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# RELATIONSHIPS BETWEEN THE TISSUE STRUCTURE AND β-ENDOGLUCANASE LOCALISATION IN GERMINATING TRITICALE SEEDS

Abstract

The mechanism of some enzyms taking part in the degradation of grain polysaccharides during the initial period of triticale germination is discussed. This concerns the function of the high pI α-amylase which is suggested to be the most important isoform active in the starch hydrolysis as well as the 1-3, 1-4-β-glucane-4-glycosidase which seems to be one of the most important enzymes degrading the cell wall structural components - β-glucanes. The use of two triticale varieties differentiated in their susceptibility to pre-harvest sprouting allowed to discuss both the enzyme activities and the internal structure changes evidenced in the light and electron microscope in relation to the various processes taking part in those samples. Most interesting observations seem to be those concerning the aleurone layer where the characteristic channels and perforations in the cell wall were formed specially in case of samples demonstrating the elevated activities of β-endoglucanase. Similar effects od starch digestion were observed in samples where the high pI-α-amylase isoform was present. Microscopic observations on the loosening of the cell wall structure seem to be particularly interesting as this could be the mechanism facilitating the enzyme and metabolite translocation through the grain tissues, thus accelerating the metabolic processes.

There are many factors which can more or less directly influence the seed germination. Thus, they are connected with the pre-harvest sprouting, quite common in cereals, particularly in rye and triticale. These factors are either of metabolic character, bound with the synthesis or activation of hydrolytic enzymes or of structural character connected with the tissue permeability to the enzymes and metabolites. The most important enzymes participating in the degradation of the storage components in order to produce low molecular weight metabolites needed for the embryo development are: the α- and β-amylases [EC 3.2.1.1 and 3.2.1.2] degrading starch, endo- and exopepti-

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dases, hydrolyzing the storage proteins to form the amino acids as well as the acid phosphatases which mobilize the $P_i$ form from their organic complexes. However, recently attention was also paid to the other hydrolases, the activity of which might influence the changes in the internal tissue structure of seeds by the degradation of some insoluble cell components. The enzymes of this type may include the β-1→3; 1→4-endoglucane-4-glycosidase [β-endoglucanase - EC 3.2.8.73] together with the related ones of other specificity to decompose the glycosidic bonds as well as the phytate hydrolase [phytase - EC 3.1.3] [26].

Starch is the basic energy substrate, which degradation is needed for the developing embryo as the ATP source. Its hydrolysis is carried out in the plant cell by the α– and β-amylases (together with some related enzymes, e.g. α-glucosidase). α-Amylase, the enzyme playing main part in the starch degradation, is synthetized to some extent already during the cereal grain maturation (Beck, Ziegler 1989; Sitarski et al. 1992; Andrzejczuk-Hybel et al. 1994). This synthesis is taking place in the scutallum and the enzyme is quickly transferred to the aleurone layer. However, the predominant increase of the β-amylase activity takes place at the very initial germination stages, mostly as the result of the transformation of the bound to the active form and, to a lesser extent, due to the enzyme protein synthesis (Sitarski et al. 1992). The main α-amylase isoform taking part in the starch hydrolysis during the germination process is supposed to be that of the high isoelectric point (hpI-α-amylase). The synthesis of that isoform also begins in the last period of grain maturation, parallel to some other isoforms of low pI (Maresh, Gale 1990; Prabucka et al. 1995). As it has already been demonstrated by the above mentioned authors, some balance of both α-amylase isoform exists in the mature grain, which, however, depends on the variety resistance to the pre-harvest sprouting; the susceptible Malno variety demonstrates the predominance of the hpI-α-amylase activity as compared to the resistant Lasko variety. It results from the cited investigations (Table 1 – Prabucka et al. 1995) showing that the hpI-α-amylase reveals the higher affinity to the native potato starch as compared to the soluble one, a fact that was additionally confirmed on the commercial barley high pI-α-amylase Sigma type VIIIA. Using this preparation it can be demonstrated that this isoform presents a 2.6 times higher affinity to the native potato starch as compared to the soluble one. This behaviour might be connected with the partial unfolding of the helical structure of the native starch (as a result of breaking numerous hydrogen bonds in the course of its preparation). The observed affinity difference of hpI-α-amylase to both starch forms and the various values of this affinity determined after the successive germination times prove indirectly that the level of the hpI isoform varies depending on the variety, milling fraction and germination time. Therefore, the appli-

cation of the definite starch sample as the substitute should be of high importance in the determination of the total α-amylase activity (or the total amylolytic one). In selecting the substrate form the native starch should be anticipated as the one producing higher results. This may be of a special significance when activities of the amylase isolated from the germinating or spouting grains are determined.

