Effect of protein concentrates, emulsifiers on textural and sensory characteristics of gluten free cookies and its immunochemical validation

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Abstract The effect of 5, 7.5 and 10 % protein concentrates namely soya protein isolate (SPI), whey protein concentrate (WPC) and addition of 0.5 % emulsifiers such as glycerol monostearate (GMS), sodium stearoyl- 2- lactylate (SSL) and lecithin (LEC) on the rheological, sensory and textural characteristics of cookies with rice flour and its immunochemical validation was studied. The results showed that the use of 7.5 % SPI/WPC along with GMS significantly improved the quality characteristics of cookies with rice flour. Dot-Blot and Western-blot studies of cookies with 7.5 % of SPI or WPC confirmed that the anti-gliadin did not recognize these proteins. Carry- through process using ELISA kit confirmed that gluten was within the permissible limit in all the stages of processing and hence these cookies can be consumed by people suffering from celiac disease.

Keywords Cookie · Rice flour · Soy protein isolate · Whey protein concentrate · Emulsifiers · Western blot

Introduction

There is an increasing interest in gluten-free (GF) products as the prevalence of celiac disease increased. Its mean prevalence is estimated to be 1 to 2 % of the world population (Reilly and Green 2012). The preparation of GF bakery products requires

application of different flours, so the resulting taste very often does not resemble that of classical, gluten products. In recent years, researchers have been working more on GF products involving a diverse approach which has included the use of starches, dairy products, hydrocolloids, other non-gluten proteins, prebiotics and combinations, thereof as alternatives to gluten, to improve quality of GF bakery products (Gallagher et al. 2004). To ensure that GF based products are acceptable, with baked and sensory characteristics similar to that of wheat flour. Many non-wheat flours like rice flour, oats, and buckwheat are tried. Among these, rice flour is well accepted and one of the most used cereal flour for the production of GF products due to its bland taste, white colour, high digestibility, and hypoallergenic properties (Marco and Rosell 2008). Regarding nutritional quality, rice-based GF formulations have, low contents of vitamins, minerals, proteins and dietary fibre (Sciarini et al. 2010; Thompson et al. 2005). Arendt et al. (2002) studied the effects of rice, corn, soya, millet, and buckwheat and potato starch, in combination with different fat sources on the formation of GF biscuits. The addition of hydrocolloids to GF products provides the necessary network for obtaining fermented bakery products (Kang et al. 1997).

Dairy and soy- bean proteins are most used proteins in GF products (Gallagher et al. 2003; Ribotta et al. 2004; Moore et al. 2006; Marco and Rosell 2008). Marco and Rosell (2008) reported that addition of soya and egg proteins to GF products showed strong gel-forming behaviour. In addition, it has also been reported that addition of soybean or pea proteins to rice flour modified the mechanical properties of the rice-proteins blend dough.

GF products should meet specific requirements regarding declaration of the gluten content. Hence, a worldwide Codex Standard on Food Labelling has recently been established which resolved the health hazard posed by unknown gluten intake due to insufficient declaration of gluten-containing ingredients and food additives (Codex-Alimentarius

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Commission 2008) and a threshold for the gluten level in GF products of 20 mg/kg was defined (Deutsch et al. 2008). However contamination of these food products by gluten containing cereals can take place at various stage of flour production or stage of production of the final product (Olexova et al. 2004). Recent research is focused 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 GF foods.

With this background, the present study was aimed at developing GF cookies by utilizing rice flour, protein concentrates in combination with emulsifiers and to carry out its biochemical and immunological validation.

Materials and methods

Raw materials

Commercially available wheat flour, rice flour, sugar powder, skimmed milk powder (Gujarat Co-operative Milk Marketing Federation Ltd, Anand, India), salt (common food grade sodium chloride), shortening (Marvo, Hindustan Lever Ltd, Mumbai, India), sodium and ammonium bicarbonate (S.D. Fine Chemicals, Mumbai, India), whey protein concentrate, WPC-70 (Mahaan Proteins Ltd., New Delhi), soya protein isolate, SPI (The Solae Company, Gurgaon, Haryana), sodium stearoyl-2-lactylate (SSL), glycerol monostearate (GMS) and Lecithin (LEC) (PD Brothers, Bangalore, India) were used for the study. Gliadin was procured from SIGMA Chemicals, USA. Molecular marker for SDS-PAGE, and Goat anti-rabbit IgG-alkaline phosphatase conjugate were procured from Bangalore Genie, India. All other chemicals used were of analytical grade.

