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Effect of pasta drying temperature on gastrointestinal digestibility and allergenicity of durum wheat proteins

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Abstract

The effects of different drying temperatures (20, 60, 85, 110 and 180 °C) on digestibility and potential allergenicity of durum wheat proteins were studied in model pasta samples, cooked in boiling water (MPSs). Increasing the drying temperature resulted in increased protein denaturation and aggregation. *In vitro* treatment of MPSs with pepsin and pancreatin showed similar protein degradability up to a drying temperature of 110 °C, resulting in the disappearance of the main prolamin components. In contrast, the MPS treated at 180 °C was much less digestible, due to the presence of Maillard-type protein aggregates.

By using sera of patients with food allergy to wheat, the potential allergens of MPSs were detected by IgE-immunoblotting and IgE dot blotting before and after digestion. The results indicated that the digestion process, although sufficient to degrade the wheat proteins in the MPSs dried up to 110 °C, was not able to completely abolish the presence of IgE-reactive peptides, irrespective of the level of the heat-treatment.

Keywords: Food allergy; Allergens; Wheat; Pasta; Drying; Digestion

1. Introduction

Pasta is commonly eaten, not only in the Mediterranean area, but also in most of the European countries and in the United States. Pasta is a basic food, particularly in Italy, where its consumption reaches about 28 kg/person/year (Wu, Hareland, & Warner, 2001). Pasta is mainly produced with (tetraploid) durum wheat (*Triticum turgidum* subs. *durum*) semolina and, in Italy, the name "pasta" can be used only for 100% durum wheat products. The technological ability to produce pasta is due the unique ability of wheat semolina to give rise, after mixing with water, to a dough with a peculiar viscoelastic behaviour. This property, as in the case of (hexaploid) bread wheat, is largely due to the water/salt-insoluble gluten proteins, also known as wheat prolamins, which can be divided into gliadins and glutenins. Gliadins are monomeric proteins, accounting for about 30% of the total proteins of the wheat kernel. According to their amino acid sequences and electrophoretic mobility in acid-polyacrylamide gel electrophoresis, these components are classified into α/β-, γ- and ω-gliadins. Glutenins are very high molecular weight polymeric proteins in which the subunits (high- and low-molecular weight glutenin subunits, HMW-GS and LMW-GS, respectively) are linked together by disulfide (SS) bonds (Mac Ritchie, 1992).

Wheat proteins belonging to both the soluble and insoluble fractions can act as allergens in sensitised individuals. While the soluble proteins seem to be responsible for respiratory allergy, such as baker's asthma arising from flour inhalation, the insoluble gluten proteins are found to be

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involved in IgE-mediated allergy after ingestion of wheat foods (for a review see Palosuo, 2003).

Once ingested, the wheat allergens are modified in the gastrointestinal (GI) tract by the action of the digestive enzymes (gastric pepsin, chymotrypsin, trypsin and various types of peptidases) before being adsorbed through the gut epithelium. Therefore, the physical-chemical and biological properties, including solubility and immunogenicity, of these proteins can be completely changed before coming into contact with their potential site of allergenic action in the gut (Simonato, Pasini, et al., 2001). In considering the effects of the GI processing of wheat proteins, however, the temperature levels reached by the wheat food during its preparation must be taken into account, because heating can affect protein digestibility, as previously demonstrated by studying bread crumb and crust (Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001).

Although a large quantity of information can be found on the allergens of (hexapolid) bread wheat (*T. aestivum*) (Palosuo, 2003), a few studies have been devoted to the allergological characterisation of only some proteins of (tetraploid) durum wheat (Kusaba-Nakayama et al., 2000; Sanchez-Monge, Garcia-Casado, Lopez-Otin, Armentia, & Salcedo, 1997; Simonato, Pasini, De Zorzi, Vegro, & Curioni, 2004; Simonato, Pasini, Giannattasio, & Curioni, 2002). The proteins of durum wheat are very similar to those of bread wheat in terms of both molecular characteristics (Autran, Lew, Nimmo, & Kasarda, 1979) and allergological properties, as demonstrated by IgE immunoblotting with sera of wheat allergic patients (Simonato, De Lazzari, et al., 2001; Simonato, Pasini, et al., 2001). However, bread and pasta preparation involves different temperature treatments, which, as mentioned above, can influence the digestibility and the allergological properties of the wheat proteins (Simonato, Pasini, et al., 2001).

The pasta production process involves extrusion and drying to reach about 12.5% of moisture. Over the past few years, technological changes in pasta processing have involved an increase in drying temperatures from 75 °C to 100 °C and even above, thus reducing the drying time from the original 48 h to only 2–3 h. The main advantages deriving from high temperature drying are related to the reduction of microbial contamination and the improvement of cooking quality of pasta (Acquistucci, 2000; Dexter, Matsuo, & Morgan, 1981). This is because the high temperature treatment reinforces the wheat protein network, thus preventing starch leaching during cooking and maintaining satisfactory surface conditions in cooked pasta (Feillet, Ait-Mouth, Kobrehel, & Autran, 1989). On the other hand, the occurrence of Maillard-type reactions caused by high drying temperatures leads, among other things, to protein modifications due to cross-linking and reactions with other compounds (Acquistucci & Quattrucci, 1993; Erbersdobler, 1986), which can affect both protein digestibility and antigenicity (Davis and Williams, 1998; Simonato, Pasini, et al., 2001).

While the effects of high temperature drying on wheat proteins have been described in relation to pasta quality, almost nothing is known regarding the consequences of the drying temperature on the gastrointestinal degradability and allergological properties of wheat proteins.

In view of these facts, by using laboratory-prepared MPSs, we here study the effects of drying temperature on the digestibility and potential allergenicity of durum wheat proteins as they are ingested by humans by eating pasta.

