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Author: Emna Chichti Matthieu George Jean-Yves Delenne
Valérie Lullien-Pellerin



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**Changes in the starch-protein interface depending on common wheat grain
hardness revealed using Atomic Force Microscopy**

Emna Chichti^{a1}, Matthieu George^b, Jean-Yves Delenne^a, Valérie Lullien-Pellerin^a

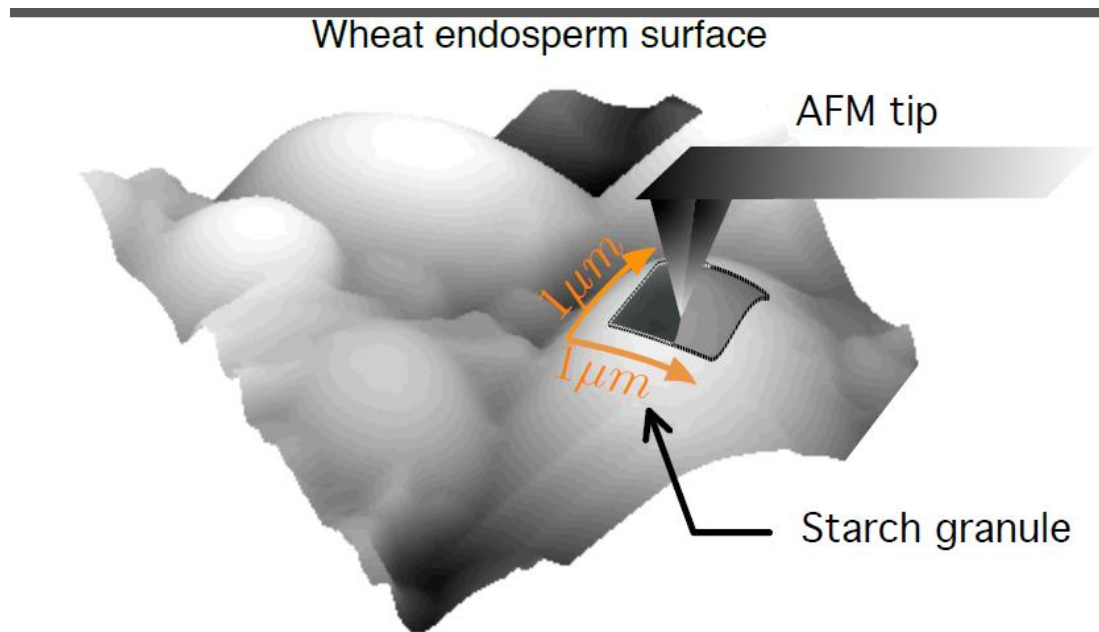
^aINRA, UMR 1208, Ingénierie des Agropolymères et Technologies Emergentes, 2
Place Viala, 34060 Montpellier Cedex 02, France, chichti@supagro.inra.fr,
delenne@supagro.inra.fr

^bInstitut Charles Coulomb, UMR 5221, CNRS-UM2, Place Eugène Bataillon, 34095
Montpellier Cedex, France, mgeorge@univ-montp2.fr

Corresponding author: Lullien-Pellerin V. UMR IATE, 2 Place Viala, 34060
Montpellier Cedex 02, France. Tél : 33 (0) 4 99 61 31 05, Fax : 33 (0) 4 99 61 30 76.
E-mail : lullien@supagro.inra.fr

¹Present address UMR 95 QualiSud, TA B95/16, 73 rue Jean-François Breton, 34398
Montpellier Cedex 5, France.

Graphical abstract



HIGHLIGHTS

- Explores the starch surface by nano-scratch AFM in the wheat grain endosperm
- Shows differences in mechanical properties related with grain hardness
- Relates the differences to distinct puroindoline b allele with near-isogenic lines
- Demonstrates for the first time an effect of puroindolines in grain mechanics
- Identifies a potential lubricant interface between starch and gluten in soft grains

ABSTRACT

The Atomic Force Microscope tip was used to progressively abrade the surface of non-cut starch granules embedded in the endosperm protein matrix in grain sections from wheat near-isogenic lines differing in the puroindoline b gene and thus hardness. In the hard near-isogenic wheat lines, starch granules exhibited two distinct profiles corresponding either to abrasion in the surrounding protein layer or the starch granule. An additional profile, only identified in soft lines, revealed a marked stop in the abrasion at the protein-starch transition similar to a lipid interface playing a lubricant role. It was related to the presence of both wild-type puroindolines, already

suggested to act at the starch-protein interface through their association with polar lipids.

This study revealed, for the first time, *in situ* differences in the nano-mechanical properties at the starch-protein interface in the endosperm of wheat grains depending on the puroindoline allelic status.

Abbreviations: (AFM), atomic force microscopy; (A_T), projected frontal abrasion area; (ESEM), Environmental Scanning Electron Microscopy; (F_N), normal force; (F_T), tangential force; (H), polymer hardness value; (PIN), puroindoline; (N), number of abrasive scans; (n_l), number of AFM tip scan lines; (NILs), near isogenic lines; (R), tip radius; (V), abraded volume; (V_T), tip velocity; (z), abrasion depth; (z_{norm}), normalized depth.

Keywords: endosperm; nanomechanics; microstructure; puroindoline; *T. aestivum*

1. INTRODUCTION

The major part of common wheat (*Triticum aestivum* L.) grains corresponds to the starchy endosperm (80-85 % of the dry grain mass) and is composed of cells filled with at least two distinct size populations of starch granules, between 8 and 30 μm , embedded in a protein matrix, mainly composed of storage proteins able to form the so-called gluten network[1]. Changes in the interface between the endosperm composing polymers are now recognized as the major factor to explain distinct hardness wheat classes[2,3]. It was found to be related to differences in the amount and allelic form, at the starch granule surface[4], of basic low-molecular weight cysteine rich proteins carrying a unique tryptophan domain[5], called Puroindolinea(PINA) and Puroindolineb(PINB), whose corresponding genes were found to be located within the main genetic locus (*Ha*) controlling hardness in the short arm of chromosome 5D[6-8]. Expression of the wild-type alleles *Pina-D1a* and *Pinb-D1a*, encoding PINA and PINB respectively, was found to lead to a soft mechanical behavior whereas mutation or deletion in one or both of the PIN encoding genes results in a harder grain texture[9]. In Europe and North America, the most common mutation in PINB is encoded by the gene allele *Pinb-D1b* and corresponds to a single amino acid change (Gly46Ser), which leads to a hard grain phenotype[10-12].

