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ULTRASTRUCTURAL ASPECTS OF SPUN PEA AND FABABEAN PROTEINS

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Abstract

The ultrastructure of pea and fababean spun proteins has been studied by SEM and TEM as a function of dope pH and washing bath salt concentrations. The textural properties (mechanical resistance, moisture content) and diameter of the fibres have been determined.

Spinning was only possible when dope pH was higher than 11. An increase in dope pH from 11.5 to 13 induced a shear strength increase whereas the moisture content and the diameter of the fibres decreased. The structure of the fibres became more compact and changed from an aggregate of spherical particles to a tridimensional network. When dope pH was equal to 12.6, the increase in washing bath salt content from 2 to 10 % NaCl produced more compact fibres.

At high dope pH, the protein aggregates were dissociated and the polypeptide chains were unfolded, which favoured the lining up of the macromolecules during the spinning process and increased the protein-protein interactions in the fibres.

High salt concentration in the washing baths produced a salting out effect which probably also enhanced the chain-chain interactions. Contrary to previous studies, protein strand orientation along the fibre axis and a double cortex-core structure have been demonstrated.

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Key Words : fababean, pea, spun proteins, scanning electron microscopy, transmission electron microscopy.

Introduction

According to Flint (1979), the spinning technique for making edible spun protein fibres is the exact imitation of a technology already used in the manufacture of textile fibres such as rayon. This technology has been applied to the processing of all kinds of food proteins (Young and Lawrie, 1975; Culioli et al., 1981; Tuoby et al., 1983) and the vocabulary has even been taken directly from the textile industry. Although the U.S. patent of Boyer (1954) for spinning proteins is not very recent, edible spun protein fibres are still not produced on a large scale. This is why spun proteins have been presented by De Man (1976) as very new textured protein food, used to replace existing meat products. The process as it was outlined by Culioli (1981), consists (Fig. 1) of pumping a dope (alkaline solution of protein isolate) through a spinneret (a die containing many hundreds of pin-holes, from 50 to 250 µm in diameter) into a coagulation bath containing acid (acetic, phosphoric or lactic, at various concentrations) and salt (most often 2 to 20% NaCl) to form insoluble protein fibres. Coagulated fibre bundles are removed under tension to improve the rheological fibre properties. They are then neutralized and washed in various baths, spin-dried and immersed in binding agents (ovalbumin, gluten, soybean proteins or polysaccharides). All these agents are coagulated either by heat, pH changes or evaporation. Acceptable simulated meat products, with added fats, coloring and flavors, are cut and either frozen or dried.

Culioli (1981) has hypothesized the molecular arrangement during protein spinning as schematized by Ziabicki (1967) in the case of the synthetic spun polymers (Fig. 2). The native globular structure of isolate protein is unfolded and denatured in the alkaline dope medium. Then molecular orientation results from the shearing flow in the spinneret where the velocity profile assumes a parabolic distribution (Fig. 2a). Just after emerging from the spinneret, the protein molecules become disorganized by the expansion of the dope flow; expansion results from restitution of the elastic energy stored during spinneret

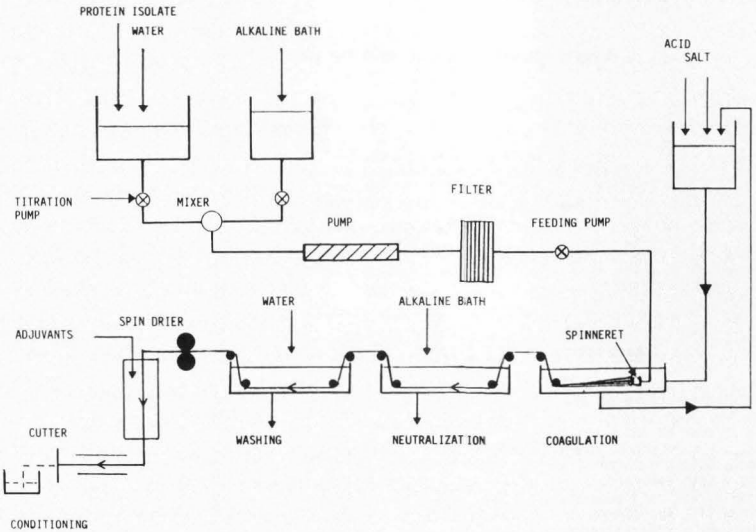

Fig. 1. Spun protein processing diagram (Culioli, 1981).

flow. Then the macromolecules are again orientated by streaming in the elongational flow (Fig. 2b). Precipitation under stretching increases the orientation parallel to the axis of the fibre (Fig. 2c), intensifying the intra- and intermolecular protein bonds (hydrogen, ionic or covalent).