2. Materials and methods

2.1. Materials

Pepsin (E.C. 3.4.23.1) from hog stomach (≈3000 U/mg) was from Fluka. Pancreatin from porcine pancreas, anti-human IgE peroxidase-conjugate antibody, anti-rabbit IgG alkaline-phosphatase conjugate antibody and BCIP/NTB alkaline phosphatase substrate tablets were from Sigma. Nitrocellulose membranes were from Sartorius. The Gel Code Glycoprotein staining kit and the Super Signal detection kit were from Pierce. All other chemicals were of analytical grade. The polyclonal rabbit antibody specific for the prolamin group (HMW-GS, LMW-GS and α/β- and γ-gliadins) was that described as anti-12 by Curioni, Dal Belin Peruffo, Pressi, and Pogna (1991).

2.2. Patients

Nine wheat allergic patients who reported GI symptoms (diarrhoea, abdominal pain and/or bloating) after ingestion of foods containing wheat, including pasta, were selected as previously reported (Simonato et al., 2001). These patients showed positive in both wheat-specific IgE detection with the CAP-FEIA system (Pharmacia, Uppsala, Sweden) and challenge tests with pasta (Simonato, De Lazzari, et al., 2001). The sera of these patients were pooled and used for IgE immunoblotting.

2.3. Methods

2.3.1. Preparation of model pasta samples (MPSs)

Durum wheat semolina (6 g) was mixed with water (1.8 ml) by hand to obtain a dough with the typical consistency of those prepared for making pasta at home according to the Italian tradition. The obtained dough was divided into five parts and each part was sheeted, cut in stands (as for preparing "tagliatelle") and dried at different temperatures (20, 60, 85, 110 and 180 °C) to reach 10% of moisture. The moisture content of each sample was determined immediately after drying and was calculated as the weight loss. Dried samples were cooked in salted boiling water for 10 min. After removing the cooking water, the samples were freeze-dried, reduced to a fine powder by grinding in a mortar and sieved.

2.3.2. Protein extraction

Powdered MPSs (1 g) were extracted in 0.05 M acetic acid (20 ml) for 3 h at room temperature by continuous stirring (Aktan & Khan, 1992). After centrifugation (12,000*g* for 15 min), the pellets were freeze-dried, whereas the supernatants were kept for SDS–PAGE analysis.

Total proteins were extracted from 100 mg of powdered MPSs with 4 ml of 25 mM Tris–HCl buffer, pH 8.3, containing 6 M urea, 2.5% (w/v) sodium dodecyl sulphate (SDS), 2.5% (v/v) 2-mercaptoethanol (2-ME) and 10% (w/v) glycerol and heated for 5 min at 100 °C. After centrifugation (12,000*g*, 20 min), the supernatant (total protein extract) was analysed by SDS–PAGE.

2.3.3. In vitro protein digestion of MPSs dried at different temperature

In vitro protein digestion was performed as previously described (Pasini et al., 2001). Briefly, MPSs (60 mg) were suspended in 4 ml of 0.2 N HCl (pH 2.2) containing 0.05 mg/ml of pepsin. After 30 min, 1.15 ml of 1 M boric acid, 0.5 N NaOH, adjusted to pH 6.8 with 5 N HCl and containing 0.25 mg/ml of pancreatin, was added. The resulting pH was 7.6. Both the reactions were performed at 37 °C in a shaking water bath and stopped at different times (0, 30 min of pepsin attack and 30, 60 and 120 min of pancreatic digestion) by addition of 0.5 volumes of 0.6 M Tris–HCl pH 7.4, containing 30% (w/v) glycerol, 6% (w/v) SDS and 6% (v/v) 2-mercaptoethanol. Samples were immediately heated at 100 °C for 5 min, centrifuged and then analysed by Tricine–SDS–PAGE.

Alternatively, the digestion reactions were stopped at different times (0, 15, 30 min of pepsin attack and 30, 60 and 120 min of pancreatic digestion) by addition of 1 volume of 20% (w/v) trichloroacetic acid (TCA). After standing for 1 h, the samples were centrifuged (8000*g*, 10 min) and the pellet was analysed for nitrogen content.

2.3.4. Nitrogen quantification

Samples were mineralized according to the method of Hach, Brayton, and Kopelove (1985) and nitrogen was quantified by the AOAC method 33.056 (1984).

2.3.5. Electrophoresis

Total protein extracts were analysed by SDS–PAGE according to Laemmli (1970), with a total polyacrylamide concentration in the gel of 16%.

To analyse (by SDS–PAGE) the acetic acid-soluble proteins, an aliquot of acetic acid extract was diluted with 0.3 volumes of 1.33 M Tris–HCl buffer, pH 7.4, containing 8% (w/v) SDS, 10% (v/v) 2-ME and 40% (w/v) glycerol and heated for 5 min in a boiling water bath. The same samples were also analysed in non-reducing conditions by omitting 2-ME from the above solution.

Electrophoresis was run at 50 mA constant current. Digested MPSs and undigested controls were analysed by Tricine–SDS–PAGE, as previously described (Pasini et al., 2001), in a 16% total polyacrylamide gel. In this case, electrophoresis was run at 30 mA constant current.

Gels were stained with Coomassie Brilliant Blue (CBB) or used for semi-dry blotting (Curioni et al., 1991). Molecular weight standard proteins (Bio Rad) were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

2.3.6. Immunoblotting

Immunoblotting experiments were carried out as previously described, with an appropriate dilution (1:200) of the anti-prolamin antibody (Curioni et al., 1991). Anti-rabbit IgG alkaline phosphatase-conjugate goat antiserum was used as the secondary antibody. Immunoenzymatic activity on blots was revealed with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/NBT tablets).

A pool of sera from allergic patients, previously characterized as suffering from GI symptoms after ingestion of wheat-based foods (Simonato, De Lazzari, et al., 2001), was also used in immunoblotting experiments. Sera IgE binding on blots was detected with an anti-human IgE peroxidase-conjugate antibody and peroxidase activity was revealed by chemiluminescence using the SuperSignal detection kit, following the manufacturer's instructions (Simonato, De Lazzari, et al., 2001; Simonata, Pasini, et al., 2001). Pooled sera from both healthy subjects and patients with allergy not related to wheat (i.e. allergic to kiwi and some other fruits, but not to cereals) were used as negative controls.

