JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 199- 203

Jordan Journal of Biological Sciences

Microscopic Analysis of *in vitro* Digested Milled Barley Grains: Influence of Particle Size Heterogeneity

Ghaid J.Al-Rabadi*

Department of Animal Production, Faculty of Agriculture, Mutah University, Al-Karak 61710 , Jordan

Received: May 21, 2014 Revised: June 12, 2014 Accepted: June 19, 2014

Abstract

In this study, Scanning Electron Microscopy (SEM) is used to characterize the structure of ground and whole barley grain before and after the exposure to *in vitro* enzymatic digestion at different incubation times (0, 0.5, 1, 2, 6 and 24 h). SEM analysis showed that digestion started to take place in barley grain fragments after 0.5 h of incubation time. SEM indicated that complete starch digestion is dependent on grain fragment size in barley. Starch digestion seems to be completed after 24 hours of digestion in small fragments of barley grains (<0.5 mm) which was not the case for larger fragment size (>1.0mm). In case of whole barley grain, SEM showed that alpha amylase was not capable of penetrating and diffusing through barley grain husk after 24 h of incubation. In conclusion, microscopic examination for *in vitro* digested milled and unprocessed barley fragments differ in particle size, indicating that the extent of starch digestion is dependent on fragment particle size.

Keywords: Scanning Electron Microscopy, Starch granules, *in vitro* digestion , Barley fragments

1. Introduction

Grains usually represent the main energy source in animal's diets where starch represents the main nutrient components (Svihus *et al.,* 2004). From a processing prospective, grains, such as barley, should be ground in order to facilitate further processing steps such as mixing and enhancing nutritive value by increasing digestibility (Al-Rabadi *et al.,* 2009). Hammer mill is widely used in the feed industry in order to mill grains as it is characterized by high production capacity and lower maintenance requirements (Amerah *et al.,* 2007). However, grains milled using hammer mill have been reported to produce wide variation in grain particle size (Audet, 1995). Heterogeneity of particle size within milled grains has been reported to influence nutrient digestibility even when the average particle size was the same (Wondra *et al.,* 1995). Within grain type, different grain fragment size, after being fractionated by sieving process, have been reported to posses different surface area per unit mass and different chemical composition (Al-Rabadi *et al.,* 2013). These factors have been reported to extensively influence the magnitude of starch digestion (Al-Rabadi *et al.,* 2012). Scanning electron microscopy (SEM) have been extensively used to track structural changes that occur into starch granules after being exposed to thermo mechanical treatments and amyloytic digestion (Srikaeo, 2008; Srikaeo *et al.,* 2006). The first objective of this study is to examine the influence of adding enzymes mixture (amylase, glucosidase, pepsin and proteases) in a sequence that mimic the digestion process *in vivo* with taking into consideration the heterogeneity of different size fragments of barley. A previous study reported that starch digestion of milled grains by alpha amylase is controlled by a diffusion process (Al-Rabadi *et al.,* 2009). However, this study aims at confirming the capability of alpha amylase to diffuse through barley grain husk using SEM.

2. Materials and Methods

2.1. Barley Grain Milling

Barely grains were milled using 4 mm hammer mill screen size when constant motor load was recorded. Ground and whole barley grains were collected and were sealed into plastic bags and stored at 4 ˚C until visual examination by using scanning electron microscopy and further being digested using in vitro starch digestibility method.

2.2. In vitro Starch Digestibility

In vitro starch digestion method was used as previously described by Al-Rabadi *et al.* (2009). In vitro digestion method was performed in a three-step enzymatic digestion to mimic digestion in the mouth, in the stomach and the small intestine in a closed system. Different digestion times (0, 0.5, 1, 2, 6 and 24 h) were used to simulate digestion process in monogastric animals and

* Corresponding author. : e-mail: ghaid78@yahoo.com.

young ruminates before weaning. The zero hour digestion was started at the start of the small intestinal simulation step (where most of starch digestion take place).

