



**Macromolecular traits in the African rice *Oryza glaberrima*
and in *glaberrima/sativa* crosses, and their relevance to
processing**

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1 **Macromolecular traits in the African rice *Oryza glaberrima* and in glaberrima/sativa crosses,**
2 **and their relevance to processing**

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23 **Molecular traits of *Oryza glaberrima***

26 **Abstract**

27 Molecular properties of proteins and starch were investigated in two accessions of *Oryza*
28 *glaberrima* and *Oryza sativa*, and in one NERICA cross between the two species, to assess traits
29 that could be relevant to transformation into specific foods. Protein nature and organization in *O.*
30 *glaberrima* were different from those in *O. sativa* and in NERICA. Despite the similar cysteine
31 content in all samples, thiol accessibility in *O. glaberrima* proteins was higher than in NERICA or
32 in *O. sativa*. Inter-protein disulphide bonds were important for the formation of protein aggregates
33 in *O. glaberrima*, whereas non-covalent protein-protein interactions were relevant in NERICA and
34 *O. sativa*. DSC and NMR studies indicated only minor differences in the structure of starch in these
35 species, as also made evident by their microstructural features. Nevertheless, starch gelatinization in
36 *O. glaberrima* was very different from what was observed in *O. sativa* and NERICA. The content
37 of soluble species in gelatinized starch from the various species in the presence/absence of
38 treatments with specific enzymes indicated that release of small starch breakdown products was
39 lowest in *O. glaberrima*, in particular from the amylopectin component. These findings may explain
40 the low glycaemic index of *O. glaberrima*, and provide a rationale for extending the use of *O.*
41 *glaberrima* in the production of specific rice-based products, thus improving the economic value
42 and the market appeal of African crops.

43

44

45 **Practical Application:** The structural features of proteins and starch in *O. glaberrima* are very
46 different from those in *O. sativa* and in the NERICA cross. These results appear useful as for
47 extending the use of *O. glaberrima* cultivars in the design and production of specific rice-based
48 products (e.g., pasta), that might, in turn, improve the economic value and the market appeal of
49 locally sourced raw materials, by introducing added-value products on the African market.

50

51 Introduction

52 Rice is one of the main crops cultivated worldwide, and is a primary source of food for more than
53 two-thirds of the world's population (Singh and others 2013; Gayin and others 2015a). The genus
54 *Oryza* comprises two distinct types of domesticated rice: *Oryza sativa* (Asian rice) and *Oryza*
55 *glaberrima* (African rice). Whereas *O. sativa* is globally consumed, *O. glaberrima* is peculiar to the
56 West Africa sub-region (Sweeney and McCouch 2007) and is characterized by specific qualitative
57 and quantitative traits (Gayin and others 2015a), such as a locally preferred taste, excellent weed
58 competitiveness, and the ability to grow in a wide range of difficult ecosystems (Agnoun and others
59 2012). For these reasons, *O. glaberrima* is adopted by many African farmers, regardless of its low
60 yields and market value, of the susceptibility to shattering, and of poor resistance to lodging (Gayin
61 and others 2015b; Manful and Graham-Acquaah 2016).

62 In the 1990s the Africa Rice Center (AfricaRice), a leading pan-African rice research
63 organization, developed through conventional cross-breeding between *O. sativa* (upland lines) and
64 *O. glaberrima* and distributed to local farmers a group of rice varieties, called NERICA (New Rice
65 for Africa) (Nwanze and others 2006). These new varieties combine the high-tillering ability, early
66 maturity, and adaptability to local agronomical conditions of *O. glaberrima* to the high-yields of *O.*
67 *sativa*, thus allowing for improvement sub-Saharan African farmers' livelihoods.

68 As for the molecular and rheological characterization of African-grown rice varieties, a high
69 number of studies have focused on starch, the major component of rice grains, and have highlighted
70 its physical, molecular, and thermal properties (Gayin and others 2015a, b). However, especially for
71 *O. glaberrima*, more information is still required, not only on starch properties and digestibility, but
72 also on what attains protein structure and their overall organization in the rice grains, that remain
73 almost uncharacterized. These investigations may pave the way to the full exploitation of this
74 indigenous variety, and eventually to meet consumers' preferences by introducing added-value
75 products from locally sourced raw materials on the African market.

76 The objective of this study was to address the molecular properties of rice protein fractions
77 by combining molecular-based approaches with starch digestibility measurements and with a study
78 on starch thermal and structural properties. This combined information may contribute to the
79 current understanding of the molecular basis of the potential use of different rice species in specific
80 food products.

81

82 **Materials and methods**

83

84 *Materials*

85 Two varieties of rice belonging to the species *O. glaberrima* (G-766 and G-995), two varieties
86 belonging to the species *O. sativa* (SAHEL 208 and WITA 8), and the interspecific rice NERICA
87 L-19 were provided by the Africa Rice Center (Cotonou, Benin). Images of representative samples
88 of *O. glaberrima* and *O. sativa* are shown in Figure 1A. When appropriate, samples were ground
89 with a laboratory mill (IKA Universal Mühle M20; Janke & Kunkel GmbH & Co KG, IKA
90 Labor Technik, Staufen, Germany), fitted with a water cooling jacket in order to avoid overheating
91 during grinding. Apparent amylose content was measured using the standard iodine colorimetric
92 method ISO 6647-2-2011, using an Auto Analyzer 3 (Seal Analytical, Germany) and well-known
93 standard rice varieties (IR65, IR24, IR64 and IR8) as standards. Proximate analyses (humidity,
94 proteins) were carried out according to AACC standard methods, as detailed elsewhere (Marti and
95 others 2010, 2014; Gayin and others 2015a, b). Scanning electron microscopy images of starch
96 granules from representative samples of *O. glaberrima* and *O. sativa* are shown in Figure 1B, and
97 show a similar morphology of starch granules in the two rice species.

98

99 *Protein characterization*

100 The solubility of proteins in rice samples was determined by using buffers of various composition,
101 essentially as described by Marti and others (2014). Proteins were extracted by dispersing 0.5 g of

102 finely ground samples (≤ 0.25 mm) in 10 mL of 0.05 M sodium phosphate buffer, pH 7.0,
103 containing 0.1 M NaCl. After stirring at room temperature for 60 min and removal of insoluble
104 materials by centrifugation ($10000 \times g$, 20 min, 20 °C), the protein content in the supernatant was
105 assessed by a dye-binding method (Bradford 1976). Where indicated, the buffer used for protein
106 extraction also contained 6 M urea or 6 M urea and 10 mM dithiothreitol (DTT). Results are
107 expressed as mg soluble proteins [g rice flour]⁻¹.

