe et al in 2000 studied the IgE binding capacity using pooled sera from wheat allergic patients. They reported that actinase treated flour retained the small degree of allergenicity whereas bromelain cross reacted with pooled sera. IgE binding capacity of flour treated with other enzymes were below the
detectable range. Berti et al, 2007 reported that, similar to human tissue transglutaminase, deamidation catalyzed by microbial transgluaminase also generates the molecular structures that are recognized by the IgA class anti-gliadin antibodies. Hence, it is evident from the results that protease enzyme treated pasta showed lowest immunogenicity compared to other pasta variations. Other enzymes when used with protease enzyme showed similar reduced immunogenicity than when used individually.

4.3.3 Validation of low gluten pasta

Apart from these chemically modified pasta variations and enzymatically modified pasta variations, low gluten pasta was prepared by replacing the 40% of wheat flour with other non-gluten flours such as maize, sorghum and oats. Quality attributes of pasta are discussed in 3rd chapter. Many researchers have established that use of oats increases the nutritional value of gluten free diet and also will not produce the T-cell stimulatory activity in celiac patients as it is distinctly related to wheat. To study the immunochemical characteristics, low gluten pasta was subjected to biochemical and immunochemical validation.

4.3.3.1 Electrophoretic and HPLC pattern of low gluten pasta

SDS-PAGE analysis of the hypoallergenic flour and pasta was carried out in 12% acrylamide gel as presented in Fig 4.11. The electrophoretic pattern of control flour and control pasta as presented in lane B and C respectively clearly shows the molecular distribution of gluten proteins. The pattern of non-gluten blend as presented in lane D showed some less intense protein bands at 97.4 kDa and 66 kDa and also lesser than 23 kDa may be coming from other non-gluten flours. Low gluten pasta also showed similar and less intense protein bands corresponding to allergen profile as shown in the non-gluten blend. Kieffer et al (1982) observed the less intense bands of other cereal prolamines in the same region as wheat omega and gamma gliadins, but little or nothing in the β region. They reported that maize, oat, rice prolamins are characterized by different less intense bands in the regions other than the gliadin region and reported low antigenic activity.
Fig 4.10 Immunogenicity of pasta treated with different enzyme

P1-Protease treated pasta, Tg2- Transglutaminase treated pasta, Xy3 – Xylanase treated pasta, Act- Actinase treated pasta, P+Act4- Protease with Actinase treated pasta, P+Xy- Protease with Xylanase treated pasta, P+Add- Protease with additive, MWP-pasta with microwave treated flour and protease enzyme
HPLC elution pattern of control flour and control pasta are shown in Fig 4.12 A and B. Control pasta had different elution pattern than control flour where slight early elution at 1.3 min was shown with the area of 8.4% compared to control flour where the same peak eluted at 1.6 min with the area of  21.2%. This clearly indicated polymerized proteins due to temperature and extrusion process. Chromatographic pattern of low gluten pasta is presented in 4.12 C. The pattern was changed in low gluten pasta where peak eluted at 1.3 min, had 5.5% area and peak eluted at 2.0 min had the larger area of 61.79%. Slight increase in the area of lower molecular weight fractions was observed and concomitant decrease in the size of higher molecular weight glutenin fractions.

4.3.3.2 Immunological characterization of low gluten pasta

The Fig 4.13 shows the pattern of colour developed when low gluten pasta was subjected to Dot-blot method. The intensity of the colour developed in spot of hypoallergenic pasta was less compared to spot of control pasta and gliadin alone. This indicated that immunological features of the wheat flour are affected when replaced by the non-gluten flours. Low gluten pasta was subjected to invitro protein digestion and was analyzed for immunoreactivity. After pepsin and pancreatin digestion immunogenicity of LGP was significantly reduced. Similar kind of observations was made by Pasini et al, 2001 in their study. They reported that both physicochemical and immunological features of the wheat flour and its digested products were affected after modifications with heat treatment. IgG reactivity of Low gluten blend and low gluten pasta was analyzed using ELISA and presented in Fig 4.14. Results indicated slight reduction in the immunogenicity of low gluten pasta when compared to control pasta. Non-gluten blend also showed decreased immunoreactivity than control flour. But this further reduced when extruded into pasta. This supports the results of electrophoretic and HPLC pattern of protein profile and indicates that the optimized LGP contains less allergen compared to control pasta.
Fig 4.11 SDS-PAGE pattern of low gluten pasta