These differences in grain mechanical resistance has a main impact on the wheat grain end-use properties as it plays a role in the energy required for starchy endosperm isolation during processing, in the obtained particle sizes and final flour yield, as well as in starch granule dissociation from the protein matrix or starch granule damage[13-17]. More recently, construction of a numerical model mimicking the endosperm granular structure showed that lowering the adhesion

strength between starch granules and the protein network leads to a decrease of the starch damage as observed in soft grains[18,19].

If differences in a soft and a hard grain phenotype have been clearly linked to the presence of both of the wild-type PIN genes thanks to the use of near-isogenic lines (NIL)[20,21]and complementation of null[22]or mutated alleles[23], introduction of PIN genes in other cereals, such as durum wheat[24], rice[25]or corn[26] lacking the corresponding genes, was moreover found to reduce grain hardness. In addition, RNAi-mediated silencing of one of the PIN genes was shown to decrease the expression of the others and led to a significant increase of grain hardness[27]. However, the mechanisms by which PINs impact on the grain texture and have a role in the starch protein interface remain unclear.

The main hypothesis suggests that interaction of PINs with the starch granule surface is mediated by polar lipids from the remnant amyloplasts [28]. This hypothesis is supported by the differences in polar (phospho- and glyco-) lipid amount found associated with starch depending on PIN composition[29-31]and by *in vitro* demonstration of the tight bonding of PINs with polar lipids[32], as well as possible insertion into the lipid bilayer[33,34]. PINs are thus believed to impair starch-protein adhesion via their lipid interaction and thus affect grain hardness, but evidence of mechanical changes at the starch granule interface was still missing. Probing the starch granule interface in the wheat endosperm from near-isogenic lines differing in hardness thus appears interesting to study to better understand the differences in the interactions between the main endosperm polymers. As a method to investigate both the local structure and mechanical properties[35,36], Atomic Force Microscopy (AFM) appears appropriate to investigate the endosperm structure and hardness properties. Indeed it was used to reveal imaging differences

between hard and soft endosperm structure [37]but no mechanical studies were pursued. Recently, using an AFM tip as an abrasive tool to abrade the surface of tablets made of starch or the storage protein matrix isolated from wheat grains, distinct mechanical properties of the two main endosperm components were determined and revealed relatively high respective hardness values of around 2.4 and 0.6 GPa [38].Therefore,this original AFM method which proceeds by scratching of the surface layer-by-layer appeared the most appropriate to both explore the wheat endosperm polymer properties *in situ*andinvestigate the starch granule interface with the surrounding protein matrix.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

NIL of *Triticumaestivum* L. displaying either a or b allelic forms of *Pinb-D1* encoding the wild-type or mutated (Gly46Ser) PINB protein, respectively, and thus differing in grain hardness were described previously [39]. Genetic similarity (> 97 %) between the NILs was confirmed with the use of Diversity Array Technology markers [40] generated by Triticarte Pty.Ltd (www.triticarte.com.au). Grains were harvested in summer 2008, cleaned to remove broken kernels and impurities, and then stored at 4°C before analysis. Grain hardness was evaluated using a Percon NIRS apparatus (method 39-70A [41]) on randomly chosen grain sub-samples of each near-isogenic line and results were respectively equal to 17 for soft NIL (*Pina-D1a/PinbD1a* genotype) and 63 for hard NIL (*Pina-D1a/PinbD1b* genotype).

2.2. Sample preparation for microscopy

Wheat grain sections were obtained with a cryotome (Microm HM 520 Cryostat, Thermo Scientific, USA). Grains were picked up randomly in the grain population of each near-isogenic line and cut at both ends to eliminate the germ and the brush then glued on a sample holder using a Frozen Section Medium (Neg-50, Richard-Allan Scientific, Thermo Scientific, USA). The grains were fixed from the widest part corresponding to the germ and placed into the cryotome at -28°C before sectioning, until the glue was frozen (2 min). Sections of 50 µm were removed progressively to reach the middle of the grain. Then continuous cutting of the sample surface was performed by removing thinner sections of 5 µm. The remaining 500 µm grain sections were finally fixed on glass slides, protected with parafilm and kept under

controlled temperature and humidity conditions (20°C, 30% relative humidity) before microscopy analysis.

2.3. Environmental Scanning Electron Microscopy (ESEM) and AFM Microscopy

ESEM (Fei Quanta 200 FEG, FEI Co, Hillsboro, OR, USA) without sputter coating was used for grain section imaging.

AFM assays were performed as previously described[38]with a Nanoscope V atomic force microscope (Bruker instruments, Santa-Barbara, CAUSA), operating in the contact mode under controlled conditions of temperature and humidity (20°C, 30% relative humidity). Commercial silicontips (Ref. FMV, 2.8N/m, Bruker, Camarillo, CA, USA) mounted on a rectangular cantilever with stiffness ranging between 1 and 5 N/m were chosen to preserve reasonable measurement sensitivity and to exert sufficiently large forces to abrade the samples. Before each measurement, the normal (F_N) and tangential forces (F_T) were calibrated and the contacting areas of the tips were characterized before and after calibration and at the end of AFM measurements, as described previously[38]. The contacting areas of the tips were characterized through reverse imaging obtained with AFM on a calibrating grid of equally-spaced sharp points of apex radius around 10 nm (TGT01, Mikromasch, Inc., Estonia). These measurements clearly showed that the AFM tip apex can be well fitted after calibration by a sphere from the extremity to 20 nm high, with an average radius for the set of tips $R = 82 \pm 32$ nm. This value is significantly above the initial tip radius of 10 nm due to wear off occurring during the calibration process and was checked to remain stable during abrasion assays.

