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

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Molecular traits of *Oryza glaberrima*

Abstract

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

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

Introduction

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

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

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

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

Materials and methods

Materials

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

Protein characterization

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

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

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

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

Pasting properties

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

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

In vitro Starch Digestibility

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

Characterization of starch fragments from enzymatic hydrolysis by SE–HPLC

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

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

NMR experiments

NMR spectra were acquired at room temperature on a Bruker AVANCE-600 spectrometer (Bruker Spectrospin GmbH, Rheinstetten, Germany), operating at 600.1 MHz (proton frequency) and equipped with a 4 mm broad-band Cross-Polarisation Magic Angle Spinning (CP-MAS) probe for solid state measurements. Flour samples were directly pressed into a 4 mm ZrO_2 rotor without preliminary treatment. Natural abundance ^{13}C spectra were acquired at 150.9 MHz while spinning samples at 10 kHz (Pines and others 1973). Proton decoupling was achieved with a GARP-based composite pulse. Relevant acquisition parameters were: spectral width 45.4 kHz; acquisition time 11 ms; relaxation delay 2s (fast acquisition conditions); contact time for Cross Polarization 1.5 ms; number of scans 3600. Adamantane was used as external ^{13}C chemical shift reference, by setting the resonance of the most intense band at 38.56 ppm.

DSC measurements

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

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

Statistical analysis

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

Results and Discussion

Pasting properties

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

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

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

Organization of the protein network

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

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

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

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

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

Structural features of starch: DSC and NMR measurements

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

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

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

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

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

Accessibility of gelatinized starch to hydrolytic enzymes and characterization of fragments from 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 glycemic response of foods (Englyst et al 1999; EFSA 2011). Glycemic
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

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

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

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

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

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

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

Conclusions

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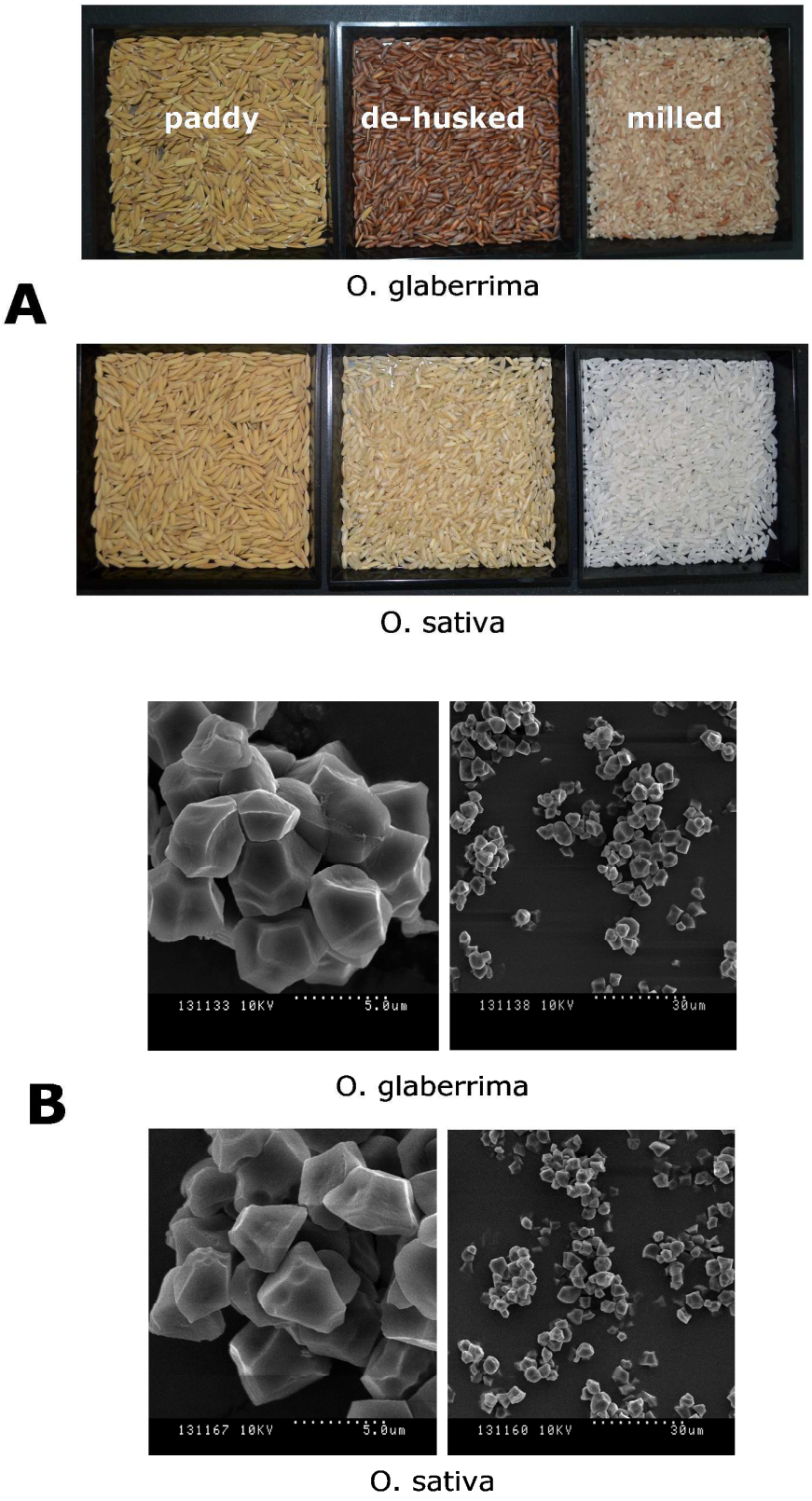