A-Molecular marker, B- Control flour, C-Control pasta, D-Low gluten blend, E- Low gluten pasta

Fig 4.12 HPLC of low gluten pasta

A-Control flour, B-Control pasta, C- Low gluten pasta
Fig 4.13 Dot-Blot pattern of low gluten pasta

A- Control flour, B- Control Pasta, C- Low gluten blend, D- Low gluten pasta, E- Invitro digested low gluten pasta

Fig 4.14 IgG reactivity of Low gluten blend and low gluten pasta

CF- Control flour, CP- Control pasta, NGB- Low gluten blend, LGP- low gluten pasta

4.3.4 Validation of Gluten free pasta
From more than 50 years gluten free diet has been the main choice of treatment for celiac disease. Development of gluten free product is a real challenge for food technologists as gluten is an essential component in most of the bakery and pasta products. As part of “Development of modified gluten pasta” along with chemically modified pasta, enzymatically modified pasta and low gluten pasta, gluten free pasta formulation was also optimized. The product quality of optimized gluten free pasta is discussed in chapter 3. The biochemical and immunochemical characteristics were studied and results are discussed below.

4.3.4.1 Gel Electrophoresis.

SDS-PAGE patterns of gluten free pasta along with control flour are shown in the Fig 4.15. The lane A is presented with control flour B showed the protein profile of gluten free blend, the lane C showed the protein profile pattern of gluten free pasta, which was distinct from control flour (lane A). Bands corresponding to allergen profile of wheat flour (HMG, LMG and Gliadin) were not observed in gluten free pasta. Gluten free flour showed very intense bands of all molecular sizes because of high protein content of the blend which may be due to addition of soya flour and channa flour, which have high protein content. Some of the bands at molecular weight lower than 23 kDa were also observed with more intensity. This profile pattern was slightly changed in gluten free pasta. Only few bands which were of high intensity in gluten free flour were observed with less intensity in gluten free pasta. Other bands were of very less intensity or were not visible. This could be due to the starch protein and fiber interaction during pasta processing and drying temperature which may be reduce the size of the protein or polymerized to reduce the extractability of the non gluten proteins. For further confirmation gluten free pasta was taken for immunological studies.

4.3.4.2 Immunological characteristics
Immunological validation was carried out to confirm the immunoreactivity of the gluten free pasta. The Dot-Blot pattern of gluten free pasta is shown in Fig 4.16. The pattern shows no colour development in the gluten free spots. The spot A to J shows gluten free pasta with different additives. This confirmed that there was no immunogenicity in gluten free pasta. The ELISA pattern of the gluten free pasta supports the results of Dot-Blot by showing the very less OD values, which were closer to blank OD value as shown in Fig 4.17. This confirms the absence of gluten protein in the flours used for pasta preparation. Storsrud et al, 2003 studied the beneficial effects of oats in the gluten free diet of adults and concluded that allowing oats in the gluten free diet increases the nutritional value and also makes it pleasant for patients without aggravating the disease condition. Several studies have already reported the use of rice, corn, soya, millet, buckwheat, and potato starch in combination along with pea flour, sorghum flour in making gluten free products like biscuits and pasta (Arendt et al, 2002). In this trial combination of soya flour, chickpea flour and sorghum flour along with WPC was used in formulating gluten free pasta. Immunological validation of developed gluten free pasta using these flours, confirms that proteins/prolamins present in these flour are not recognized by the anti-gliadin antibodies.

4.3.5 Validation of optimized pasta using patients’ sera

As an alternative food product, modified gluten pasta using chemical and enzymatic modification was developed. Along with this low gluten pasta and gluten free pasta were also formulated. These modified gluten pasta were subjected to product quality analysis and biochemical and immunochemical validation. For further confirmation of their immunogenicity these pasta samples were subjected to further immunochemical validation using pooled sera from celiac disease patients.
Fig 4.15 SDS-PAGE pattern of Gluten free blend and gluten free pasta

*M-Molecular marker, A-Control flour, B-Gluten free blend, C-Gluten free pasta*
Fig 4.16 Dot- Blot pattern of Gluten free pasta

A- GF Pasta with xanthan gum, B- GF Pasta with HPMC, C- GF Pasta with Guar Gum, D- GF Pasta with xanthan gum, HPMC and Guar Gum, E- GF Pasta without any additive F- GF Pasta without WPC, G- NC1, H- NC2, I- Control pasta.