2.3. Scanning Electron Microscopy (SEM)

Milled barley grains fragments (before and after digestion at different incubation times) were placed onto aluminium stubs with carbon tabs. Fragments then were sputter coated (10-15 nm layer) of platinum using an Platinum Sputter Coater (model EIKO IB-5). Digested and undigested barley fragments were examined in either a JEOL 6300 or JEOL 6400 field emission scanning electron microscope. Micrographs were chosen by taking many pictures (i.e., 5 to 10 pictures) for the selected samples to obtain representative Scanning electron micrographs. The selected sample contains many barley grain fragments on the carbon tabs. Comparable appearance was selected as a representative picture. Many pictures (6-10 pictures) were taken at different magnifications to find any main structural difference at both grain fragment size level and starch granule size level.

3. Results and Discussion

Scanning electron micrographs for milled raw barley samples by using 4 mm hammer mill screen size is shown in Figure 1. Milling process resulted in breaking barley grains into different levels of fragment sizes that ranged from very fine particles to quarter and half broken grains (Figure 1). Previous studies showed that milling grains using hammer mill resulted in high heterogeneity in grain particle size distribution when compared with other milling equipments such as roller mill (Seerley *et al*., 1988; Douglas *et al.,* 1990; Audet, 1995). It can be also seen from Figure 1 that barley grains milled by using hammer mill produce spherical shape fragments. In his report, Kim (2002) reported that the hammer mill produces spherical shape grain fragments while the roller mill produces more rectangle grain fragments after milling.

The effect of alpha amylase on starch granules digestion, at different incubation times (0, 0.5, 1, 2, 6 and 24 h), using three enzymatic step models were examined (Figures 2, 3, 4, 5, 6, 7 and 8). As expected, there was no enzymatic activity on starch granules at 0 hour incubation time (Figure 2) although starch granules were exposed to salivary alpha amylase. It is hardly for any enzymatic digestion to take place on starch granules after 30 minutes of the incubation time (Figure 3). It has been previously shown that the enzymatic digestion by amylase is controlled by diffusion process through channels present on granules surface (Helbert *et al.,* 1996) and this may suggest that starch digestion may take place inside starch granules. Magnitude of diffusion coefficient for amylase has been previously quantified in barley starch granules (1.7 x 10-7 cm2 s-1) (Al-Rabadi *et al*., 2009). Extant of starch digestion for different grain fragment size, ranging from 0.045-2.8 mm, have been reported to range from 23-1%, respectively after a 30-minute incubation time (Al-Rabadi *et al.*, 2012).

Enzymatic digestion by amylase started to take place on starch granules surface after one hour incubation time (Figure 4). However, enzymatic activity by alpha amylase does not seem to be associated with every starch granules. On the other hand, digestion by alpha amylase seems to be associated with every starch granules after 2 h of incubation time (Figure 5). A number of holes on starch granules resulted from enzymatic activity increased as the incubation time progresses (i.e., digestion 6 h) as shown in Figure 6. Integrity of oval shape structure of starch granules starts to disappear after 24 h of incubation time for large fragment size (>1mm) as shown in Figure 7. However, starch granules in smaller fragment size disappeared after a 24-h digestion time, as shown in Figure 8. Complete starch digestion was achieved for barley fragment.

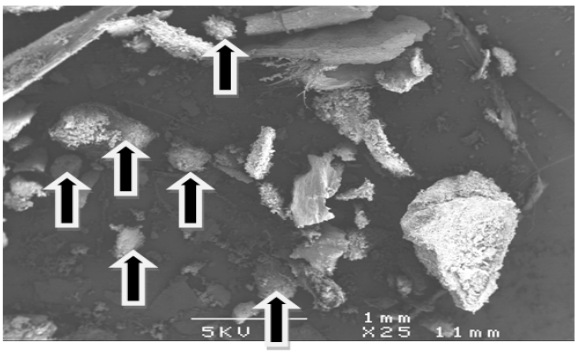

Figure 1. Raw milled barley grain fractions using 4 mm hammer mill screen size (heterogeneity of milled grain particle size range from very fine particles to half broken grains). Round oval shape particles indicated by black arrows.