Proximate analysis

Determination of proximate composition of wheat flour, rice flour, SPI and WPC was carried out using standard methods. Moisture, ash and protein contents were estimated by approved methods (AACC 2000).

Preparation of blends

Blends were prepared using mixtures of rice flour and SPI/WPC in the ratios of 100/0; 95/5; 92.5/7.5 and 90/10 w/w.

Cookies preparation

Cookies from blends containing 0, 5, 7.5 and 10 % SPI/WPC were prepared according to AACC micro method (No. 10-52, 2000). The formulation for the preparation of cookie was rice

flour/5, 7.5 and 10 % SPI/WPC blend-100 g; 0.5 g GMS/SSL/LEC, sugar powder – 60 g, shortening (Marvo, India) - 30 g, skimmed milk powder – 3 g, sodium bicarbonate - 1.0 g, ammonium bicarbonate - 0.75 g, sodium chloride - 1.0 g and water according to requirement. All the ingredients were mixed in a Hobart mixer (Model N-50, Ontario, Canada). Cookie dough was sheeted to a thickness of 1.0 cm and cut using circular cutter of 6.5 cm diameter. Cookies were baked at 200 °C for 13 min, cooled and evaluated for physical and sensory characteristics.

Physical analysis and sensory characteristics of cookies

Cookies were evaluated for physical characteristics, including diameter (mm) and thickness (mm) and spread ratio. Diameter and thickness were measured with caliper, average of 4 cookies were recorded for each batch. The spread ratio of cookies was calculated by dividing values of diameter by the thickness values. The breaking strength, a measure of texture of cookies was determined using the texture analyser (Model TA-HDi, stable Micro Systems, Surrey, U.K.) according to a triple beam snap (also called 3-point break) technique described by Gaines (1991) at a crosshead speed of 50 mm/min and with a load cell capacity of 10 kg. The peak force (g) required to a break a single cookie was recorded and the average of three replicates is recorded.

Color of cookie was measured using Color measuring system (Hunter Lab Color measuring Labscan XE system, USA). Color readings were expressed by Hunter values for L, a and b. L values measure black to white (0 to 100), a values measure redness when positive and b values measure yellowness when positive.

Sensory evaluation of cookies was carried out by a panel consisting of baking technologists. The panelists were trained in four sessions involving 2 h of training in each session. Four samples of cookies in four replicates were evaluated by each panelist following a score card consisting of various quality parameters like surface color, surface cracking pattern, crumb color, texture, mouth feel and flavor. The scores assigned in the score card for these parameters were as follows: Surface colour: 1=dull white, 10=golden brown; Surface cracking pattern: 1=absence of islands/very small/very large islands, 10=medium-sized islands; crumb colour: 1=brown color, 10=creamish white; texture: 1=less crisp, 10=crisp; mouth-feel: 1=residual, 10=no residue; and flavor: 1=dominating flavor, 10=pleasant. The OQS (max. 60) was taken as the total score of all the six quality parameters.

Texture profile analysis of cookie dough

Since the studies showed that the combination of 7.5 % SPI/WPC and 0.5 % GMS produced the best results further studies such as textural evaluation of cookie dough and biochemical

and immunological validation were carried out using only these samples. Texture Profile Analysis of cookie dough with WF/RF/RF+7.5 % SPI/WPC; RF+7.5 % SPI/WPC+0.5 % GMS was carried out at room temperature by using a LR- 5 K Texture Analyser (Lloyds Instruments Ltd, Hampshire, England) with 5 kg load cell. The cookie dough samples (4 cm diameter, 1 cm thickness) were compressed by using an aluminium 80 mm diameter circular disc probe. The texture parameters were determined with cross head speed of 50 mm/min, compression distance of 50 % cookie dough's and 5-s delay between two bites. The data were analyzed by using Nexygen Version 4.0 Software (LR-5 K) to measure cookie dough's hardness, cohesiveness, adhesiveness, gumminess and springiness as described by Bourne (1978).