Table 1

Activities of α-amylase determined on the native – n and soluble – s starch as well as β-endoglucanase activitities in selected milling fractions of Triticale grains of various pre-harvest sprouting resistance: Lasko (resistant) and Malno (susceptible)[a]

| Germination time h | α-amylase activity mg starch · $min^{-1}$ · g dry $matter^{-1}$ | | | | | | β-endoglucanase u · g dry $matter^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lasko | | | Malno | | | | | |
| | native | soluble | n/s | native | soluble | n/s | Lasko | Malno | M |
| | Milling bran | | | | | | | | |
| 0 | 52.8 | 75.1 | 0.70 | 100.0 | 77.7 | 1.25 | 3.7 | 5.7 | 1.5 |
| 24 | 81.2 | 88.8 | 0.91 | 135.1 | 79.2 | 1.70 | 13.9 | 19.4 | 1.4 |
| 48 | 242.1 | 168.2 | 1.44 | 348.1 | 183.4 | 1.90 | 25.4 | 35.8 | 1.4 |
| 72 | 542.6 | 261.0 | 2.02 | 707.5 | 413.9 | 1.71 | 32.2 | 36.5 | 1.1 |
| | Outer endosperm | | | | | | | | |
| 0 | 34.3 | 92.7 | 0.37 | 49.4 | 76.8 | 0.64 | 2.7 | 2.8 | 1.0 |
| 24 | 31.9 | 81.4 | 0.39 | 51.0 | 77.4 | 0.66 | 3.2 | 3.6 | 1.1 |
| 48 | 144.4 | 133.0 | 1.08 | 220.9 | 127.4 | 1.73 | 9.9 | 12.1 | 1.2 |
| 72 | 332.5 | 256.2 | 1.30 | 476.4 | 363.9 | 1.31 | 15.7 | 15.0 | 0.9 |

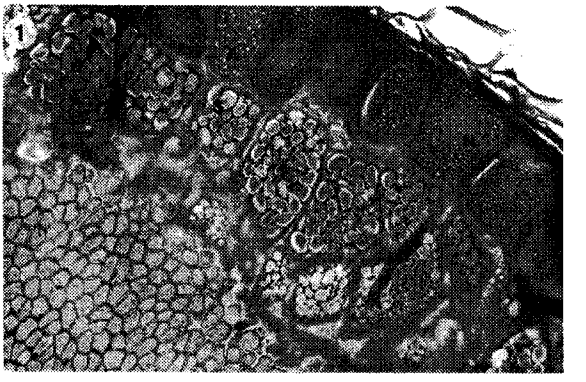
[a] In case of α-amylase the n/s ratio is the measure of the hpI-α-isoform as compared to the total, the pure hpI-α-amylase from barley (Sigma) has the n/s ratio = 2.60.

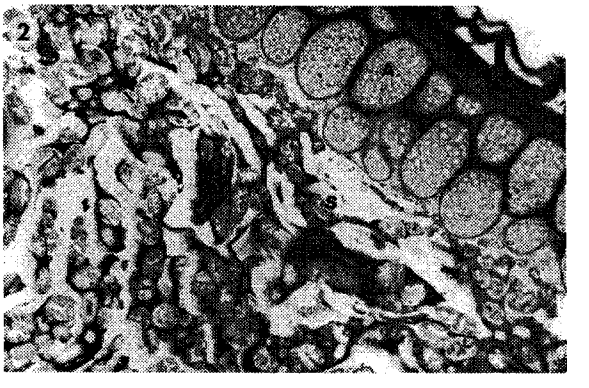
Our recent data obtained in the experiments on the triticale pre-harvest sprouting have confirmed that some amounts of the hpI-α-amylase are really formed during the final maturation period (Sitarski et al. 1992; Andrzejczuk-Hybel et al. 1994). However, the highest increases were observed during the initial 24 h (48 h in Lasko) of germination depending on the varietal susceptibility to the pre-harvest sprouting. Comparing the two investigated varieties it was demonstrated that the metabolic activity was at least one day slower in grains of the resistant variety Lasko as compared to the susceptible Malno. It seems, therefore, that the synthesis velocity of the isoform hpI-α-amylase may be the important factor affecting the pre-harvest sprouting resistance trait (Prabucka et al. 1995). At present, some more detailed investigations on the α-amylase isoform composition (together with some related enzymes) during grain germination were carried out in our laboratory.