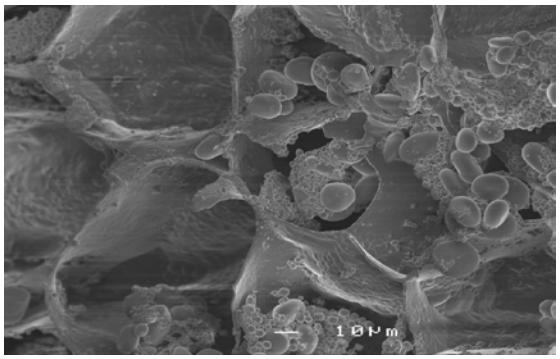

Figure 2. Undigested starch granules embedded in protein matrix.

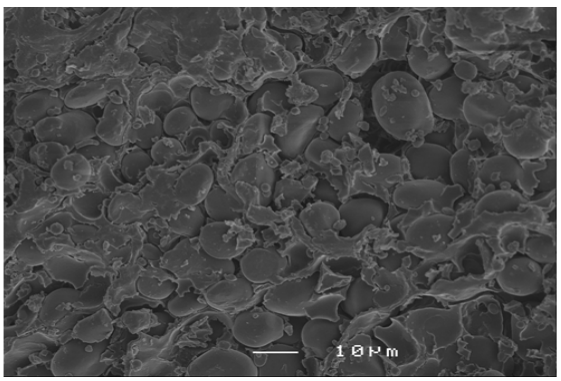

Figure 3. Digested starch granules after 0.5 h incubation time (no appearance for any enzymatic activity on starch granules). Image was taken from small fragment size (<0.5mm)).

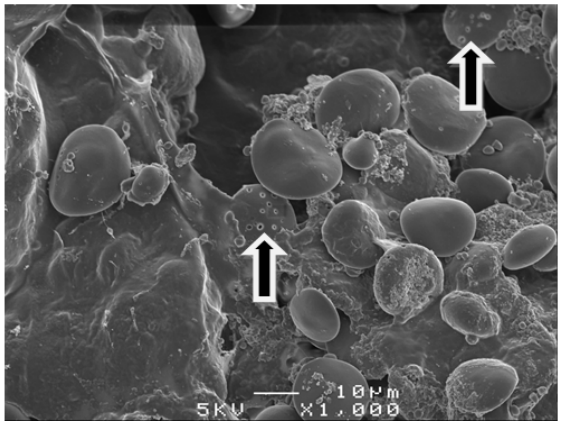

Figure 4. Digested starch granules after 1 h incubation time (initial enzymatic activity (holes) on certain starch granules as indicated by arrow). Image was taken from small fragment size (<0.5mm)).

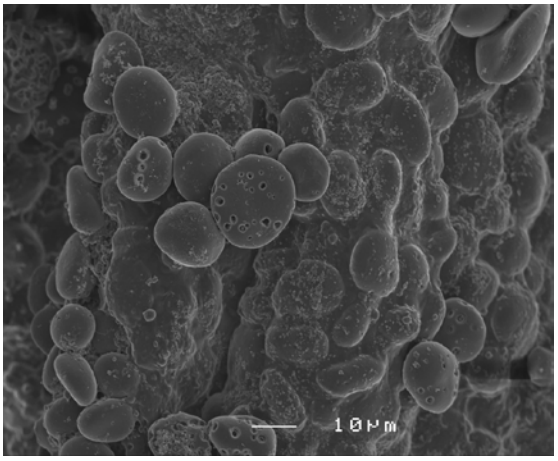

Figure 5. Digested starch granules after 2 h incubation time (initial enzymatic activity (holes) on most starch granules). Image was taken from small fragment size (<0.5mm))