In vitro protein digestion

The defatted cookies was incubated with pepsin and pancreatin according to the method described by Akeson and Stahmann (1964), with slight modifications. Freeze-dried samples of about 2 g were incubated with 1.5 mg of pepsin in 15 ml of 0.1 mol equiv./L HCl at 37 °C. After 3 h the samples were neutralized with 0.5 mol equiv./L NaOH. The sample was then incubated with pancreatin (4 mg) in 0.2 mol/L phosphate buffer (pH 8) and then 0.005 mol/L sodium azide was added. After overnight incubation, TCA was added and centrifuged at $955.89 \times g$ for 20 min. The supernatant was subjected to protein estimation by Kjeldhal method.

Electrophoresis

Defatted cookie samples were analyzed by Electrophoresis. SDS-PAGE was carried out as per the method adopted by Prabhasankar (2002). 12 % acrylamide gel was used to separate the protein fractions of GF cookies. Gel was stained with coomassie brilliant blue R250.

Dot-blot analysis

Dot-Blot analysis was carried out according to the method described by Susanna and Prabhasankar (2011). Extract of defatted cookies (4 µl/spot) were spotted on 0.2 µm Nitrocellulose membrane (SIGMA chemicals, USA) followed by blocking with 2 % gelatin in PBS-T (Phosphate-Buffered Saline, pH 7.4 containing 0.05 ml Tween 20) followed by treating with rabbit anti-gliadin antibodies. Then, the blot was washed with PBS-T treated with Goat anti-rabbit IgG-ALP conjugate. Finally, the blot was treated with BCIP/NBT substrate.

Western blot

Western Blot was carried out according to the method described by Ma (2006) with slight modification.

Immunoblotting was carried out by transferring separated proteins on electrophoretic gel. Transferring was carried out using Biotech semi dry blot (BIOTECH, R & D Laboratories, India) with the power supply of 6 mA constant current for 2 h. 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. Then blot was 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 1 h at 37 °C then was treated with BCIP/NBT substrate in alkaline phosphatase buffer for 15- 20 min.

Enzyme linked immunosorbent assay (ELISA)

Gluten analysis was performed using the RIDASCREENR-7001Gliadin ELISA (R Biopharm, Germany). Extraction procedure described in the kit insert using Cocktail (R Biopharm, Germany) was followed for the analysis. Each homogenized sample (0.25 g) was weighed into a 15 ml centrifuge tube, 2.5 ml of cocktail solution was added, and the tubes were mixed well followed by incubation for 40 min at 50 °C. Samples were brought to room temperature and added 7.5 ml of 80 % ethanol and incubated for 60 min. on a shaker followed by centrifugation at room temperature for 10 min. at 2,500 g (Eppendorf, 5810R). 80 µl of supernatant was removed and diluted with 920 µl of sample diluent. 100 µl of this solution was used in the assay. Standard and samples were added in wells on the plate and allowed to incubate for 30 min. at room temperature followed by three step washing. At this point substrate and chromogen were added to each well and allowed to react for 30 min, followed by addition of stop reagent. The absorbance was read at 450 nm and data was analysed to determine gluten concentration. The gliadins concentration in µg/kg (ppb) was read from the calibration curve, and further multiplied by dilution factor, then multiplied by 2 in order to obtain the gluten concentration.

Statistical analysis

The data pertaining to chemical and nutritional characteristics were expressed as mean±standard deviation. Sensory mean scores were analyzed using Duncan's new MRT with different experiment groups appropriate to the completely randomized design with three replicates each, as described by Steel and Torrie (1960). The significant level was established at $P < 0.05$.