108 A given amount (typically, 0.015 mg, as assessed by the dye-binding protein assay) of the
109 proteins solubilised from rice samples in the presence/absence of urea and DTT (see above) was
110 diluted (1/1 v/v) with SDS-PAGE denaturing buffer (0.125 M Tris-HCl, pH 6.8, 50% glycerol,
111 1.7% sodium dodecyl sulphate; 0.01% Bromophenol Blue) containing 1% (v/v) 2-mercaptoethanol
112 when indicated, and heated at 100 °C for 10 min. The electrophoretic runs were carried out in a
113 12% monomer fixed porosity gel using a MiniProtean apparatus (Bio-Rad, Richmond, VA, USA).
114 Gels were stained with Coomassie Brilliant Blue (Barbiroli and others 2013).

115 Accessible thiol groups were determined as in Barbiroli et al. (2015), by suspending 0.5 g of
116 finely ground rice samples in 10 mL of 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.0, containing
117 0.2 mM 5,5'-dithiobis-(2-nitrobenzoate) (DTNB, Ellman, 1959), in the presence/absence of 6 M
118 urea. After stirring for 60 min at 25 °C, the suspension was centrifuged ($10000 \times g$, 20 min, 20 °C)
119 and the absorbance of the supernatant was read at 412 nm against a proper blank. Results are
120 expressed as μmol thiols [g rice flour]⁻¹.

121

122 *Pasting properties*

123 Rice pasting properties were measured in a Brabender Micro-ViscoAmyloGraph (Brabender,
124 Duisburg, Germany) on finely ground samples, according to a slight modification of the procedure
125 in Marti et al. (2010). An aliquot of rice flour (12 g) was dispersed in 100 mL of distilled water,
126 scaling both flour and water weight on a 14% flour moisture basis. The pasting properties were
127 evaluated under constant instrumental conditions (speed: 250 rpm; sensitivity: 300 cmg_f) by using

128 the following time-temperature profile: heating from 30 °C up to 95 °C; holding at 95 °C for 20
129 min; cooling from 95 °C to 30 °C. Heating and cooling were carried out at a rate of 3 °C/min.
130 Pasting parameters were calculated by using a specific software (Viscograph, version 2.3.7).

131

132 *In vitro Starch Digestibility*

133 The method of Englyst (Englyst and others 2000) was used to assess in vitro carbohydrate
134 digestibility on cooked rice grains by means of the estimation of rapidly (RDS) and slowly (SDS)
135 digestible starch fractions that are likely to become available for rapid or slow absorption from the
136 small intestine, thus modulating glycemic response. Rapidly (RDS) and slowly (SDS) digestible
137 starch fractions were calculated from the glucose released at 20 min and between 20 and at 120 min,
138 respectively, as determined by HPLC (Marti and others 2017). Hydrolytic enzymes were from
139 Sigma Aldrich (St. Louis, MO, USA): pancreatin from porcine pancreas, EC 232.468.9, Sigma
140 P7545; amyloglucosidase from *Aspergillus niger*, EC 3.2.1.3, Sigma A7095. Two sets of data from
141 independent cooking trials were averaged. RDS and SDS fractions are expressed as percentage of
142 total available starch (RDS + SDS).

143

144 *Characterization of starch fragments from enzymatic hydrolysis by SE-HPLC*

145 Rice flour samples were heated up to the microviscoamylographic gelatinization peak (see above).
146 At this point, the run was stopped and the samples were immediately frozen in liquid nitrogen. After
147 freeze-drying, an aliquot of each sample (100 mg) was dispersed in 3 mL of 0.05 M sodium acetate
148 buffer, pH 6.0, and incubated for 24 h at 37°C in the absence of enzymes or in the presence of either
149 10-11 U of pullulanase (EC 3.2.1.41, from *Bacillus acidopullulyticus*, Sigma P2986) or of 10-11 U
150 of α -amylase (EC 3.2.1.1, from *Bacillus spp.*, Sigma A6814). At the end of the incubation period,
151 samples were spun for 10 min at 10,000 \times g, 20°C. Supernatants were filtered through a 0.22 μ m
152 filter, and 0.2 mL of the filtrate were loaded into a HPLC system (515 pump, Dual Absorbance
153 detector 2487, Waters Co., Milford, MA, USA), connected in series to a differential refractometer

154 (Optilab T-rEX, Wyatt Co., Santa Barbara, CA, USA) and to a Multi Angle Light Scattering
155 (MALS) instrument (DAWN HELEOS, Wyatt Co., Santa Barbara, CA, USA). Polysaccharides
156 were fractionated on a size-exclusion column (UltrahydrogelTM Linear 7.8 × 300 mm, Waters Co.,
157 Milford, MA, USA), by using 0.05 M sodium acetate, pH 6.0 as the eluant, at a flow rate of 0.4
158 mL/min. The ASTRA software (ASTRA V 5.1.9.1, Wyatt Technology Co., Santa Barbara, CA,
159 USA) was used for data analysis.

160

161 *NMR experiments*

162 NMR spectra were acquired at room temperature on a Bruker AVANCE-600 spectrometer (Bruker
163 Spectrospin GmbH, Rheinstetten, Germany), operating at 600.1 MHz (proton frequency) and
164 equipped with a 4 mm broad-band Cross-Polarisation Magic Angle Spinning (CP-MAS) probe for
165 solid state measurements. Flour samples were directly pressed into a 4 mm ZrO₂ rotor without
166 preliminary treatment. Natural abundance ¹³C spectra were acquired at 150.9 MHz while spinning
167 samples at 10 kHz (Pines and others 1973). Proton decoupling was achieved with a GARP-based
168 composite pulse. Relevant acquisition parameters were: spectral width 45.4 kHz; acquisition time
169 11 ms; relaxation delay 2s (fast acquisition conditions); contact time for Cross Polarization 1.5 ms;
170 number of scans 3600. Adamantane was used as external ¹³C chemical shift reference, by setting the
171 resonance of the most intense band at 38.56 ppm.

172

173 *DSC measurements*

174 Differential Scanning Calorimetry (DSC) measurements were carried out in the 20–150°C range at
175 a scanning rate of 2.0 °C/min in a Perkin-Elmer DSC6 calorimeter (Waltham, Massachusetts,
176 USA). Indium was used for calibration and distilled water as reference. An aliquot of flour (5 g)
177 was added to distilled water to give 73% moisture and thoroughly manually mixed. A 30 mg aliquot
178 of the dough mass was placed in a 0.06 mL measuring cell. Raw calorimetric data were analyzed
179 with the dedicated software IFESTOS (Fessas and Schiraldi 2000). Two heating-cooling cycles

180 were applied to each sample. The average trend of the DSC record of the immediate re-heating run
181 was used as the base-line for elaboration of each given DSC trace. The instrument output signal was
182 converted into apparent specific heat and was scaled with respect to the baseline to obtain the trend
183 of the excess (with respect the pre-gelatinization state) specific heat trace, $Cp^{ex}(T)$ [$J \cdot K^{-1} \cdot g^{-1}$]⁻¹ of
184 the sample (per gram of dry matter), which in turn allowed evaluation of the enthalpy drop ΔH by a
185 straightforward integration of the corresponding trace (Fessas and others 2008). Gelatinization onset
186 was calculated as the flex point tangent interception with the temperature in the gelatinization peak.
187 Errors were evaluated on at least three replicates.