AFM assays were performed on wheat grain sections after selection of a working area i.e at the surface of a small starch granule. A first topographic image ($5 \times 5 \mu\text{m}$) was acquired at a low applied normal force ($F_N=100\text{nN}$) and a scan tip velocity $V_T = 10\mu\text{m/s}$ (512×512 pixels) and was used as the reference of the undamaged surface. The abrasion process was then initiated in the central area ($L \times L=1 \times 1\mu\text{m}$) with an increase of the applied normal force ($F_N > 200\text{nN}$) and a decrease of the scan velocity $V_T = 2\mu\text{m/s}$ (256×256 pixels). Both the trace and retrace F_T force maps were acquired (respectively scanning from left side to right side of the image and from right side to left side) to determine the average force sustained by the sample in the direction of displacement. Then, the normal force was lowered back to its initial value (100nN) and a second topographic image ($5 \times 5 \mu\text{m}$) was recorded at $V_T=10\mu\text{m/s}$ before again increasing the normal force to further abrade the material. A progressive and controlled abrasion of the sample was ensured by repeating the abrasion step at least twenty times with a mean number of abrasion scans $N=50$, depending on the abraded area, interrupted by regular acquisitions of larger topographic images. Large topographic images ($5 \times 5 \mu\text{m}$) were acquired after each scan until five scans were reached and then after each group of five scans. Repetitions of the abrasion process were made on different starch granules (at least twelve) chosen in the middle of the grain cheek section from randomly selected soft or hard NIL samples. The acquired AFM images were visualized and analyzed using the Gwyddion 2.26 software (Department of Nanometrology, Czech Metrology Institute, Brno, CZ) in order to evaluate the abrasion depth and the friction force F_T . The depth increment between two successive abrasion steps was obtained by subtracting the two corresponding topographic images after careful lateral positioning through correlation techniques and sensible leveling of the height to

account for instrumental drift. The evolution of abrasion depth was then determined by summing all depth increments. More details on the measurement protocol and the error estimations can be found in Chichti et al [38].

2.4. Statistical analysis

Analysis of variance was performed with the XLSTAT software (2008 version, Addinsoft, Paris) using the Tukey test at 5 % probability for significance between differences.

3. RESULTS

3.1. Microstructure analysis of the grain endosperm depending on hardness

Microstructure of the starchy endosperm from cryo-cut near-isogenic wheat grains differing in hardness was either observed using ESEM or AFM (Fig. 1). Both types of observation highlighted clear differences in grain endosperm microstructure according to wheat hardness. Endosperm from soft grains (*Pina-D1a-Pinb-D1a* genotype) was shown to display a loose structure (Fig. 1A) in comparison with hard grains (*Pina-D1a-Pinb-D1b*), which presented a more tightly compacted endosperm with microcracks bypassing the starch granules (Fig. 1B). Differences were more pronounced using AFM, and at a lower scale (10 versus 50 μm), where the endosperm from soft grains was found to present more uncut starch granules (Fig. 1C). Starch granules also appeared less embedded in the protein matrix and even dislodged from the protein matrix in comparison with the endosperm structure from hard grains where the sectioning entailed cutting of the major part of the large starch granules and some of the small ones, depending on their position in the endosperm (Fig. 1D).

Figure 1

Therefore, only small starch granules which were observed as undoubtedly uncut in AFM topographic images (mean diameter around 5 μm), were selected, as shown in Fig. 1E, to explore the starch-protein interface. As revealed in Fig. 1F, the AFM topographic image did not allow the identification of the polymer nature at the surface of the selected starch granule.

3.2. Probing of the polymer present at the starch granule surface during endosperm abrasion

A square of $L \times L = 1 \times 1 \mu\text{m}$ (Fig. 1 F) was selected in the middle of each small starch granule in order to avoid the curvature and border effect. Its surface was abraded using an AFM tip according to the conditions recently described [38]. According to these conditions, the abrasion process could be interpreted as a linear scratching test to evaluate the resistance of the analysed material. As already described in Chichti et al. [38], a tribological model was developed, to assess the material hardness which is related to the normal applied force F_N and the depth of the abraded area z . The projected frontal area in contact with the AFM tip, A_T , corresponds to the surface of the removed material obtained for each scratching line and can be determined from the equation of removed volume, taking into account the total depth z , the length of the abraded scan L , the number of abrasion scans N ($20 < N < 70$) and n the number of scan lines in the acquired image (256 lines):

$$A_T = \frac{V}{2n_l N L} = \frac{zL}{2n_l N} \text{ Eq. 1}$$

Considering the definition of hardness (ratio between the applied normal force and the area under the tip) and assuming a spherical approximation of the tip apex, we get that A_T is also a function of the applied normal force F_N , the tip radius R and the hardness H of the abraded material:

$$A_T = \frac{F_N^{3/2}}{2R(\pi H)^{3/2}} \text{ Eq. 2}$$

According to equations 1 and 2, the depth of the scratched area z should increase linearly with the scan number N when abrasion occurs in a homogeneous material, at a given applied force F_N and for a determined tip radius R . As AFM assays were performed at different applied forces F_N and with different AFM tips to explore potential differences in polymer hardness and to avoid tip wear, the depth z was

normalized (z_{norm}) according to F_N and R for comparison between distinct abraded areas:

$$z_{norm} = \frac{zR}{F_N^{3/2}} = \alpha N \text{ Eq. 3}$$

Changes in the normalized depth z_{norm} as a function of the abrasive scan numbers N was thus relevant to characterize the different polymer layers which can be encountered during the progressive abrasion of the heterogeneous endosperm microstructure.

In a homogeneous material, the slope α describing the linear variation between the normalized z_{norm} and the number of abrasive scan N could be considered as a direct indication of the material hardness H since the dimension of the abraded area L and the number of scan line n_l were kept constant in all of the measurements:

$$\alpha = \frac{n_l}{L(\pi H)^{3/2}} \text{ Eq. 4}$$

This slope will be used to identify the nature of the polymers encountered during progressive abrasion at the starch surface. According to the previously determined hardness of the endosperm constitutive polymers [38], reported in Table 1, values for the curve slope α were expected to be equal to $0.012 \pm 0.005 \text{ nm}^2/\text{nN}^{3/2}$ and $0.09 \pm 0.04 \text{ nm}^2/\text{nN}^{3/2}$ for starch and protein network, respectively, in our working conditions.