Results and discussion

Proximate composition of WF, RF, SPI and WPC

Proximate composition of ingredients used in the cookie preparation is discussed here. Moisture content of wheat flour and rice flour was 10.25 % and 10.40 % respectively. Moisture content of protein concentrates was less ranging from 5.67 (SPI) to 6.53 % (WPC). SPI had highest protein content of 89.0 % and WPC had 69.2 %. Lowest protein content was reported in RF (7.67 %). Highest ash content was reported in WPC (5.2 %) and lowest was observed in RF (0.56 %) which was comparable with WF (0.58 %).

Effect of protein concentrates and emulsifiers on the quality characteristics of cookies

Texture analysis of cookie dough

Textural properties are important characteristics affecting the moulding characteristics of dough and quality of the end products. In this study, these properties including hardness, cohesiveness, chewiness, springiness and gumminess were determined using TPA and presented in Table 1. Based on the results, the cookie dough made exclusively from RF had more hardness, adhesiveness, gumminess and less cohesiveness and springiness when compared to cookie dough with WF. Hou et al. (1996) observed that the quality of the wheat flour containing gluten has a direct effect on the dough mixing properties. Use of 7.5 % SPI/WPC+0.5 % GMS significantly improved the textural characteristics of cookie dough with RF as shown by the decrease in the hardness, adhesiveness, gumminess and increase in the cohesiveness and springiness. These results confirm that combination of protein concentrates and emulsifier is building up the structure of RF cookie dough similar to gluten proteins. Brewer et al. (1992) also found that higher soy protein content resulted in higher bread toughness.

Color characteristics of cookies

The color characteristics of cookies are presented in Figs. 1 and 2. The L values of 61.91 for WF cookies and 69.01 for RF cookies show that RF cookies are lighter than WF cookies. Lower a and b values representing redness and yellowness were observed for RF cookies. Addition of SPI decreased L increased a and b. With WPC, L and b value decreased while a increased. It is clear from the Figs. 1 and 2. that the cookies with WPC are darker than with SPI. This may be due to the Maillard reaction between proteins and reducing sugars, contributing to significantly dark brown product color. Huyghebaert (1984) noticed that formation of brown polymers/melanoidins is a result of browning reactions due to protein incorporation. Cocup and Sanderson (1987) also reported that lactose in dairy powders contributes to Maillard and caramelisation reactions. Singh and Mohamed (2007) reported similar results for the cookies containing soya protein isolate. Taha et al. (2006) reported that fortification of cookies with isolates of soy protein and chickpea results in increased darkness of the biscuits. With the addition of emulsifiers to RF cookies with 7.5 % SPI, L and b values decreased, the most significant effect was observed with SSL. Use of emulsifiers in RF cookies with 7.5 % WPC increased L and b while a value decreased indicating opposite effect of emulsifiers in the presence of WPC when compared to SPI. It is clear from these results that emulsifiers decreased the redness of cookies with WPC.

Physical characteristics of cookies

The spread ratio of WF was 8.3, RF (7.2), with addition of 5–10 % SPI to RF decreased the spread ratio from 7.2 to 5.5–6.4, while WPC increased it from 7.2 to 7.9–9.1 (Fig. 1.) The breaking strength value of cookies with WF was 72.87 N and RF–80.50 N. These results indicate that the texture of cookies with RF was harder than WF cookies. Use of SPI and WPC improved the texture of cookies with RF as indicated by the decrease in the breaking strength values from 80.50 to 52.72–58.48 N and 52.40–57.64 N respectively.

Table 1 Texture profile analysis of cookie dough

Attributes	WF	RF	RF+SPI (7.5 %)+GMS (0.5 %)	RF+WPC (7.5 %)+GMS (0.5 %)
Hardness (N)	53.04±0.62 ^{ab}	88.41±6.67 ^d	49.97±0.00 ^a	62.68±5.43 ^c
Cohesiveness	0.08±0.00 ^{ab}	0.05±0.02 ^a	0.06±0.05 ^{ab}	0.10±0.01 ^c
Adhesiveness (Nmm)	0.01±0.03 ^a	0.11±0.60 ^c	0.06±0.04 ^{ab}	0.87±0.72 ^d
Gumminess (N)	6.19±0.42 ^a	9.62±0.94 ^c	5.82±0.25 ^a	7.66±0.38 ^b
Springiness (mm)	0.84±0.05 ^{cd}	0.49±0.06 ^{ab}	0.64±0.29 ^c	0.30±1.53 ^a

