1 **Process conditions affect starch structure and its interactions with proteins in rice pasta**

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17 **Abbreviations: DTNB**, 5,5-dithiobis(2-nitrobenzoate); **DTT**, dithiothreitol; **GI**, glycaemic index;

18 **M**, protein markers; **P1**, pasta made from native rice flour by extrusion-cooking; **P2**, pasta made

19 from parboiled rice flour by conventional extrusion; **P3**, pasta made from parboiled rice flour by

20 extrusion-cooking; **PRF**, parboiled rice flour **RF**, untreated rice flour; **RS**, resistant starch; **TS**,

21 total starch.

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23

ABSTRACT

Structural changes of starch and proteins in rice pasta were investigated as a function of raw-materials and pasta-making conditions, and their impact on cooking behavior and glycaemic index was assessed. Rice pasta was prepared from untreated or parboiled rice flour by conventional extrusion or by extrusion-cooking. Starch structure was studied by assessing starch accessibility to specific enzymes (α-amylase and pullulanase), and by evaluating the molecular properties of fragments from enzymatic action. Protein solubility in presence/absence of chaotropes and accessibility of protein cysteine thiols allowed to evaluate the intensity and nature of inter-protein interactions. Parboiling stiffens the protein network in rice flour and makes starch more accessible to hydrolysis. Pasta-making induced further changes in the starch structure, that were most evident in pasta made from untreated rice and were mainly related to the amylopectin fraction. Thus, the interplay among structural modifications on starch and/or proteins affect the features of products.

Highligths:

1) Novel treatments and processes to prepare rice pasta with satisfactory properties were compared

2) Sensory properties of rice pasta relate to structural features of starch and proteins

3) Amylopectin structure was most sensitive to treatments used for production of rice pasta

4) Parboiling affected rice protein reticulation to a lesser extent than extrusion-cooking

5) Process-related structural changes had an impact on the glycaemic index of rice pasta

Keywords: rice pasta, parboiling, extrusion-cooking, starch structure, enzymatic starch hydrolysis, protein structural rearrangements

48 **1. Introduction**

49 Consumption of rice pasta is increasing also outside the traditional Asian markets, mostly
50 because of health-related issues. Rice-based products are low in allergens and fat, easily digestible,
51 and suited for gluten-free diets (Rosell & Marco, 2008). Pasta with appropriate cooking behavior
52 can be obtained from rice flour either by adding additives (Marconi & Carcea, 2001), or by
53 applying appropriate heating/cooling treatments during pasta processing (Pagani, 1986). However,
54 development of these processes to improve the sensory features of products and to satisfy market
55 requirements has been based almost invariably on semi-empiric approaches (Parker & Ring, 2001).

56 The ability to relate process-dependent modifications in some macromolecular properties or
57 in the interactions among different or similar macromolecules to product features may represent the
58 starting point for engineering rice pasta with suitable features in terms of texture and nutritional
59 properties on a somewhat more scientific basis. Various approaches have been developed to predict
60 the macromolecular behavior during the process (Petitot, Abecassis, & Micard, 2009; De Noni &
61 Pagani, 2010; Cabrera- Chávez et al., 2012), and some studies have tried to addres non-gluten
62 matrices, where the main structural backbone is provided by a three-dimensional starch network
63 (Marti, Seetharaman, & Pagani, 2010; Marti, Pagani, & Seetharaman, 2011).

64 The aim of this study was to investigate in detail the organization of macropolymers in
65 native and heat-treated rice flours used as starting ingredients for rice pasta, and the modifications
66 caused by pasta-making itself. The structural and nutritional effects ensuing from the different
67 organization of proteins and polysaccharides as induced by the various processes were also
68 investigated on the cooked pasta.

69

70 **2. Materials and Methods**

71

72 ***2.1 Flour and pasta samples***

73 Rice flour (RF; total carbohydrate: 84% db; protein: 7% db; particle size < 250 μm) was

74 produced in an industrial plant by directly grinding native dehulled rice (*Oryza sativa,* cultivar
75 Indica; amylose 25 g/100 g total starch; Riso Viazzo s.r.l., Crova, Italy). Parboiled rice was
76 produced from the same paddy rice in an industrial plant (Riso Viazzo s.r.l., Crova, Italy) by
77 steeping at 70°C, followed by steaming at 100°C, and by a final drying step at 50°C for 5 hours.
78 The parboiled rice was ground into flour (PRF; total carbohydrate: 83% db; protein: 8% db; particle
79 size < 250 μm).

80 Pasta from RF (P1) was prepared by an extrusion-cooking process (Marti, Seetharaman, &
81 Pagani, 2010). Rice flour (RF) and water at 60 °C were blended to a final moisture of 40%. The
82 mixture was heated by steam at 2.5 atm (> 120 °C) for 10 minutes in a gelatinization tank. The pre-
83 gelatinized dough was then subjected to a first extrusion at 120 °C in a Progel extruder (single-
84 screw type, Braibanti, Milano, Italy) to generate pellets (small cylinders, 2-3 mm diameter). The
85 wet pellets were immediately transferred to a continuous press for semolina pasta (Braibanti, Milan,
86 Italy), in which a second and final extrusion step was carried out at 50 °C.

87 Pasta from parboiled flour was produced using two different extrusion conditions. Pasta P2
88 was produced by applying to a PRF/water mixture (40% final moisture) the conventional extrusion
89 process used for preparing durum wheat semolina pasta, at an extrusion temperature of 50 °C. Pasta
90 P3 was produced from a PRF-water mix (40% final moisture) that underwent a double extrusion
91 step, as described for sample P1.

92 All the samples were formed into macaroni shape (7 mm o.d.) and dried in a pilot-scale
93 plant using a low-temperature drying cycle (50 °C max, 14 hours) (Marti, Seetharaman, & Pagani,
94 2010). When appropriate, pasta was ground (particle size < 250 μm) in a laboratory mill (IKA
95 Universalmuhle M20, Janke and Kunkel GmbH & Co KG, IKA Laborteknic, Staufen Germany).

96

97 ***2.2 Chemical analysis***

98 Analysis of the various rice flours was performed according to AOAC (2005) for moisture
99 (934.01), protein (960.52) and to AACC (2001) for carbohydrates (AACC 76-13). Data are from

100 triplicate determinations.

101

102 ***2.3 Properties of the polysaccharide network***

103 *2.3.1 Enzymatic susceptibility to α-amylase*

104 Susceptibility to α-amylase was determined by using the Starch Damage Assay Kit (AACC
105 76-31, 2001; Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow,
106 Ireland) with some modifications. The sample (100 mg) was dispersed in 3 ml of 0.1 M sodium
107 phosphate buffer, pH 6.9. Alpha-amylase (1.1 U) from *Bacillus subtilis* (EC 3.2.1.1, 449 U/mg) was
108 added, followed by incubation at 20 °C. The amount of amylase was increased to 11.1 U when
109 treating RF samples for short incubation times, since lower amounts of enzyme did not produce
110 measurable hydrolysis.

111 At appropriate times, samples were centrifuged (12000 × *g*, 10 min, 20 °C), and
112 amyloglucosidase was used to convert into glucose all soluble starch material from the previous
113 hydrolytic step. An 1-ml aliquot of the supernatant was diluted with 4 ml of 0.2 M sodium acetate
114 buffer pH 4.5, prior to adding 0.1 ml of amyloglucosidase (E.C. 3.2.1.3, 3300 U/ml, from
115 *Aspergillus niger*, Megazyme International Ireland Ltd). After incubation for 30 minutes at 50 °C,
116 volume was adjusted to 100 ml with water. A 0.1 ml aliquot of the diluted sample was added to 3
117 ml of the GOPOD reagent solution (Megazyme International Ireland Limited), and the absorbance
118 at 510 nm was read against a reagent blank after incubation at 50 °C for 20 min, using glucose for
119 calibration. Results are the average of four replicates, and are expressed as mg glucose/g sample.