Figure 1 – A: morphology of *O. glaberrima* and *O. sativa* grains. B: Scanning electron microscopy images of starch granules from *O. glaberrima* and *O. sativa*.

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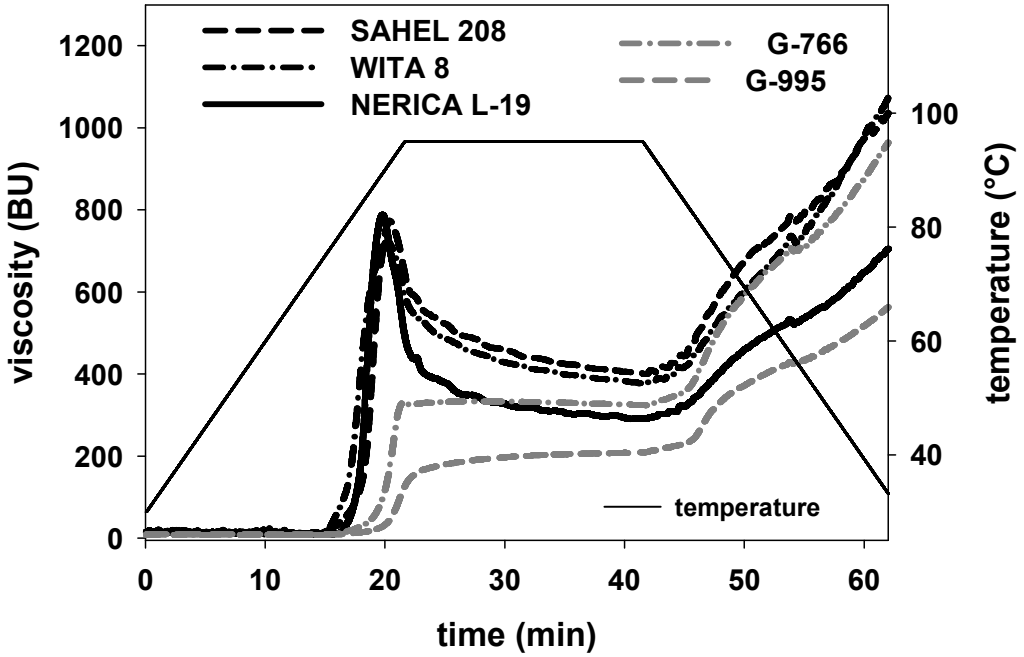

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531 Figure 2 - Pasting properties of rice samples. The thin black line is the temperature profile. The

532 thick lines are the pasting profiles: grey, *O. glaberrima*; solid black line, NERICA; other black

533 lines, *O. sativa*.