188

189 *Statistical analysis*

190 All tests and measurements were carried out at least in triplicate. Analysis of variance (ANOVA)
191 was performed with Statgraphics XV version 15.1.02 (StatPoint Inc., Warrenton, VA, USA).
192 Samples were used as factor. When the factor effect was significant ($p \leq 0.05$), differences among
193 the respective means were determined using Fisher's Least Significant Difference (LSD) test.

194

195 **Results and Discussion**

196 *Pasting properties*

197 Pasting properties provide information on starch properties and mutual arrangement of starch
198 components during gelatinization and retrogradation and might allow prediction of the starch
199 behaviour during processing and its suitability for food-related applications (Marti and others
200 2011). The pasting profiles of the various rice varieties are presented in Figure 2, and values of the
201 most representative properties are reported in Table 1. Samples from *O. sativa* varieties SAHEL
202 208 and WITA 8 gave viscoamylographic tracings with a high viscosity peak (774 ± 27 and $723 \pm$
203 16 Brabender Units (BU) respectively), suggesting the presence of starch granules with a high
204 swelling capacity, as previously observed in other rice flours (Marti and others 2010).

205 In spite of a similar amylose content and of similar morphological traits, a specific viscosity
206 peak is either almost absent (G-995) or very low (G-766) in the case of *O. glaberrima*. In the
207 absence of microstructural differences among starch granules in the two species (Fig. 1B), this may
208 relate to the properties of amylopectin chains (Park and others 2007; Vandeputte and others 2003).
209 In the case of NERICA, the viscosity peak (788 ± 21 BU) appears similar to that of *O. sativa*, but
210 viscosity dramatically decreases immediately after the gelatinization peak to values similar to those
211 of *O. glaberrima*, suggesting a lower ability of starch in NERICA to withstand heating and shear
212 stress during cooking.

213 Species-specific differences were assessed during the holding period at 95 °C, when high
214 temperatures and mechanical shear may lead to starch granule disruption and amylose leaching.
215 Indeed, during the holding time, viscosity decreases with a similar tendency in the case of *O. sativa*
216 samples, whereas it appears stable in the case of the two *O. glaberrima* varieties. Although viscosity
217 increases for both cultivars because of retrogradation, this increase is more evident in G-766. This
218 may result from the different starch gelatinization level detected during the heating step, that in turn
219 might be related to a cultivar-dependent organization within the starch granules, as pointed out in
220 recent studies on various *O. glaberrima* accessions (Gayin and others 2015) and in studies that
221 compared cultivars not considered here (Gayin and others 2016 a, b).

222

223 *Organization of the protein network*

224 Protein aggregation studies provide information on protein structural features and on the nature of
225 inter-protein interactions in cereal-based materials (Moroni and others 2010). In particular, the role
226 of hydrophobic interactions and of disulfide bonds in the stabilization of protein aggregates can be
227 addressed by extracting proteins in the absence/presence of denaturants and of disulfide-breaking
228 agents (Marengo and others 2015; Barbiroli and others 2013) in various media. Results from *O.*
229 *sativa* (SAHEL 208, and WITA 8), *O. glaberrima* (G-766, and G-995), and NERICA L-19 are
230 shown in Figure 3A.

231 The amount of soluble proteins appears comparable in all samples in the presence/absence
232 of urea, whereas the presence of dithiothreitol (DTT, a disulfide-breaking agent) increases the
233 amount of proteins solubilized from *O. glaberrima* much more than in other samples. This
234 highlights a major role of inter-protein disulfide bonds in stabilizing a protein network in *O.*
235 *glaberrima*. The effect of DTT on the amount of proteins extracted from *O. sativa* and NERICA
236 was comparable, suggesting that this feature is controlled by *O. sativa* genetic traits in the NERICA
237 hybrid. Given the modest differences in the overall protein pattern between *O. glaberrima* and *O.*
238 *sativa* (see below), it is possible that these traits govern the rate of synthesis or deposition of
239 individual components. Different rates of synthesis for specific proteins may affect intermolecular
240 interactions, as observed in wheat (Iametti and others, 2006; 2013).

241 Protein attitude to network formation - relevant to technological behaviour - is affected by
242 the amount and accessibility of protein thiols that can undergo disulphide exchange reactions when
243 with existing protein disulfides (Barbiroli and others 2013). The amount of accessible thiols in the
244 different rice samples as a function of presence/absence of urea is shown in Figure 3B. Since the
245 total cysteine contents in *O. glaberrima* and *O. sativa* is comparable (3.37 and 3.13 percent of total
246 aminoacids, respectively, equivalent to 0.31 and 0.26 g cysteine in 100 g of the original sample), the
247 observed marked differences should relate to structural features of the proteins in each species. The
248 number of accessible thiol groups in the presence/absence of denaturant is highest in *O. glaberrima*
249 samples. Cysteine thiols in *O. glaberrima* are more exposed - regardless of the presence of
250 chaotropes - and participate to a higher number of interprotein disulfide bonds (as indicated by the
251 conditional solubility studies reported in Figure 3A) than in *O. sativa*. The amount of thiols detected
252 in NERICA L-19 in the presence and absence of chaotropes confirms that protein aggregation in
253 this hybrid strongly depends on *O. sativa* traits.

254 To assess the nature of proteins present in the various samples and to verify whether some
255 specific proteins were preferentially involved in aggregation events, proteins solubilized in the
256 various media were separated by SDS-PAGE. As shown in Figure 4, differences between rice

257 species under investigation were mostly limited to the relative abundance of individual components
258 within a given protein family. Thus, the same polypeptides contribute to the protein pattern of the
259 different rice species. However, the conditional solubility and thiol accessibility studies reported
260 above suggest that these proteins may acquire a species-specific folding (as indicated by thiol
261 accessibility data), and may interact among themselves with species-specific bonds (as indicated by
262 the solubility data in Fig. 3A). Hydrophobic bonds play a major role in stabilizing buffer-insoluble
263 aggregates in *O. sativa* and NERICA, whereas disulfide bonds are preferentially responsible for
264 aggregate formation and/or stability in *O. glaberrima*.