120

121 *2.3.2. Susceptibility to pullulanase*

122 A sample aliquot (100 mg) was dispersed in 3 ml of 50 mM sodium acetate buffer, pH 6.0,
123 to which 200 U of pullulanase (EC 3.2.1.41, ≥400 U/mL; from *Bacillus acidopullulyticus*; Sigma
124 P2986) were added. After 1, 2, and 24 h incubation at 37 °C, the amount of soluble hydrolysis
125 products was quantified as glucose according to the procedure presented above.

126

127

128 ***2.4. Characterization of starch fragments from enzymatic hydrolysis by SE–HPLC/Light***
129 ***Scattering***

130 Supernatants from enzymatic treatments (prior to the treatment with amyloglucosidase for
131 glucose quantitation) were filtered through a 0.22 μm filter, and 0.2 ml of the filtrate were loaded
132 into a HPLC system (515 pump, Waters Co., Milford, MA, USA); UV detector (Dual Absorbance
133 detector 2487, Waters Co., Milford, MA, USA), connected in series to a differential refrattometer
134 (Optilab T-rEX, Wyatt Co., Santa Barbara, CA, USA) and to a Multi Angle Light Scattering
135 instrument (DAWN HELEOS, Wyatt Co., Santa Barbara, CA, USA). Polysaccarides were
136 fractionated on a size-exclusion column (Ultrahydrogel^{TM} Linear 7.8 x 300 mm, Waters Co.,
137 Milford, MA, USA), using as eluant either 0.1 M sodium phosphate buffer, pH 6.9 (for samples
138 deriving from α-amylase hydrolysis) or 0.05 M sodium acetate, pH 6 (for samples deriving from
139 pullulanase hydrolysis), at a flow rate of 0.4 mL/min. The Astra software (ASTRA V 5.1.9.1, Wyatt
140 Technology Co., Santa Barbara, CA, USA) was used for data analysis.

141

142 ***2.5 Pasting properties of starch***

143 Pasting properties were measured in a Brabender Micro-Visco-AmyloGraph (MVAG)
144 (Brabender OHG, Duisburg, Germany). Each sample was finely ground, and 15 g of the resulting
145 powder were dispersed in 100 mL of distilled water. The pasting properties were evaluated, in
146 triplicate, under constant conditions (speed: 250 rpm; sensitivity: 300 cm g_f). according to Marti,
147 Seetharaman & Pagani (2010).

148

149 ***2.6 Properties of the protein network***

150 *2.6.1 Protein solubility*

151 Protein solubility in native and denaturing conditions was determined by suspending 0.5 g of

152 finely ground sample in 10 mL of 50 mM phosphate, 0.1 M NaCl, pH 7.0 containing 8 M urea or 8
153 M urea and 10 mM dithiothreitol (DTT) when indicated. Suspensions were stirred for 30 and 60
154 minutes at 25 °C. After centrifugation (10000 × *g* for 20 min, 20 °C) the amount of protein in the
155 supernatant was determined by a dye-binding method (Bradford, 1976) using bovine serum albumin
156 as a standard. Results are expressed as mg proteins/g sample.
157

158 *2.6.2 SDS-PAGE*

159 The protein profile in various samples and extraction conditions mentioned above was
160 analysed by SDS-PAGE after denaturation in the presence of 2-mercaptoethanol on a 12% gel using
161 a Miniprotein Apparatus (Biorad, Richmond, VA) as described by Cabrera-Chávez et al. (2012).
162 Low molecular weight markers (Amersham Biosciences, Amersham, UK) were used for calibration.
163

164 *2.6.3 Protein thiols*

165 Accessible –SH groups were measured by suspending 0.5 g of finely ground sample in 10
166 mL of 50 mM sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl and 0.2 mM 5,5'-
167 dithiobis(2-nitrobenzoate) (DTNB; Ellman, 1959). After 15 min at room temperature, insoluble
168 material was removed by centrifugation at 12000 × *g* for 10 minutes at 15 °C, and the absorbance at
169 412 nm of the supernatant was read against a DTNB blank. Total accessible thiols were measured
170 according to the same protocol outlined above, but adding 8 M urea to the DTNB-containing buffer.
171

172 ***2.7. Cooked pasta characterization***

173 Pasta was cooked in natural spring water (pasta/water ratio 1:10, no salt added) until the
174 optimal cooking time, evaluated according to D'Egidio, Mariani, Nardi, Novaro, & Cubadda
175 (1990). Optimal cooking times were 9, 10, and 9 minutes for P1, P2, and P3 respectively. The
176 amount of material leached into cooking water (cooking loss) was measured according to Marti,
177 Seetharaman, & Pagani (2010) and expressed as grams of matter lost/100 g of dry pasta. Weight

178 increase of pasta due to water absorption during cooking was evaluated gravimetrically.

179 Textural characteristics of cooked pasta were determined by using a TA.HD-plus Texture 180 Analyzer (Stable Micro System Ltd., Godalming, UK), equipped with a Kramer cell, according to 181 Marti, Seetharaman & Pagani (2010). The following indices were considered: compression energy, 182 as the area under the part of the curve related to the compression phase; firmness, as the maximum 183 strength necessary to pack the sample; shear force, as the force necessary for blades to pass through 184 the sample. Total (TS) and resistant (RS) starch were determined by using the enzymatic procedure 185 proposed by Brighenti, Casiraghi & Baggio (1998).

186

187 ***2.8 In vivo studies***

188 This study was approved by the Research Ethics Committee of the University of Milan. Ten 189 (five male and five female) healthy volunteers aged 21–25 years, with normal body mass index 190 (22.7 ± 2.6 kg/m²) and basal glycemia (4.2 ± 0.1 mmol/L) participated in this study, after signing 191 informed consent. Each volunteer took part in the experiment on two non-consecutive days per 192 week. Each subject consumed, in separate meals, each of the pasta samples (40 g of carbohydrates 193 portions), or glucose solutions (40g/500ml water). Foods were eaten within 10–12 min and 500 ml 194 of water were drunk with the meals. Capillary blood samples were taken using a softclix plung 195 (Glucolet® 2, Bayer Diagnostics) in the fasting state and 15, 30, 45, 60, 90, and 120 min after each 196 meal. Blood samples were taken in capillary tubes (Microvette® CB 300 Sarstedt, Germany) and 197 stored at -18°C until analysis. Blood samples were analysed with a semi-automatic analyzer for 198 glucose (YSI 2373, Yellow Spring Instruments, OH, USA), results been given as mmol glucose/L. 199 The Glycemic Index (GI) was estimated from the 120 min incremental areas under post-prandial 200 glucose responses, calculated geometrically, ignoring the area beneath the baseline (ISO, 2010), and 201 using the mean IAUC of the three glucose trials as the reference for calculating GI.