The structure of spun proteins has generally been studied either with light microscopy or SEM (De Man, 1976; Saio, 1979 and 1981; Wolf and Baker, 1980). Fibres were thus shown to be analogues of the meat or fish muscle fibres; such fibre bundles combined well with unstructured gluten and fats in spun soybean meat analogues (Flint, 1979; Culioli, 1981). SEM observations were also correlated with physical measurements such as shear strength, diameter and fibre water content (Culioli et al., 1983) when the dope pH or the salt content of washing baths were varied. The SEM of spun soybean protein isolate showed cylindrical morphology with surface striations running along their length (Wolf and Baker, 1980) that suggests longitudinal orientation of the protein structure.

In TEM studies of spun proteins Tombs (1970), and Young and Lawrie (1975) noted that there was no orientation of protein molecules along the axis of the fibre. In cross sections, the fibre ultrastructure was shown to consist of spherical particles linked together in strands forming a three-dimensional network incorporating pores of various sizes, with random aggregation in some areas.

There were no signs of orientation in TEM micrographs of extruded soybean protein fibre (Smith, 1979). Such observations seem contradictory to the scheme of molecular arrangement in Figure 2.

In order to understand both the spinning process and molecular orientations of the proteins, the ultrastructure of longitudinal sections from the periphery and central core of pea and fababean protein fibres are shown. Structural modifications (protein strand orientation, particle size and alveolation) were studied as a function of dope pH and washing bath salt concentrations.

Materials and methods

Spinning

Pea or fababean protein isolates were prepared at the pilot plant of our Research Center (Food Technology Center - INRA Nantes - France) from hulled pea or fababean flour. The nitrogen was determined to be 15% (pea) and 15.9% (fababean) of the isolate dry matter. Protein dopes were prepared at room temperature in a mixer with two horizontal blades. The dope pH was adjusted to the 11.6 to 13.2 range by adding caustic soda. Extrusion was carried out at 20°C through a spinneret containing 300 holes of 100 µm each. The coagulating bath contained 4% acetic acid and 20%

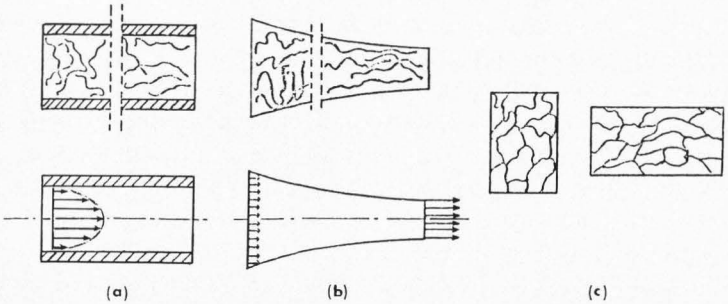

Fig. 2. Diagram of molecular arrangement during spinning: a) molecular orientation from shear flow in the spinneret; b) molecular orientation from elongated flow at spinneret exit and c) molecular orientation after deformation of a three-dimensional viscoelastic network (Ziabicki, 1967).

sodium chloride (pH 1.7 to 1.8). Bundles of fibres were then washed in sodium chloride solutions varying from 2 to 10%.

Physical measurements

Water content. The bundles were centrifuged on fritted glass for 15 min at 200 g. The moisture content was then determined by weighing the centrifuged samples before and after drying at 105°C for 12 h.

Fibre Mean Diameter. Determined on wet fibres with light microscope using an ocular micrometer.

Mechanical Resistance. The fibres were characterized by their shear strength using the method of Laroche and Salé (1976).