Parallel to the hpI-α-amylase synthesis the changes of 1→3, 1→4-β-glucane-4-glycosidase (β-endoglucanase - EC 3.2.8.73) were investigated in the developing and germinating triticale grains. They are enzymes degrading β-glucanes which are the main components of the cell wall. The opinion exists that this enzyme (together with many related ones of other specificities) takes part in the preliminary digestion of the cell wall glucanes and pentosans (Stuart et al. 1986; Cordes, Henry 1989; Fincher 1989; Fry 1995). Their actions can, therefore, facilitate the transport of other digestive enzymes and their contact with substrates. Their action against the cell aleurone layer may be of particular significance facilitating the communication between the scutellum and endosperm. Admittedly the preliminary analysis did not exhibit much difference between the β-endoglucanase activities in the whole grains of various pre-harvest sprouting resistance during the first two days of germination (Niziołek et al. 1994). However, when these activities had been examined in particular milling fractions, some high differences were observed, particularly between those fractions which contained scutellum and aleurone cells; the β-endoglucanase activity in the susceptible variety Malno after 18 h germination was about 50 % higher than in the resistant Lasko (14.8 u and 10.4 u respectively per g dry matter but also at the zero time the difference amounted to 5.7 and 3.7 u, respectively). Hence the activity increase during the initial 18 h amounts to 9.7 u in Malno and 6.7 u in Lasko, i.e. it is about 1.5 times higher.

These results allowed to expect some consequent structural changes between the cross-sections of cell fragments of both varieties and imbibition times (0 and 18 h) which might be registered as different microscope images. The investigations were carried out in the light microscope UN 2, Zeiss, Jena and electron microscope JEM 100C (Jeol) and on respective enlargements of 150-450x and 5,000-50,000x. The material for the investigation included kernels germinated in the conditions described earlier (Bartoszewicz et al. 1993); the grain humidity for Malno amounted to 7 and 44 % and for Lasko 7 and 33 %, respectively at the zero and 18 h.

There were observed at least two changes differentiating the investigated samples in relation to the susceptible Malno variety after 18 h of germination which could not be observed either in both samples at zero h or in the resistant Lasko variety after 18 h of germination. At least 50 different elements of each sample were analysed in order to confirm the preliminary observations. The typical border cells between the aleurone layer starchy endosperm in the Malno variety at zero time and after 18 h germination are presented in Figs. 1 and 2, respectively. The basic differences can already be observed in the light microscope after 18 h germination as compared to the ungerminated sample. They include the reduced thickness of the cell wall, the partial digestion of the

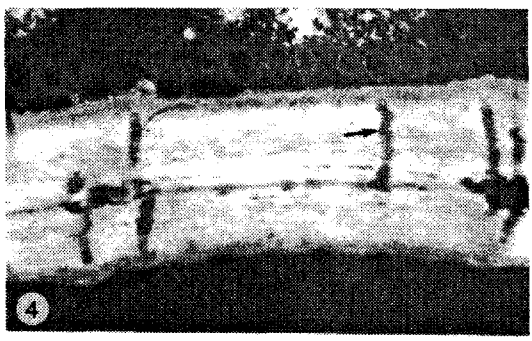



Figs. 1, 2. The cross section of the aleurone cells of variety Malno t = 0(1) and t = 18 h(2); light microsciope, enlargement 150 x.

aleurone granules as well as the partial digestion and desorganization of the starch globules.