202

203 ***2.9. Statistical analysis***

Results are expressed as mean values and standard deviation of their mean. Data were analyzed using an one-way analysis of variance (ANOVA). In vivo results (expressed as mean ± SEM) were submitted to Repeated Measures Analysis of Variance (RM-ANOVA). The significance of differences between products and/or time were checked by Tukey's Honest Significant Differences post-hoc test. The analyses were performed by using the StatSoft Statistica Package (release 5, Statsoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1 Properties of the polysaccharide network in the starting materials and in uncooked rice pasta

3.1.1. Accessibility to hydrolytic enzymes and characterization of hydrolytic fragments

Investigating the accessibility of starch to various and specific enzymatic activities can provide insights into the possible structural differences among samples. The accessibility of rice flour samples to α-amylase action is shown in Fig. 1A. RF showed the lowest susceptibility to α-amylase, even if the amount of enzyme used here was ten times higher than that used for all other samples, and low susceptibility to α-amylase persisted after 24 h of exposure to the enzyme, underscoring the starch structural compactness in RF.

Parboiling promoted starch granule gelatinization (Bhattacharya, 2004) making the structure more susceptible to α-amylase action, as shown for PRF in Fig. 1A. However, the initial steps (1h) of glucose release from PRF upon hydrolysis were slow, suggesting conservation of starch organisation in PRF, as gelatinized starch granules reassociate in the parboiled kernels after the cooling and drying steps (Ong & Blanshard, 1994).

229 The α-amylase susceptibility increased markedly after processing into pasta, also as a
230 function of the absence/presence of a pre-treatment step on the raw material (Fig. 1A). In all pasta
231 samples, glucose release after 1 h was higher than in the corresponding starting materials. The most
232 marked effects were observed on P1, where most of the starch solubilised by α-amylase action was
233 released within 1 h. The starch solubilised from P1 after 24 h was lower than from P2 and P3, both
234 obtained from PRF. This suggests that extrusion-cooking of untreated flour do not allow complete
235 accessibility of starch to α-amylase. Colonna, Tayeb, & Mercier (1989) mentioned that extrusion-
236 cooking causes macromolecular degradation of starch leading to different digestibility profiles and
237 to increased starch accessibility in extrudates. This was related to the severity of the extrusion-
238 cooking, as the rupture of individual granules made the starch more accessible and facilitated the
239 amylolytic hydrolysis in vitro (Hagenimana, Ding, & Fang, 2006).
240 On the other hand, the fraction of glucose released after 1 h treatment with α-amylase
241 decreased in pasta obtained from parboiled flour, and was lowest in P3, where an extrusion-cooking
242 step was involved also in pasta-making. This implies that parboiling (carried out on paddy rice) and
243 extrusion-cooking of flour had different and non-synergistic effects on starch structure. The
244 interplay of intrinsic factors such as the degree of retrogradation or recrystallization, the starch–
245 protein interactions, and the degree of crystallinity are known to play a significant role in the rate of
246 enzymatic starch hydrolysis (Hagenimana, Ding, & Fang, 2006). Impaired enzyme degradation of
247 extrusion-cooked pasta (as evident here by comparing the glucose release at 1 h from P2 and P3)
248 has been attributed to the formation of amylose-lipid complexes, to starch-protein interactions, and
249 to limited water availability, as observed in starchy systems by Guha, Ali, & Bhattacharya (1997).
250 However, our observations can be explained also by the changes in starch organisation after its
251 gelatinisation during the extrusion-cooking process, that leads to irreversible loss of the crystalline
252 regions with concomitant transition to an amorphous state. Cooling of the product results in starch
253 recrystallisation (Haralampu, 2000), forming a network with novel crystalline properties. Both the

254 crystallite structure and the packing of the amorphous phase reportedly affect enzymatic
255 susceptibility (Colonna, Leloup, & Buleon, 1992).

256 The susceptibility to pullulanase of the same samples is reported in Fig.1B. Pullulanase is a
257 debranching enzyme with the ability to cleave α-1, 6 linkages in amylopectin molecules (Lin &
258 Chang, 2006). The amount of material made soluble by pullulanase hydrolysis could give
259 information of the amount and organization of branches in amylopectin and on possible effects of
260 the processing conditions on specific regions of its structure.

261 RF showed the lowest susceptibility to pullulanase, suggesting dense packing of
262 amylopectin branches. The parboiling process increased sensibly the amount of hydrolyzable α-1, 6
263 linkages. In the pasta sample made from RF (P1), amylopectin debranching within 1 h of
264 pullulanase action increased >10-fold, suggesting the presence of a high portion of α-1, 6 linkages
265 in the external regions of the amylopectin structure. Such an increase was much less evident in
266 pasta samples made from PRF (P2 and P3). The amount of soluble material released by pullulanase
267 was higher in P3 than in P2, suggesting that extrusion-cooking of previously parboiled flour could
268 generate a starch structure characterized by less organised (and therefore more accessible) regions.
269 This suggests that the pullulanase-accessible amylopectin fraction may be responsible for a good
270 deal of the differences in starch arrangement in the various pasta samples.

271 SE-HPLC was used in this study in combination with Static Light Scattering to obtain
272 information about the nature and size of soluble products of enzymatic hydrolysis. The separation
273 of fragments released by α-amylase is shown in Fig. 2. Alpha-amylase hydrolysis of RF (used on
274 this substrate at concentrations ten-fold higher than those used for other materials) gave a small
275 peak at 23 min and a larger one at 24 min, likely related to di- and trisaccharides (Fig. 2A).
276 Hydrolysis of PRF gave a peak at 22 min (10-40 kDa, 60-250 glucose molecules, as assessed by
277 Static Light Scattering) along with a large peak at 24 min. The intensity of the peak at 22 min
278 remained unchanged at 24 h hydrolysis of PRF (Fig. 2B), suggesting that these soluble polymeric
279 species represent a hydrolytic intermediate. These hydrolytic intermediates were also observed after

280 α-amylase action on pasta samples, regardless of the starting material and/or the pasta-making
281 process. After 1 h hydrolysis, their abundance in the various samples increased in the order
282 P3<P2<P1 (Fig. 2C), whereas at 24 h the abundance of these intermediates followed the order
283 P2>P3>>P1 (Fig. 2D). The lower amount of hydrolytic fragments in P3 with respect to P2 at either
284 incubation times may be a consequence of the higher extent of starch retrogradation in P3.

285 The size distribution of molecules released in solution by pullulanase is shown in the various
286 panels of Fig. 3. Pullulanase action on RF for 1 h gave three different peaks (Fig. 3A). The one
287 corresponding to largest fragments (at 22 min) was no longer present after 24 h (Fig. 3B).
288 Conversely, pullulanase action on PRF gave a time-dependent accumulation of large soluble
289 polymers, with a constant content in di- and trisaccharides (Fig. 3B). The effects of parboiling on
290 starch susceptibility to pullulanase seem more evident when comparing the area of chromatographic
291 peaks in Fig. 3B than when measuring the amount of carbohydrates present as glucose after
292 complete hydrolysis of the released fragments (Fig. 1B).

293 The pasta-making process promoted important changes in the distribution of soluble
294 polysaccharides produced by pullulanase action, in particular as a function of the hydrolysis time
295 (Fig. 3C and D). Starch in P1 - made from RF by extrusion-cooking - showed a greater release of
296 di- and trisaccharides than the corresponding starting flour at both 1h and 24 h. No major effects of
297 the pasta-making process were evident when considering the larger polymeric species in pasta made
298 from PRF or RF after 1 h exposure to pullulanase. However, release of di- and trisaccharides from
299 P3 was very low, suggesting once again a significant impact of the extrusion-cooking step on the
300 amylopectin component and suggesting once again the absence of a synergistic effect between
301 parboiling and extrusion-cooking. This is confirmed by analysis of the products released after 24 h
302 exposure to pullulanase. The chromatographic profile from pasta P2 again closely resembled that of
303 PRF, whereas the products of prolonged pullulanase action on pasta P3 indicated a modest release
304 of hydrolytic intermediates and the presence of minimal amounts of di- and trisaccharides.