2.3.7. Dot blotting

10 μl of pasta samples, taken after 150 min of pepsin-pancreatic digestion, were spotted, in both reducing and non-reducing conditions, on a 0.22 μm nitrocellulose membrane and air-dried. The membrane was stained with a solution of 0.1% (w/v) Ponceaus in 3% (w/v) trichloroacetic acid to check transfer and destained in water. The membrane was then probed with pooled patients sera, as described above. The negative control was bovine serum albumin (BSA).

2.3.8. Carbohydrate detection on gels

Carbohydrates were stained on electrophoretic gels by the periodate method, using the GelCode Glycoprotein staining kit from Pierce.

3. Results

3.1. Effect of pasta drying temperature on semolina proteins

Protein solubility of durum wheat proteins in 0.05 M acetic acid was used as a measure of protein modification in MPSs dried at different temperatures (Aktan & Khan, 1992). Increasing the drying temperature caused a significant increase in the amount of protein which could not be solubilised in acetic acid (insoluble proteins, IP). As

compared to the IP content in the MPS dried at 20 °C, approximately 5%, 9%, 23% and 39% of the total proteins became insoluble in acetic acid in the MPSs dried at temperatures of 60, 85, 110 and 180 °C, respectively (Fig. 1).

The acetic acid-soluble proteins (SP) of durum wheat semolina and MPSs were analysed by SDS–PAGE in both reducing and non-reducing conditions (Fig. 2). Under reducing conditions (Fig. 2A), the MPSs treated at 20 °C and 60 °C (lanes 2 and 3, respectively) had protein patterns similar to that of semolina (lane 1), comprising the main wheat prolamins, i.e. HMW- and LMW-GS (*Mr* value of ≈ 100 and 31–45 kDa, respectively), ω-gliadins (*Mr* ≈ 60 kDa), α/β and γ gliadins (*Mr* ≈ 30–40 kDa), and also some bands belonging to the albumin/globulin fraction (*Mr* ≈ 14–16 kDa). In the MPSs treated at 85 °C and 110 °C, the protein pattern of the SP fraction (Fig. 2A, lanes 4 and 5) was rather similar to that of the MPSs dried at lower temperatures, although the bands were fainter, confirming a decrease of protein extractability, which involved most of the different components belonging to semolina SP fraction. In contrast, the bands of the SP fraction of the MPS treated at the highest temperature (180 °C) were only barely detectable after SDS–PAGE (Fig. 2A, lane 6) and the protein bands corresponding to members of the α-amylase inhibitors protein family (*Mr* of about 16 kDa) (Carbonero & Garcia-Olmedo, 1999) completely disappeared.

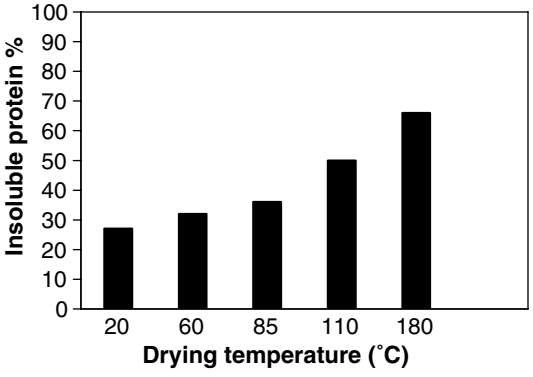

Fig. 1. Acetic acid-insoluble protein (percent on total protein of the sample) in model pasta samples dried at different temperatures.

When analysed by SDS–PAGE under non-reducing conditions (Fig. 2B), the SP fractions of semolina and MPSs showed a heavy streaking at the top of the resolving gel, indicating the presence of protein aggregates unable to enter the gel. These aggregates should correspond to glutenin polymers with such a molecular weight as to be soluble in acetic acid (Mac Ritchie, 1992). The aggregates detected in both the semolina and the MPSs dried at 20 and 60 °C progressively decreased in the MPSs treated at 85, 110 and 180 °C (Fig. 2B, lanes 4, 5 and 6, respectively). This result suggests that high temperature treatments caused extensive aggregation of the gluten proteins, leading to insolubility in acetic acid (Hansen, Johnston, & Ferrel, 1975; Pasini et al., 2001).

The extracts obtained by treating MPSs with a strongly denaturing solvent (SDS/2-ME/6 M urea) were analysed by SDS–PAGE (Fig. 3). The proteins of the MPSs treated up to 110 °C (T_{20-110}) became totally soluble in the SDS/2-ME/6 M urea system, as assessed by measuring the nitrogen content of the un-dissolved material (not shown). No protein aggregates were detected by SDS–PAGE in these MPSs (Fig. 3, lanes 1–4), indicating that the forces involved in their stabilisation were disrupted by the solubilisation system. Therefore, these forces should be identified as hydrophobic interactions and disulfide (SS) bonds, which are broken by SDS/6M urea and 2-ME, respectively. On the other hand, it was not possible to completely solubilise the proteins of the MPS treated at 180 °C (T_{180}) by the SDS/2-ME/6 M urea system (data not shown). Therefore, it is likely that heating at 180 °C led to the formation of protein aggregates stabilised by strong irreversible interactions, which had to be different from SS bonds and/or hydrophobic interactions. As a matter of fact, when analysed by SDS–PAGE, the material solubilised by SDS/2-ME/ 6 M urea from the T_{180} MPS contained a fraction

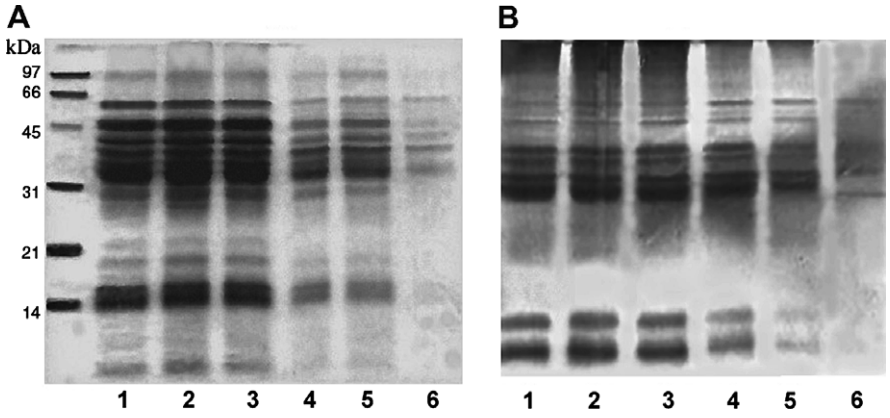

Fig. 2. SDS–PAGE analysis under reducing (A) and non-reducing (B) conditions of the acetic acid-soluble proteins of durum wheat semolina (lanes 1), and model pasta samples dried at 20 °C (lanes 2), 60 °C (lanes 3), 85 °C (lanes 4), 110 °C (lanes 5) and 180 °C (lanes 6). Molecular weight standard proteins are on the left.