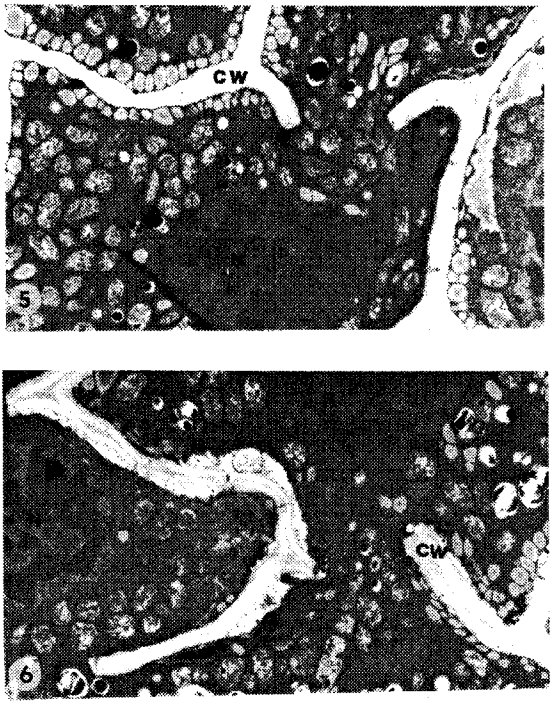
The phenomenon clearly visible in the electron microscope concerns the formation of the numerous channels penetrating into the walls of the aleurone cells at various angles (Figs. 3 and 4) which is assumed to be generated by the partial digestion of the β-glucane wall components. No such channels can be found either in both Lasko samples (0 h and 18 h) or at 0 h in the Malno variety. In the latter case only the plasmodesmata of similar shape could be observed. The even more interesting second phenomenon seems to be the appearance of a distinct discontinuity of the cell walls resulting from the complete breaking of the β-glucane components which is demonstrated in Figs. 5 and 6. Such an impairement allows the direct contact between the neighbouring cells and the mechanical exchange of the cell contents such as cytomyxia. Some direct translocations of starch granules through the recently formed slits in the cell wall were also clearly visible (Figs. 5 and 6). It could be presumed that the


Figs. 3, 4. Two objects of the cell wall sections, variety Malno 18 h showing the channels penatrating them. Electrone microscope, enlargement 50000 x.

demonstrated structural changes (the perforations of the cell wall) resulted from the action of β–endoglucanase which is 50 % more active in those samples. For comparison, Figs. 7 and 8 demonstrate that in the Lasko variety neither the channels nor the perforations of the cell wall can be observed.

Finally the comparison of Figs. 9 and 10 presenting the border region between the scutellum and endosperm in both Lasko and Malno varieties after 18 h germination shows the significant differences in the degree of digestion of the external starch granules. The enzyme layer is strictly adhering to the starch granules which in the Lasko variety are not much changed in their shape whereas in the susceptible Malno variety they are almost fully digested forming the almost amorphous matter. Similar comparison is presented in Figs. 1 and 2 showing the starch granules in the Malno variety at 0 and 18 h of germination but in this case the granules are digested to the lesser extent as compared to Figs. 9 and 10. The main site of the α–amylase and β-endoglucanase synthesis as well as peptidases particularly on the initial day of germination is located in the scutellum cells. During 2nd and 3rd day of germination the site of the second highest activity of the mentioned enzymes was situated in almost all

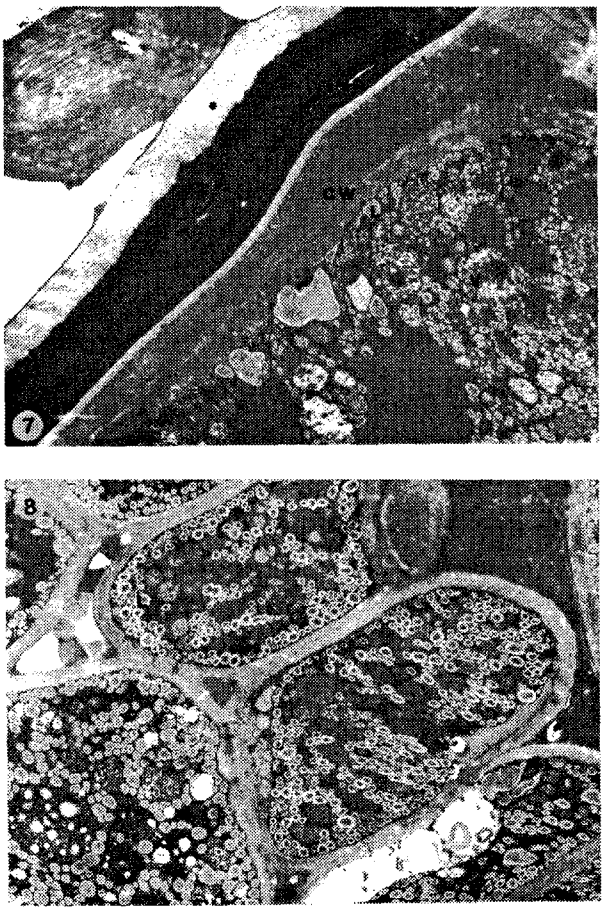



Figs. 5, 6. Two objects of the cell wall sections, variety Malno 18 h with the holes. Electron microscope enlargement 5000 x; the flow of starch granules trought the recently formed holes.