305 This may stem from the occurrence of organized clusters within the starch structure,
306 proposed to stem from extrusion-cooking of a pre-treated rice flour (Marti, Seetharaman & Pagani,
307 2010). These organized regions, characterized by a poorly packed external region and by a compact
308 core, may be responsible for the higher RS in extrusion-cooked pasta in comparison to pasta made
309 by conventional extrusion, as discussed in what follows and in agreement with reports indicating
310 that starch containing both amorphous and partially ordered regions is digested slowly (Guraya,
311 James & Champagne, 2001; Miao, Jiang, & Zhang, 2009).

312

313 *3.1.2 Pasting properties of starch in rice pasta*

314 The pasting profiles in Fig. 4 may provide information on macromolecule arrangement in
315 the products considered here. Samples P2 and P3 had the lowest pasting temperature and P1 the
316 highest, a result that could be related to the strength of bonds in the starchy network (Eliason &
317 Karlson, 1983). Only sample P1 showed a peak viscosity (at ~ 90 °C), suggesting the presence of
318 starch granules with a high swelling capacity, as previously observed in rice flours (Marti,
319 Seetharaman & Pagani, 2010).

320 During the holding period at 95 °C, the product slurries were subjected to high temperatures
321 and mechanical shear stress, causing starch granule disruption and amylose leaching, which led in
322 turn to a slight decrease in viscosity in P1, whereas viscosity in P2 and P3 continued to increase
323 while being held at 95 °C. Upon cooling, P1 gave the highest setback values, suggesting the highest
324 retrogradation tendency among all the samples, followed by P3 and P2 in the order. Also
325 noteworthy is the absence of viscosity changes in the starch paste formed by samples P2 and P3
326 while being stirred at 50 °C, in contrast with the decreased viscosity observed for P1, that stems
327 from physical breakdown of the starch gel formed upon cooling of this particular sample.

328

329 ***3.2 Properties of protein network in the starting materials and in uncooked pasta***

330 Structural features of proteins in rice pasta were evaluated by extraction in buffers with

331 different dissociating ability towards covalent and non-covalent interprotein bonds, and by
332 accessibility of specific residues under the same conditions. As shown in Fig. 5A, protein solubility
333 in buffer was low in P1 (≤ 3.2 mg/g pasta), and almost nil in P2 and P3, confirming aggregation of
334 soluble protein components (albumins and globulins) upon parboiling (Bhattacharya, 2004).
335 Addition of urea to the buffer/saline extractant resulted in a significant increase in solubilized
336 protein from all samples, suggesting that hydrophobic interactions are relevant to the structure of
337 whatever protein matrix in rice pasta. A similar behaviour was also detected in commercial rice and
338 corn pasta (Mariotti, Iametti, Cappa, Rasmussen, & Lucisano, 2011) and in amaranth-enriched pasta
339 (Cabrera-Chávez et al., 2012). When both urea and DTT were present in the extraction medium, the
340 amount of soluble proteins increased further, most notably in samples (P2 and P3) prepared from
341 PRF. This suggests that inter-protein disulphides play a fundamental role in the structure of the
342 protein network in pasta made from parboiled flour, almost regardless of the pasta-making process.
343 The minor but significant differences observed between P2 and P3 may be related to the different
344 starch structure in these samples. It has been suggested that sequential starch
345 gelatinization/retrogradation cycles may result in protein entrapment in an organized starch
346 structure (Cabrera-Chávez et al., 2012).

347 The nature of the proteins solubilised in the different media from the various pasta samples
348 was investigated by SDS-PAGE. As shown in Fig.1S, buffer-soluble albumins (25, 18, and 16 kDa;
349 Shih, 2004) were present in P1 but absent in P2 and P3, confirming the data in Fig. 5A. Some of the
350 same low-molecular weight species were solubilized by urea in P2 and P3, along with another
351 polypeptide at 36 kDa, also present in urea extracts of P1 together with bands at high molecular
352 weight (60 and 65 kDa). These bands were absent in urea extracts of P2 and P3, but the one at 60
353 kDa was present in both samples when extraction with urea was carried out in the presence of
354 disulfide-reducing agents. These latter conditions also allowed solubilization of the 18 kDa species,
355 but not of the one at 65 kDa, possibly as a consequence of easier physical entrapment of the latter
356 and larger species into process-modified starch structures, as discussed above.

357 Thus, the pasta-making process results in almost all proteins being linked through
358 hydrophobic interactions. A previous parboiling step on rice flour provides further stabilization of
359 these interactions by intramolecular disulfide bonds, whose formation seems insensitive to the
360 conditions used for pasta making and apparently involving specific proteins in a preferential way.
361 Quantification of accessible –SH groups, that may be carried out independently of protein
362 solubility, has been applied to understanding the nature and evaluating the extent of modification
363 induced in cereal-based foods by processing (Elkhalifa et al., 2006; Mariotti, Iametti, Cappa,
364 Rasmussen, & Lucisano, 2011; Cabrera-Chávez et al., 2012). As shown in Fig. 5B, the number of
365 accessible thiols decreased in the order P1>P2>P3 both in the absence and in the presence of urea,
366 confirming the impact of parboiling on the compactness of the protein matrix. The lower content of
367 accessible thiols in P3 vs P2 indicates that the effects of extrusion-cooking on protein structure
368 rearrangements are more dramatic than those of conventional extrusion. The relevance of
369 hydrophobic interactions to the compactness of the protein matrix is made evident by the increase in
370 accessible thiols upon addition of urea. Altogether, thiol accessibility data confirm the fundamental
371 role of the pasta-making process in establishing the compactness of the protein aggregates, as
372 pointed out by the solubility approaches described above.
373
374 ***3.3 Properties of cooked pasta as related to the starting material and the pasta-making***
375 ***process***
376
377 *3.3.1 Physical and chemical properties of cooked pasta*
378 Data summarizing the cooking performance of the various rice pasta samples are presented
379 in Table 1. Pasta P3, made by extrusion-cooking of flour from previously parboiled rice (PRF),
380 gave the lowest cooking losses and the lowest water absorption, and was by large the most resilient
381 product. The impact of extrusion-cooking is evident when considering that P2, made from the same
382 starting material by conventional extrusion, had the highest cooking losses, although it retained

383 some of the texture characteristics of the much harder P3. On the other hand, extrusion-cooking of
384 an otherwise untreated rice flour gave pasta P1, that upon cooking gave the highest water
385 absorption, with cooking losses only slightly lower than those measured for P2, and showing by far
386 the lowest values for all textural indices.

387 As reported in Table 2, retention of protein components and of total starch upon cooking
388 increased in the order P3>P2>P1. The differences in protein and starch content among the samples
389 can be related solely to the pasta-making process, as the parboiling process had been shown to leave
390 starch (Bhattacharya, 2004) and protein content (Bhattacharya & Ali, 1985) totally unaffected.
391 Conversely, the lower sugar content measured in pasta made from parboiled flour (P2 and P3, in
392 comparison to P1) seem to relate to the parboiling process, where sugars from the bran layers leach
393 into the soaking water (Ali & Bhattacharya, 1980; Lamberts, Brijs, Mohamed, Verhelst, & Delcour,
394 2006; Lamberts, Rombouts, Brijs, Gebruers, & Delcour, 2008).