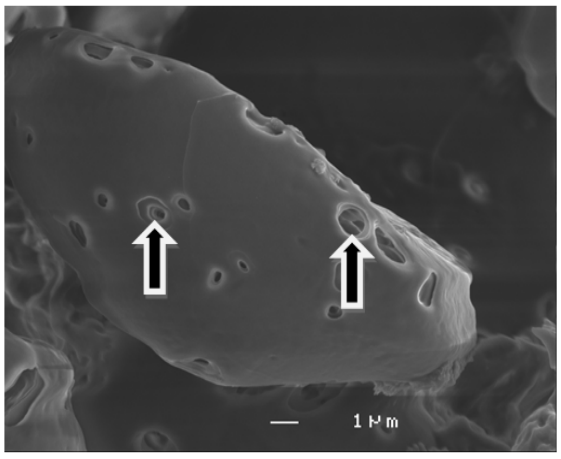

Figure 6. Digested starch granule after 6 h incubation time (increase the number and size of digestion holes compared to starch granules digested at 2 h incubation time as indicated by black arrows). Image was taken from small fragment size (< 0.5mm)).

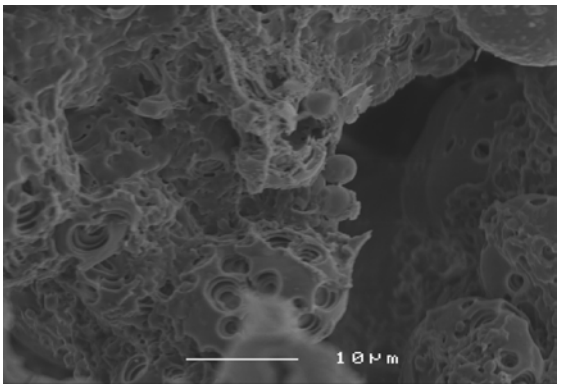

Figure 7. Digested starch granule after 24 h incubation time (increase the number and size of digestion holes). Image was taken from large fragment size (>1.0 mm)).

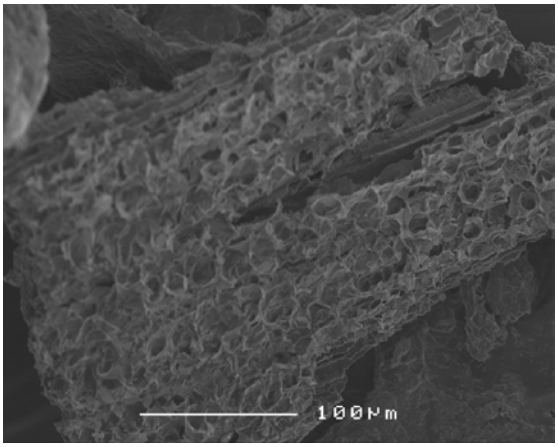

Figure 8. Absence of any starch granules after 24 h incubation time. Image was obtained from particles < 0.5mm.

It was found that the electron micrographic features of the granules after treatment with alpha amylase and glucosidase possesses synergistic influance (Matsubara *et al.,* 2004). The synergetic influences by both enzymes were explained by Sun and Henson (1990) and Robertson *et al.* (2006). The ability of the alpha-glucosidases to breakdown glucosidic bonds other than alpha-1,4- and alpha-1,6- that are present at the granule surface can eliminate bonds which were barriers to digestion by alpha-amylases. In addition, the presence of protease in the current in vitro digestibility method may enhance indirectly the synergy influence of both alpha amylase and glucosidase by increasing the exposure of starch granules to enzymatic digestion. It has been reported that the interaction between protein and starch granules can decrease the exposure of raw starch granules to enzymatic digestion by alpha amylase (Rooney and Pflugfelder, 1986). The interactions between the protein granules (size range 5–60 kDa) and starch may affect starch digestibility; it is important to take into consideration that protein digestion usually precedes starch digestion (Svihus *et al.,* 2005).