Table 1

3.3. AFM abrasion at the starch surface in the endosperm of hard wheat grain sections

Distinct areas selected in apparent uncut starch granules surfaces were scratched using the AFM tip in the endosperm of grains from the hard NIL displaying the *Pina-*

D1a/Pinb-D1b genotype. Evolution of the normalized depth z_{norm} as a function of the number of abrasive scans N revealed two distinct types of abrasion profiles summarized in Fig. 2 depending on the analysed starch granule.

In one out of three AFM scratching assays, a constant linear progression of the normalized depth according to N was observed consistent with the removal of a homogeneous material (Fig. 2A). Considering the curve slope α ($\alpha = 0.013 \text{ nm}^2/\text{nN}^{3/2}$ as an example in Fig. 2A), the abraded material can be considered as starch polymer in accordance with its previously determined hardness value [38]. The considered abraded area thus appeared to correspond to the naked starch granule.

Figure 2

Normalized depth according to the number of AFM tip scans at the surface of other analysed starch granules in the endosperm of hard grains was found to present non-linear curves characterized by a progressive transition from a linear regime with a high slope α ($\alpha = 0.05 \text{ nm}^2/\text{nN}^{3/2}$ as for example in Fig. 2B), consistent with that calculated with protein hardness, to another linear regime with a lower slope ($\alpha = 0.009 \text{ nm}^2/\text{nN}^{3/2}$) corresponding to the expected slope for starch polymer (Fig. 2B). Indeed as the mechanical resistance of protein polymer is lower than starch it was more easily removed with a lower number of AFM tip scans in comparison with the starch polymer. This material heterogeneity revealed that AFM abrasion in these cases occurred at a starch granule covered with a protein layer. Mean thickness of this protein layer was estimated to be in the order of a hundred nanometers, ranging from 48 to 188 nm, depending on the selected starch granule.

The hardness H was determined with equation 4 using the slopes α of each linear part of the curve for each abraded area and compared, in Fig. 3, to hardness values previously determined in similar AFM conditions on tablets made of isolated starch and protein polymers [38]. Taking into account the uncertainty of our measurements (calculated and represented by bar errors in Fig. 3), hardness values obtained for starch and protein from assays with hard wheat grain sections were in good agreement with hardness values obtained on isolated biopolymers whatever the applied normal force used in the AFM assays. However, in a few cases, determined hardness values appeared outside the range of the data corresponding to the extracted polymers and were found between those of the protein matrix and starch.

Figure 3

Comparison of protein and starch hardness mean values and relative errors calculated with data obtained from endosperm samples of hard wheat grains were summarized in Table 1 and showed no significant differences with the values determined with isolated polymers.

3.4. AFM abrasion at the starch surface in the endosperm of soft wheat grain sections

Distinct starch granules were abraded with the AFM scratching method in endosperm from soft near-isogenic grains carrying both wild-type PIN genes *Pina-D1a* and *Pinb-D1a*. Results are expressed as described before and compared with those obtained in endosperm from NIL carrying the mutated allele encoding PINB. In half of the AFM assays, the obtained curves of normalized depth as a function of

the number of abrasion scan presented a linear curve corresponding to pure starch as pointed out in Fig. 2A, whereas around a quarter displayed an abrasion profile, similar to that observed in Fig. 2B, consistent with a starch granule covered with a protein layer.

Interestingly, a distinct abrasion profile, not previously observed in the endosperm from hard wheat grains, was revealed in four of the abraded areas and reported in Fig. 4A. In this case, abrasion started with the removal of a protein polymer and ended by digging into the starch granule as determined by the corresponding values of respective curve slopes α . But, a marked transition between the two parts of the profile, characterized by a “stop” of the AFM tip abrasion, was observed. Indeed, for up to twenty abrasion scans, normalized depth remained fairly constant and thus no material removal could be measured.

Figure 4

At this non-abrasive step, a significant drop of the lateral force F_T was observed, as reported in Fig. 4B by the study of the F_T/F_N ratio as a function of the abrasive scan number N . Indeed, at this interface step, the F_T/F_N ratio was found to be close to the friction coefficient while imaging the sample topography and therefore confirmed the absence of abrasion. After a number of AFM tip scans without detectable abrasion, an increase in the F_T/F_N ratio was observed, when abrasion was recovered and the starch polymer was beginning to be removed. No significant depth variation and thus no measurable volume of removed material were therefore possible at this interface step in comparison with those corresponding to protein or starch abrasion (inserts, Fig. 4A). However superficial damage of the scratched surface must have occurred as

further abrasion of the endosperm material could be continued. Depending on the selected scratching area, the mean thickness of the protein layer covering the starch surface was found to range from 7 to 40 nm.

Hardness values of protein and starch obtained from the distinct scratched endosperm surface in soft NIL were also reported in Figure 3 and Table 1 and compared to values obtained from hard NIL and for isolated polymers. Results confirmed the higher mechanical resistance of wheat starch polymer in comparison with the endosperm protein network. Moreover, no significant differences were noted for the polymers in wheat grains whatever their hardness, nor with the extracted polymers.

4. DISCUSSION

The origin of the mechanical differences between soft and hard wheat grains was generally recognized to be linked to changes in the interactions between starch granules and the surrounding protein matrix in the endosperm and to be mediated by either the presence of both wild-type PINs or the mutation or deletion of one of them[28]. In this study, NILs carrying either the wild-type alleles *Pina-D1a/Pinb-D1a* or the mutated version of the gene encoding PINB (*Pina-D1a/Pinb-D1b*) were intentionally chosen to investigate the local differences between hard and soft endosperm.

ESEM, as well as AFM, observation of the starchy endosperm microstructure after grain cutting clearly confirmed the looser structure of the soft NIL in comparison with the hard, which displayed a more compact microstructure. Indeed, cutting in the endosperm from soft grains revealed local spaces in the protein matrix and possible sites of dislodged starch granules whereas starch granules in the endosperm from hard grains were firmly bound to the matrix and were more often cut, especially the large ones. Differences in cohesion between starch and the protein matrix were already pointed out in cultivated common wheat cultivars[42] and further clearly related to differences in PIN genotype by others[23,37,43,44]. It was also in accordance with the attributed role of wild-type PINs as non sticking agents and correlates well with endosperm modelling which results in more starch damage when adhesion between starch granules and the protein matrix increased[18,19]. As a consequence only the interface between the non-cut small starch granules and the surrounding protein matrix could be explored, in soft and hard NIL grains, using an original AFM scratching method, previously described[38], and found to differentiate

between the respective mechanical properties of wheat starch and the protein matrix (extracted as gluten).

