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EXTRUSION-COOKING OF PEA FLOUR: STRUCTURAL AND IMMUNOCYTOCHEMICAL ASPECTS

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Abstract

Pea flour was submitted to extrusion-cooking under various conditions. The progressive structural transformation was investigated by light microscopy and immuno-gold transmission electron microscopy. Each of the three major compounds, i.e., starch granules, protein bodies, and cell wall fragments, develop a specific, independent structure. Protein bodies aggregate and fuse giving a protein matrix. Starch granules swell, deform, come into contact with each other, and ultimately also fuse together. The resulting gel expands giving a honeycombed structure. Consequently, the protein matrix is arranged into dense strata, i.e,. protein fibers in cross or longitudinal sections, disrupted by the expanded starch gel. Cell wall fragments are clustered together and seem to be intact. This structural segregation is shown to be related to the fact that protein bodies fuse before starch granules. The use of pressure and heating models in conjunction with scanning electron microscopy confirm this observation. On the other hand, immuno-gold labelling has shown that the legumin fraction was localized in the protein bodies as well as in the protein fibers. This indicates that, during extrusion-cooking, some of the antigenic determinants of this protein were not affected.

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Key words: Pea, extrusion-cooking, protein bodies, starch granules, structural changes, immuno-gold labelling, protein fibers.

Introduction

The extrusion-cooking process can be considered as a combination of a number of unit operations (i.e., conveying, mixing, shearing, cooking, pressurizing, texturization, shaping...) in a relatively simple machine, the extruder (Stanley, 1986). Simultaneous or subsequent effects of these unit operations on the initial ingredients (generally starch- and/or protein-based materials) result in products with a specific texture (Kinsella, 1978; Stanley, 1986). For many years, this technology had been applied to the production of shaped pasta products (extrusion without cooking) and ready-to-eat breakfast cereals (Ledward and Mitchell, 1988). More recently, its application was extended to the production of a large number of human foods (snacks, flat bread, biscuits, meat extenders, etc...), and animal feeds using a wide variety of raw materials (Cheftel, 1986; Harper, 1989).

This extrusion-cooking process had been studied extensively over the last fifteen years. Microstructural data of extruded products had been published by many researchers (Cumming *et al.*, 1972; Aguilera *et al.*, 1976; Taranto *et al.*, 1978; Mercier *et al.*, 1979; Rhee *et al.*, 1981; Kazemzadeh *et al.*, 1982; Colonna *et al.*, 1983; Fletcher *et al.*, 1984; Brenner *et al.*, 1986; Gwiazda *et al.*, 1987; Stanley, 1989; Noguchi, 1989). In most cases, the raw material used was a soybean- or a maize-based flour and the method employed for microstructural studies was scanning electron microscopy (SEM). To follow the progressive structural changes in the raw material during extrusion-cooking, some researchers practiced dead-stop experiments, a procedure which consists of stopping the extruder at the steady state, opening the barrel and taking samples from several points along the screw. The samples are then microscopically and/or physico-chemically characterized. Using soybean grits, Aguilera *et al.* (1976) reported that, at the last turns of the screw, cells were completely disrupted and formed strands that oriented into thin fibrils at the die. Kazemzadeh *et al.* (1982) noted that the most significant changes in soybean flour were the fusion of proteins into a continuous matrix and the

formation of texture in the last third of the extruder. Colonna *et al.* (1983) reported that granular and crystalline structures of maize starch disappeared, intrinsic viscosity decreased and macromolecular breakdown occurred as the material progressed along the screws. Gwiazda *et al.* (1987) used soy meal/potato starch mixture to demonstrate texture formation along the screw of the extruder. Fletcher *et al.* (1984) used SEM for recording deformation of starch granules during extrusion-cooking of maize grits. They reported that the formation of a textured structure occurred at the die. Thus, dead-stop experiments demonstrated that the most significant changes in the raw material occurred in the last third of the extruder where the effects of shear, heat and pressure are combined. Also, some model systems had been developed to study the effect of shear, heat and pressure on the physico-chemical and structural properties of starchy and proteinaceous materials. The pressure applied was lower than 4 kN/cm^2 and did not have any significant effect on the microstructure and biochemical properties of the material used (Aguilera *et al.*, 1976; Fujio *et al.*, 1988; Noguchi, 1989). However, the combination of pressure and heat induced protein reactions (Rhee *et al.*, 1981; Fujio *et al.*, 1988; Noguchi, 1989) and the development of textural properties (Taranto *et al.*, 1978; Rhee *et al.*, 1981). A combination of shear and heat led to products which were similar to those obtained from extrusion-cooking (Vergnes *et al.*, 1987).