395 P3 showed the highest content of RS among the pasta samples, suggesting that RS content
396 was affected from both the parboiling process and the extrusion conditions, in agreement with
397 previous reports (Singh, Dartois, & Kaur, 2010). Higher amounts of RS starch were found in
398 parboiled rice than in raw rice (Casiraghi, Brighenti, Pellegrini, Leopardi, & Testolin, 1993;
399 Eggum, Juliano, Perez, & Acedo, 1993). Extrusion-cooking was conducive to a compact product
400 structure (Marti, Seetharaman, & Pagani, 2010), also due to reorganization of the crystalline
401 structure of resistant starch. This behavior is consistent with the viscoamilographic data in Fig. 3,
402 suggesting the presence of starch granules with a high swelling capacity in P1. Indeed, sample P1
403 absorbed the greatest amount of water (Table 1), consistent with high swelling capacity of starch in
404 this pasta, and with reports relating the granules swelling to the tendency to leach contents into
405 cooking water (Sisson & Bately, 2003).

406

407 *3.3.2 In vivo glycemic response*

408 Incremental post-prandial blood glucose curves after consumption of the various rice-based

409 pasta samples by healthy volunteers are shown in Fig. 6. No significant differences were detected
410 among all the samples at any time of these tests. However, in the case of P1, the glycemic peak
411 shifted toward a longer time (45 min) than in pasta prepared from parboiled flour (P2 and P3). This
412 suggests that parboiling affected the starch digestion rate and glucose intestinal absorption. Changes
413 in accessibility to degradation of starch in samples that underwent one or more steps of thermal
414 treatment (see Fig. 1 and related comments) translate into a lower glycemic index for P2 and P3 (61
415 and 65, respectively) in comparison with P1 (71), although these differences are not statistically
416 significant. The similar trend of the glycemic response in P2 and P3, that have been shown to
417 display quite different starch structures (see Fig. 3 and related comments), may in turn be related to
418 the fact that the different protein organization in these two samples may counteract - at least to
419 some extent - the effects of extrusion-cooking on specific starch fractions.
420
421
422 **4. Conclusions**
423 This study shows that individual treatments (or their combination) had a markedly different
424 impact on the structural features of macromolecules in rice-based pasta. Pasta made from parboiled
425 rice through an extrusion-cooking process (P3) was extremely firm after cooking. This seems to be
426 consequent to the presence in the uncooked pasta of a peculiar amylopectin organization, as made
427 evident by the limited accessibility of this polysaccharide to pullulanase, that hydrolyzes alpha-1-6
428 glycosidic bonds. At the opposite end of the sensory spectrum was the pasta made through
429 extrusion-cooking of untreated rice flour (P1), that was extremely soft. This stems from limited
430 protein reticulation, and from part of the starch being present in a compact native-like conformation,
431 as evidenced by its limited accessibility to alpha-amylase action in the uncooked pasta and by the
432 delayed increase in blood glucose when this pasta is eaten.
433 Pasta made from parboiled rice flour through conventional extrusion (P2) had satisfactory
434 sensory parameters. Amylopectin in the uncooked material was loosely structured, as indicated by

435 its sensitivity to the action of pullulanase, whereas starch as a whole had a structure comparable to
436 that of pasta P3. These structural considerations may explain the observation that the time course of
437 post-prandial glucose levels were almost overlapping in subjects consuming P2 or P3, where
438 treatments increased the content of resistant starch.
439 From a methodological standpoint, the approaches reported here seem able to provide useful
440 insights as for improving our current molecular-level understanding of the effects of treatment
441 conditions (and of their combination and temporal sequence) on overall product quality.
442
443 **Acknowledgements**
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449 **REFERENCES**

450 Ali, S. Z., & Bhattacharya, K. R. (1980). Changes in sugars and amino acids during parboiling 451 of rice. *Journal of Food Biochemestry, 4*, 169-179.

452 American Association of Cereal Chemists (AACC), Approved Methods of the AACC 2001, St 453 Paul, MN.

454 Association of Official Analytical Chemists (AOAC), Approved Methods of the AOAC 1999, 455 Washington DC, MD.

456 Bhattacharya, K. R. (2004). Parboiling of rice. In E. T., Champagne (Ed.), *Rice: chemistry and* 457 *technology* (pp. 329-404). St. Paul: The American Association of Cereal Chemists.

458 Bhattacharya, K. R., & Ali, S. Z. (1985). Changes in rice during parboiling and properties of 459 parboiled rice. In Y., Pomeranz (Ed.), *Advances in cereal science and technology* (pp. 105-167). St. 460 Paul: The American Association of Cereal Chemists.

461 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram 462 quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry, 72*, 248- 463 254.

464 Brighenti, F., Casiraghi, C., & Baggio, C. (1998). Resistant starch in the Italian diet. *British* 465 *Journal of Nutrition, 80*, 333-341.

466 Cabrera-Chávez, F., Calderón de la Barca, A. M., Islas-Rubio, A. R., Marti, A., Marengo, M., 467 Pagani, M. A, Bonomi, F., & Iametti, S. (2012). Characterization of amaranth-enriched gluten-free 468 pasta. *LWT - Food Science and Technology, 47*, 421-426.

469 Casiraghi, M. C., Brighenti, F., Pellegrini, N., Leopardi, E., & Testolin, G. (1993). Effects of 470 processing on rice starch digestibility eveluated by in vivo and in vitro methods. *Journal of Cereal* 471 *Science, 17,* 147-156.

472 Colonna, P., Leloup, V., & Buleon, A. (1992). Limiting factors of starch hydrolysis. *European* 473 *Journal of Clinical Nutrition, 46*, S17-S32.

474 Colonna, P., Tayeb, J., & Mercier, C. (1989). Extrusion cooking of starch and starchy products.

475 In C., Mercier, P., Linko, & J. M., Harper (Eds.), *Extrusion Cooking* (pp. 247-319). St. Paul: The
476 American Association of Cereal Chemists.

477 D'Egidio, M. G., Mariani, B. M., Nardi, S., Novaro, P., & Cubadda, R. (1990). Chemical and
478 technological variables and their relationship: a predictive equation for pasta cooking quality.
479 *Cereal Chemistry, 67*, 275-281.

480 De Noni, I., & Pagani, M.A. (2010). Cooking properties and heat damage of dried pasta as
481 influenced by raw material characteristics and processing conditions. *Critical Reviews in Food*
482 *Science and Nutrition, 50*, 465-472.

483 Eggum, B. O., Juliano, B. O., Perez, C. M., & Acedo, E. F. (1993). The resistant starch,
484 undigestible energy and protein contents of raw and cooked milled rice. *Journal of Cereal*
485 *Science,18*, 159-170.

486 Eliason, A. C., & Karlson, R. (1983). Gelatinization properties of different size classes of
487 wheat starch granules measured with differential scanning calorimetry. *Starch/Stärke, 35,* 130-133.

488 Elkhalifa A. E. O., Bernhardt, R., Bonomi, F., Iametti, S., Pagani, M.A., & Zardi, M. (2006).
489 Fermentation modifies protein/protein and protein/starch interactions in sorghum dough. *European*
490 *Food Research and Technology, 222*, 559-564.

491 Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and. Biophysics, 82*,
492 70-77.

493 Guha, M., Ali, S. Z., & Bhattacharya, S. (1997). Twin-screw extrusion of rice flour without die:
494 effect of barrel temperature and screw speed on extrusion and extrudate characteristics. *Journal of*
495 *Food Engineering, 32*, 251-267.