The size of the starch granules within grain type can influence the starch digestion process when examined using SEM. Large starches granules displayed massive degradation and were described by sever corrosion toward the radial axis of granule (Franco and Preto, 1992). On the other hand, small starch granules showed a surface

attrition and, later on, followed by solubilization (Franco and Preto, 1992). The difference in the behavior of starch digestion between small and large particles could be attributed to many factors. A previous study showed that large and small starch granules possess different chemical compositions and endothermic properties and thus possess a different enzymatic response to digestion by alpha amylase (Szczodrak and Pomeranz, 1991). Chiotelli and Le Meste (2002) reported that large starch granules have a lower water affinity due to more compact structure (i.e., higher crystallinity) than small starch granules and this could increase their susceptibility to enzymatic hydrolysis by alpha amylase. In addition, small starch granules

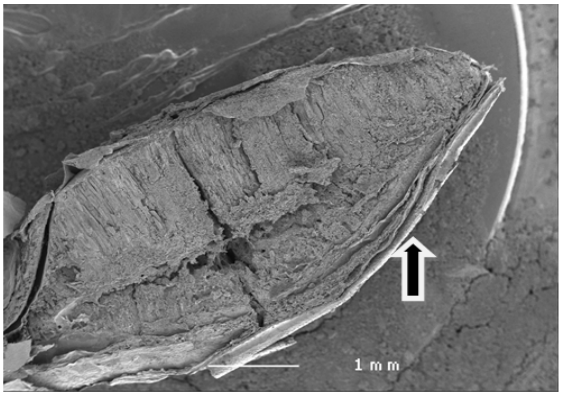

Figure 9. Cross section of whole barley grain after 24 h incubation time (barley grain husk is indicated by arrow).

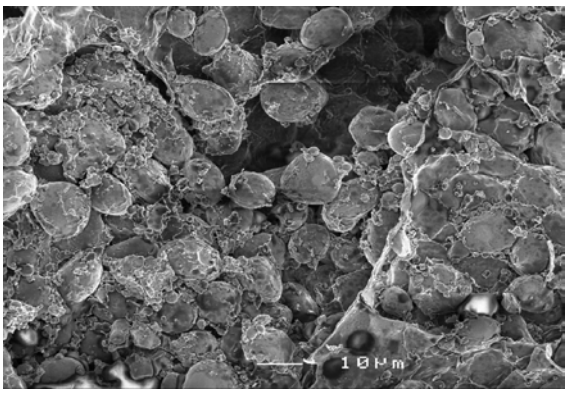

Figure 10. Absence of any enzymatic activity of whole barley grain .

have a higher surface area to weight ratio and this may suggest that alpha amylase binding to starch granules and the potential hydrolysis would be higher compared to large starch granules when all other factors being the same (Tester *et al.*, 2004).

In this study, the presence of large fragment size of barley fragments and the absence of fiber digesting enzymes may inhibit the synergetic influence of both alpha amylase and glycosidase. To confirm the capability of both alpha amylase and glycosidase to diffuse through barley grain husk which is mainly composed of cellulose, hemicelluloses and lignin (Adrados *et al*., 2005). Whole barley grain was incubated into digestion solution for 24 h to investigate whether any enzymatic digestion can take place (Figure 9). As shown in Figure 10, no enzymatic digestion took place on starch granules after cutting the whole barley grains into two halves, which indicates that barley husks work as a strong barrier against enzymatic diffusion of both amylase and glycosidase. Large particles have been shown to survive ruminal attack and pass to small intestine for digestion (Owens *et al.*, 1986).

In conclusion, microscopic examinations for in vitro digested milled barley fragments differ in particle size and this indicates that the extent of starch digestion is dependent on fragment particle size (i.e., heterogeneity of particle size distribution). SEM for whole barley grain revealed that the presence of barley husk prevents any enzymatic diffusion and thus no starch digestion takes place.

References

Adrados BP, Galbe M and Zacchi, G. 2005. Pretreatment of barley husk for bioethanol production. *J Chem Technol Biotechnol.*, **80**:85–91.

Al-Rabadi GJ. 2012. Influence of hammer mill screen size on processing parameters and starch enrichment in milled sorghum. *Cereal Res Comm.*, DOI:10.1556/CRC.2013.0016.