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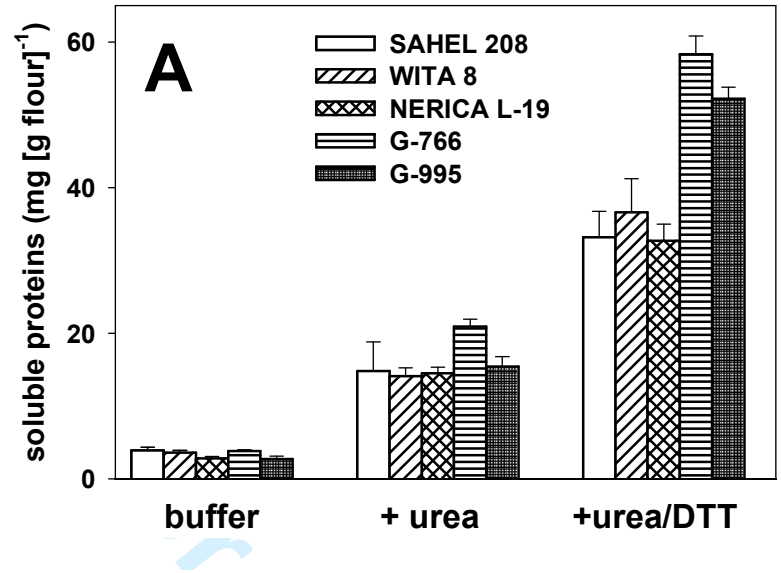

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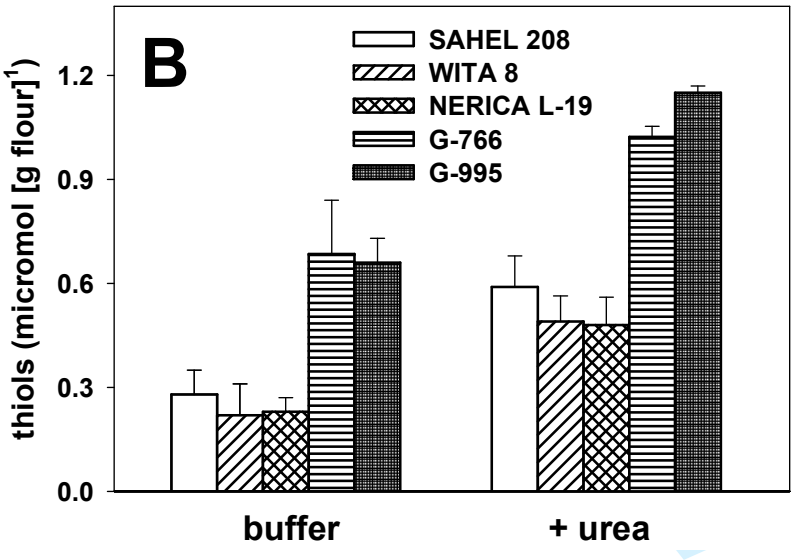