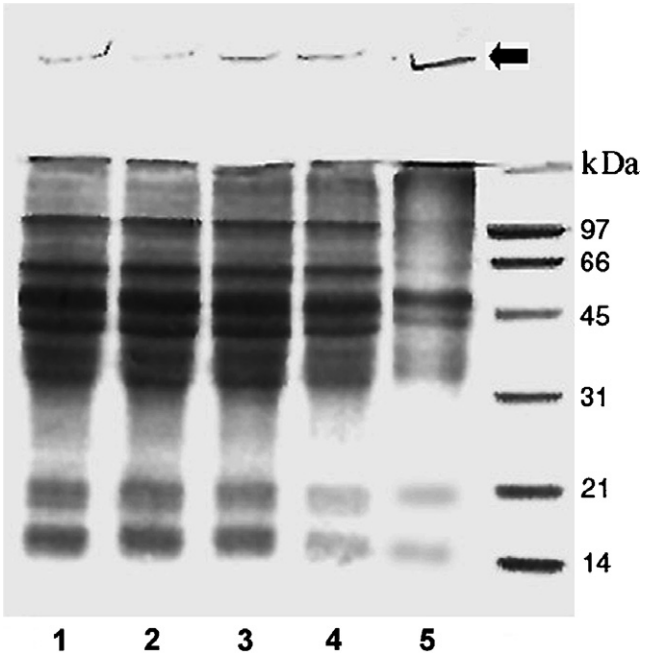

Fig. 3. SDS–PAGE analysis in reducing conditions of the proteins extracted with SDS/2-mercaptoethanol/urea from the model pasta samples dried at 20 °C (lane 1), 60 °C (lane 2), 85 °C (lane 3), 110 °C (lane 4) and 180 °C (lane 5). Protein aggregates are indicated by arrow. Molecular weight standard proteins are on the right.

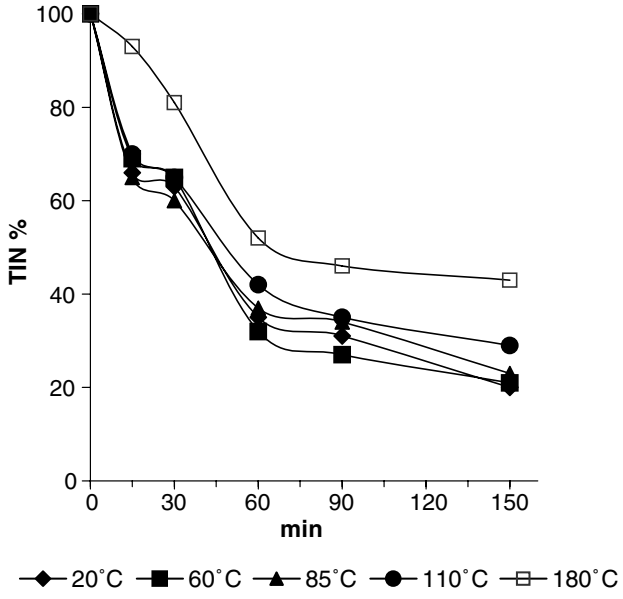

Fig. 4. TCA-insoluble nitrogen (TIN) during a 150 min *in vitro* digestion with pepsin (30 min), followed by pancreatin (120 min) of the model pasta samples dried at different temperatures.

of protein aggregates which were unable to penetrate the pores of the separating gel (arrow in Fig. 3), confirming that protein cross-linking through covalent bonds occurred during heating at 180 °C (Ames, 1992; Gerrard, 2002).

3.2. In vitro protein digestion of MPSs dried at different temperatures

Since wheat protein digestibility can be affected by heating (Hansen & Johnson, 1976; Pasini et al., 2001), MPSs treated at different temperatures were digested by an *in vitro* multi-enzymatic system, simulating the digestion process occurring in the gastrointestinal (GI) tract. MPSs were treated sequentially with pepsin at pH 2.2 (gastric digestion) and pancreatin at pH 7.6 (duodenal digestion). Samples were taken out at different digestion times and their TCA-insoluble nitrogen (TIN) was quantified. This method allows a measure of the amount of polypeptides with a molecular weight >400 Da, thus giving an idea of the course of the protein degradation during the digestion process (Pasini et al., 2001). Two different behaviours of the digested MPSs were noted: the first typical of the MPSs dried at temperatures up to 110 °C ($T_{20–110}$) and the second specific for the MPS treated at 180 °C (T_{180}) (Fig. 4). After 15 min of pepsin proteolysis, the TIN of the $T_{20–110}$ MPSs was reduced to ≈65% of the initial value, remaining as such also after 30 min of enzyme action while the TIN of the T_{180} MPS remained much higher during the pepsin digestion. After 120 min of pancreatin attack, the TIN of the MPSs treated up to 85 °C tended to approach the same value of ≈ 20% of the initial one, while a slightly lower digestibility (TIN ≈ 28%) was measured for the MPS dried at 110 °C. This result indicates that, at this latter temperature, the thermal modifications have begun to affect protein digestibility. In contrast, at the end of the digestion process, the TIN of the T_{180} MPS reached only about 50% of the initial value, confirming that heat-processing affects the digestibility of the wheat proteins in relation to the temperature level used (Pasini et al., 2001).