In our experiments, we used a raw material that included starch granules, protein bodies and cell wall fragments (i.e., pea flour). Such material may give further information about the passage of cell organelles from an individualized structure to a texturized matrix.

Furthermore, our approach to the problem of progressive raw material transformation was based on the structural heterogeneity of the extrudates produced under moderate conditions. Since the light microscopy observations of a large number of fields in the same extrudate revealed a great structural heterogeneity, we adopted a procedure consisting of establishing a relationship between this structural heterogeneity and the thermomechanical history of the product. We subsequently studied the effects of pressure stress and thermal treatment on the cell organelles by submitting the pea flour to axial compression (pressure applied was higher than 4 kN/cm^2) and heat treatment respectively.

In addition, it is usually assumed that the formation of new inter- and/or intra-molecular bonds is the key process of formation of the protein fibers during extrusion-cooking texturization of plant proteins (Kinsella, 1978; Rhee *et al.*, 1981; Sheard *et al.*, 1984; Hager 1984; Stanley, 1989; Noguchi, 1989). However, this possible mechanism give any indication as to the conformation of proteins within the fibers. Results based on a protein solubilization step are unavoidably incomplete because a part of the protein remains insoluble in water and normal buffers (Noguchi, 1989). A method which may give direct evidence of conformational modifications of protein is based on the specific, "in situ" recognition of antigens by their corresponding antibodies. With this method, both soluble and insoluble proteins are accessible to antibodies. To our knowledge, no work had been done using "immunocytochemical" techniques to study proteins in extruded products. For this purpose, we employed the immuno-gold transmission electron microscopy (TEM) technique for its high resolution and the quantitative comparison it offers.

Material and Methods

Material

Smooth pea seeds (*Pisum sativum* L., Var. Amino) were dehulled and ground. The resulting flour (particle size < 500 μm) contained 12.5% free water and 25% proteins (Nx6.25 on dry basis).

Extrusion-cooking

The pea flour was extruded in a Clextral BC-45 twin screw extruder under the following conditions: screw length was 1 m, reverse pitch was just before the die, screw speed was 210 rpm, operating temperature ranged from 100 to 200 °C, flour moisture ranged from 12.5 to 39.5% and feed rate ranged from 10 to 50 kg/h.

The resulting temperature and pressure were measured by a thermocouple and a pressure transducer, respectively, placed just before the dies. The temperature ranged from 120 to 190 °C and the pressure ranged from 1 to 12.5 MPa.

Mechanical treatment.

15g of pea flour were moistened by adding 20% water (total moisture: 32.5%) and submitted to pressure stress, i.e., axial compression, using a laboratory-built hydraulic press (Perrier press) as described by Le Deschault de Monredon (1990). The axial pressure applied was 4.4, 8.8 or 12.4 kN/cm^2, respectively. The cylindrical samples obtained were 4.9 cm^2 in cross-section and 2.7 cm in length. Blocks (1 cm^3) were taken from these samples and prepared for SEM examination.

Thermal treatment

50g of pea flour were moistened by adding 20% water (total moisture: 32.5%) and packed in an aluminium cylindrical cell (inside diameter = 10 cm, inside height = 1 cm, metal thickness = 0.5 cm) equipped with a thermocouple. The cell was closed tightly and placed on a hot-plate. To produce a decreasing vertical heat transfer through the 1 cm thick flour layer, only

one side of the cell was exposed to the heat source. When the temperature in the middle of the flour layer reached 150 °C (as measured with the thermocouple incorporated in the cell), the cell was removed and rapidly cooled in water at 20 °C. The treated flour, in the form of a girdle-cake, was then removed, cross-fractured (areas exhibited were perpendicular to the two surfaces of the girdle-cake) and prepared for SEM examination.