cases in the milling bran fraction. This fraction contains the majority of all the grain aleurone cells (Niziołek et al. 1994; Prabucka et al. 1995).

The presented results in relation to both the enzymatic character and microscope observations reveal that the accelerated production of the hydrolytic enzymes which are active in the degradation of the energy substrate (starch) as well as the storage proteins takes place at the very beginning of germination which is demonstrated in Figs. 1 and 2. This fact is well known from literature (Beck, Ziegler 1989; Kermode 1990; Shutov, Waintcaub 1987). Recently also the action of other hydrolases was reported which take part in the decomposition of the structural components of the cell wall, e.g. the β-endoglucanase (Cordes, Henry 1983; Fincher 1989; Prabucka et al. 1995). There was also suggested the ability of these enzymes to loosen the cell structure and thus to facilitate the internal transport of enzymes from the site of their synthesis (scutellum) to the regions of their substrate localisation as well as the metabolite translocations between the tissues of the germinating grains.

The investigations of the triticale resistance to the pre-harvest sprouting has been carried out for five years revealing, among others, that the initiation time of at least

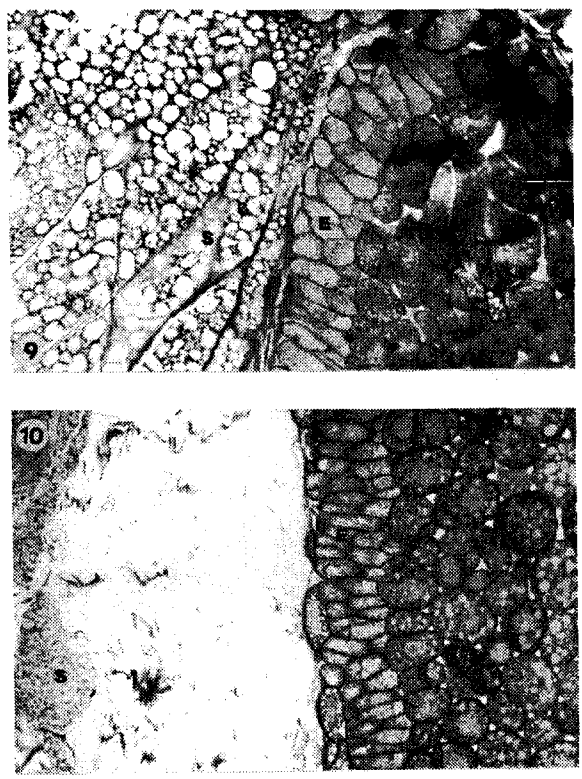



Figs. 7, 8. For comparison the two objects of Lasko variety t = 0 are presented. Electrone microscope; no channels holes penetrating the cell walls can be observed.

some processes directly connected with germination is simply related to the grain morphology changes and the resistance of the variety. The further experiments were carried out on two varieties well differentiated in relation to that feature – the susceptible Malno and resistant Lasko, which were developed in the direction of the activity and action mechanisms of some glycosidases. The comparison of the activity determination of the high pI α-amylase on two types of starch substrates – native and soluble potato starch allows the conclusion reached in the indirect way that the synthesis of this isoform (most important to starch degradation) in the susceptible variety overtakes that of the resistant variety by at least one day. Therefore, the isoform hpI in the initial germination period (up to 48 h) predominates in the aleurone layer but later it is translocated to the endosperm. This transport is also faster in the susceptible variety than

in the resistant one. The increase faster by about 50 % of the β-endoglucanase activity in the aleurone layer was also demonstrated during the first 24 h of germination of the susceptible variety as compared with Lasko. However, also in that case the enzyme translocation to the endosperm cells was delayed though not as much as in case of α-amylase. That last observation could suggest that the function of β-endoglucanase in the endosperm cells should be rather of limited significance if any at all.