SEM

Wet spun fibres were frozen either directly in liquid nitrogen or in isopentane cooled to -150°C with liquid nitrogen, then freeze-dried and cross fractured. The following technique was also used: fixation 2h in a solution containing 6 % glutaraldehyde and 5 % NaCl and ethanol dehydration (2 h) with a graded ethanol series . Samples were examined in a JEOL 50A at 20 keV, $5X10^{-13}A$.

TEM

Wet spun fibres were fixed 1 h in 6% glutaraldehyde and postfixed 30 min in 1% OsO_4, the two fixatives being prepared with the washing bath solutions (2, 5 or 10% NaCl in distilled water). Then the bundles were rinsed in distilled water, acetone dehydrated and embedded in Epon 812. Blocks were prepared for longitudinal sectioning as shown in Figure 3. The silver sections, which were cut using a diamond knife on the JEOL JUM7 ultramicrotome, were stained for 30 min in 2.8% uranyl acetate (50% methanol) at 48°C and for 5 min in lead citrate (pH 13). Sections were observed in a JEOL 100S at 80 keV.

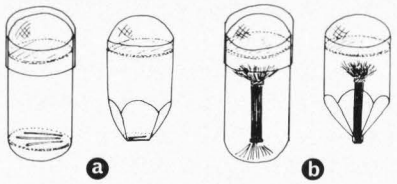

Fig. 3. Embedding and sectioning: a) longitudinal section, sample position and block preparation and b) cross section, sample orientation and block preparation.

Results

Physical measurements

Fababean and pea protein spinning is possible only when the dope protein content exceeds 10% and the pH is higher than 11 (Culioli and Salé, 1981a). When the fababean protein dope pH varied from 11.6 to 13.2, the shear strength of the spun fababean fibres increased, whereas their moisture content and diameter decreased as shown in figure 4a. Similar behaviour was found with spun pea protein isolate. The salt content of washing baths had a great influence (figure 4b). Fibre diameter decreased from 67 to 47 µm, water content decreased from 61 to 48% and shear strength increased from 1.4 to 5.6 x 10^5 N/m^2 as the salt content increased from 2 to 10%.

SEM

As observed with spun soybean proteins (De Man, 1976; Wolf and Baker, 1980), spun fababean proteins (Figs. 5 and 6) and spun pea proteins (Fig. 7) showed a cylindrical morphology with surface striations running along their length. Freeze-drying after freezing in isopentane cooled by liquid nitrogen was the best preparation technique (Fig. 5a). On freezing the fibers directly in liquid nitrogen (Fig. 5b) gas turbu-

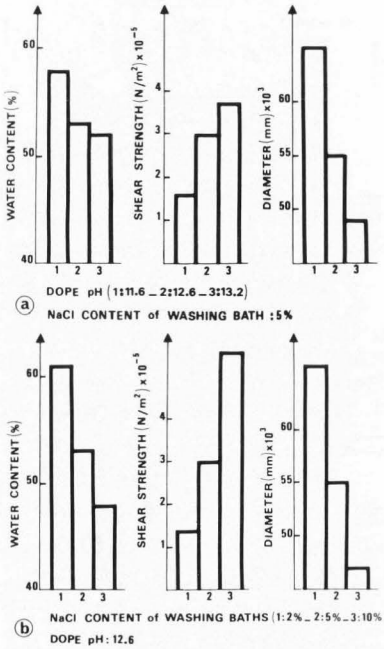

Fig. 4. Physical measurements on spun fababean protein fibres: a) evaluation of fibre bundle water content, shear strength and fibre diameter as a function of the dope pH (NaCl content of washing bath: 5%) and b) evaluation of fibre bundle water content, shear strength and fibre diameter as a function of bath NaCl content (dope pH:12.6).

lence caused surface holes and some structural deformations, but the fibre displayed some longitudinal strand-like structure. In Figure 5c, fixation with 4% glutaraldehyde and dehydration in the presence of 5% NaCl, two different parts were apparent : 1) a granular central core; and 2) a more compact, strand-structured cortex.

