POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

Pol. J. Food Nutr. Sci. 1998, Vol. 7/48, No 3

EFFECT OF ENZYMATIC TREATMENT ON THE CELL WALL MICROSTRUCTURE OF *G. AVELLANA* SEEDS: OPTICAL MICROSCOPY AND GRANULOMETRIC STUDIES

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INTRODUCTION

The recovery (extraction) and use of vegetable oils have played an important role in the manufacture of a large number of industrial products and human food items [Christensen, 1991]. Pressing and/or solvent extraction (conventional processes) are the most widely used techniques, owing to their high efficiency in oil recovery [Domínguez *et al.*, 1996].

The quest for new and improved methods of oil extraction, based on clean and economically feasible technologies, using non-toxic, renewable solvents, has lead researches to the utilisation of enzymes. Several authors have reported an increment in oil recovery with enzyme treated seeds, when compared to traditional and aqueous processes [Bocevska *et al.*, 1993; Che Amin *et al.*, 1996; Domínguez *et al.*, 1995; Tano-Debrah & Otha, 1994, 1995; Tano-Debrah *et al.*, 1996].

However, enzymatic preparations must have a very broad spectrum of activity, as the plant cell walls, that must be hydrolysed to extract the oil, have a complex structure [Christensen, 1991].

In this work, preliminary data is shown on the characterisation by optical microscopy of the cell wall of *G. avellana* seeds, and on the fragmentation effects caused by an enzymatic cocktail of enzymes, as studied by granulometry.

MATERIAL AND METHODS

Seeds and Enzymes: in this work *G. avellana* seeds were used. These seeds were dehulled, ground in a coffee grinder and stored at 4°C, wrapped in a plastic bag, until use.

A mixture of a cellulase (Celluclast 1.5 L), pectinase (Pectinex Ultra SP-L), protease (Neutrase) and a high xilanase activity enzyme (Ecostone L), from Novo Nordisk, were used.

Enzymatic treatment: two samples were prepared, each containing 0.5 g of ground seeds in 2 mL of distilled water. To one of the suspensions ("Enzyme"), a mixture of the above-mentioned enzymes (0.25 mL of each) was added. Both suspensions (Enzyme and Control) were incubated in an end-to-end shaker, at 50°C, for 24 h. Afterwards, they were heated in order to denaturate the added enzymes.

Granulometric characterisation: it was effected in a Galai laser granulometer. The above suspensions were centrifuged and re-suspended in 20 mL of distilled water. A third suspension ("Reference"), not submitted to any of these treatments, was prepared directly in 20 mL of distilled water.

150 μL of each suspension, were diluted in 2 mL of distilled water and placed in a cuvette. The particle size distribution was analysed in two distinct ranges: 0.5–150 μm and 2–600 μm. For each sample 10 independent and unique assays were performed.

Optical microscopy: according to the standard preparation procedures, all samples (Reference, Control and Enzyme) were fixed by the *FAA* (Formalin-Acetic acid-Alcohol) method, dehydrated though a graded series of ethanol and incubated in *tert*-butanol/paraffin (1:1). Then, they were incubated in paraffin and sectioned (5 μm in thickness).

Staining. The prepared sections were deparaffinized and re-hydrated in decreasing concentrations of ethanol. They were examined by the *PAS-Amido Black 10B* method (periodic acid-Schiff base – Amido Black 10 B) to localise complex polysaccharide (Schiff reagent) and proteins (Amido Black 10 B).

RESULTS AND DISCUSSION

The results obtained from the granulometric analysis are shown in Table 1, in terms of median values (and standard deviations). Figure 1 shows an example of the probable volume distribution of *G. avellana*, within the range of 0.5–150 μm.

Photo 1 shows the *G. avellana* cells analysed by optical microscopy. The pink areas, as stained by Schiff's reagent, correspond to the cell wall (complex polissacarides). The dark regions, stained by Amido Black 10 B, correspond to proteins.

The size of *G. avellana* particles was strongly reduced by the action of

TABLE 1. Median particle size of *G. avellana* seeds for Reference, Control and Enzyme samples.