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538 Figure 3 - A: Solubility of proteins from rice in different media. Aliquots of the various samples
539 were suspended under stirring in 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.0, in the
540 presence/absence of 6M urea and of 10 mM DTT, as indicated.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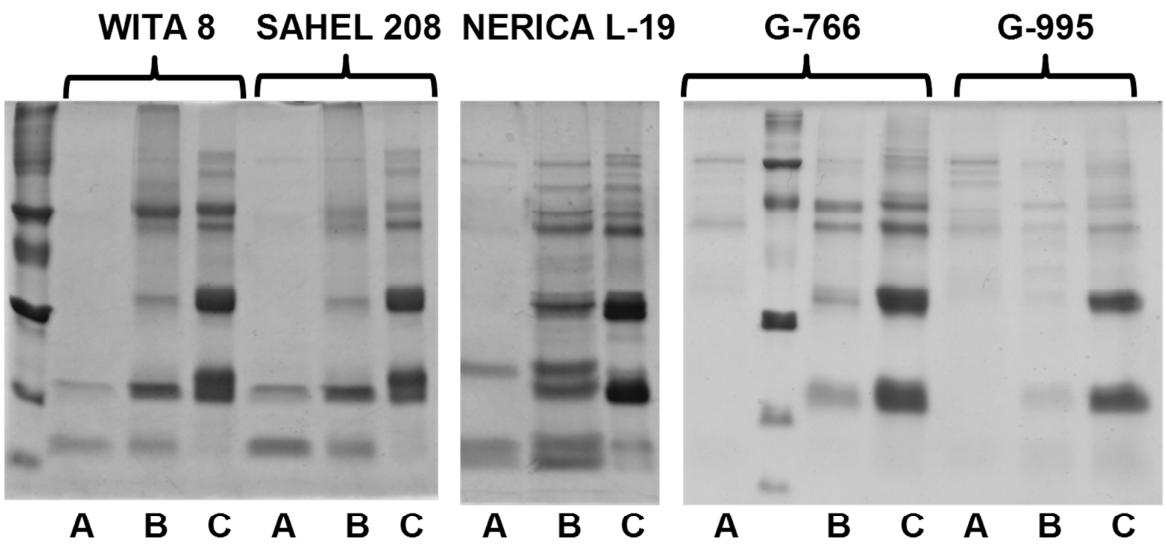

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547 Figure 4 - SDS-PAGE of proteins solubilized in different media. Proteins were extracted by using
548 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.0 (A), to which 6M urea (B), or of 6 M urea and 10
549 mM DTT (C) were added.

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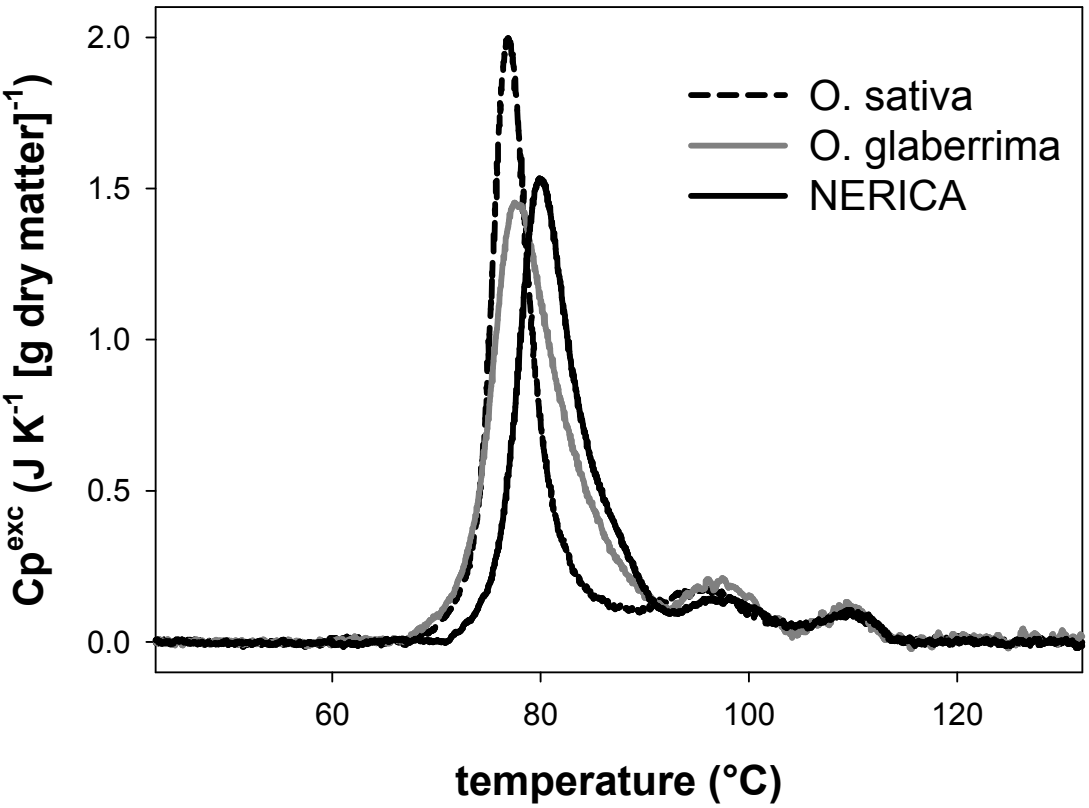