Al-Rabadi GJ. 2013. Effect of hammer mill screen size on processing parameters and starch enrichment in milled barley. *Jordan J Agri Sci*., **9:**162-168.

Al-Rabadi GJ, Gilbert R and Gidley M. 2009. Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine alpha-amylase. *J Cereal Sci.*, **50** (2): 198-204.

Al-Rabadi GJ, Torley P, Williams BA, Bryden WL and GidleyM.J. 2012. Particle size heterogeneity in milled barley and sorghum grains: Effects on physico-chemical properties and starch digestibility. *J Cereal Sci*., **56**: 396-403.

Amerah AM, Ravindran V, Lentle RG and Thomas DG. 2007. Feed particle size: Implications on the digestion and performance of poultry. *World Poultry Sci J*., **63:** 439-455.

Audet L. 1995. Emerging feed mill technology: keeping competitive. *Animal Feed Sci Technol*., **53**: 157-170.

Douglas JH, Sullivan TW, Bond PL, Struwe FJ, Baier JG and Robeson LG. 1990. Influence of grinding, rolling, and pelleting on the nutritional-value of grain sorghums and yellow corn for broilers. *Poultry Sci.*, **69**: 2150-2156.

Franco CML, Preto, SJR and Ciacco CF. 1992. Factor that affect the enzymatic degradation of natural starch granules-effect of the size of granules. *Starch/ Stärke*. **44**: 422-426.

Helbert W, Schu lein M and Henrissat B. 1996. Electron microscopic investigation of the diffusion of *Bacillus licheniformis* α-amylase into corn starch granules. *Inter J Biol Macromolecules*, **19**: 165-169.

Kim K. 2002. Hammer mills or roller mills. Feed Manufacturing (MF-2048). Manhattan, Kansas State University.

Matsubara T, Ammar YT, Anindyawati S, Yamamoto K, Ito Mand MN. 2004. Degradation of raw starch granules by α-amylase purified from culture of *Aspergillus awamori* KT-11. *J Biochem Molecular Biol*., **37**: 422-428

Owens FN, Zinn RA and Kim YK. 1986. Limits to starch digestion in the ruminant small intestine. *J Animal Sci*., **63**: 1634-1648.

Robertson HG, Wong DWS, Lee CC, Wagschal K, Smith MR and Orts WJ. 2006. Native or raw starch digestion: a key step in energy efficient biorefining of grains. *J Agri Food Chem*., **54**:353-365.

Rooney LW and Pflugfelder RL. 1986. Factors affecting starch digestibility with specialemphasis on sorghum and corn. *J Animal Sci.,* **63**: 1607-1623.

Seerley RW, Vandergrift WL and Hale OM. 1988. Effect of particle size of wheat on performance of nursery, growing and finishing pigs. *J Animal Sci.,* **66**: 2484-2489

Srikaeo K. 2008. Microscopy and image analysis techniques for quality control in food industry: A case study of wheat grain cooking process. *J Microscopy Soc Thailand.* **22**: 46-49.

Srikaeo K, Furst JE, Ashton JF and Hosken RW. 2006. Microstructural changes of starch in cooked wheat grains as affected by cooking temperatures and times. *LWT*. 39: 528-533.

Sun Z and Henson A. 1990. Degradation of native starch granules by barley glucosidases. *Plant Physiol*., **94**: 320-327

Svihus B, Uhlen AK and Harstad OM. 2005. Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. *Animal Feed Sci Technol*., **122**: 303-320.

Szczodrak J and Pomeranz, Y. 1991. Starch and enzyme resistance starch from high amylose barley starch. *Cereal Chem.*, **68**: 589-596.

Tester RF, Karkalas J and Qi X. 2004. Starch structure and digestibility enzyme-substrat relationship . *World's Poultry Sci Asso*., **60**: 186-195.

Wondra K, Hancock J, Behnke K and Stark C. 1995. Effects of mill type and particle size uniformity on growth performance, nutrient digestibility, and stomach morphology in finishing pigs. *J Animal Sci*., **73** (9): 2564-2573.