Light microscopy

Small pieces were taken from several zones of rehydrated, unprocessed or extruded samples, fixed in 2% paraformaldehyde and 1% glutaraldehyde in 23 mM phosphate buffer, pH 7.2, for 1 hour and then embedded in glycol methacrylate (GMA) resin according to Leduc and Bernhard (1967).

Thin sections were prepared using an LKB glass knife mounted on a JEOL JUM-7 ultramicrotome, transferred to slides, dried, and then treated with a fast green-iodine mixture for proteins and starch staining. Observations were performed on a Olympus Vanox light microscope.

Preparation of the antiserum to legumin

Legumin, one of the two major storage proteins of pea seeds, was purified from the pea flour by a two-step chromatography procedure on DEAE-Sepharose (Pharmacia Fine Chemicals) and ACA 34 Ultrogel (IBF Pharmindustrie), followed by preparative ultracentrifugation. The antiserum against this protein fraction was produced in a rabbit by intramuscular injections (1 mg protein/ml each) at 10-day intervals. Ten days after the last injection, the rabbit was bled. The collected antiserum was tested by immunodiffusion techniques and shown to be specific. For further details see Gueguen *et al.* (1984).

Immunogold labelling

Following light microscopy examination, areas which presented interesting structural indicators were selected for TEM observation. Ultrathin sections were prepared using a diamond knife, then transferred to parlodion-carbon coated gold grids, and processed for immunogold labelling.

Ultrathin sections on grids were rehydrated for 15 minutes in PBS (23 mM phosphate, 225 mM NaCl, pH 7.2) containing 0.1% Tween 20 and 0.1% BSA to prevent non-specific binding of antibodies. The grids were then incubated in 1/20 PBS diluted antiserum or pre-immune serum (control grids) for 1 hour at room temperature. They were washed four times (5 minutes each) in PBS-Tween and incubated in 1/20 PBS diluted 10 nm gold-labelled-goat IgG anti-rabbit IgG (Sigma Chemical). After incubation for 30 minutes, the grids were washed in PBS-Tween four times (5 minutes each), rinsed with distilled water to remove excess buffer and post-stained with uranyl acetate (2.7% in 50% ethanol) followed by a saturated lead citrate solution (in 0.1N NaOH). Observations were performed on a JEOL 100S TEM. The density of gold particles on the microphotographs was determined by manually counting the number of gold particles per μm^2 of protein matrix area. A stereo microscope was used to magnify the size of gold particles.

Scanning electron microscopy

Samples from unprocessed moistened pea flour (total moisture: 32.5%), pressure stress treated pea flour and heat treated pea flour were dehydrated by critical point drying (Cohen, 1974) using an acetone/carbon dioxide substitution series in a Polaron apparatus. The specimens were then gold coated in a Jeol Fine coat ion-sputterer JFC-1100 and examined in a Jeol JSM 840A SEM.

Results and Discussion

Mature pea seed storage tissues consist of polyhedric cells which essentially contain large, elliptic starch granules and small, nearly spherical protein bodies (Figs. 1a and 2a). In the pea flour used, large particles (entire cells) can be observed (Fig. 3a).

When GMA-sections of storage cells were reacted with the anti-legumin serum followed by the gold-labelled secondary antibody, gold particles were located in the protein bodies (Fig. 2a). There were no gold particles in starch granules or cell walls. Also, few gold particles were observed in protein bodies or the cytoplasm network when the anti-legumin serum was replaced by a pre-immune serum. These results verify the specificity of the immunological binding and demonstrate that the legumin of pea seeds is exclusively localized in protein bodies (in agreement with results reported by Craig *et al.*, 1980).