Even if the number of AFM experiments on each type of NIL grains was necessarily low due to the time of data acquisition, between 12 and 20 independent starch granule surfaces were explored from each near-isogenic line, at different locations within the wheat endosperm and with distinct grains randomly selected. No differences between the mechanical properties of the two main endosperm polymers were measured *in situ* whatever the grain hardness, in accordance with previous studies [2,38,45], clearly highlighting the importance of local investigations of the starch-protein interface. Indeed, distinct behaviors at the starch surface were revealed between hard and soft NILs using an AFM tip as an abrasion tool.

In hard wheat grain sections, probed starch granule surfaces were mainly found covered with a protein layer of significant thickness (48-188 nm) whereas, in fewer cases, the AFM tip directly abraded the naked starch granule. When the starch granules were coated with the protein matrix, the abrasion profile presented a gradual transition from the mechanical strength of proteins toward that of starch as abraded depth increased. This transition fits well with the traditional area-law-of-mixtures or volume-law-of-mixture models used to describe the mechanical hardness of a coated substrate [46-48]. When the adhesion between the coating and the substrate is strong enough, stresses are therefore transferred from the coating to the substrate and the apparent hardness of the coated material is the average hardness of both materials weighted by the respective areas/volumes loaded in each of them. The mechanical reinforcement of the protein layer as the underneath starch sustains increasing strain by the AFM tip seems to fall well inside this frame and appears thus to indicate a sufficient adhesion at the protein/starch interface to allow for the stress/strain

transfer. This law of mixtures could also explain the relative out of range values of a few protein or starch hardness data if obtained too close to the interface. In the endosperm from soft wheat grain sections, the surface of starch granules was mainly found naked revealing a non-cohesive interface with the protein network. Additionally, if in some positions, the starch granule was found to be covered with a protein layer, this layer was thinner (7-40 nm) than that observed in the endosperm from hard NIL grains. Moreover, an additional abrasion profile was never observed in hard grains and displaying a marked 'stop' of the AFM tip abrasion, along with a significant drop of the lateral forces, at the interface between starch and the surrounding protein matrix was observed. In this last case, the mechanical properties of both protein and starch were well defined within the expected hardness values and were not affected by the presence of the interface. This behavior thus appears coherent with a lack of adhesion between the protein matrix and the starch granule at the interface and thus suggests that the protein network in this case was not adhesively coupled with the starch granule. Furthermore, the observed stronger resistance to the tip abrasion concomitant with a drop of the lateral friction forces at the interface between the endosperm polymers appears to correlate well with the lubricating properties observed when exploring a non-punctured polar lipid covered surface using AFM [49,50]. Taking into account the genetic difference between soft and hard NILs, these observed distinct abrasion patterns at the surface of the starch granule can be related to the PIN nature (wild-type versus mutant of PINB). The higher frequency for the observation of a naked starch surface in soft endosperm also confirms the lower interaction between starch and the surrounding protein network and higher decohesion of the starch granule with the protein matrix, already pointed out by ESEM or AFM imaging. Furthermore, a specific sliding interface was

only found in soft grains in one out of four analyzed samples. Taking into account the number of samples analyzed with AFM in each near-isogenic line and considering a similar frequency to encounter such a sliding interface in both types of wheat grain, it is possible to estimate that the probability to find a specific mechanical behavior in one quarter of the samples from the soft line, but never for the samples from the hard line, is in the order of a few per cent. Therefore, even if the sampling appears small to perform a complete statistical analysis of the encountered abrasion behavior at the starch/protein interface, the observed rheological differences between starch and protein interface depending on hardness appear relevant and robust with a level of confidence higher than 95 %. Thus, it is the first time that significant mechanical differences at the protein-starch granule interface are experimentally evidenced.

As the peculiar sliding behavior at the protein-starch interface was not observed in each of the explored positions in soft grains, it thus implies that changes in the PIN nature only impact in some places and not around the overall granule surface. This appears coherent with previous PIN detection at the starch granule surface by immunolabelling[51], which clearly showed non-continuous location at the starch granule surface even if precise location and sub-cellular distribution in grains was not consensual[28]. Unfortunately, AFM abrasion could not be coupled with immunolocalisation.

Most importantly, the peculiar abrasion profile only observed at certain positions at the starch granule surface in the endosperm from soft NIL grains revealed a lipid interface between the protein matrix and the starch surface displaying a mechanical role like a lubricant. Taking into account the strong interactions with polar lipids pointed out for the wild-type PINs[32-34] and their suggested mode of action at the starch surface via polar lipids[9,28], this observation appears particularly relevant.

Indeed it is believed that wild-type PINs bind to remnant amyloplast polar lipids at the starch surface via their tryptophan-rich domain resulting in a soft mechanical behavior of the endosperm. However, the mechanisms were still not identified. Our AFM observation unambiguously revealed possible mechanical differences at the starch granule surface mediated by lipids in the soft endosperm. As shown previously, a Glycine to Serine mutation at position 46 in PINB, next to the tryptophan rich domain, reduces its lipid-binding capacity[52], leading to a lower amount of the deposited PINs at the starch granule surface and to a hardness increase in comparison with wheat grains containing the wild-type allele[4,53-55]. A higher association and stabilization of polar lipids around the starch granule with wild-type PINs was previously demonstrated[31] which could explain the unique AFM abrasion profile found in soft NIL grains. However, it is difficult to determine if this unique profile observed in soft endosperm results from a higher amount of PINs linked to the starch granule in grains via a lipid stabilization or to the higher amount of polar lipids found at the starch surface when both wild-type PINs are present[30]. Indeed, recent experiments suggested that PINs need lipids for binding to the starch granule surface[56].