IgG reactivity

OD at 405nm

Control     IX IIH IIIGG IVxhg VC VI NC1 NC2

Fig 4.17 ELISA pattern of gluten free samples

Ix-GF Pasta with xanthan gum, IIH-GF Pasta with HPMC, IIIGG-GF Pasta with Guar Gum, Ixhg-GF Pasta with Xanthan gum, HPMC and Guar Gum, VC-GF Pasta without any additive VI-GF Pasta without WPC.
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4.3.5.1 Immunogenicity of pasta samples

Serological screening tests such as (Anti-gliadin antibody) AGA-IgG to detect IgG class antibody against gliadin, and AGA-IgA detects IgA class antibody against gliadin, IgA-EMA endomysium antibodies against gliadin and IgA-tTG which detects IgA antibodies to tissue transglutaminase are best markers to diagnose CD apart from the intestinal biopsy. Hence, used patients’ sera were used to detect the presence of allergenic epitope in the developed pasta. Optimized pasta samples which had showed lowest immunogenicity when analyzed using anti gliadin antibody, raised in rabbits were selected for further validation, using CD patients’ sera. IgA, IgG and IgE reactivity was analyzed. For analyzing IgA and IgE reactivity pooled patients’ sera was used as primary antibody and anti-human IgA-ALP conjugate and anti human IgE-ALP were used as secondary antibody. IgG reactivity was also analyzed using rabbit antisera to compare the results. The Fig 4.18 showed ELISA pattern of all optimized pasta. The graph reveals the IgA, IgE and IgG reactivity of all optimized pasta samples. The results indicated that, among all modified pasta samples from different modification processes, pasta with alkaline pH (CM-9.00), pasta with spinach puree (CM/sp-9.00), protease treated pasta (P1), pasta with both spinach puree and protease treated (P+Sp/9.00) and gluten free pasta, documented the lowest IgA, IgE and IgG reactivity. Pasta modified to alkaline condition OD value equal to blank value confirms that the IgA antibodies present in the patients’ sera failed to recognize the altered structure of antigen present in pasta due to
modification that may have occurred as a result of alkaline pH, as explained earlier. Pasta prepared with spinach also showed very less immunoreactivity. Protease treated pasta and pasta with both spinach and protease treatment also showed marked reduction in the immunoreactivity. Gluten free pasta showed no reactivity when analyzed for IgA, IgE and IgG reactivity. This confirms that modification by altering pH 9.00 and protease treatment is more effective in reducing the allergenicity of wheat flour. For further confirmation of immunogenicity of these modified pasta samples, gliadin was extracted using two step sequential gluten method and the extract was subjected for ELISA and immunoblotting.

4.3.5.2 SDS-PAGE of gliadin extracted by sequential extraction method

Gliadin from different modified pasta samples was extracted using sequential extraction method and electrophoresis was carried out. Results indicated that gliadin extract from low gluten pasta showed similar protein profile compared to control but the intensity of bands were lesser than the gliadin extract from control pasta and control flour as presented in the lane C of Fig 4.19. The lane D and E shows CM-9.00 and CM/sp-9.00 where, protein profile were entirely different from protein profile of control flour and control pasta. These lanes showed only few bands at higher molecular weight range but lower molecular weight protein bands were not visible. These bands at higher molecular range may be of glutenin subunits where some amount of glutenin subunits also can be extracted by 50% aqueous isopropanol. Similar protein pattern was observed in gliadin extract of both protease treated pasta and P+Sp/9.00. Very few bands with less intensity at higher molecular weight range were observed in these protease treated pasta samples. This clearly indicates that both chemically modified (pH altered) pasta and enzyme treated pasta showed absence of gliadin which may be because of deamidation or polymerization of peptide sequences that are responsible for celiac disease. This is supported by the immunoblotting analysis where these bands of higher molecular weight were not recognized by the anti human IgA antibody tested against serum of celiac diseased patients. Isopropanol extract of gluten free pasta sample also showed two bands at lower molecular weight range, which
may be coming from prolamins of other non wheat cereals. But these bands were not recognized by the anti human IgA antibody indicating the absence of gliadins responsible for celiac disease.