Among the numerous extrudates we have produced, those extruded under moderate conditions (high moisture, high feed rate, low temperature) were characterized by thick air cell walls and structural heterogeneity, as illustrated in Figures 1 and 2. This structural heterogeneity indicates that the thermomechanical history of the same product was different from one zone to another. Such more or less modified zones can be used as indicators for the delineation of the progressive structural transformation of the raw material during extrusion-cooking.

Figure 1b shows entire cells found in a pea flour extrudate. Starch granules are more voluminous and bear marks of deformation. Their concentric layer structure is not visible. Some of them appear fused together (opposing arrows). The cell wall is locally disrupted. Protein bodies cannot be seen individually but, at their

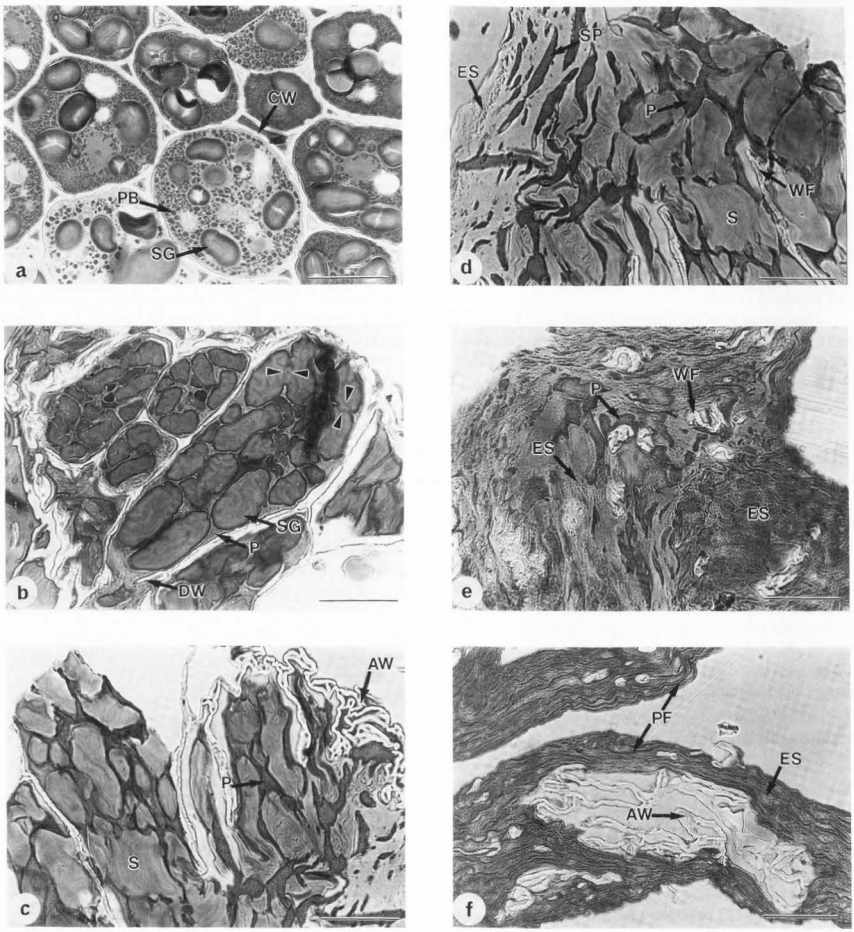

Figure 1. Light microscope photographs of: **a)** storage cells of pea seed, **b-f)** extruded pea flour showing several stages of transformation during extrusion-cooking (water added: 22.4%, feed rate: 39 kg/h, temperature: 150 °C, screw speed: 215 rpm). SG: starch granule, PB: protein bodies, CW: cell wall, P: proteins, DW: disrupted cell wall, S: starch, AW: assembled cell walls fragments, ES: expanded starch, WF: cell wall fragment, SP: spindle-shaped protein, PF: protein fiber. Opposing arrows show fused starch granules. Bar = 40 μm.