Thus, our AFM experiments highlighted for the first time, differences in mechanical properties at the starch granule surface depending on the PIN nature and consequently grain hardness. Observed abrasion profiles at the surface of starch granules in hard endosperm were in accordance with the suggested close association with the surrounding matrix as in the major part of the assays, starch granules were found to be covered with a thick protein layer even if cutting grain before AFM analysis could also lead to naked starch granules. By contrast, when wild-type PINs were present, a naked granule was preferentially found, as a loose interaction with

the protein matrix. In some cases, identification of a lubricant interface highlights the possible mechanisms underneath the lower adhesion between the main wheat endosperm polymers found in soft NIL. A few assays also pointed out starch granule surface covered with a thinner protein layer in accordance with the fact that PINs were not uniformly distributed and more probably form patches at the starch surface[34,51]. However, if our AFM scratching method was found efficient to probe *in situ* polymer interfaces and explore the local mechanical properties, complementary studies will be necessary to better define the nature of the interactions and to more precisely identify the molecular partners.

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Figure Captions

Figure 1: ESEM images of soft endosperm from *Pina-D1a/Pinb-D1a* NIL (A) or hard endosperm from *Pina-D1a/Pinb-D1b* NIL(B) grain sections. Topographic images of the same soft (C) or hard (D) grains but different area obtained with AFM. White arrows in C indicate the dislodged starch granules commonly observed in soft endosperm whereas black arrows in D indicate cut starch granules, which occurred when hard wheat grains were sectioned with the cryotome. Example of a selected AFM abrasion area corresponding to a small starch granule surface is shown in E(dotted square) and F (lower scale). The grey levelbar scale shows the relative height of the explored area.

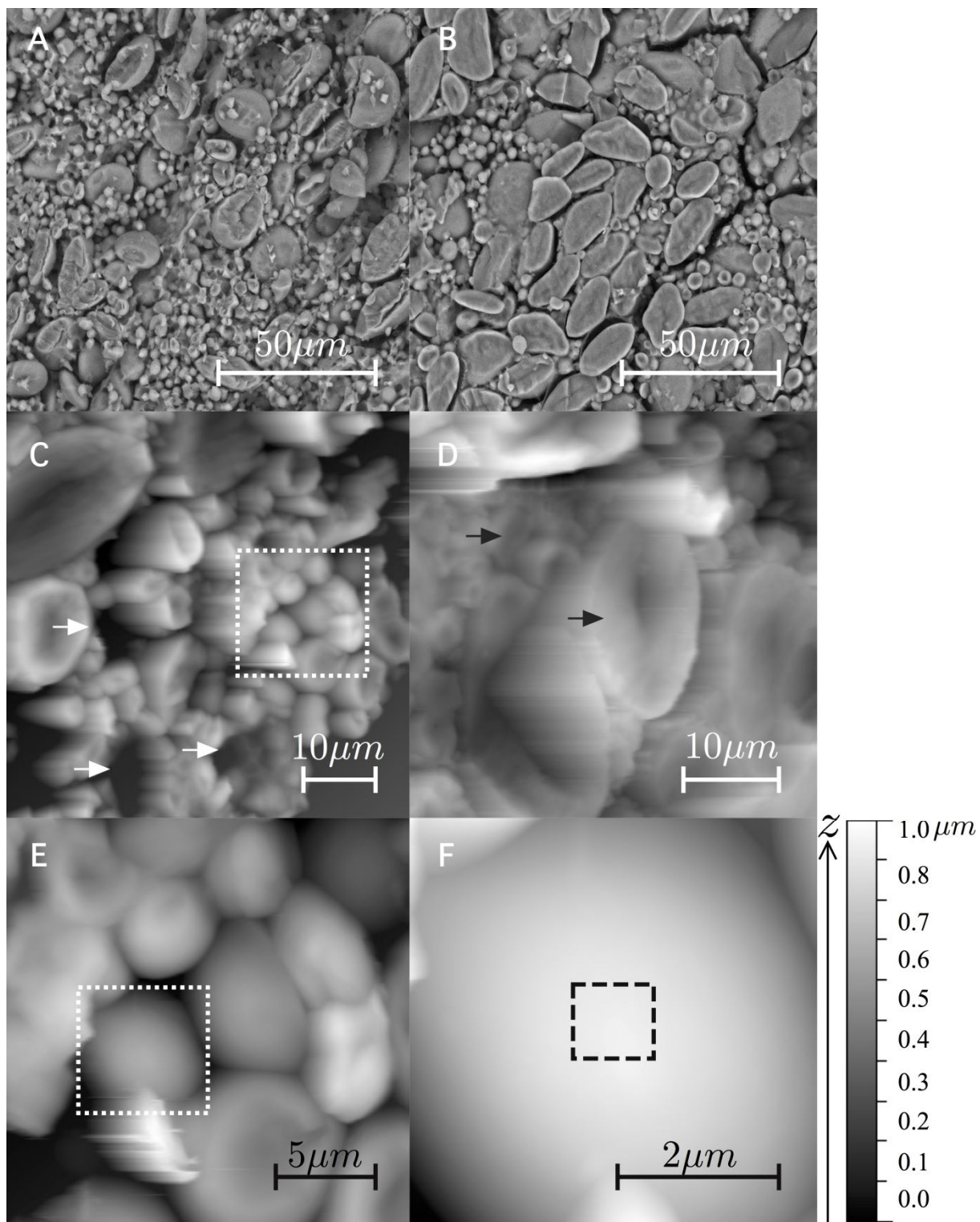
Figure 2: Example of normalized depth z_{norm} progression as a function of AFM abrasive scan numbers N for two distinct (A, B) abraded areas displaying contrasted behavior and observed in endosperm from hard wheat NIL (*Pina-D1a/Pinb-D1b* genotype). Different line styles and colors are used to point out changes in the fitting curve slope α corresponding to distinct type of encountered material along endosperm abrasion: dashed line for the fitting curve of the softer material (protein) and dotted line for the harder material (starch).

Figure 3: Hardness values obtained for the easily abraded protein material (circles) and the harder starch material (diamonds) encountered along endosperm abrasion from hard (empty symbols) and soft (full symbols) wheat NIL. Already determined hardness mean values and relative errors of pure extracted polymers [38], starch and gluten, were marked with continuous (mean values) and dotted lines (relative errors) respectively.