265

266 *Structural features of starch: DSC and NMR measurements*

267 Since pasting properties and protein overall structural studies highlighted species-specific
268 differences, one sample for each species (namely, WITA 8, G-766, and NERICA L-19) underwent
269 further investigation of details of the starch structure and behaviour by using both DSC and solid-
270 state ^{13}C CP/MAS NMR. These approaches have been widely used to characterize starches in native
271 food products (Bertocchi and Paci 2008; Cheetham and Tao 1998; Tester and others 1998).

272 As shown in Figure 5, all the DSC profiles present signals ensuing from starch gelatinization
273 (at low temperature) and from amylose-lipid dissociation (at high temperature). Both these events
274 are strongly dependent on water availability (Schiraldi and Fessas, 2003). Here, the presence of
275 excess water and the slow scanning rate allowed good discrimination of the observable transitions.

276 Although the overall enthalpies were similar (14.3 ± 0.7 , 15.6 ± 0.7 , 13.7 ± 0.7 J/g, for
277 WITA 8, G-766, and NERICA L-19, respectively), the gelatinization profile for *O. sativa* was
278 markedly sharper than that observed for the other two samples (half-height width of 4.5, 6.0, 8.0 °C,
279 for *O. sativa*, NERICA L-19, and *O. glaberrima*, respectively). This may be taken as an indication
280 of a more homogenous distribution of smaller-sized starch granules in *O. sativa*, in accordance with
281 results from enzymatic hydrolysis activities (see below).

282 The gelatinization onset temperatures (72.8 ± 0.5 , 73.1 ± 0.5 , 75.5 ± 0.5 °C, for *O. sativa*,
283 NERICA L-19, and *O. glaberrima*, respectively) essentially confirm recent and detailed reports
284 (Gayin and others 2016a, 2016b). Some of these recent studies also provided evidence of a
285 difference in the macromolecular organization within *O. glaberrima* starch granules, that had higher
286 ratio of absorbance to scattering than *O. sativa* when exposed to iodine vapor. This result was
287 interpreted in terms of greater flexibility and availability of glucan chains to form complexes with
288 iodine in *O. glaberrima* as compared to *O. sativa* (Gayin and others 2016b). Finally, the amylose-
289 lipid dissociation process seems very similar in all samples.

290 On these basis, it seemed worth to explore whether the subtle differences reported in
291 previous studies could be interpreted and/or explained in improved detail by using solid-state ^{13}C
292 NMR. Figure 6 shows an expansion (in the C_1 carbon region) of the CP-MAS ^{13}C -NMR spectra
293 taken at room temperature on untreated rice flours obtained from some of the same cultivars of *O.*
294 *glaberrima*, *O. sativa*, and NERICA used for DSC studies. Resonances corresponding to the various
295 individual polymorphs appear to be present in varying amounts. Both A- and B-type starch are
296 distinguishable on the basis of their characteristic resonance patterns (respectively, a triplet and a
297 doublet of resonances with even intensities). Such features arise from the different number of non-
298 equivalent glucose monomers within the crystalline cell unit. Residues at the interface of the helical
299 regions of both A- and B-type polymorphs also give another well resolved resonance at 102.4 ppm,
300 accompanied by a broad signal at about 98 ppm (Paris and others 1999). The structural differences
301 made evident by room-temperature CP-MAS ^{13}C -NMR studies may affect the behaviour of starch
302 when exposed to higher temperatures in the presence of enough water to allow starch gelatinization.
303 Detailed studies on the kinetics and equilibria of these events are currently underway.

304

305 *Accessibility of gelatinized starch to hydrolytic enzymes and characterization of fragments from*
306 *enzymatic starch hydrolysis*

307 The susceptibility of starch to specific enzymatic attack can provide information on structural
308 differences among samples (Miao and others 2011), that may depend on starch granule size,
309 composition (i.e., the amylose/amylopectin ratio), and on physical architecture and porosity
310 (Lehmann and Robin, 2007; Naguleswaran and others 2014). Accessibility to amylase action in the
311 cooked whole grains, as estimated by the Englyst's method, allows to distinguish between readily
312 digestible (RDS), slowly digestible (SDS), and resistant starch (RS). These parameters may be used
313 to estimate the potential glycaemic response of foods (Englyst et al 1999; EFSA 2011). Glycaemic
314 response appear to be directly related to RDS, whereas insulin demand was shown to be inversely
315 correlated to SDS (Garsetti et al, 2002).

316 In spite of the microstructural similarity between starch granules in the two species (Fig.
317 1B), *O. glaberrima* had a much lower RDS content than *O. sativa* (69.8 ± 2.8 vs 80.9 ± 2.6 g/100 g
318 available starch). NERICA showed an intermediate RDS content (75.7 ± 4.3 g/100 g available
319 starch). Thus, *O. glaberrima* has a much lower predicted glycaemic index than *O. sativa*, in
320 accordance with other very recent reports (Gayin and others 2017),

321 In a different approach, aiming at assessing the behaviour of starch in rice flours used as
322 ingredient of other food products, suspensions of rice flours were heated until gelatinization, as
323 assessed by microviscoamylographic tests (see Fig. 2). The gelatinized samples were then treated
324 overnight with α -amylase or with pullulanase, a debranching enzyme that cleaves α -1,6 linkages in
325 amylopectin molecules (Lin and Chang 2006). Effects of the various enzymatic treatments were
326 then studied by analysing the chromatographic pattern of soluble polysaccharides.

327 The size distribution of soluble molecules released by action of the two enzymes on the
328 gelatinized products was addressed by SE-HPLC in combination with Static (Multi Angle) Light
329 Scattering, and compared to the pattern of soluble polysaccharides in samples incubated in the same
330 condition, but in the absence of enzymes. This approach is useful to highlight differences in the
331 hydrolysis pattern of the rice samples that may result from a different structural organization of
332 starch components in the various rice species (Barbiroli and others 2013). Indeed, differences in the

333 distribution and length of amylopectin lateral chain length may lead to different hydrolysis patterns
334 with pullulanase, that is reportedly more effective on short amylopectin branches (Liu and others
335 2015).

336 As shown in the top panel in Figure 7, the SE-HPLC pattern of soluble polysaccharides
337 present in the gelatinized materials in the absence of enzymatic treatments is different. Although the
338 response from the refractive index detector used to analyse the chromatographic profile is not
339 strictly quantitative, there is some indication that only a modest amount of large-sized soluble
340 fragments is present in the gelatinized starch from *O. glaberrima*, confirming indications from
341 cooking losses studies (Gayin and others 2017). Sensibly smaller fragments – present in larger
342 amounts – formed the majority of the soluble material solubilized from gelatinized *O. sativa*
343 samples, whereas NERICA gave a chromatographic profile intermediate between that of *O. sativa*
344 and of *O. glaberrima*.