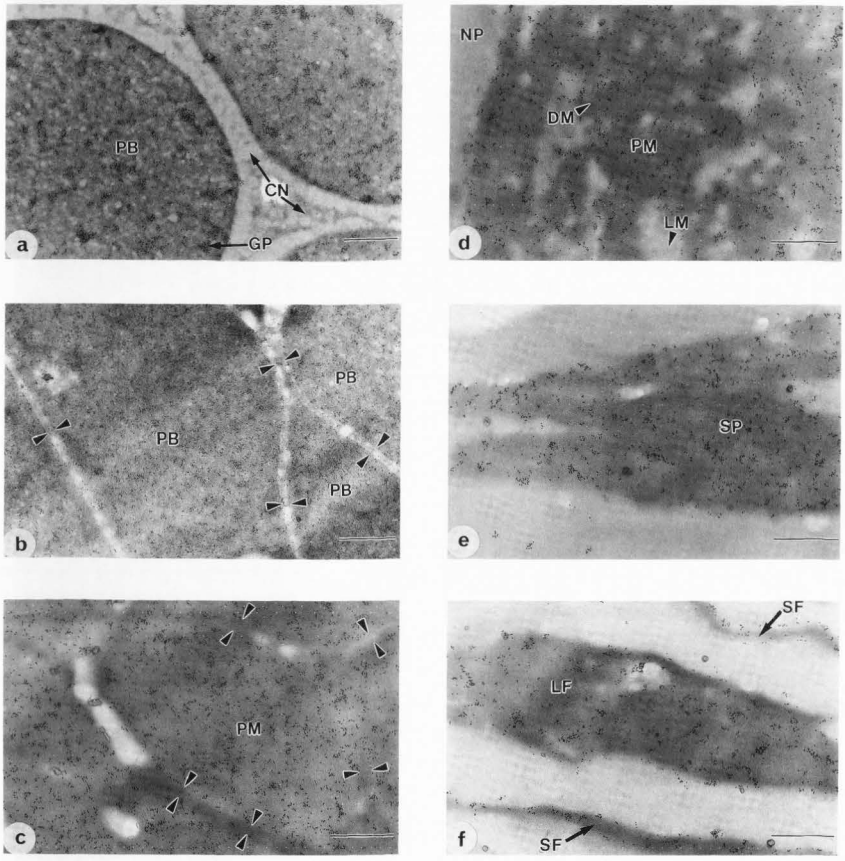

Figure 2. Transmission electron micrographs after immunogold labelling of legunin of the same fields of Figure 1: a) native protein bodies, b-f) progressive transformation steps of protein bodies into protein fibers. PB: protein bodies, GP: gold particles, CN: cytoplasm network, PM: protein matrix, DM: electron dense material, LM: electron transparent material, NP: non protein material, SP: spindle-shaped protein, LF: large fiber, SF: small fiber. Opposing arrows show condensed protein bodies. Bar = 0.5 μm.