The above considerations could be confirmed to some extent by the microscopic investigation in which the unsprouted (t = 0) and germinating samples (18 h) were compared as well as those of both susceptible and resistant varieties. From the technical point of view the preparation of kernels more humid than 40 % was too difficult. The significant structural differences were demonstrated between samples of t = 0 and 18 h of each variety as well as between parallel times and inter-sections of Malno and Lasko. It seemed to be most interesting to investigate the suspected effects of the β-endoglucanase action on the aleurone cell walls, expressed by the formation of charac-




Figs. 9, 10. Two objects of bordering cells between the scutellum and endosperm of Lasko (9) and Malno (10) varieties t = 18h; the differences in the digestion degrade are observed. Light microscope enlargement 150 x.

teristic channels visible on the wall sections as well as the decline of the cell fragments (perforations) providing the exchange of the contents of the neighbouring cells such as the cytomyxia. The findings of such changed structures among the elements of the susceptible variety Malno after 18 h of germination and the total lack of such structures at 0 time in Malno and at both times (0 and 18 h) in Lasko allow the suggestion that those channels or holes can be treated as the effect of the higher activity of the enzymes degrading the cell wall components (β-glucane) during the initial period of germination of the variety susceptible to the pre-harvest sprouting. The facilitated translocation of enzymes and metabolites in the grains of the susceptible variety is confirmed not only by the changes in their activities determined chemically in the milling fractions but also by comparing the structures shown in Figs. 1 and 2 or 9 and 10, where significant starch digestion in Malno cells after 18 h of germination is compared to that in Lasko at 0 time and 18 h of germination. The presence of channels formed in the cell wall as the effect of the β-endoglucanase action had already been suggested (Stuart, Fincher 1987) but the microscopic documentation of this phenomenon concerning the cereal seeds is lacking so far. Thus the comparison of structures presented in our research can serve as the possible confirmation of that suggestion also in relation to other seeds. On the other hand, the information of the existence of holes or wanes in the cell wall formed as the result of the hydrolytic degradation of polysaccharides can already be found in the literature, however it was suggested to be rather the effect of the cellulose hydrolysis (Fry 1995). Nevertheless, the observations of such structural modification facilitating the exchange of the cell contents of the cytomyxia type seems to be very interesting. However, in our opinion the existence of many glycosidases situated inside the plant cell wall makes the lysis of its components much more complicated but probably β-endoglucanase is playing a rather important part in that process. All these and similar phenomena have not been so far connected with the grain susceptibility to the pre-harvest sprouting. The investigations of these relations should be further continued as the possible diagnostic tool or in some other directions.

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## ZALEŻNOŚĆ MIĘDZY STRUKTURĄ KOMÓRKI I UMIEJSCOWIENIEM β-ENDOGLUKANAZY W KIEŁKUJĄCYCH ZIARNACH TRITICALE

Streszczenie

Omówiono mechanizm działania pewnych enzymów uczestniczących w degradacji polisacharydów ziaren w czasie początkowego kiełkowania triticale. Dotyczy to roli wysokiego pI α-amylazy co sugeruje, że jest ona w hydrolizie skrobi najważniejszą aktywną izomorfą oraz 1-3,1-4-β-glukano-4-glikozydazy, która zapewne jest najważniejszym enzymem degradującym β-glukany będące składnikami strukturalnymi ścian komórek. Badanie dwu odmian triticale różniących się podatnością na wzrost do momentu zbioru pozwoliło poznać rolę aktywności obu enzymów. Badania mikroskopowe, także z użyciem mikroskopu elektronowego, pokazały wewnętrzne zmiany strukturalne zachodzące pod wpływem poszczególnych procesów. Najbardziej interesujące obserwacje dotyczą warstwy aleuronowej, w której powstały charakterystyczne kanały i perforacje, szczególnie widoczne w próbkach o podwyższonej aktywności β-endoglukanazy. Podobne efekty trawienia skrobi obserwowano w próbkach z wysoką izomorfą pI α-amylazy. Obserwacje mikroskopowe zaniku struktury ścian komórkowych pozwalają przypuszczać, że ten zanik jest częścią mechanizmu ułatwiającego przemieszczanie się enzymu i metabolitów przez komórki ziaren, co przyspiesza procesy metabolityczne.