345 The action of α -amylase on gelatinized samples resulted in the formation of soluble
346 polymers with a very similar size distribution (centered around 4000-5000 Da, equivalent to about
347 25-30 glucose units). With the limitations discussed before, the intensity of the peak in the *O.*
348 *glaberrima* tracings in the middle panel of Figure 7 is lower than in *O. sativa* and in NERICA,
349 supporting previous observations on the lower sensitivity of *O. glaberrima* starch to α -amylase as a
350 consequence of its crystallinity (Gayin and others 2017).

351 As shown in the bottom panel of Figure 7, pullulanase action on the various gelatinized
352 samples gave two major peaks - at 23 and 25 minutes - for all samples, indicative of the release of
353 small-sized oligosaccharides in addition to larger species (Barbiroli and others 2013). However, the
354 amount of these peculiar fragments appears species-specific, and the highest amount of the smallest
355 released molecules was detected in the case of *O. glaberrima*. This is consistent with the reported
356 presence of a higher level of short B-chains of the so-called fingerprint-type in *O. glaberrima* with
357 respect to *O. sativa* and NERICA (Gayin and others 2016a). In all cases, the release of soluble
358 relatively small-sized glucose oligomers appears to stem from the action of pullulanase on

359 otherwise insoluble amylopectin components, and offers circumstantial support for external
360 amylopectin chains being longer in *O. glaberrima* than in the other two species, as suggested by
361 Gayin and others (2016a).

362 Indeed, a comparison between the tracings in the various panels of Figure 7 also makes it
363 clear that both pullulanase and amylase are capable to break down the large soluble polysaccharides
364 that are already present in the gelatinized materials before any enzymatic treatment. This
365 breakdown is almost complete in the case of amylase, as discussed above. Residual soluble
366 fragments of apparent size around 10^5 Da are still present in all cases after treatment with
367 pullulanase, but these large species are much more evident after treatment of gelatinized samples
368 from *O. sativa* and NERICA.

369

370 **Conclusions**

371 The approaches presented here provide useful insights as for the structural features of proteins and
372 starch in rice species grown in Africa. The overall protein structural organization in *O. glaberrima*
373 appears much different from the one in *O. sativa*. Proteins in *O. glaberrima* are organized in
374 polymeric forms mainly stabilized by inter-protein disulphide bonds, at contrast with hydrophobic
375 interactions being the dominating type of interaction in protein aggregates in both *O. sativa* and
376 NERICA, where these interaction impairs thiol accessibility much more than in *O. glaberrima*.
377 Differences in molecular starch structure among the various species are reflected in their starch
378 gelatinization behaviour. Structural features of the gelatinized starch in the various species indicate
379 that soluble fractions were least present in *O. glaberrima*, in particular when considering the
380 amylopectin component. This provides additional molecular-based rationale for the reportedly low
381 potential glycaemic index of *O. glaberrima*. All together, the properties of starch and proteins in the
382 NERICA variety appear very similar to those of *O. sativa*. The results presented here may provide
383 some useful guidelines for extending the use of *O. glaberrima* cultivars (and of their crosses) in the

384 design and production of new types of rice-based products, that could expand the market for locally
385 sourced materials in Africa.

386

387 **Acknowledgements**

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389 Partnership (GRiSP).

390

391 **Authors' Contributions**

392 Authors M. Marengo, A. Barbiroli, J. A. Hogenboom, and S. Iametti conducted the experiments
393 dealing with protein characterization. Authors M. C. Casiraghi, A. Marti, M. A. Pagani, and A.
394 Barbiroli conducted the experiments related to starch characterization. Authors J. Manful, S.
395 Graham-Acquaah, F. Bonomi, and S. Iametti designed the experiments. Authors E. Ragg and D.
396 Fessas conducted the NMR and DSC experiments, respectively. Authors S. Iametti, A. Marti, J.
397 Manful and F. Bonomi interpreted the results and wrote the manuscript.

398

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497

Table 1

498

Features of starch in the various rice samples

499

| Sample | Amylose, g/100g total starch | Pasting temperature (°C) | Max viscosity during heating (BU) | Final viscosity (BU) | Breakdown (BU) | Setback (BU) |
|-------------|------------------------------------|--------------------------------|---|----------------------------|-------------------|-------------------|
| SAHEL 208 | 28.4 ± 0.9 ^b | 76.6 ± 0.1 ^b | 774 ± 27 ^d | 1029 ± 25 ^d | 373 ^c | 627 ^c |
| WITA 8 | 27.7 ± 1.0 ^{ab} | 75.1 ± 0.3 ^a | 723 ± 16 ^c | 1050 ± 14 ^d | 365 ^c | 661 ^d |
| NERICA L-19 | 28.7 ± 0.9 ^b | 77.3 ± 0.3 ^c | 788 ± 21 ^d | 715 ± 31 ^b | 476 ^d | 433 ^b |
| G-766 | 26.2 ± 0.7 ^a | 80.6 ± 0.6 ^d | 331 ± 29 ^b | 966 ± 23 ^c | 9 ^b | 641 ^{cd} |
| G-995 | 26.2 ± 0.4 ^a | 85.9 ± 0.8 ^e | 199 ± 18 ^a | 565 ± 11 ^a | 0 ^a | 356 ^a |

500 Values in the same column with the same letters are not significantly different (LSD; p≤0.05)

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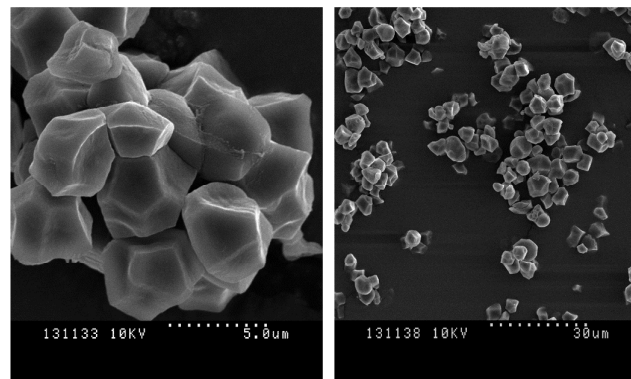
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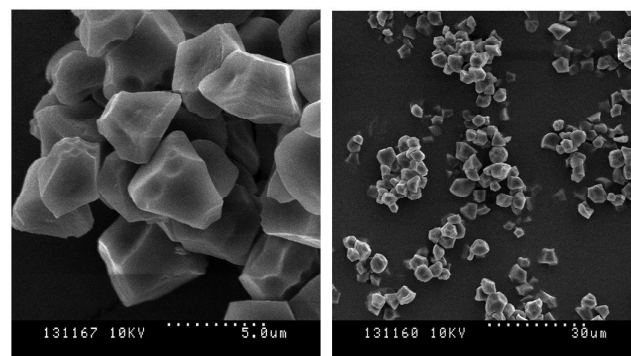
A *O. glaberrima*