Figure 6 shows spun fababean protein fibres prepared with increased salt concentration in the washing bath. At low salt concentration (2%), the central core was finely alveolated (Fig. 6a). At intermediate concentration (5%), the fibres appeared granular in structure as was also noted at high concentration (10%) for which the diameter was practically the same (Figs. 6b, 6c). It was noteworthy that NaCl crystals remained on the fibre surface as was demonstrated using micro-analysis.

Figure 7 shows spun pea protein fibres prepared from dopes at various pH values. At low pH

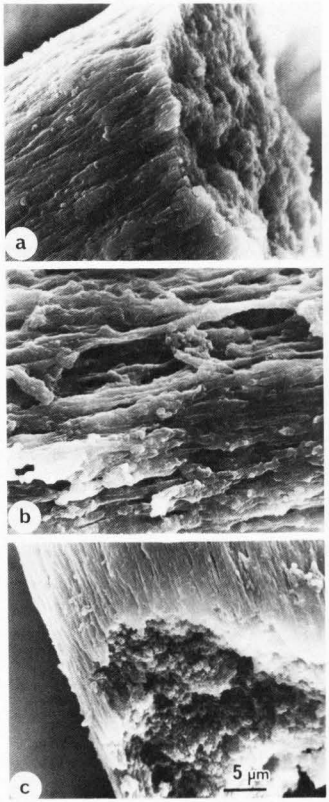

Fig. 5. SEM preparations of spun fibres: a) fractured spun protein fibre after freezing in isopentane (-150°C) and freeze-drying; b) structural deformation after freezing directly in liquid nitrogen and then freeze-drying and c) fractured spun protein fibre after glutaraldehyde fixation and alcohol dehydration.

(Fig. 7a), the cortex was very thin (1 to 3 µm) and the central core appeared granular and very dispersed. At intermediate pH (Fig. 7b), the cortex was thicker (around 5 µm) and the internal structure was porous. At high pH (Fig. 7c), the entire fibre appeared compact with a few large vacuoles in the core. Under the same conditions, pea protein fibre diameters were thicker than those of fababean fibres.

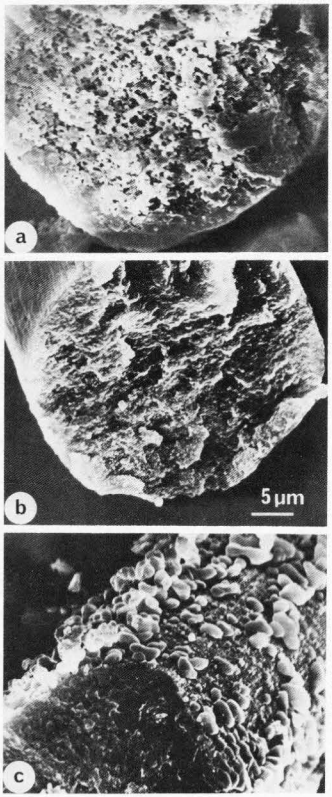

Fig. 6. SEM of spun fababean protein fibre cross-section (dope pH: 12.6). Washing bath NaCl content: a) 2%; b) 5% and c) 10%.

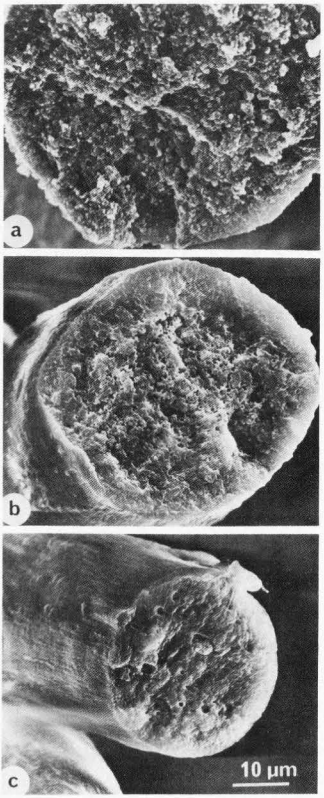

Fig. 7. SEM of spun pea protein fibre cross-section (NaCl content of washing bath: 5%). Dope pH: a) 11.09; b) 12.05 and c) 13.20.