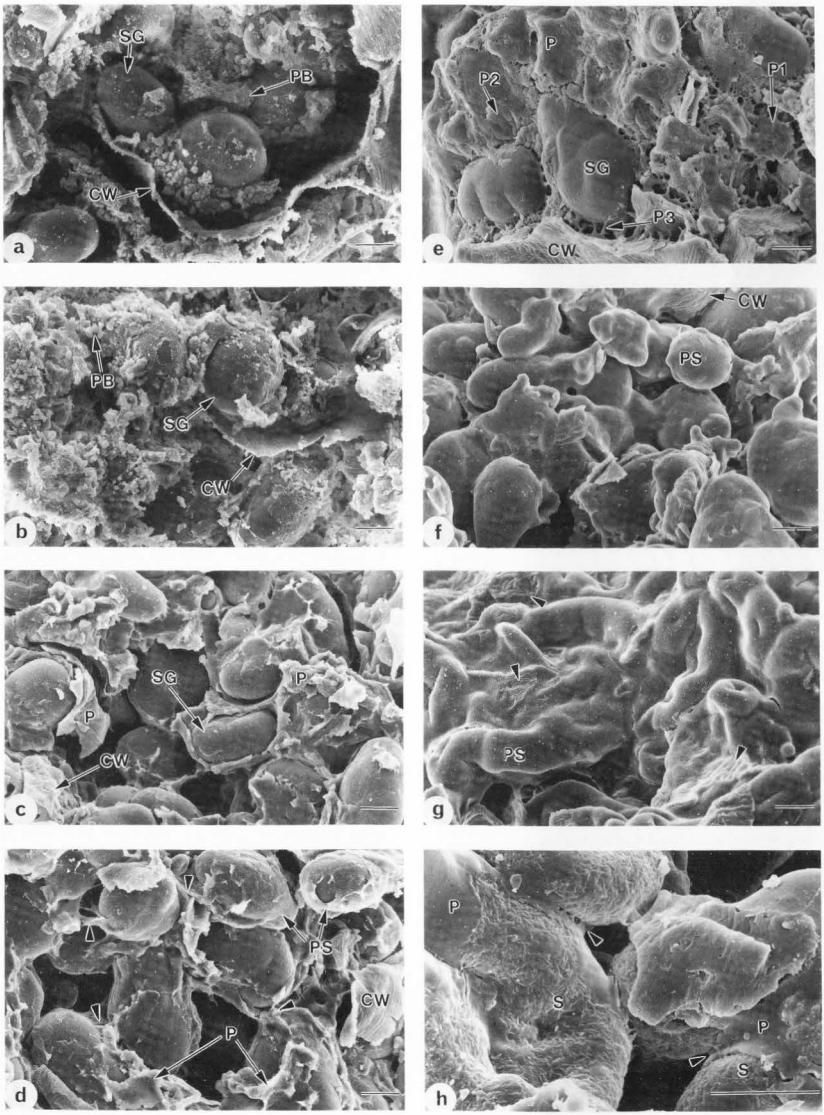
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Figure 3 (facing page). Scanning electron micrographs of: **a)** unprocessed flour, **b-d)** pressure stress treated pea flour (4.4, 8.8 and 12.4 kN/cm^2 respectively) showing the progressive transformation steps of protein bodies into protein matrix, **e-h)** four states of proteins and starch as a function of increasing vertical heat transfer through a 1 cm thick pea flour layer (decreasing distances from the heat source were approximately 8, 6, 4 and 2 mm, respectively). SG: starch granule, PB: protein bodies, CW: cell wall, P: proteins, PS: protein coated starch granule, S: starch, P1: protein body aggregate, P2: protein body fusion, P3: protein network. Arrows indicate protein link between starch granules (micrographs d and h) and surface of gelatinized starch (micrograph g). Bar = 10 μm.

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presumed location, a granular structure can be observed. TEM (Fig. 2b) reveals that protein bodies of the same field are condensed but remain separated by narrow spaces (opposing arrows).

In Figure 1c, starch granules are more deformed and some of them are fused. The cell walls seem to be unmodified. As shown in Figure 2c, protein bodies now form a protein matrix but the zones of contact between some of them remain distinguishable (arrows). At this stage, it can be noted that the protein bodies fuse before starch granules.

Figure 1d shows the continuous phase of starch which begins to expand and include spindle-shaped protein strands (cross or longitudinal sections). However, TEM observation reveals that there is no orientation inside these structures (Fig. 2d). This protein matrix contains electron dense material which is gold-labelled and unlabelled non-protein electron transparent material.

As shown in Figure 1e, expansion of the starch leads to a honeycombed structure. The protein matrix is now very condensed and arranged into dense strata (spindle-shaped structures in cross or longitudinal sections), but no internal orientation is visible and the legumin is still gold-labelled (Fig. 2e). The structural segregation between proteins and starch can be related to the fact that the protein bodies fuse before the starch granules.