O. sativa



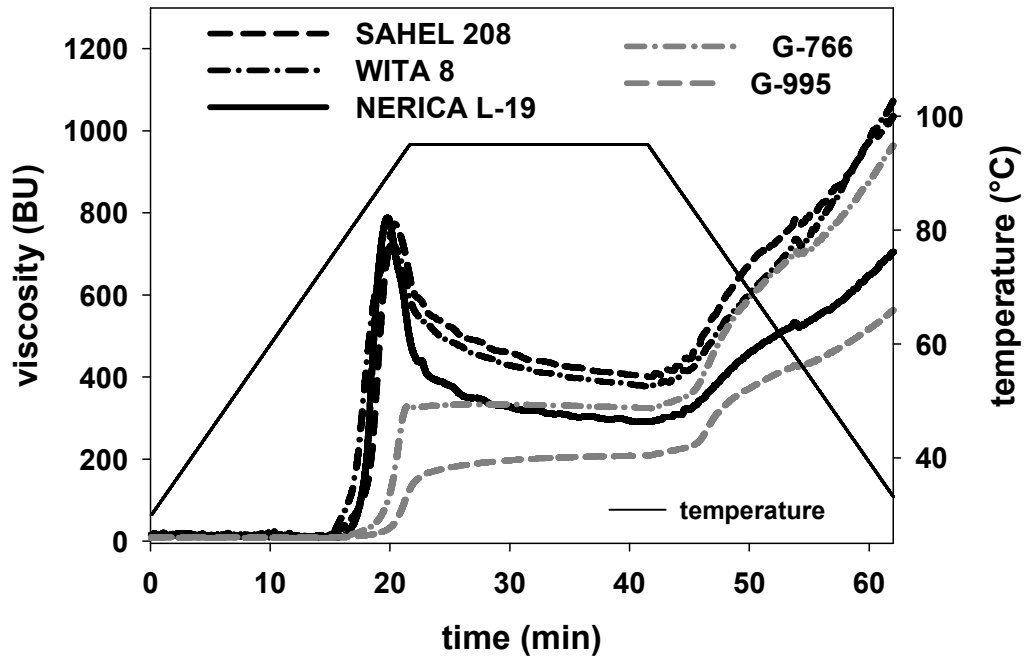
B *O. glaberrima*



O. sativa

Figure 1 – A: morphology of *O. glaberrima* and *O. sativa* grains. B: Scanning electron microscopy images of starch granules from *O. glaberrima* and *O. sativa*.

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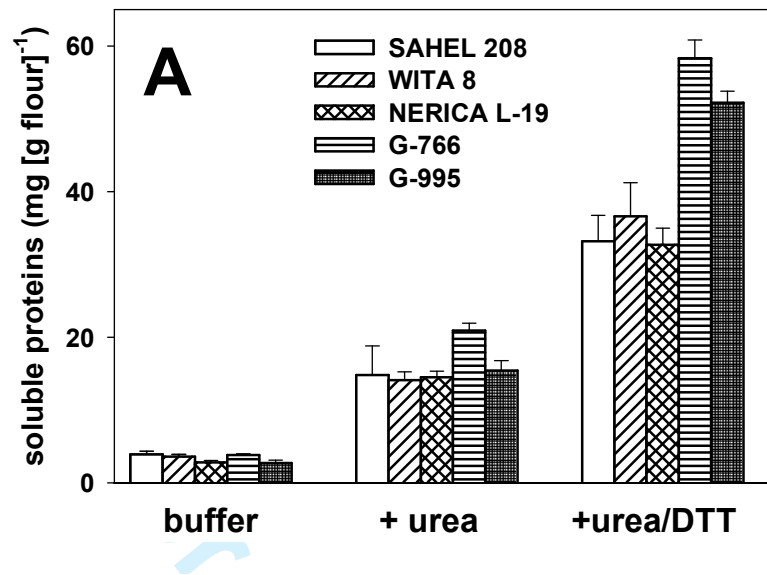


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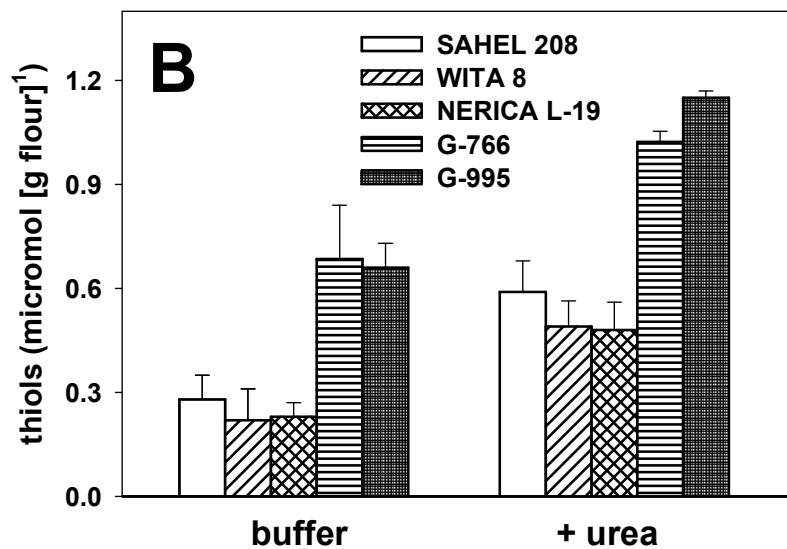
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531 Figure 2 - Pasting properties of rice samples. The thin black line is the temperature profile. The
 532 thick lines are the pasting profiles: grey, *O. glaberrima*; solid black line, NERICA; other black
 533 lines, *O. sativa*.