TEM

Electron micrographs showed, as observed on spun proteins produced from meat waste (Young and Lawrie, 1975), that the fibres were constituted of spherical protein particles associated together in chains or strands. These chains were arranged in a three-dimensional network entrapping fat globules of various sizes and numerous vacuoles more or less anastomosed.

In the cortex where oblong fat globules and vacuoles were observed, orientation of protein strands along the fibre axis was quite evident (Figs. 8a, 8c, 9a, 9c). This observation is in opposition to those of Young and Lawrie (1975). In the central core of the same fibre (Figs. 8b, 8d, 9b, 9d) strands were randomly oriented. In fibres made from dope at pH 13.2 (Figs. 8e, 8f) protein strands were no longer visible. The structure with minute fat globules and very small vacuoles was very confused. Probably protein-protein interactions were higher at such pH giving hardening to the fibre and lower diameter.

Comparing spun proteins from dopes at pH 12.6, thinner strands were found in 2% NaCl (Figs. 8c, 8d) than in 5% NaCl (Figs. 9a, 9b) and 10% NaCl (Figs. 9c, 9d), the latter showing folded strands penetrating the fat globules and twisted-kinked strands hinting of higher viscosity of the dope. But protein strands alone were not responsible for these different structures.

Young and Lawrie (1975) reported that "poorly defined structures and large pores were found

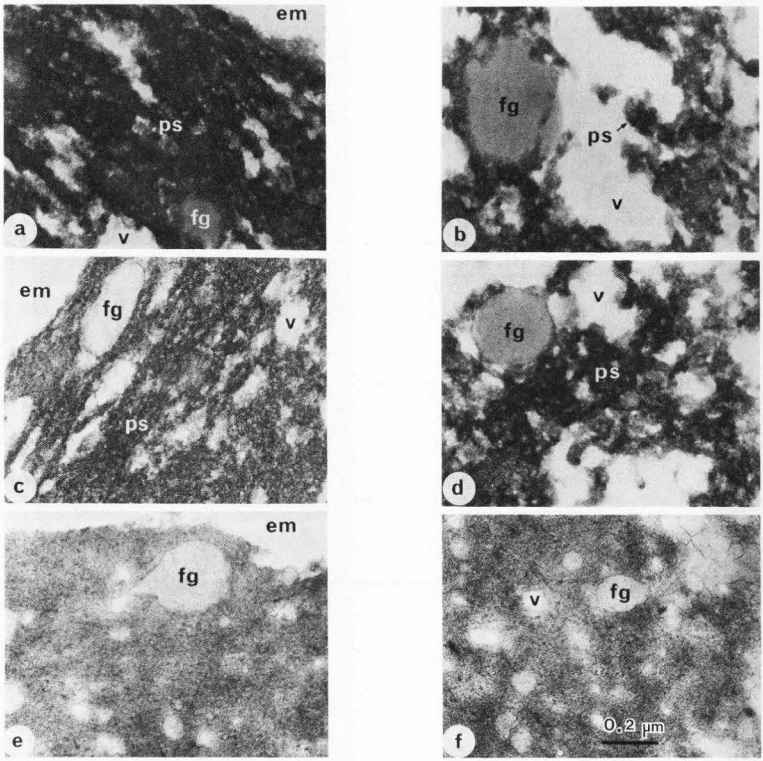

Fig. 8. TEM of longitudinal sections of spun fababean protein isolate (NaCl content of washing bath : 2%). Sections a, c and e are from the fibre periphery and sections b, d and f are from the central core. Structural changes as a function of the dope pH : a-b) 11.6; c-d) 12.6; e-f) 13.2. em : embedding medium; fg : fat globule; ps : protein strand; v : vacuole.

in the fibre giving rise to low mechanical strengths". In fact, the vacuolar system remained a major factor. At a low pH (11.6) the central core and the periphery were very alveolated. At an intermediate pH (12.6) the central core was more alveolated (larger vacuoles) than the cortex. At a high pH (13.2) pores were smaller. The vacuole ratio as a function of total volume of the fibre has been estimated on the TEM pictures (40,000 X) using statistical image analysis. Under the same conditions of salt concentrations (5% NaCl), vacuole ratios of spun fababean protein were 39%, 23% and 16% respectively for corresponding dope pH values of the spinning dopes (11.6, 12.6 and 13.2). At the same dope pH (12.6) vacuole ratios were 40%, 23% and 14% for corresponding values of 2%, 5% and 10% NaCl in the washing baths.