WF- Wheat flour, RF- Rice flour, SPI- Soya protein isolate, WPC- Whey protein concentrate, GMS- Glycerol monostearate

Data represent the mean of three replicates±standard deviation; Values in the row with the same letter in superscript are not significantly different from each other at $p \leq 0.05$

Fig. 1 Effect of SPI and WPC on quality characteristics of cookies

The results are in agreement with Taha et al (2006) who reported a decrease in spread ratio of biscuits with increasing amount of chickpea, broad beans and soya protein isolates were substituted with WF. (McWatters 1978) also noticed a decrease in spread ratio of cookies when wheat flour was supplemented with non wheat flours. Reduction in the spread ratio was noticed when soy flour was substituted for wheat flour (Singh et al. 1996). (Kissel and Yamazaki 1975) observed that composite flour form aggregates with increased number of hydrophilic sites available to compete for the limited free water in cookie dough which results in decrease in spread ratio of cookies and affected the top grain formation of cookies (Claughton and Pearce 1989).

It was observed that as the amount of WPC increased the spread ratio of cookies was also increased. This was in agreement with earlier studies reported by (Kadarmestan et al., 1998). Hosene and Rogers (1994) noticed that the interaction of proteins and starch by hydrogen bonding was responsible for the hardness of cookies.

Sensory characteristics of cookies

The surface color of WF cookie was golden brown and cookie with RF showed dull brown color. There was an improvement in the crust color of RF cookies from dull brown to golden brown with the use of SPI and WPC (Table 2). The crumb color of WF cookies was creamish white whereas RF cookies showed yellowish white crumb color. The crumb of RF cookies showed bigger crumb cells with thick cell wall and tunnels. Addition of SPI did not change the crumb color of cookies with RF. However the crumb showed closed and compact cells without any tunnels in it. Cookies substituted up to 10 % WPC had brownish white crumb color, the crumb showed very big cells and tunnels. The size of the cells increased and tunnels became more deep with increase in WPC.

WF cookies possessed medium sized islands while RF cookies showed bigger and superficial thin islands (Fig. 3). Cookies substituted with SPI also had uniform small sized islands at all the levels of replacement. But in case of cookies substituted with WPC, the island size was bigger which is undesirable. The texture of WF cookie was crisp, showed

Fig. 2 Effect of emulsifiers on quality characteristics of cookies

Table 2 Effect of protein concentrates on sensory characteristics of cookies

Attributes	WF	RF	RF			RF		
			SPI (%)			WPC (%)		
			5	7.5	10	5	7.5	10
Surface color (10)	8.5 ^{cd}	7.5 ^c	7.0 ^c	7.5 ^c	8.0 ^{cd}	7.0 ^c	6.0 ^{ab}	5.0 ^a
Surface cracking pattern (10)	8.5 ^c	6.5 ^c	6.5 ^c	7.5 ^{cd}	7.0 ^c	5.0 ^b	4.0 ^a	3.0 ^a
Crumb colour (10)	8.0 ^c	5.5 ^{ab}	6.5 ^b	6.5 ^b	6.5 ^c	5.0 ^a	4.0 ^a	4.0 ^a
Texture (10)	8.0 ^c	5.0 ^c	6.0 ^d	6.5 ^d	6.0 ^d	4.0 ^b	3.0 ^a	2.5 ^a
Mouthfeel (10)	8.0 ^c	5.5 ^c	6.0 ^d	7.0 ^d	6.5 ^d	4.5 ^b	4.0 ^a	3.5 ^a
Flavour (10)	8.5 ^b	7.5 ^{ab}	7.5 ^{ab}	7.5 ^{ab}	7.0 ^a	7.0 ^a	7.0 ^a	7.0 ^a
Overall quality (60)	49.5 ^b	37.5 ^d	39.5 ^c	42.5 ^g	41.0 ^{ef}	32.5 ^c	28.0 ^b	25.0 ^a