496 Guraya, H. S., James, C., & Champagne, E. T. (2001). Effect of enzyme concentration and
497 storage temperature on the formation of slowly digestible starch from cooked debranched rice
498 starch. *Starch/Stärke, 53*, 131-139.

499 Hagenimana, A., Ding, X., & Fang, T. (2006). Evaluation of rice flour modified by extrusion
500 cooking. *Journal of Cereal Science, 43*, 38-46.

501 Haralampu, S. G. (2000). Resistant starch - a review of the physical properties and biological
502 impact of RS3. *Carbohydrate Polymers, 41*, 285-292.
503 International Standard ISO 26642:2010(E): Food products - Determination of the glycaemic
504 index (GI) and recommendation for food classification.
505 Lamberts, L., Brijs, K., Mohamed, R., Verhelst, N., & Delcour, J. A. (2006). Impact of
506 browning reactions and bran pigments on color of parboiled rice. *Journal of Agricalture and Food*
507 *Chemistry, 54*, 9924-9929.
508 Lamberts, L., Rombouts, I., Brijs, K., Gebruers, K., & Delcour, J. A. (2008). Impact of
509 parboiling conditions on Maillard precursors and indicators in long-grain rice cultivars. *Food*
510 *Chemistry, 110*, 916-922.
511 Lin, J. H., & Chang, Y. H. (2006). Effects of type and concentration of polyols on the
512 molecular structure of corn starch kneaded with pullulanase in a Farinograph. *Food Hydrocolloids,*
513 *20*, 340-347.
514 Marconi, E., & Carcea, M. (2001). Pasta from nontraditional raw materials. *Cereal Foods*
515 *World, 46*, 522-530.
516 Mariotti, M., Iametti, S., Cappa, C., Rasmussen, P., & Lucisano, M. (2011). Characterization of
517 gluten-free pasta through conventional and innovative methods: Evaluation of uncooked products.
518 *Journal of Cereal Science, 53*, 319-327.
519 Marti, A., Pagani, M. A., & Seetharaman, K. (2011). Understanding starch organisation in
520 gluten-free pasta from rice flour. *Carbohydrate Polymers, 84*, 1069-1074.
521 Marti, A., Seetharaman, K., & Pagani, M. A. (2010). Rice-based pasta: a comparison between
522 conventional pasta-making and extrusion-cooking. *Journal of Cereal Science, 52*, 404-409.
523 Miao, M., Jiang, B., & Zhang, T. (2009). Effect of pullulanase debranching and
524 recrystallization on structure and digestibility of waxy maize starch. *Carbohydrate Polymers, 76*,
525 214-221.
526 Ong, M. H., & Blanshard, J. M. V. (1994). The significance of the amorphous-crystalline

527 transition in the parboiling process of rice and its relation to the formation of amylose-lipid
528 complexes and the recrystallisation (retrogradation) of starch. *Food Science and Technology Today,*
529 *8*, 217-226.

530 Pagani, M. A. (1986). Pasta products from non-conventional raw materials. In C., Mercier, &
531 C., Cantarelli (Eds), *Pasta and extruded products* (pp. 52-68). London: Elsevier Applied Science.

532 Parker, R., & Ring, S.G. (2001). Aspects of the physical chemistry of starch. *Journal of Cereal*
533 *Science, 34*, 1-17.

534 Petitot, M., Abecassis, J., & Micard, V. (2009). Structuring of pasta components during
535 processing: impact on starch and protein digestibility and allergenicity. *Trends in Food Science and*
536 *Technology, 20*, 521-532.

537 Rosell, C.M. & Marco, C. (2008). Rice. In E. K., Arendt, & F., Dal Bello (Eds.), *Gluten-free*
538 *cereal products and beverages* (pp. 81-100). London: Elsevier Applied Science.

539 Shih, F. F. (2004). Rice Proteins. In In E. T., Champagne (Ed.), *Rice: chemistry and technology*
540 (pp. 143-157). St. Paul: The American Association of Cereal Chemists.

541 Singh, J., Dartois, A., & Kaur, L. (2010). Starch digestibility in food matrix: a review. *Trends*
542 *in Food Science and Technology, 21*, 168-180.

543 Sisson, M. J., & Bately, I. L. (2003). Protein and starch properties of some tetraploid wheats.
544 *Cereal Chemistry, 80,* 468-475.

545

546 Figure legends

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Table 1

Cooking behavior of rice pasta

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552

	Cooking loss (g/100 g)	**Water absorption (g/100g)**	**Compression energy (N*mm)**	**Firmness (N)**	**Shear force (N)**
P1	9.8 ± 0.2^{b}	90.7 ± 4.2^{b}	328.4 ± 6.9^{a}	190.6 ± 6.9^{a}	150.4 ± 4.6^{a}
P2	12.6 ± 0.7^{c}	79.5 ± 3.8^{a}	553.3 ± 30.9^{b}	275.3 ± 8.2^{b}	259.1 ± 15.1^{b}
P3	5.6 ± 0.1^{a}	77.3 ± 3.5^{a}	1914.8 ± 364.3^{c}	901.6 ± 119.3^{c}	524.7 ± 70.6^{c}

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556 Means and standard deviation followed by different superscript letters in a column are significantly
557 different at $p<0.05$.
558

559 **Table 2**

560 **Chemical composition of cooked rice pasta**

561

	Protein (%)	Sugars (%)	TS (%)	RS (%)
P1	3.01 ± 0.05^{a}	0.80 ± 0.0^{b}	37.21±1.99^{a}	2.73±0.18^{a}
P2	3.51 ± 0.05^{b}	0.10 ± 0.0^{a}	40.11±0.22^{b}	3.34±0.10^{b}
P3	4.49 ± 0.02^{c}	0.20 ± 0.0^{a}	48.78±1.15^{c}	4.62±0.22^{c}