The samples taken at increasing times of *in vitro* digestion were also analysed by Tricine–SDS–PAGE [a technique allowing detection of polypeptides with very low *Mr* (Schägger & Von Jagow, 1987)] and compared with the corresponding undigested MPSs. Because the electrophoretic patterns of all the $T_{20–110}$ MPSs were similar (not shown), only one of these MPSs and its digestion products are shown in Fig. 5. The undigested $T_{20–110}$ MPSs showed electrophoretic patterns similar to that of unprocessed semolina, showing the protein components typical of durum wheat. During digestion, a progressive breakdown of the gluten proteins and a corresponding accumulation of stained material at *Mrs* lower than 15 kDa was detected (Fig. 5), resembling what was previously observed for commercial pasta (Simonato et al., 2004). A different behaviour was shown by the T_{180} MPS (Fig. 6A). In this case, in addition to a loss of bands and a general decrease in band definition, the presence of protein aggregates blocked at the top of both the stacking and resolving gels (Fig. 6A, lane 5, arrows) was noted. These aggregates seemed to remain unaffected during the entire digestion process, indicating that they were resistant to the action of the proteases. Moreover, carbohydrate detection (on the gel) showed the presence of sugars at the level of the protein aggregates (Fig. 6B), which, on the contrary, could

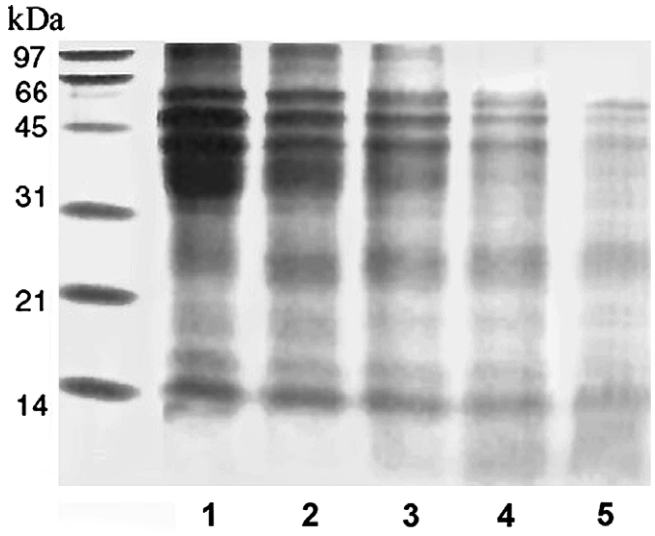

Fig. 5. Tricine–SDS–PAGE analysis under reducing conditions of the proteins of the pasta sample dried at 60 °C (lane 1) and its digestion products taken after 30 min of pepsin (lane 2) and 30, 60 and 120 of pancreatin (lanes 3, 4 and 5, respectively) hydrolysis. Molecular weight standard proteins are on the left.

not be seen when the T_{20-110} MPSs were analysed (not shown). These results suggest the occurrence of intense Maillard-type reactions only in the MPS dried at 180 °C.

3.3. Immunoenzymatic analysis of MPSs and their digestion products

Immunoblotting analyses with a polyclonal antibody specific for the wheat flour prolamins (Curioni et al., 1991) was performed to study the fate of these proteins after both peptic and pancreatic digestion of the different MPSs. The antibody recognised the same bands in all the undigested MPSs (Fig. 7), confirming the immunological heat-resistance of at least some components of the wheat prolamins (Pasini et al., 2001). The same protein components could be immuno-detected after pepsin hydrolysis, but, after pancreatic digestion, the binding of the antibody was no longer detectable in any of the T_{20-110} MPSs. This indicates that the prolamins recognised by the antibody, although showing pepsin resistance, were degraded by pancreatin to fragments with a molecular weight so low as to allow them to run off the electrophoretic gel. When the undigested T_{180} MPS was analysed by immunoblotting, in addition to the antigens recognised in the MPSs treated at lower temperatures, the appearance of a heavy streaking in the stacking gel was evident (Fig. 7 E, lane 1), indicating the presence of protein aggregates in which immuno-reactive prolamin components were involved. These aggregates were resistant to pepsin digestion but, after pancreatic treatment, were no longer immuno-detectable (Fig. 7E, lanes 2 and 3), as already observed for the bread crust (Pasini et al., 2001). Since the protein complexes of the T_{180} MPS were detected by CBB staining until the end of digestion (Fig. 6B), it would seem that the pancreatin action caused a degradation of the prolamins in the aggregates, limited to their antibody binding sites. Nevertheless, prolamin components with *Mr* between 31 and 45 kDa were recognised until the end of the digestion process in the T_{180} MPS. These results confirm that the high temperature treatment induced an increased resistance to the proteolytic degradation by pancreatic enzymes, as observed by both TIN quantification and electrophoretic analyses.

3.4. Serum IgE binding to MPSs proteins and their digestion products

The proteins extracted from MPSs and their digestion products were analysed by IgE-immunoblotting with pooled sera of patients suffering from GI symptoms after ingestion of wheat-based foods, including pasta (Simonato, De Lazzari, et al., 2001).

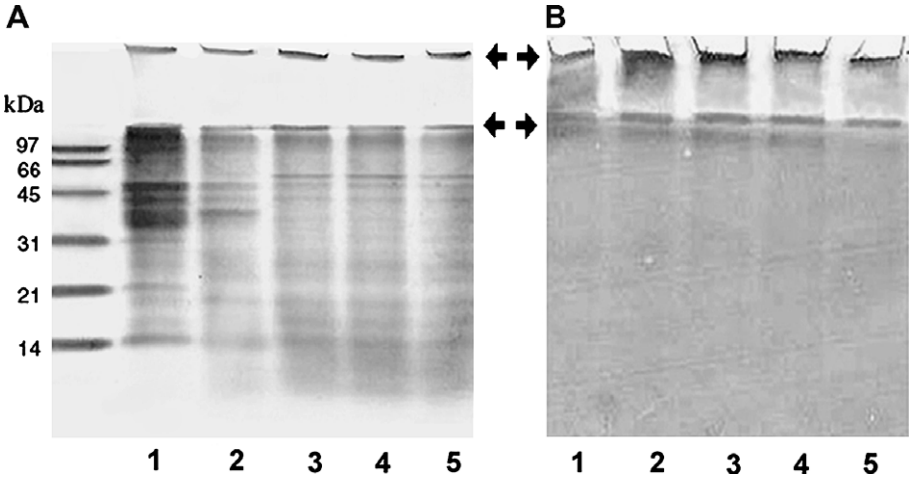

Fig. 6. Tricine–SDS–PAGE analysis under reducing conditions of the proteins of the model pasta sample dried at 180 °C (lane 1) and its digestion products taken after 30 min of pepsin (lane 2) and 30, 60 and 120 of pancreatin (lanes 3, 4 and 5, respectively) hydrolysis. Protein aggregates are indicated by arrows. Gels were stained for total protein (A) and carbohydrate detection (B). Molecular weight standard proteins are on the left.