Figure 1f shows the final structure of the wall of an air cell. Protein fibers, embedded in the expanded starch gel, are layered parallel to the air cell surface. Some cell wall fragments are clustered together in the wall of the air cell. The orientation of the cell wall fragments suggests that the structural organization of the wall of the air cell should be essentially a result of the "puffing" phenomenon. Thus, proteins and starch which were transformed into a viscous mass by the combined effects of heat, shear and pressure were easy to deform during the subsequent spontaneous evaporation of water at the die exit. Rigid cell wall fragments were then embedded in this viscous mass. Also, the legumin fraction within the protein fibers is still gold-labelled (Fig. 2f). This indicates that, during extrusion-cooking, some of the legumin antigenic determinants were not modified. Since the antibodies used are polyclonal, i.e., a mixture of antibodies directed against primary, secondary and tertiary structures, two cases can be considered: **1)** these antibodies recognize only the globular conformation; **2)** some of these antibodies may recognize the globular conformation as well as the unfolded one. On the other hand, the number of gold particles per mm^2 of protein matrix area in protein fibers is comparable to that in protein bodies. Thus, in the first case, the legumin conformation might be only slightly modified during extrusion-cooking. In the second case, the observed density of gold particles should be a result of recognition of sites on both globular and modified forms of the legumin macromolecules, but the role of each form cannot be evaluated in this experiment. The use of antibodies with defined specificity such as monoclonal antibodies may give direct evidence of conformational state of this protein in extruded products.

As shown in Figures 3b-d, by increasing pressure stress, i.e., axial compression, from 4.4 to 12.4 kN/cm^2, protein bodies aggregate and fuse. They are transformed into a protein matrix in which intact starch granules and cell wall fragments are embedded. A protein network was formed locally (Fig. 3d, arrows). This suggests that, in moistened pea flour, protein bodies are less resistant to mechanical stresses than starch granules or cell wall fragments. Thus, the protein matrix could be formed only by compression. Macroscopically, axial compression transforms powdery product, i.e., pea flour, to aggregated particles. The formation of a protein matrix, which could have a role of binding material, may explain the consolidation of the powder. Pressure lower than 4 kN/cm^2 applied to proteinaceous materials was shown to have no significant effect on protein aggregation (Aguilera *et al.*, 1976; Rhee *et al.*, 1981; Fujio *et al.*, 1988; Noguchi, 1989). On the other hand, increasing vertical heat transfer through a 1 cm thick pea flour layer induced protein body aggregation and fusion (Figs. 3e-h). In addition, it simultaneously induced swelling and deformation of starch granules. Proteins appear to have been spread over the starch granules (Figs. 3f-h). The starch granules, although protein coated (Fig. 3f), gelatinized (Fig. 3g arrows) and somewhat expanded (Fig. 3h), are still recognizable. These observations confirm that the structural segregation between proteins and starch in extruded products can be related to the fact that the protein bodies fuse before the starch granules.

From a microstructural point of view, extrusion-cooking, axial compression and heat treatment induce the formation of a protein matrix by aggregation and fusion of protein bodies. However, the mechanism of macromolecular arrangements should be different since the processing parameters involved in each treatment are different. This is supported by the fact that the behaviour of starch granules is different for the three treatments. Thus, shearing action along the screw and pressure release at the die exit may be important parameters during extrusion-cooking. Using defatted soy flour, Taranto *et al.* (1978) showed that extrusion and non-extrusion (combined effect of compression and heat treatment) processes lead to products with similar microstructures. They noted, however, that products obtained by non-extrusion processing had higher rheological properties than extruded products. Rhee *et al.* (1981) reported that, during extrusion and non-extrusion (combined effect of compression and heat treatment) processes, the formation of protein matrix was temperature dependent. They also noted, however, that changes in soy flour compounds during non-extrusion texturization were unlike those of extrusion texturization because shearing action did not occur in the former.

Conclusion

The study of the structural heterogeneity of some extrudates allowed the progressive transformation of the raw material during extrusion-cooking to be followed. Each of the three major compounds of pea flour, i.e., starch granules, protein bodies, and cell wall fragments, give a specific, independent structure. The structural segregation between proteins and starch was shown to be related to the fact that the protein bodies fuse before the starch granules. Effects of pressure stress and heat treatments on pea flour confirm this observation. The study of shearing action on pea flour may give further information about the behaviour of cell organelles during thermomechanical treatments. On the other hand, immuno-gold transmission electron microscopy was shown to be a useful technique for the localization of proteins in extruded products. Although this technique is delicate and requires a long time, its application to other processed foods may give interesting information about the distribution of proteins or any other antigenic molecules within complex matrices. However, a quantitative comparison based on the determination of the density of gold particles requires antibodies with a well defined specificity such as monoclonal antibodies.