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553 Figure 5 - DSC tracings for the various rice flours (73% moisture, scan rate 2 °C/min). Grey, *O.*

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

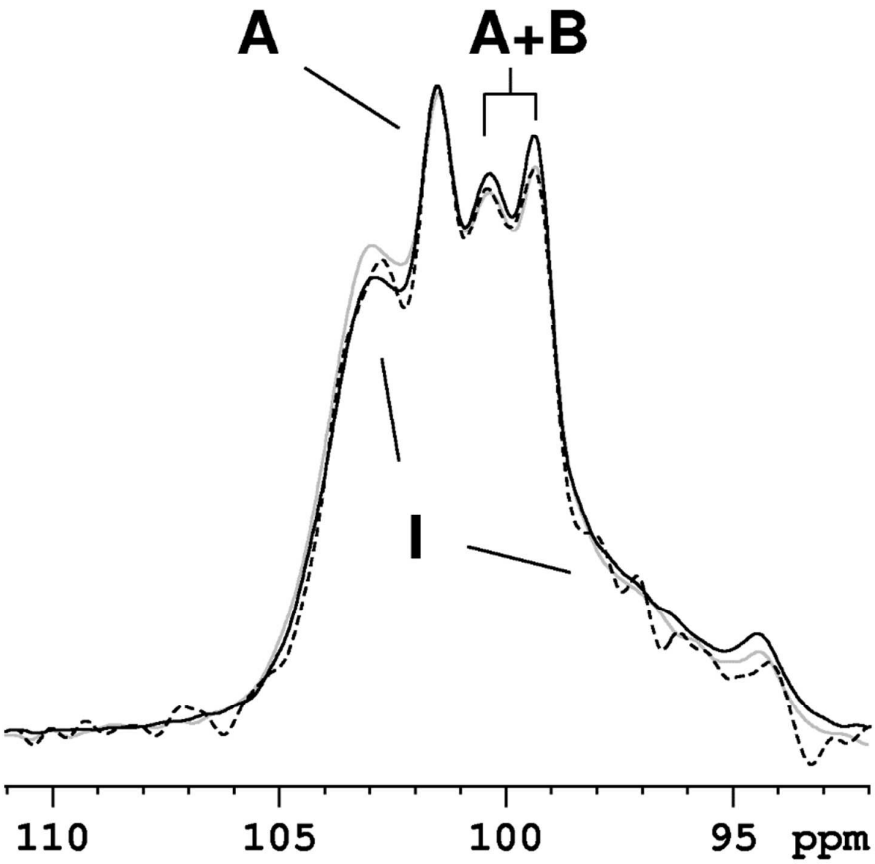
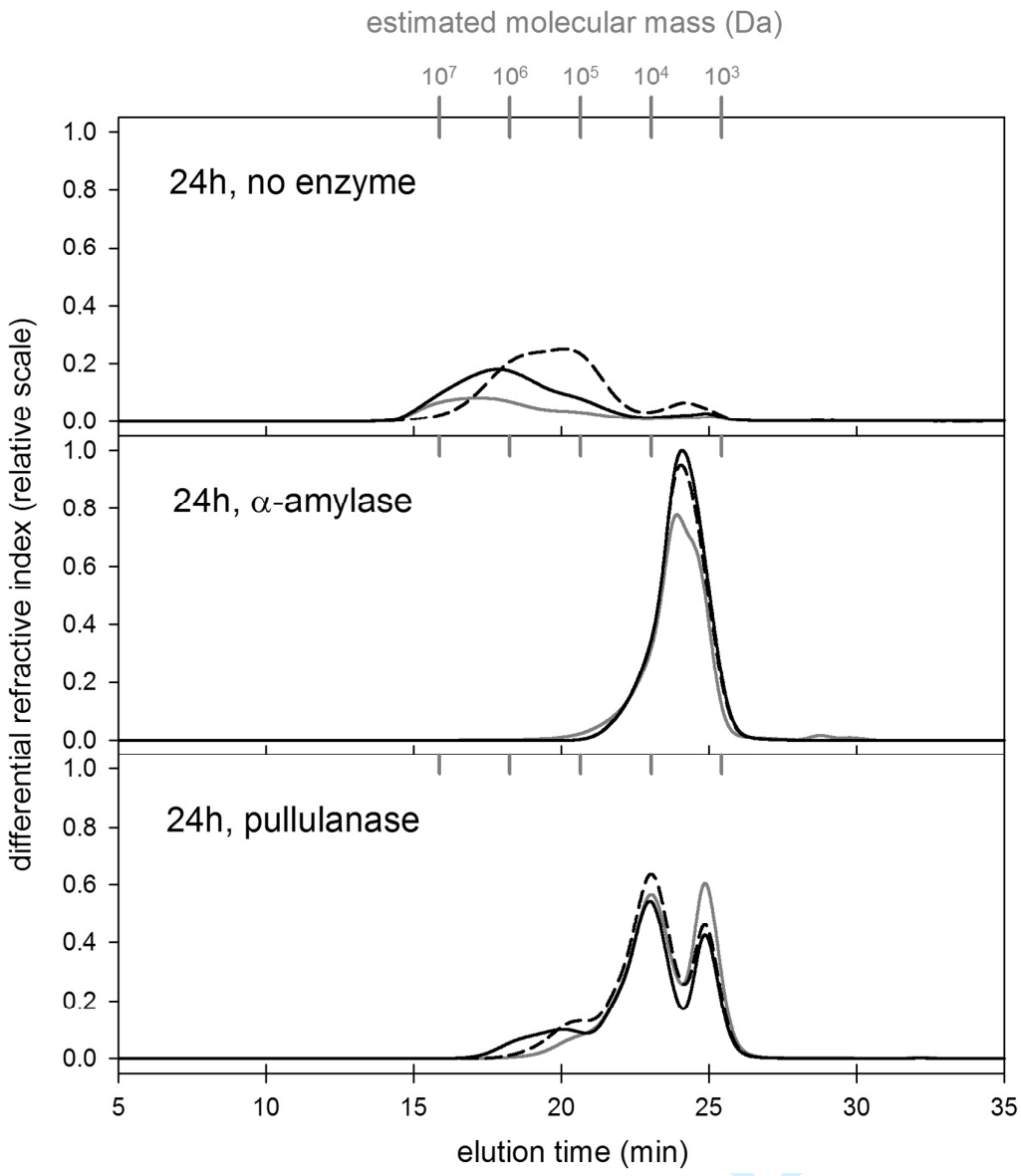


Figure 6 - Expansion of the CP-MAS ^{13}C-NMR room temperature spectra (amylose C_1 resonance region) of flours obtained from *O. glaberrima* (grey), *O. sativa* (dashed black) and NERICA (solid black). Resonance attributable to either A-type (A) or B-type (B) starch, or to "interfacial" carbons (I),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