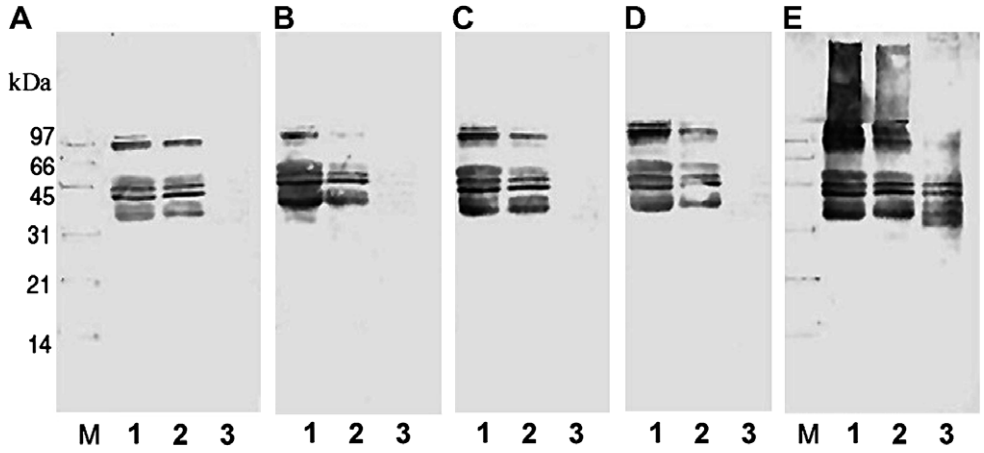

Fig. 7. Immunoblot with a prolamin-specific rabbit antibody after Tricine–SDS–PAGE under reducing conditions of the proteins of the undigested model pasta samples (lanes 1) and their digestion products taken after 30 min of pepsin (lanes 2) and 120 of pancreatin (lanes 3) hydrolysis. The samples dried at 20 °C (A), 60 °C (B), 85 °C (C), 110 °C (D) and 180 °C (E) are shown. Molecular weight standard proteins (M in A and E) were marked with a pencil before immunostaining.

The results obtained for T_{20-110} undigested and digested MPSs showed the same IgE-binding pattern (data not shown). For this reason, only the immunoblotting result of one of the above-mentioned MPSs, in comparison with that of the T_{180} MPS, is reported (Fig. 8). Undigested T_{20-110} MPSs (Fig. 8, lane 1) showed an IgE-binding pattern similar to that previously observed for commercial pasta (Simonato et al., 2004). Bands with an electrophoretic mobility corresponding to that of prolamin components (HMW- and LMW-GS and α/β- and γ-gliadins) were recognised by the IgE in undigested MPSs, while the low *Mr* albumins (≈ 16 kDa) which could be bound by the IgE in raw semolina (Simonato et al., 2004) were not detectable in processed pasta. After 30 min of pepsin digestion of the T_{20-110} MPSs, protein bands corresponding to the HMW- and LMW-GS, and, in particular, a band with *Mr* around 42 kDa, were recognised by serum IgE (Fig. 8, lane 2). These results demonstrate a partial resistance to pepsin hydrolysis of the potential pasta allergens, confirming the results obtained with the prolamin-specific antibody (Fig. 7). However, after pancreatic hydrolysis, no protein bands were recognised by the serum IgE, indicating that all the potential allergens of T_{20-110} MPSs detectable in Tricine SDS–PAGE gels were degraded during the *in vitro* digestion process. Different results were obtained for the T_{180} MPS (Fig. 8). Compared to the T_{20-110} MPSs, a strong modification of the IgE binding pattern was evident in the undigested T_{180} control, as well as in the samples deriving from its proteolytic digestion. The high molecular weight protein aggregates, blocked at the top of both the stacking and the resolving gels, were recognised by the IgE until the end of the digestion process, suggesting that these heat-induced aggregates with allergenic potential were resistant to the action of the digestive enzymes. Moreover other IgE binding bands, with *Mr* around 42 kDa, were detectable until the end of enzymatic digestion, indicating that the thermal treatment prevents the GI degradation of some potential allergens present in durum wheat semolina. These results are similar to those previously observed by studying the potential allergenicity of bread crust (Simonato, Pasini, et al., 2001).

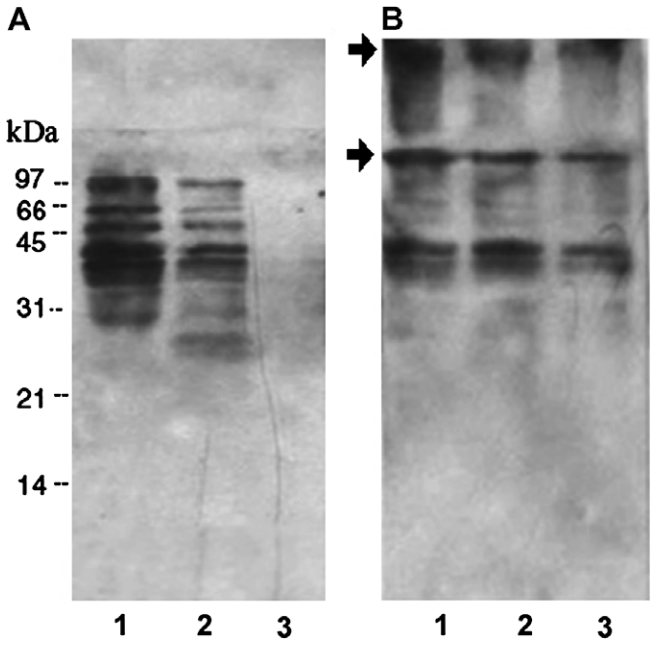

Fig. 8. IgE immunoblot with pooled sera of patients with food allergy to wheat products after Tricine–SDS–PAGE under reducing conditions of the proteins of the undigested model pasta samples (lanes 1) and their digestion products taken after 30 min of pepsin (lanes 2) and 120 of pancreatin (lanes 3) hydrolysis. The samples dried at 60 °C (A) and 180 °C (B) are shown. Protein aggregates are indicated by arrows.