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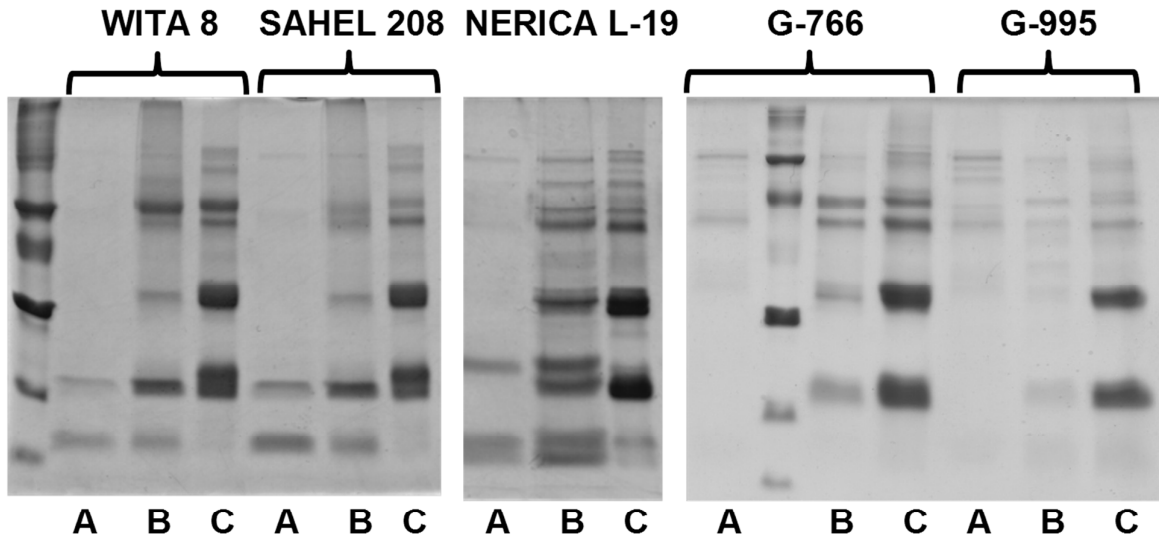
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538 Figure 3 - A: Solubility of proteins from rice in different media. Aliquots of the various samples
 539 were suspended under stirring in 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.0, in the
 540 presence/absence of 6M urea and of 10 mM DTT, as indicated. B: Accessibility of protein thiols in
 541 the various rice samples. Thiols were assessed on rice flour samples suspended in 0.05 M sodium
 542 phosphate, 0.1 M NaCl, pH 7.0, in the presence/absence of 6 M urea as indicated.

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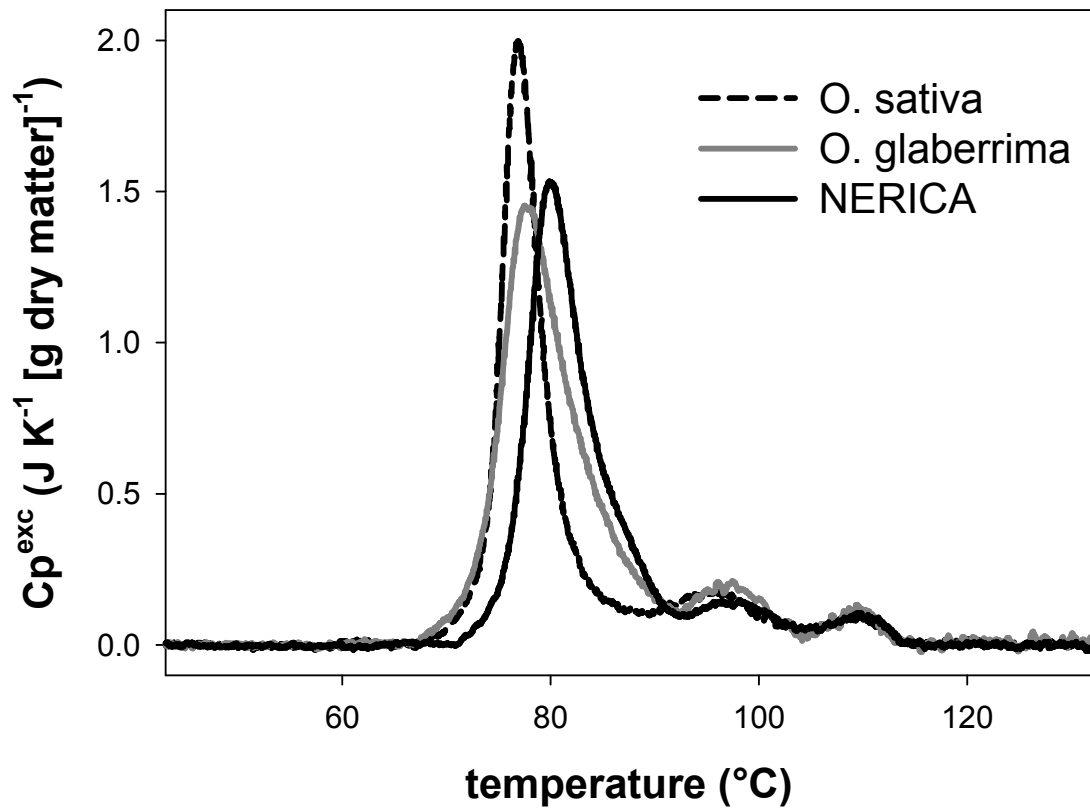


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547 Figure 4 - SDS-PAGE of proteins solubilized in different media. Proteins were extracted by using
548 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.0 (A), to which 6M urea (B), or of 6 M urea and 10
549 mM DTT (C) were added.

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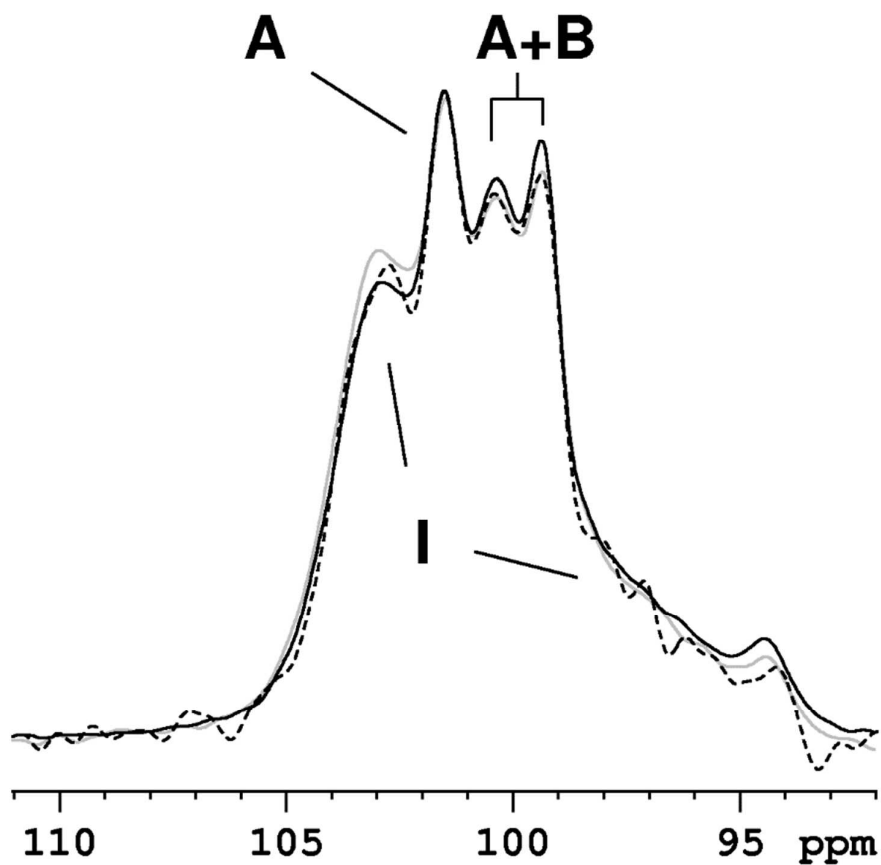


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553 Figure 5 - DSC tracings for the various rice flours (73% moisture, scan rate 2 $^{\circ}C/min$). Grey, *O.*

554 *glaberrima*; dashed black, *O. sativa*; solid black, NERICA.