WF- Wheat flour RF- Rice flour, SPI - Soya protein isolate, WPC- Whey protein concentrate

Mean values in a row with the same letter in superscript did not differ significantly at $p \leq 0.05$ by DMRT

clean mouth feel whereas RF cookies were hard and showed slightly gritty mouth feel. Addition of SPI decreased the crispness, resistance to bite and residue formation. With WPC, the cookies became brittle initially, later on became somewhat chewy. Singh et al. (1993) carried out studies on the development of high- protein biscuits from composite prepared from wheat, green gram, Bengal gram and black gram flours. They reported that 15 % level of composite flour adversely affected the top grain, texture and color of biscuits. (McWatters et al. 2003) reported that the harder texture of the cookies is due to increased protein content and its interaction

during dough development and baking. Among cookies with different levels of SPI highest OQS was observed for cookies containing 7.5 % SPI. Similarly for WPC, highest OQS was obtained at 7.5 % level. These results confirm that cookies with 7.5 % of SPI or WPC, showed highest improvement than other levels tried. According to Arendt et al. (2008), in a baked product, dairy ingredients form networks that enhance flavor and crust color, improve texture, reduce staling, and increase water absorption.

Effect of emulsifiers on the quality characteristics of cookies

Fig. 3 Photographs of cookies A- WF cookies, B- RF Cookies, C-RF Cookies with 7.5 % SPI+0.5 % GMS, D- RF Cookie with 7.5 % WPC + 0.5 % GMS. RF- Rice flour, SPI - Soya protein isolate, WPC- Whey protein concentrate GMS- Glycerol monostearate, SSL- Sodium stearoyl -2- lactylate, LEC- Lecithin

Since the cookies made with 7.5 % SPI and WPC were considered optimum, these were treated with emulsifiers - GMS, SSL and LEC to further improve the quality of cookies. The emulsifiers influenced differently the quality characteristics of RF cookies with 7.5 % SPI and WPC. The spread ratio of cookies with 7.5 % SPI ranged from 6.2 to 8.2 with addition of emulsifiers (Fig. 2.). Highest increase in the spread ratio was observed with LEC followed in decreasing order by GMS and SSL. With regard to texture addition of GMS improved the texture significantly as shown by the decrease in the breaking strength values from 80.44 to 53.61 N. While other two emulsifiers namely SSL and LEC marginally decreased the breaking strength values. Addition of GMS significantly improved the crumb color from yellowish white to creamish white, from medium size islands to slightly bigger islands. The texture of cookies was crisp, and cookies showed clean mouth feel. With SSL, the cookies were small, thick and it showed very big islands which were deep also. With LEC, the cookies showed more spread with very small sized islands. These data is confirmed by the OQS which was highest for cookies with GMS followed by SSL and LEC (Table 3).

Table 3 Effect of emulsifiers on sensory characteristics of cookies

Attributes	WF	RF	RF+SPI (7.5 %)			RF+WPC (7.5 %)		
			Emulsifiers (0.5 %)			Emulsifiers (0.5 %)		
			GMS	SSL	LEC	GMS	SSL	LEC
Surface color (10)	8.5 ^b	7.5 ^{ab}	7.5 ^{ab}	8.0 ^{ab}	7.0 ^a	6.5 ^a	6.0 ^a	7.0 ^a
Surface cracking pattern (10)	8.5 ^c	6.5 ^c	7.5 ^d	3.0 ^a	4.0 ^{ab}	5.0 ^b	3.0 ^a	3.0 ^a
Crumb colour (10)	8.0 ^d	5.5 ^b	6.5 ^c	6.5 ^c	7.0 ^c	4.5 ^a	4.0 ^a	6.5 ^c
Texture (10)	8.0 ^f	5.0 ^d	6.5 ^c	6.0 ^c	5.5 ^d	4.0 ^c	2.0 ^a	3.5 ^b
Mouthfeel (10)	8.0 ^e	5.5 ^c	7.0 ^d	6.0 ^{cd}	6.5 ^d	4.5 ^b	3.0 ^a	3.0 ^a
Flavour (10)	8.5 ^c	7.5 ^b	7.5 ^b	7.0 ^b	7.0 ^b	7.0 ^b	6.0 ^a	6.0 ^a
Overall quality (60)	49.5 ^f	37.5 ^d	42.5 ^e	36.5 ^d	37.1 ^d	31.5 ^c	24.0 ^a	29.0 ^b