Median:	0. 5±150 μm	2±600 μm
Reference	111, 40±3, 64	226, 49±10, 99
Control	109, 08±4, 22	240, 14±15, 23
Enzyme	49, 50±1, 30	70, 38±9, 71

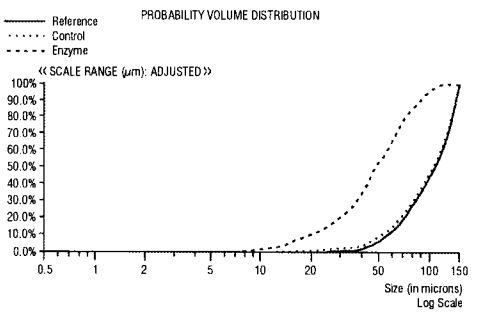
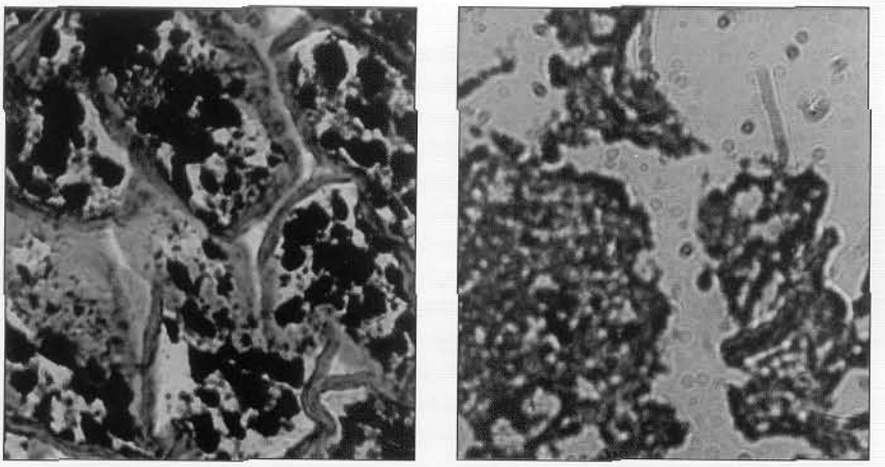
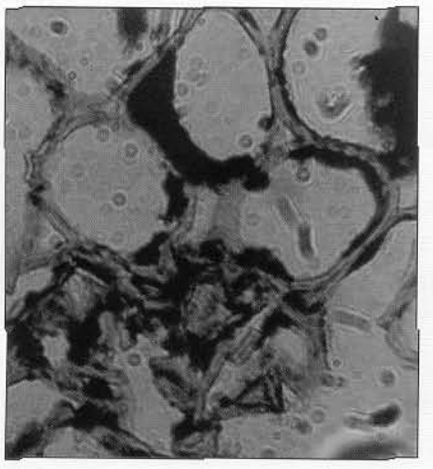


FIGURE 1. Histograms of *G. avellana* particle size distribution, obtained by granulometric analysis.



REFERENCE ENZYME



CONTROL

PHOTO 1. Light micrographs of Reference, Control and Enzyme samples from *G. avellana* seeds (400X), stained by the PAS Amido Black 10 B method.

the enzymes. These results suggest that, along with the hydrolytic action on the cell wall, enzymes may also act on the *middle lamella* therefore releasing entire groups of cells (and eventually individual cells). Alternatively, the enzymes may act from the surface of the particles, into the inner regions, gradually attacking the cells walls and releasing cell material. (This hypothesis will be confirmed later on, by performing enzymatic treatments at different scheduled periods).

The reduction in particles size, by the hydrolytic action of the enzymes, may be of great importance regarding the efficiency of oil extraction, since smaller particles size facilitate the oil release and enhance enzyme diffusion rates which can then more easily act on the substrates. On the other hand this effect may raise

problems in terms of filtration since, in industrial operations, the particles size is optimised to maximise oil extraction and not for enzyme attack. The granulometry of the samples must therefore be subject of careful evaluation, taking the two mentioned aspects in perspective.

It can be seen (Reference) that the intensity of the pink colour characteristic of the cell wall, and moderate staining of the cytoplasm, fades out following the enzymatic treatment (Enzyme). Virtually, all cells have lost their definite shapes or integrity. The black stained particles previously found inside the cells, now seem to be aggregated in large particles, which probably includes also small pieces of the degraded cell wall. As to Control, the integrity of the cell walls is in general maintained, showing some degeneration (rupture) in certain areas.

These observations confirm the fact that the enzyme treatment actually causes significant degradation of the ground seeds.

Further work will focus on the characterisation of the soluble sugars, the staining of the seeds with Sudan IV(a specific stain for lipids), and the evaluation of the enzymes on the efficiency of the oil extraction.

ACKNOWLEDGEMENT

This work is funded by an INCO-DC: 96-2205 (OLNOCO).

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