562

563

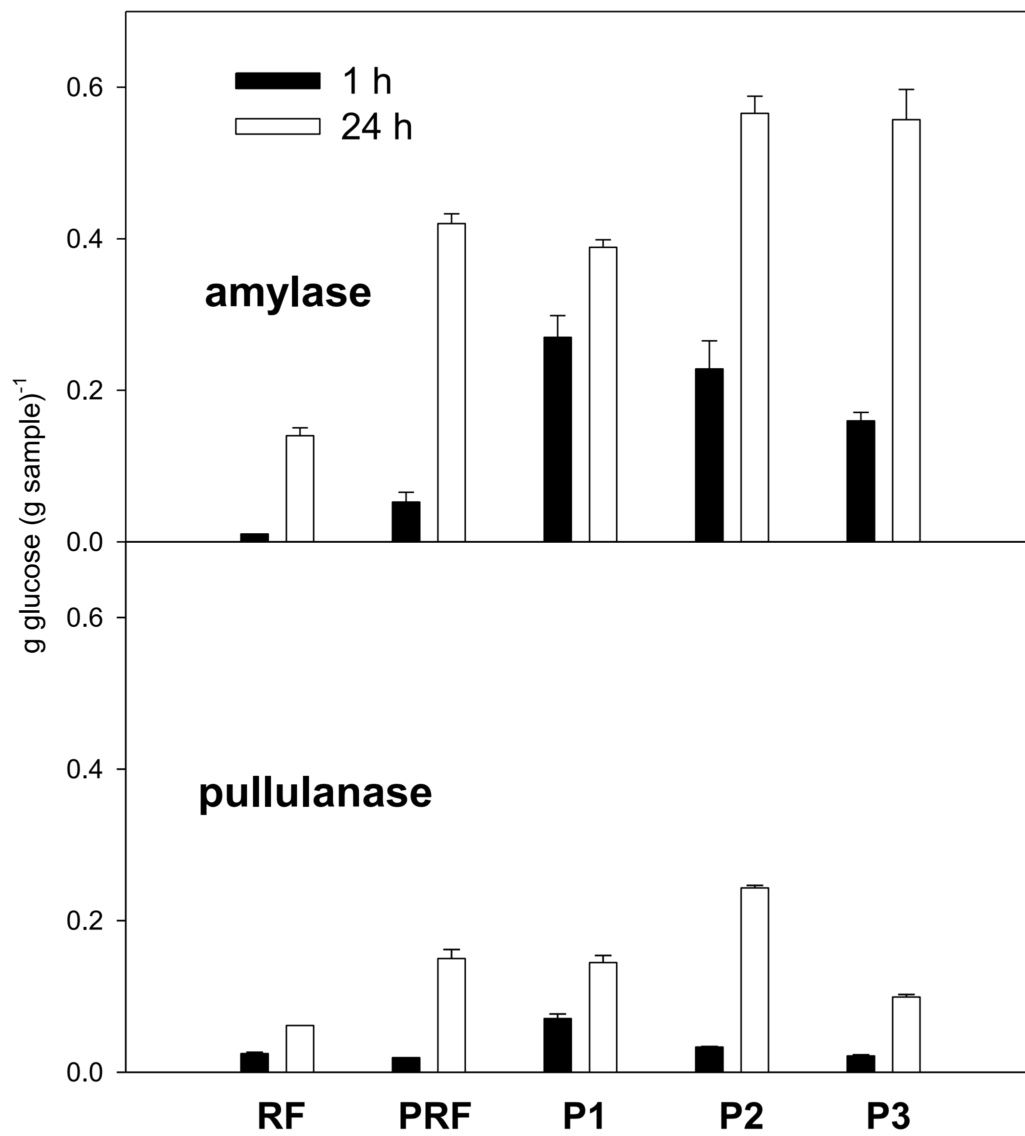
564 Means and standard deviation followed by different superscript letters in a column are significantly

565 different at $p<0.05$.

566

567

568 **Fig. 1.** Susceptibility to α-amylase (a) and pullulanase (b) hydrolysis. RF, native rice flour; PRF,
569 parboiled rice flour; P1, pasta from RF; P2, pasta from PRF by conventional extrusion; P3, pasta
570 from PRF by extrusion-cooking.
571
572 **Fig. 2.** SE-HPLC-Light Scattering chromatograms after α-amylase hydrolysis. (A) rice flours after
573 1 h of hydrolysis; (B) rice flours after 24 h of hydrolysis; (C) pasta samples after 1h of hydrolysis;
574 (D) pasta samples after 24 h of hydrolysis. Samples are identified as in the legend to Fig. 1.
575
576 **Fig. 3.** SE-HPLC-Light scattering chromatograms after pullulanase hydrolysis. (A) rice flour after
577 1 h; (B) rice flours after 24 h; (C) pasta samples after 1h; (D) pasta samples after 24 h. Samples are
578 identified as in the legend to Fig. 1.
579
580 **Fig. 4.** Pasting properties of rice pasta. Samples are identified as in the legend to Fig. 1.
581
582 **Fig. 5.** Amount of solubilized protein (A) and accessible protein thiols (B) in different buffer
583 systems. Samples are identified as in the legend to Fig. 1.
584
585 **Fig. 6.** Mean blood glucose concentrations in healthy subjects after intake of rice pasta. Samples
586 are identified as in the legend to Fig. 1.
587



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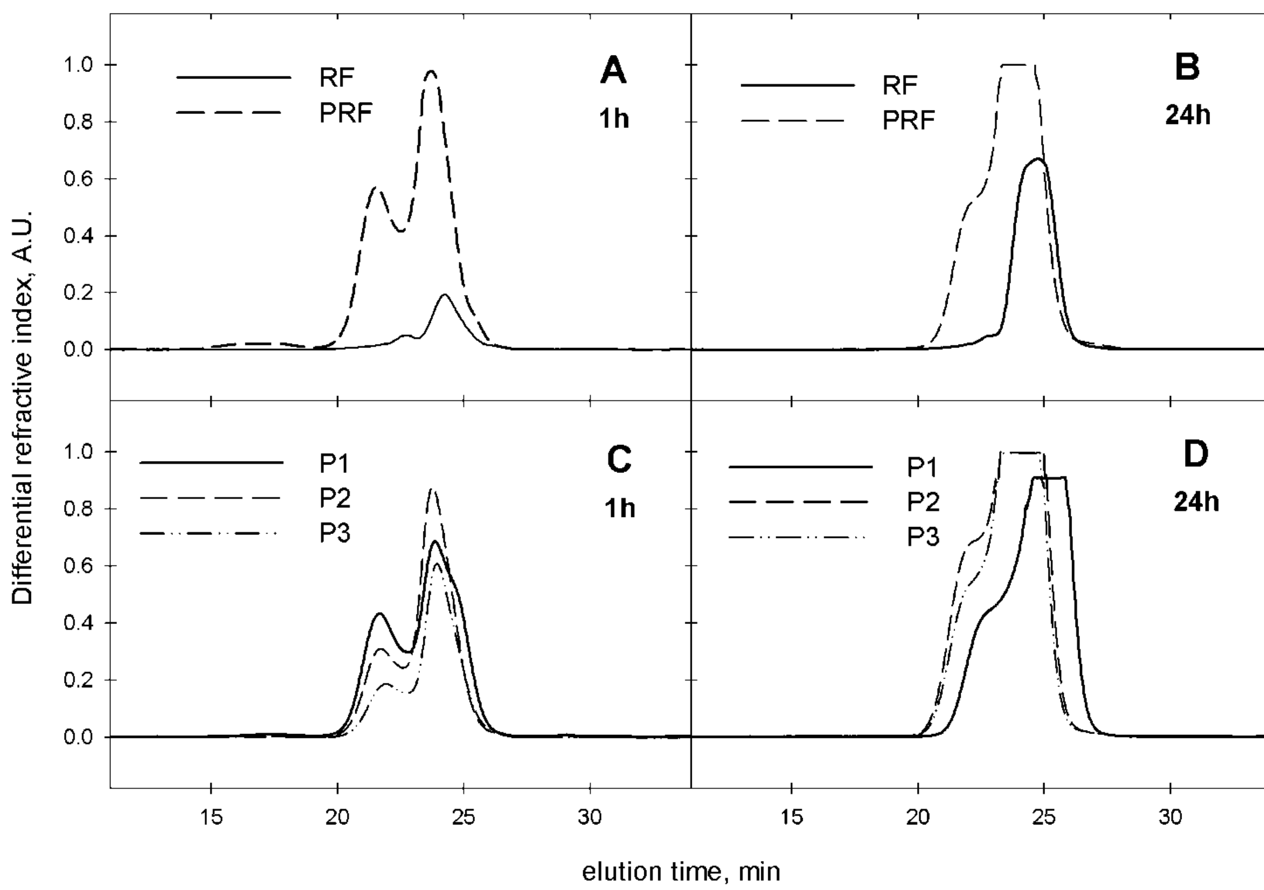
590 Figure 1