WF- Wheat flour RF- Rice flour, SPI - Soya protein isolate, WPC- Whey protein concentrate, GMS- Glycerol monostearate, SSL- sodium stearoyl -2-lactylate, LEC- Lecithin

Mean values in a row with the same letter in superscript did not differ significantly at $p \leq 0.05$ by DMRT

The spread ratio of the cookies with 7.5 % WPC treated with LEC was extraordinarily high (12.8) when compared to GMS (9.5) and SSL (7.5). Labuschagne et al. (1996) reported that in the presence of 5 % WPC, cookies diameter was increased. The increase in the diameter is the result of interaction between whey protein and gluten. The breaking strength of cookies with LEC was the lowest (17.35 N), indicating fragile nature, while GMS showed breaking strength value of 47.64 N and SSL (56.47 N). Among different emulsifiers tried, GMS brought about significant improvement in crust color, island formation, texture, mouthfeel and overall quality this is indicated by the increase in OQS. With the use of SSL, the island formation was adversely affected and the cookies were devoid of any islands. With LEC, there was an enormous increase in the spread resulting in very thin and fragile cookies. The cookies showed superficial and very thin islands. These results confirm that only the addition of GMS improved the quality characteristics of cookies with

7.5 % WPC as indicated by OQS of 31.5 followed by LEC (29) and SSL (24). GMS has been reported as a monitor of spread ratio and could impart better textural characteristics to cookies (Hutchinson et al. 1997). Sangeetha et al. (2011) reported that sugarcane bagasse based biscuit had better mouth feel, taste and the highest OQS when GMS and SSL were used in combination.

Protein quantity and quality characteristics of cookies

It is clear from the Fig. 4. that the protein content and in vitro protein digestibility value were lower in the case RF (5.4, 31.4 %) cookies than WF cookies (6.7, 58.2 %). Addition of 7.5 % SPI/WPC+0.5 % GMS to RF cookies improved the protein profile significantly. 7.5 % SPI+0.5 % GMS had highest protein content and protein digestibility (7.9, 86 %) followed by WPC (7.2, 74.1 %) Taha et al. (2006) reported that fortification of biscuits with different chickpea, broad bean and isolates of soya protein increased the protein

Fig. 4 Total protein content and in vitro protein digestibility (IVPD) of cookies

Fig. 5 a SDS –PAGE pattern of WF and GF cookies and **b** Western blotting of WF and GF cookies. A- RF cookies, B- RF+ 7.5 % SPI+0.5 % GMS, C- RF+ 7.5 % WPC +0.5 % GMS, D-WF cookies, MM- Molecular marker

content. Ribotta et al. (2004) and Sanchez et al. (2004) reported that soy has long been used to add the needed amino acids to complement grain proteins and to improve nutritional quality. It also improves mechanical behaviour of dough and textural quality.

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE patterns of GF cookies along with WF cookies are shown in Fig. 5a. In GF cookies, much intense bands were obtained than WF cookies may due to SPI/WPC having very high protein than WF. In Fig. 5a, the lane A, B, C shows the protein profile pattern of GF Cookies, which is distinct from WF cookie (lane D), and bands corresponding to allergen profile (HMG, LMG and Gliadin) are not observed in GF cookie. This result is in agreement with earlier studies reported by Susanna and Prabhasankar (2012) who evaluated GF pasta for its allergenicity and noticed that wheat allergen profile were not observed. Susanna and Prabhasankar (2011) noticed that wheat flours modified with protease showed a decrease in the intensity of protein bands corresponding to allergen profile of unmodified wheat flour. For further confirmation GF cookies was taken for immunological studies.