Although Tricine SDS–PAGE is a technique that allows detection of polypeptides with very low *Mr*, the presence of potentially allergenic peptides, with a molecular weight so low as to escape from the gel in the digested MPSs, could not be excluded. Unfractionated digests obtained from T_{20-110} MPSs were spotted on a 0.22 μm nitrocellulose membrane to detect the presence of IgE-binding material

of low molecular weight. Incubation of the spotted membrane with the sera pool showed IgE binding in all the samples (Fig. 9) demonstrating the presence of IgE-reactive peptides also in those samples that did not show any reaction in IgE-immunoblotting after Tricine SDS–PAGE (Fig. 8). The same results were obtained in both reducing and non-reducing conditions (Fig. 9) indicating that the potential allergenicity of the whole mixture of the proteolytic fragments deriving from the digestion was not affected by the presence of intra- or inter-molecular disulphide bonds.

4. Discussion

Although a large quantity of information can be found on the allergens of bread wheat (Palosuo, 2003), only a few studies have been devoted to the allergological characterisation of durum wheat (Kusaba-Nakayama et al., 2000; Sanchez-Monge et al., 1997; Simonato et al., 2004; Simonato et al., 2002), although this cereal is largely utilised for pasta production, especially in Italy.

In a previous paper we have shown that bread baking has a strong effect on the physicochemical properties of wheat proteins, resulting in a decreased protein digestibility (Pasini et al., 2001), which also involves potential neoallergens formed during heating (Simonato, Pasini, et al., 2001). In contrast, the *in vitro* digestion of a cooked commercial pasta sample resulted in the disappearance of IgE binding to durum wheat allergens, indicating that they may be completely degraded before reaching the intestine (Simonato et al., 2004). Therefore, despite a certain clinical reactivity detected for patients after eating pasta (Simonato, De Lazzari, et al., 2001), it was hypothesized that patients with food allergy to wheat may tolerate pasta better than bread (Simonato et al., 2004). Because the proteins of bread and durum wheat share almost complete homology (Autran et al., 1979), these differences between bread and pasta can be related to the different heat treatments used for their production. As a matter of fact, the potential allergenicity of a food can be modified by heat-processing (Davis and Williams, 1998; Simonato, Pasini, et al., 2001) and, in addition, these modifications can also affect food protein degradability by the GI enzymes and, therefore, their potential allergenicity in the intestine.

Although producers are normally reticent to publicize the values of the pasta drying parameters, it is well known that the drying systems used in industrial processing can involve treatments from 50–60 °C up to more than 100–110 °C, these latter temperatures being similar to those reached by the bread crumb during baking.

Taking these facts into account, the effects of the drying temperature on the digestibility and potential allergenicity of laboratory-prepared MPSs has been studied, by considering three temperatures in the range of those used industrially (60, 85 and 110 °C) whose effects were compared to those of both a traditional room temperature drying (about 20 °C) and a ultra-high temperature treatment (180 °C). This latter temperature, which obviously is not used in industrial pasta preparation, was considered in order to confirm the effects of a very drastic heating, as those previously observed for the bread crust (Pasini et al., 2001; Simonato, Pasini, et al., 2001). Moreover, in order to consider food as it is commonly eaten, each MPS was cooked in boiling water before being analysed.

Increasing the drying temperature caused a progressive loss of solubility in acetic acid of the pasta proteins (Fig. 1). Since protein solubility depends on the molecular size, which is affected by heating (Pomeranz, 1991), the decrease of durum wheat protein solubility after drying confirmed that an aggregation of semolina proteins occurred during this process (Aktan & Khan, 1992; De Stefanis & Sgrulletta, 1990; Dexter et al., 1981; Mei & Tung-Ching, 1996). For the MPSs treated at temperatures in the range of those used industrially (i.e. up to 110 °C), this aggregation should be due to disulfide bonds formation and hydrophobic interactions, because almost complete solubilisation was possible by using a solvent able to disrupt these interactions. In contrast, the same solvent did not allow complete solubilisation of the proteins of the MPS dried at 180 °C, which showed the presence of protein aggregates stabilised by irreversible protein interactions. Similar results have been previously reported in studies on the thermal modifications occurring during both heating of bread wheat gluten (Guerrieri & Cerletti, 1996) and bread baking (Pasini et al., 2001).

As in the case of the bread crust, protein aggregation in the MPS treated at 180 °C probably occurred through interpeptide cross-linking and Maillard-type reactions (Ames, 1992; Mei & Tung-Ching, 1997), as indicated by the presence of sugar residues associated with the protein aggregates (Fig. 6B).

The degradation of food proteins during digestion in the GI tract can have relevant effects on their ability to reach the intestinal mucosa in an immunologically active form

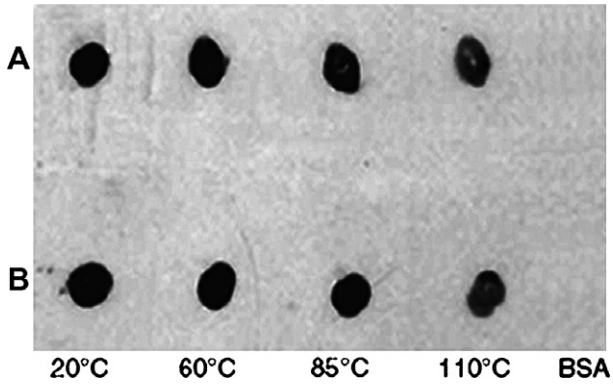

Fig. 9. Dot blot with pooled sera of patients with food allergy to wheat. Unfractionated digested model pasta samples were spotted before (A) and after (B) reduction with 2-mercaptoethanol. Negative control (BSA) is reported on the right.