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Discussion with Reviewers

A. Noguchi: It is surprising and very interesting that the legumin still maintains the immuno-gold labelling after HTST extrusion-cooking and embedding in GMA. It seems that the density of gold particles in Figure 2d-f is lower than that of Figure 2a-c in spite of the same magnification. This indicates that many legumin antigenic determinants were modified during extrusion-cooking. Please comment.

Authors: All magnifications of the TEM micrographs presented are not equal. Also, the density of gold particles was determined on numerous micrographs of several fields, taking into account the real area of protein matrix. The average density of gold particles was then determined for each zone previously defined (see light microscope photographs). All average densities were found to be comparable to those of native protein bodies. Thus, one can say that most of the legumin antigenic determinants are still recognizable with the antibodies used. Consequently, one can conclude that these antigenic determinants were only slightly modified through the formation of protein matrix.

A. Noguchi: The authors mentioned that protein bodies in moistened pea flour are less resistant to mechanical stresses than starch granules or cell wall fragments. We are preoccupied with the idea that the protein bodies are very hard. However, the starch granules are not gelatinized and the cell walls are very hard. If the protein bodies have some elasticity like raw meat, they would be fused while they are compressed among hard starch granules. In extrusion-cooking, it is likely that the protein bodies will be compressed, fused and deformed to the protein strands before the starch granules are gelatinized. Do the authors obtain similar results when they use roasted pea flour? I imagine that the heat-denatured and hardened protein bodies will give different results.

Authors: In all our experiments, the starting material was a moistened native pea flour. We have not yet used roasted pea flour, but we believe that this starting material should give different results than those obtained when native pea flour is used because the compounds are not in the same state.

A. Noguchi: Mechanical and heat treatments in this paper never accompany any shear which is very important for the transformation of components during extrusion-cooking. Can the authors elaborate on the shear?

R. Parker and A. Smith: Do the authors argue that pressure and heating alone are sufficient to model the thermomechanical history of the extruder?

Authors: The transformation of a raw material by extrusion-cooking involves combined effects of shear,

heat and pressure. When these parameters are studied separately, they would not have the same effects. Thus, in our experiments, we have tried not to model the extruder, but to show the difference between behaviours of starch granules, protein bodies, and cell wall fragments during mechanical or heat treatment. The study of shearing action alone may give further information which may complete the present results.

R. Parker and A. Smith: Do the authors consider dead-stop experiments useful in illustrating the pressure and temperature effects? For example, material could be extracted at points of increasing pressure and temperature (from feed zone to die) which could be quantified by barrel instrumentation. Similarly, extrusion experiments at low and high temperatures would be expected to provide extrudates which show protein matrix and protein/starch matrix development respectively. With the evidence given, it seems doubtful to attribute pressure and temperature as uniquely responsible for the changes.

Authors: Quantification of temperature and controlling pressure by barrel instrumentation is useful in controlling the extrusion-cooking process. However, its use in dead-stop experiments may give erroneous results because the material is still in a hot environment when the barrel is opened for sample extraction. On the other hand, extrusion experiments at low and high temperatures had been done by Rhee *et al.* (1981). These authors showed that, during extrusion-cooking of soy flour, protein fiber formation was temperature dependent. Concerning the effects of pressure and temperature alone, our results show clearly that protein bodies are less resistant to mechanical stresses and heat treatment than starch granules or cell wall fragments. Similar behaviour was observed in extrudates. However, the final organization of extruded products is, microscopically and macroscopically, very different from the ones of compressed or heat treated products. Thus, to model extrusion-cooking, a system which involves combined effects of shear, heat and pressure is required.