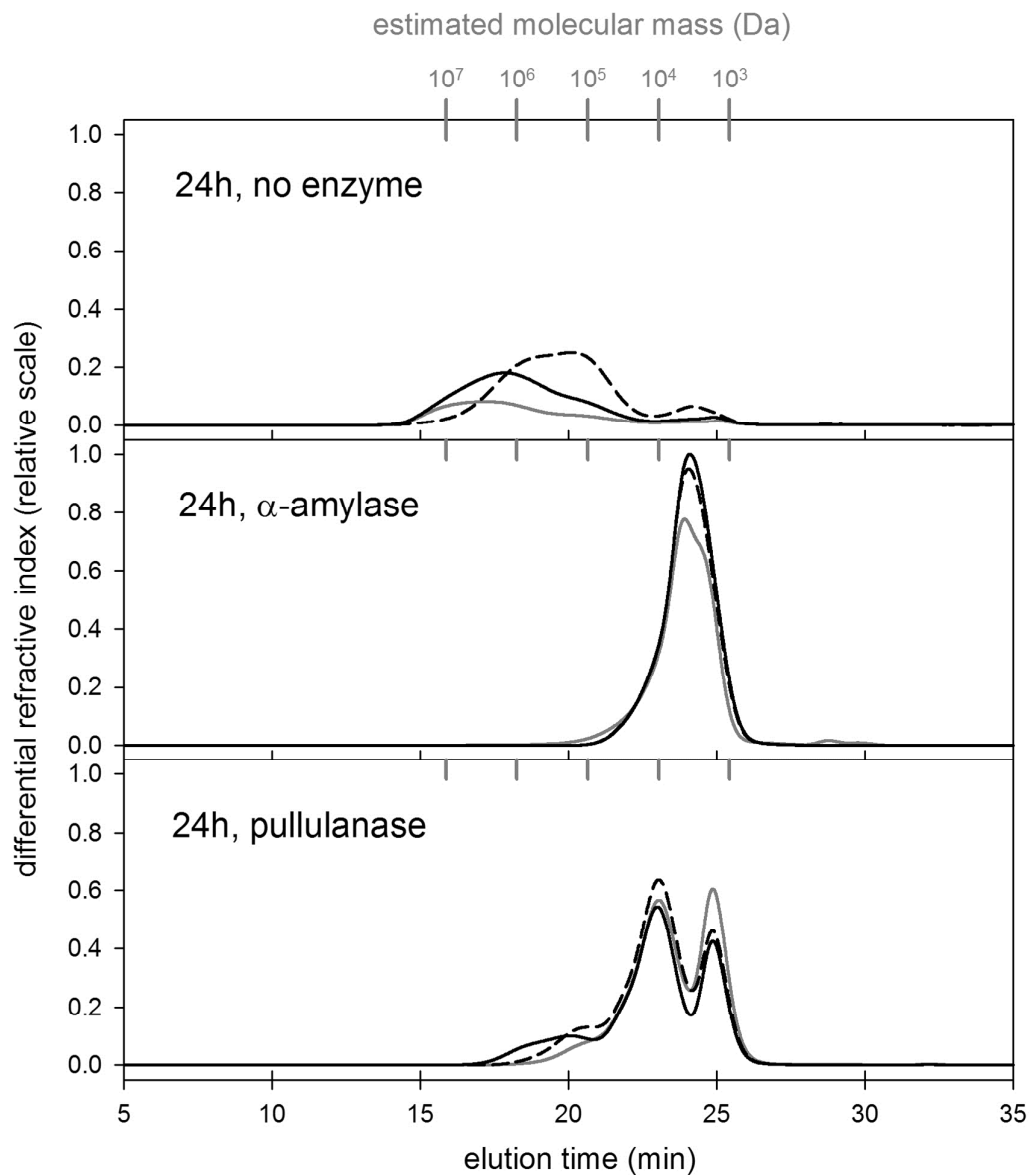


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557 Figure 6 - Expansion of the CP-MAS ^{13}C -NMR room temperature spectra (amylose C_1 resonance
558 region) of flours obtained from *O. glaberrima* (grey), *O. sativa* (dashed black) and NERICA (solid
559 black). Resonance attributable to either A-type (A) or B-type (B) starch, or to “interfacial” carbons
560 (I), are indicated.

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564 Figure 7 - SEC-HPLC profiles of soluble polysaccharides present in gelatinized starch from various
 565 rice grains after incubation at 37 °C in the absence/presence of different hydrolytic enzymes. The
 566 estimated molecular size of soluble molecules was derived from online Static Light Scattering
 567 (MALS) measurements. Grey, *O. glaberrima*; dashed black, *O. sativa*; solid black, NERICA.

Dear Dr. Youling Xiong,

This accompanies a revised version (JFDS-2017-0564.R1) of the manuscript “Macromolecular traits in the African rice *Oryza glaberrima* and in glaberrima/sativa hybrids, and their relevance to processing”.

We are grateful to both Reviewers for their positive attitude towards our work. We took into account all the remarks made by the Reviewers, and revised the text whenever appropriate. Aside from typos and language issues, that have been duly taken care of as indicated, here below are a couple of points where action was taken in response to specific points brought forward by Reviewer 2.

Q. Authors suggest that O. sativa traits may be responsible for controlling the effect of DTT on amount of proteins extracted. Could authors mention examples of such traits?

R. A sentence has been added to lines 236-240 of the revised manuscript to bring forward some evidence we had gathered on other cereals with respect to these issues, and appropriate references are also provided.

Q. Line 317-320 should be rephrased to better establish the flow of events

A: We cannot but concur that the construction of the original sentence was confusing at least. Following the Reviewers suggestion, we are now listing the various steps of this approach in temporal order and as separate sentences.

As requested, all changes made in the text are highlighted in red characters, including the newly added references.

Looking forward to hearing from you in due course, I remain,

Yours sincerely,

Stefania Iametti, PhD

Professor of Biochemistry

University of Milan