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598 Figure 2
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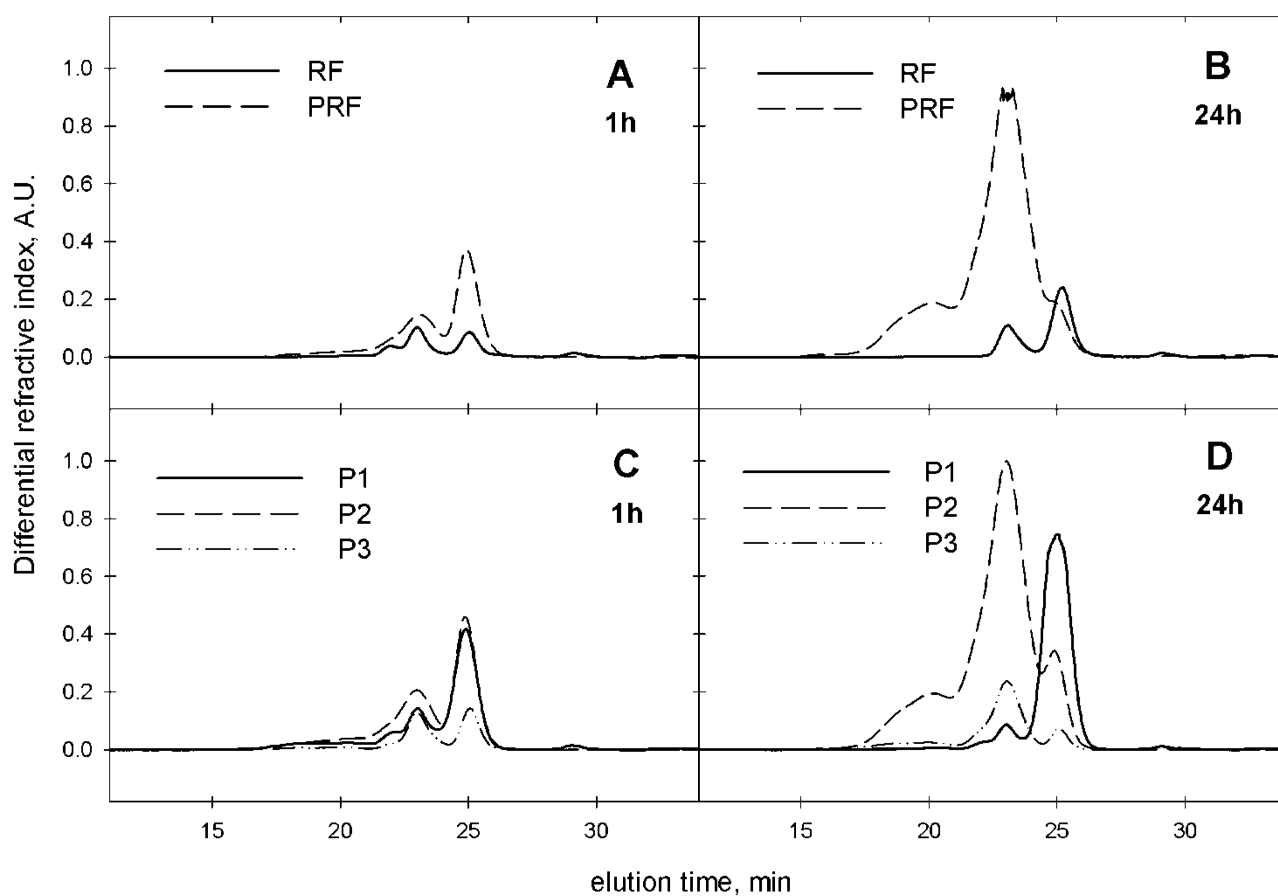

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606 Figure 3
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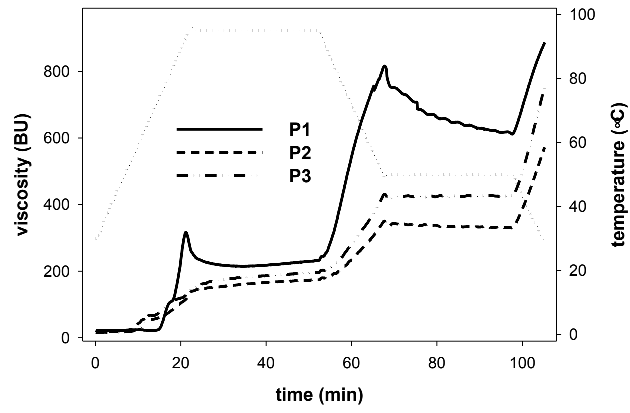

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609 Figure 4.

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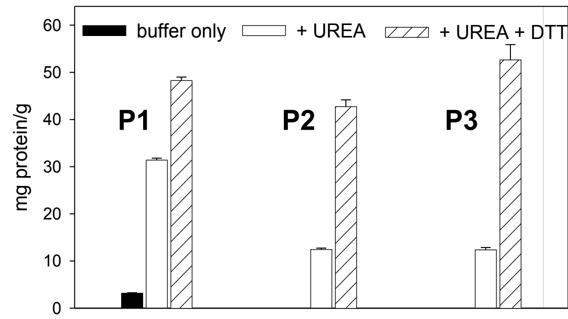
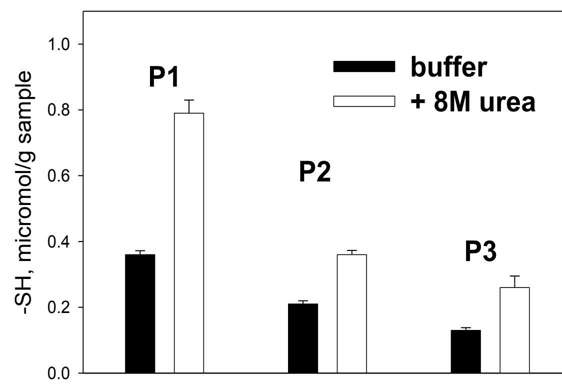
612 A



613 B



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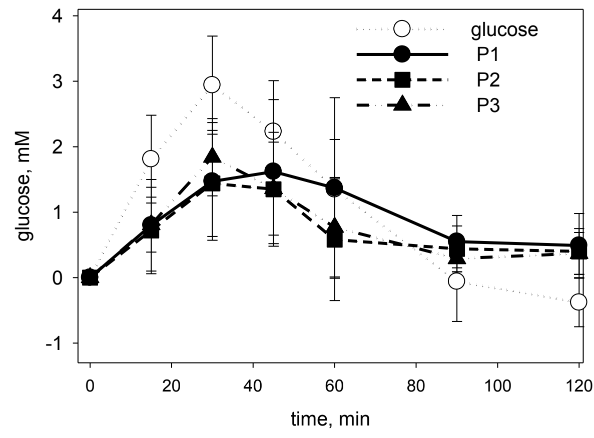

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620 Figure 6

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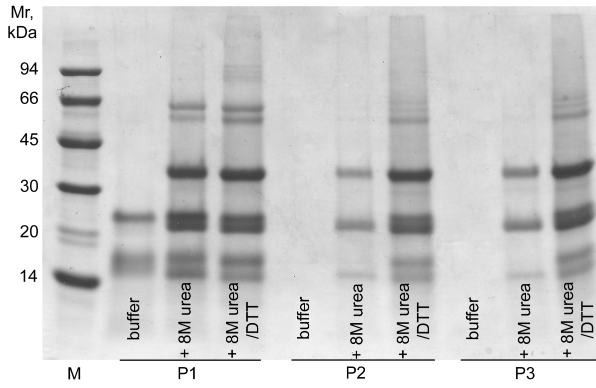

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628 **Fig. 1S.** SDS-PAGE separation of proteins solubilized in different buffer systems from rice pasta

629 samples. M, protein markers. Samples are identified as in the legend to Fig. 1.

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