Immunological characteristics

Immunological validation of GF cookies was done to confirm the immunoreactivity. The Dot-Blot pattern of GF cookies is shown in Fig. 6. The pattern shows no color development in GF cookies spot, whereas spot was observed in WF cookies. Western blot also supports the results of Dot- Blot showing no bands in GF cookies whereas a distinct band was observed in WF cookie shown in Fig.5 b. This confirms the absence of gluten protein in cookies. In this study, immune-blotting analysis with polyclonal antibody reveals that antibody recognized

the wheat protein and gives colored spots. Development of GF diet presents most formidable challenge for the food technologist. The committee for nutrition and food for special dietary uses in Codex Alimentarius allows maximal of 20 ppm in the GF products (Di Cagno et al. 2005). Several studies have already been reported on the use of non wheat flours like rice, corn, soya, millet, buckwheat, potato starch, pea flour, sorghum flour in making GF products like biscuits and pasta (Arendt et al. 2002; Wang et al. 1999). (Susanna and Prabhasankar 2011) reported the effect on immunological features on replacement of wheat flour with non-gluten flours. They also carried out immunochemical validation of GF pasta and reported no immune reactivity. GF cookies developed using RF, SPI and WPC confirms that proteins present in the flour and protein concentrates are not recognized by the antibodies developed against the gliadins. Hence cookies developed can be considered as immunologically safe for celiac disease patients.

Carry- through property of GF cookies

Carry-through property of GF cookies from raw materials to final products was estimated through ELISA format of commercially available kit. Spaenij-Dekking et al. (2005) have used the same cocktail method for detection of gluten content and expressed in ppm. At different stages of processing namely raw materials, cookie dough and finally baked cookies, gluten content was estimated. The gluten content for WF

Fig. 6 Dot- Blot pattern for GF cookies. RF cookies, B- RF+7.5 % SPI+ 0.5 % GMS, C- RF+7.5 % WPC +0.5 % GMS, D-WF cookies

Table 4 Validation of cookies and ingredients using ELISA

Samples	Gluten (ppm)
WF	68.5±0.02
RF	2.1±0.04
SPI	12.0±0.04
Cookie dough	9.5±0.02
WF Cookies	62.3±0.09
RF Cookies	8.8±0.04
RF+SPI (7.5 %)+GMS (0.05 %)	12.1±0.08
Commercial GF Cookies	8.1±0.02

WF- Wheat flour, RF- Rice flour, SPI- Soya protein isolate, GMS- Glycerol monostearate, GF-Gluten free

was 68.5 ppm, RF (2.11 ppm) and SPI (12.08 ppm); cookie dough (9.5 ppm), baked cookies with WF (62.5 ppm), RF (8.8 ppm) and 7.5 % SPI+0.5 % GMS (12.1 ppm) (Table 4). ELISA method confirms that the amount of gluten present in the final product was within the permissible limits (less than 20 ppm) and cookies were comparable with commercial GF cookies. The detection of some amount of gluten may be due to contamination during processing conditions. Some studies have been conducted on carry through property of antioxidants in biscuit processing by Nanditha et al. (2009). But so far no studies have been reported on complete validation of the product at different stages of processing in GF food products. This is the first study to show the complete validation of the product at different stages of processing right from procurement of raw materials till end product stage.

Conclusion

GF cookies were developed using rice flour and protein concentrates such as SPI and WPC along with GMS. Addition of GMS improved the textural quality characteristics of GF cookies. Sensory results indicated that overall quality of GF cookies were acceptable and comparable to wheat flour cookies. SDS-PAGE pattern of gluten free cookies showed distinct protein profile. Dot-Blot and western blot studies confirmed that antibodies developed against gliadins did not recognize any allergic protein peptides. ELISA results confirms that the amount of gluten present in the cookies was within the permissible limit (<20 ppm) and comparable with commercial GF cookies. The study concludes that the developed GF cookies using rice flour and protein concentrates can be consumed without fear of allergic symptoms. Hence GF cookies can be recommended to individual who exhibit gluten allergy or celiac disease.

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