Figure 4:A. Changes in the normalized depth z_{norm} as a function of the abrasive scan numbers N for the particular behavior only observed in soft wheat NIL. The distinct removed polymers were discriminated through the different fitting curve slopes α (dashed line for protein and dotted line for starch) as in Fig. 2. The non-abrasive step corresponding to AFM tip slipping is represented by triangles. Picture inserts correspond to the difference between AFM images taken before and after several tip abrasive scans: from left to right, in the protein matrix ($N=5$), at the interface ($N=27$) and in starch ($N=6$).

B. Changes in the lateral friction force F_T related to the normal applied force $F_N(+)$ as a function of the abrasive scan number N for the particular behavior only observed for one part of the explored starch granule surface in soft wheat NIL. Comparison with measured F_T/F_N ratio along AFM imaging of the endosperm topography (■) was reported to point out non-abrasive conditions.

Figure 1

**Fig. 1**

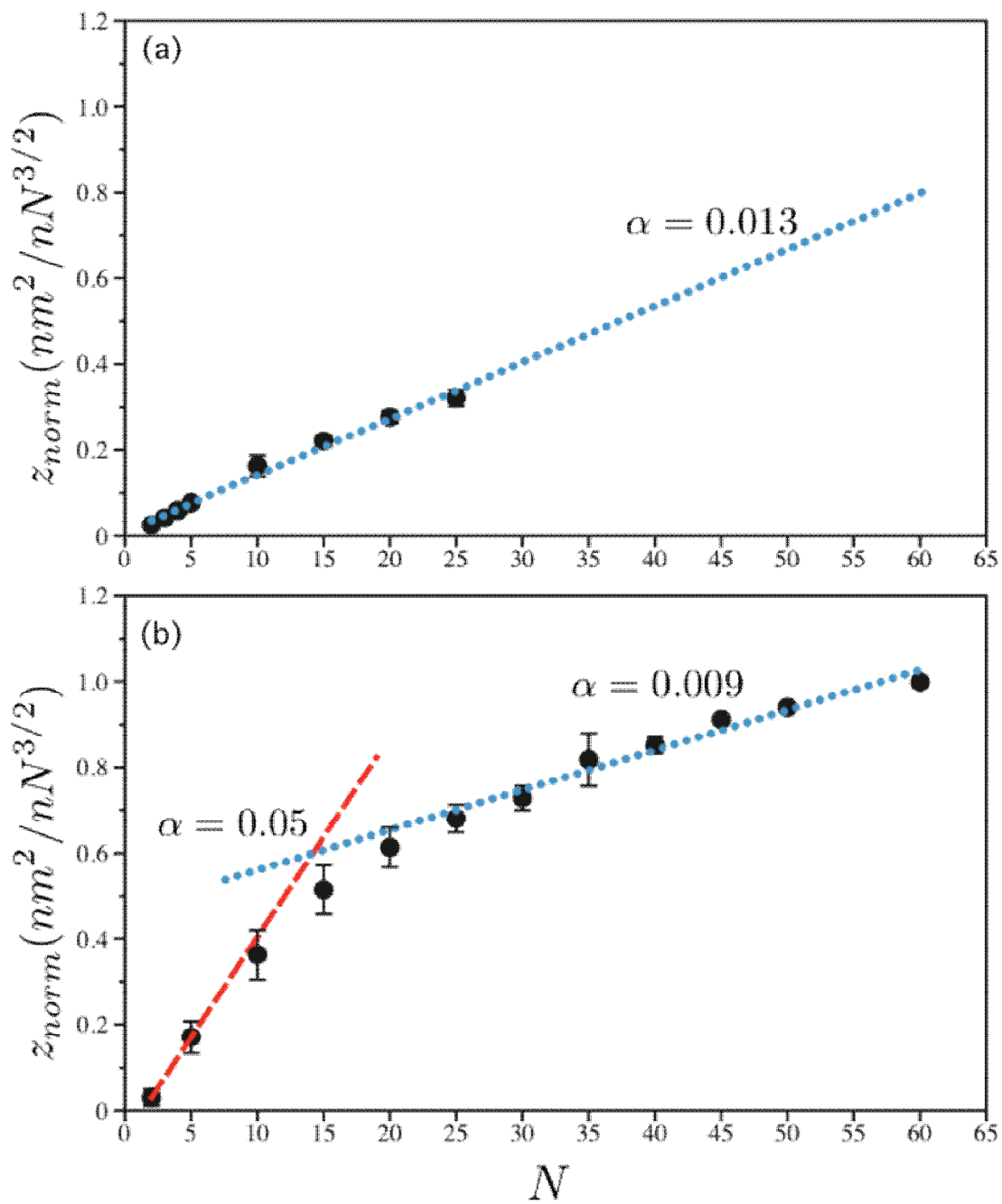


Fig. 2

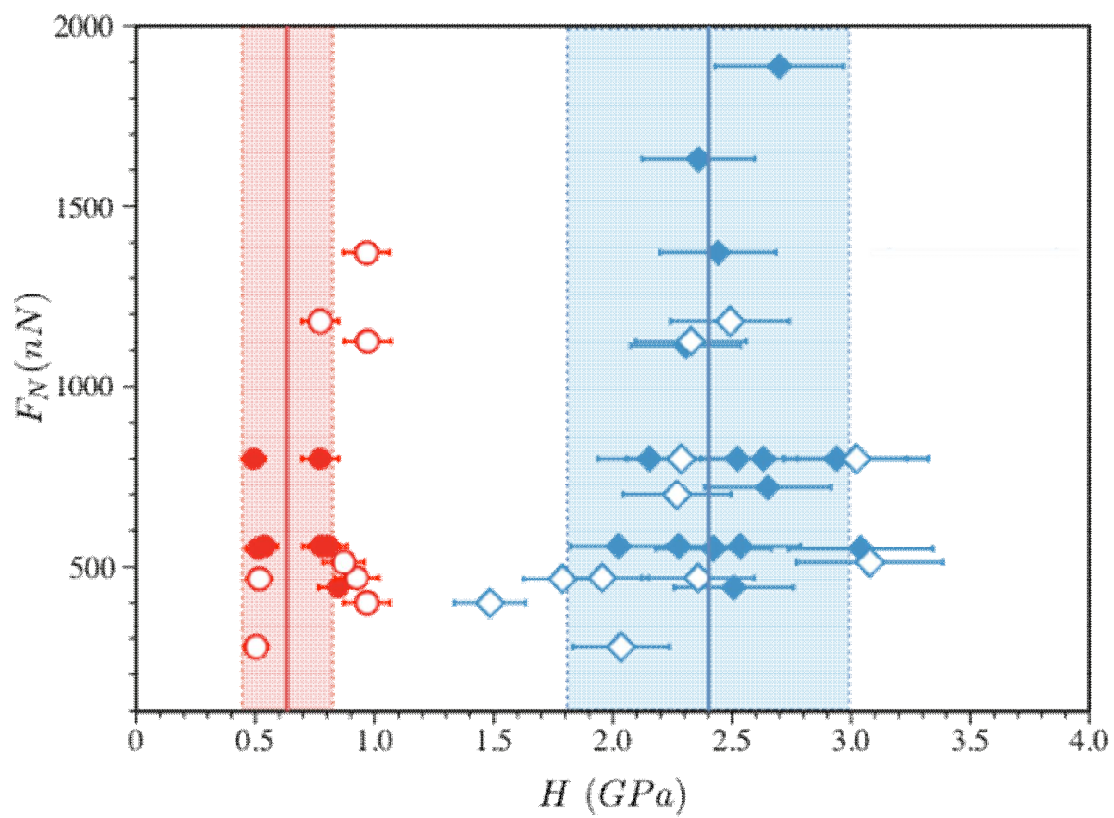


Fig. 3

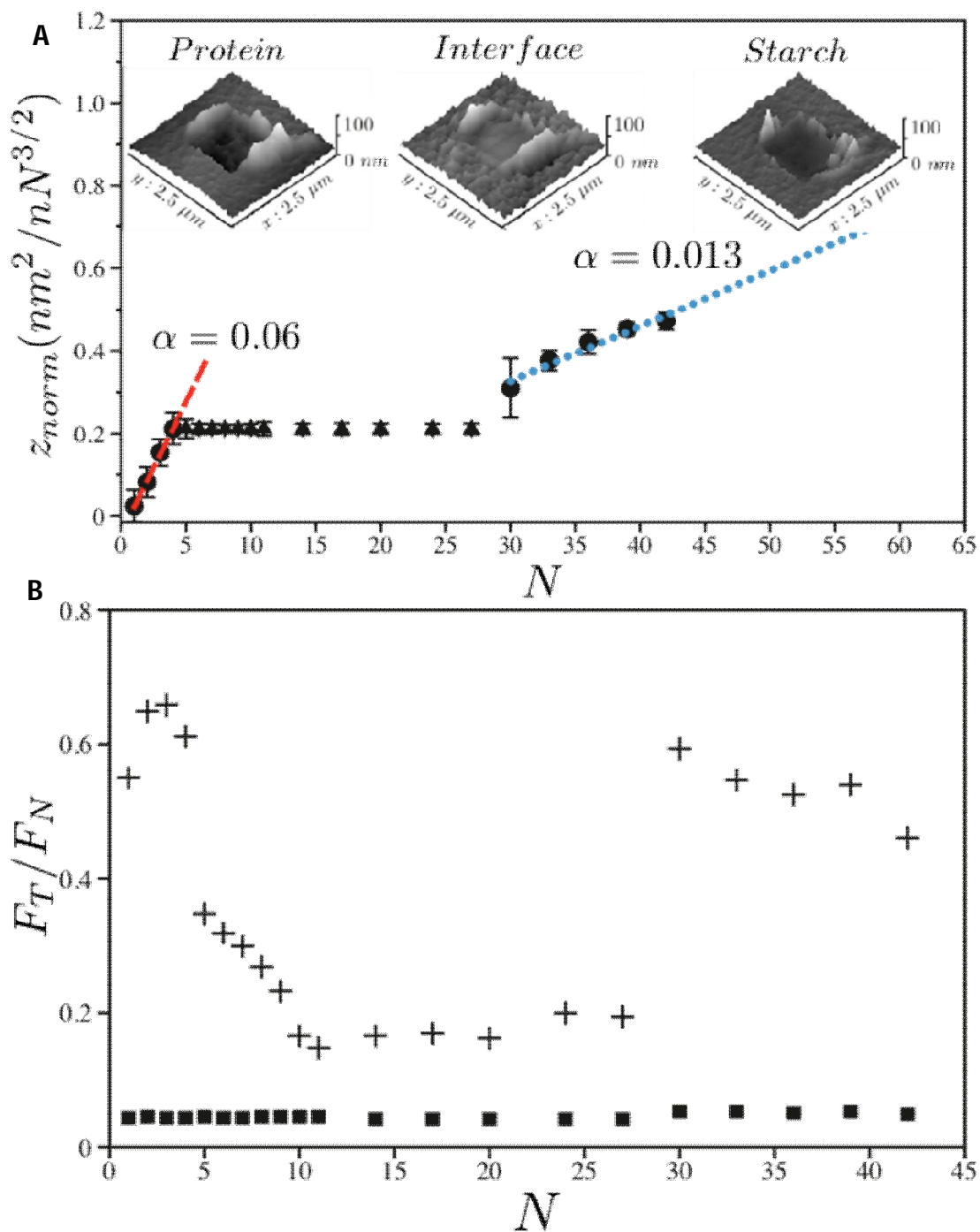


Fig. 4

Table 1. Hardness values for protein and starch obtained from soft and hard wheat grain sections and comparison with data obtained from tablets made of pure polymers using similar AFM abrasion scratching assays [38]. Different letters indicate statistically distinct values.

	Starch Hardness (MPa)	Protein Hardness (MPa)
Polymer Tablets	2400±600 ^a	640±170 ^b
Hard (<i>Pina-D1a/Pinb-D1b</i>)	2370±600 ^a	811±200 ^b
Soft (<i>Pina-D1a/Pinb-D1a</i>)	2532±300 ^a	677±150 ^b