(Mittag et al., 2004). As a consequence, the presence of IgE binding proteins in the undigested food does not necessarily mean that these potential allergens are able to elicit an allergic response at the level of the gut, because they can be degraded by the GI proteases during digestion. However, in addition to the specific resistance of a given food protein to proteolysis, the degradability of the allergens can be influenced also by the heat-treatment used during food preparation (Davis & Williams, 1998). Drying temperatures, at least up to 85 °C, seemed not to impair MPSs protein digestibility, whereas the MPSs treated at a temperature of 110 °C, which can used in very-high-temperature (VHT) pasta drying (Didonè & Pollini, 1990), tended to be less digestible than were the samples dried at lower temperatures. The effect of the temperature on protein degradability was confirmed in the MPS treated at 180 °C, which clearly showed a reduced digestibility, probably due to the presence of extensive heat-induced proteins aggregation (Fig. 6).

A joint consideration of the results suggests that the protein aggregates should be of two distinct types, depending on the temperature used during the thermal treatment. Polymers stabilised by SS bonds were typical of the MPSs dried at temperatures up to 110 °C, whereas protein aggregates also containing sugars, likely arising from Maillard-type reactions, were detectable in the MPS treated at 180 °C. The presence of these polymers seems to be related to variation of GI digestibility of the wheat proteins (Pasini et al., 2001). Therefore, as well as the effects on pasta cooking quality (De Stefanis & Sgrulletta, 1990) and nutritional value (Acquistucci, 2000), also the negative consequences on protein digestibility of the drying temperature should be taken into account in choosing the technology for industrial pasta production.

The problem of protein degradability in the GI tract is of particular relevance for proteins that act as food allergens after ingestion (Mittag et al., 2004; Simonato, Pasini, et al., 2001). In most cases, these proteins elicit the immunological response in sensitised individuals at the level of the gut associated lymphoid tissue (GALT). Therefore the GI modifications of the IgE-binding epitopes play a key role in determining the possibility of eliciting the allergic response in the gut.

To study this aspect, pooled sera from wheat allergic patients showing GI symptoms after being challenged with durum wheat pasta (Simonato, De Lazzari, et al., 2001) were tested for IgE binding to the proteins extracted from MPSs before and after *in vitro* digestion. IgE binding to the low molecular weight allergens belonging to the alpha-amylase inhibitor protein family (Gomez et al., 1990) was not detectable in any of the MPSs, confirming the heat-lability of their IgE binding epitopes (Simonato et al., 2004). In contrast, pasta processing and cooking did not affect the IgE binding to prolamin components, which have been shown by several authors to be potentially implicated in determining food allergy to wheat products (Palosuo, 2003; Simonato, De Lazzari, et al., 2001; Simonato, Pasini, et al., 2001). As mentioned above, this allergenic activity is strongly influenced by the digestion process, to a point that pepsin resistance is considered a characteristic which distinguishes the potential food allergens (Astwood, Leach, & Fuchs, 1996). However, the effects of the pancreatic enzymes should also be considered. This seems to be particularly important for wheat gluten proteins, whose allergenicity was shown even to be enhanced by pepsin, but abolished by further tryptic digestion (Aoki & Kushimoto, 1987). Moreover, gliadins showed partial stability to the action of gastric pepsin, but were rapidly digested in a simulated duodenal fluid (Mittag et al., 2004). In the case of processed pasta proteins, the potential allergens of the undigested food showed a partial resistance to the pepsin action, but seemed to be completely degraded by the pancreatic enzymes, at least in those MPSs treated with temperatures in the range of those used in industrial drying (i.e. up to 110 °C).

This would indicate that eating pasta, as it is normally prepared, should be safe for wheat-allergic patients showing symptoms after eating bread (Simonato et al., 2004). However, this seems not to be completely true from the clinical point of view, because some GI symptoms were reported by the patients used in this study also after ingestion of pasta and this was confirmed by challenging them with this food (Simonato, De Lazzari, et al., 2001). As a matter of fact, IgE binding to all the unfractionated digested MPSs dried at temperatures from 20 to 110 °C was detected when they were spotted on a nitrocellulose membrane, indicating the presence of peptides maintaining their immunological activity. Therefore, it seems that the digestion process, at least *in vitro*, although sufficient to degrade the wheat proteins to peptides with such a size as to allow their escape from the electrophoretic gel, is not able to completely abolish the presence of allergenic structures. Moreover, the IgE binding reactivity of the unfractionated digests was not affected by the presence of a reducing agent and therefore it is likely that the IgE binding epitopes correspond to sequences not involving the S-S bonds originally present in the wheat gluten. IgE binding to digested pasta was detected in all the MPSs, including that dried at room temperature. Therefore the presence of potentially allergenic peptides seems not to be related to the level of the thermal treatment, but rather to an intrinsic resistance to the GI enzymes. The presence of digestive protease-resistant wheat prolamin fragments, able to reach the intestine in a immunologically active form, has been reported previously in studies aimed to identify the peptides involved in celiac disease (CD) (Shan et al., 2002). Although the antigens/epitopes involved in the pathogenesis of CD and IgE-mediated allergy seem to be different (Constantin, Huber, Granditsch, Weghofer, & Valenta, 2005), the presence of common structural features among the wheat prolamin components (Shewry & Tatham, 1990) would justify a common behaviour with respect to the GI protease actions.

In conclusion, the results here reported indicate that the temperature treatments in the range of those used in industrial pasta drying, although having some effects on the semolina proteins, do not affect their allergenic potential, as occurs during bread baking (Simonato, Pasini, et al., 2001). However, the degradation of the potential allergens present in durum wheat semolina during GI digestion do not result in the complete disappearance of IgE binding peptides of low molecular weight, whose immunological action could sensitise and elicit type I allergic reactions in the intestine.

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