**4.3.5.3 IgA reactivity of gliadin extract of modified pasta samples**

Optimized modified pasta samples were analyzed for IgA reactivity. Proteins separated through electrophoresis were transferred to nitrocellulose membrane to stain with patients’ sera and antihuman IgA-ALP conjugate. Immunoblot of gliadin extracts is shown in the Fig 4.20. Lane A and B show the immunogenicity of control flour and control pasta. IgA antibodies present in pooled sera of CD patients’ were able to recognize the proteins of control flour and control pasta whereas these antibodies were unable to recognize the low molecular weight gliadin fractions of CM-9.00 and CM/sp-9.00. Only few bands at higher molecular range were recognized by IgA antibodies. Interestingly gliadin fractions protease treated pasta and pasta with alkaline pH and gluten free pasta were not recognized by IgA antibodies, confirming the low immunogenicity of these modified pasta samples.

**4.3.5.4 ELISA and immunoblot of gliadin extracts**

For further confirmation these gliadins extracts were subjected to Ig G reactivity also. The OD values are graphically shown in the Fig 4.21. Pasta with pH 9.00, pasta with spinach, showed very less IgG reactivity. Protease treated pasta also showed marked reduction in the IgG reactivity compared to control pasta and control flour. Pasta with both protease treatment and spinach showed comparatively less immunoreactivity compared to all other variations of modified pasta. Gluten free pasta sample also showed no IgG reactivity. This result was supported by immunoblot. Separated proteins were transferred to NCP membrane and IgG reactivity was analysed. Results are shown in Fig 4.20. Immunoblot clearly indicates that pasta with alkaline pH (CM-9.00, CM/sp-9.00), protease treated pasta (P1), pasta with spinach and protease and gluten free pasta showed no reactivity with antibodies. Low gluten pasta showed very few and very less intense bands at high molecular weight fraction. P1 and P+Sp/9.00 and GF pasta lanes were almost invisible.
This confirms that no immunogenicity was observed in gliadin extracts of modified pasta samples.

Fig 4.18 Immunoreactivity of optimized pasta samples

LGP-Low gluten pasta, MWF-Microwave treated flour, CM-9.00- Chemically modified pasta with pH 9.00, CM/sp-9.00, Chemically modified pasta using spinach puree, P1-Protease treated pasta, P+Sp/9.00-Protease treated pasta with chemical modification to pH 9.00, GF- Gluten free pasta.
Fig 4.19 SDS-PAGE of gliadin extract of different modified gluten pasta

- M: Molecular marker, A: Control flour, B: Control pasta, C: Low gluten pasta (LGP), D: CM-9.00, E: CM/sp-9.00, F: Protease treated (P1), G: Protease treated with chemically modified using spinach puree (P+Sp/9.00), H: Gluten free (GF)
Fig 4.20 Western blot- IgA reactivity and IgG reactivity of gliadin extracts of modified gluten pasta samples

A-Control flour, B-Control pasta, C-Low gluten pasta, D-CM-9.00, E-CM/sp-9.00, F-P1, G-P+Sp/9.00, H-GF

Fig 4.21 IgG reactivity of gliadin extracts of optimized pasta samples

A-Control flour, B-Control pasta, C-Low gluten pasta, D-CM-9.00, E-CM/sp-9.00, F-P1, G-P+Sp/9.00, H-GF
4.4 Conclusion

Results of validation of modified gluten pasta samples from all modification techniques revealed that the immunogenicity of pasta samples using patients’ sera was reduced markedly when its pH was adjusted to pH 9.00 using NaHCO₃ and natural source like spinach puree whereas, pH adjusted to 3.00 using lactic acid and tomato paste did not give satisfactory reduction in the immunogenicity. Biochemical studies also confirm the changes during modification process to proteins which were unable to be recognized by the anti-gliadin antibodies (IgG, IgE and IgA). Results of validation of enzymatic treatment revealed that among different enzymes, protease enzyme is more effective in reducing the immunogenicity of pasta. Results showed that multiple treatment in combination such as protease treatment and alkaline pH is also effective in reducing the immunogenicity of pasta. Biochemical evaluation by SDS-PAGE of pasta indicated distinct protein profile when compared to wheat allergen profile. Dot-Blot and ELISA confirmed non-immune response to antibodies raised against gliadin. Hence, optimized gluten free pasta using high protein, non-gluten flours can be recommended to individuals who exhibit allergic symptoms to wheat gluten.