Discussion and Conclusions

Although Tombs (1970) and Young and Lawrie (1975) considered spun fibres as cylindrical gels without any sign of orientated strands, photomicrographs of longitudinal sections of various spun fibres have shown here that the structure is quite different.

According to these results, macromolecular strands are orientated along the fibre axis,

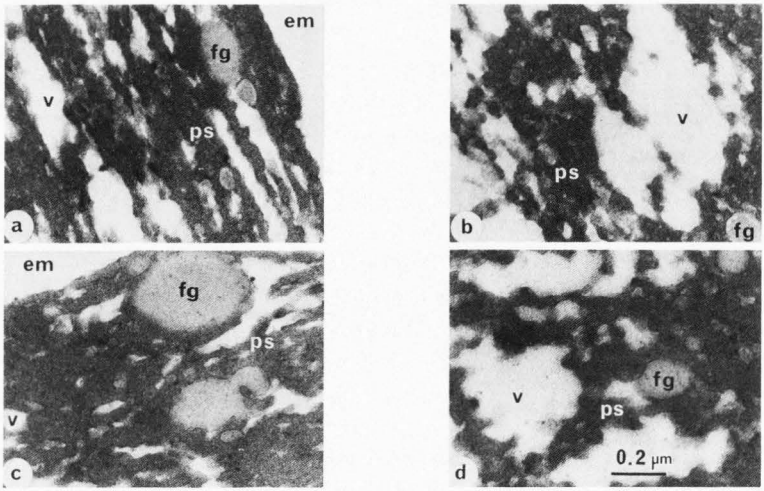

Fig. 9. TEM of longitudinal section of spun fababean protein isolate (dope pH : 12.6). Sections a and c are from the fibre periphery; sections b and d are from the central core. Structural evolution as function of washing bath NaCl content : a-b) 5%; c-d) 10%. Symbols are defined in Figure 8.

which could explain the surface longitudinal striation previously described (De Man, 1976; Wolf and Baker, 1980). This observation also confirms the hypothesis of Culioli et al. (1983) that molecular orientation results from shear and elongational flows (Fig. 2). The oriented structure of fibre cortex could be explained by: i) during the extrusion through the spinneret by greater lining-up of the macromolecules at the periphery due to higher strain rates near the wall of the pin holes and ii) at the spinneret exit by fast coagulation of the external part of the spun fibre. Under these conditions, the relaxation phenomena due to restitution of elastic energy could be reduced in the external part of the fibre and so, the structures fixed in the orientation induced by the shear flow through the spinneret. Proteins of the central core may then be randomly coagulated under relaxation into the available volume.

Culioli and Salë (1981 a,b) hypothesized about changes that occurred in the dopes. When dope pH was below 11 in the aqueous protein solution, proteins would be hydrated and agglomerated. Under these conditions spinning is very difficult. Fibres are so weak that structural investigations were practically impossible. When dope pH exeeded 11, increase in dope consistency should be related to the dissociation of protein aggregates into subunits and to the unfolding of polypeptide chains. This unfolding favours lining-up of the chains and protein-protein interactions. Then the structure of the fibres changes from an aggregate of spherical particles (Fig. 6a) to a tridimensional network (Fig. 6b) with longitudinally oriented subunit-like strands. When dope pH is above 12.5, a large decrease in the consistency of dopes is observed. As proposed by Culioli and Salë (1981 a,b) three factors should be responsible for this large decrease in consistency : i) an increase in electrostatic repulsive forces due to the deionisation of the guanidyl groups of arginine; ii) the breaking of disulfide bonds; iii) the destruction of hydrophobic areas. Increased unfolding of protein chains is then obtained in the dope which induces more protein-protein interactions during the coagulation in the bath. Resulting fibres appeared more compact, the vacuolar system being three times smaller than at pH 11.6. In the fibres from the most viscous dope, kinked strand appearance is the result of elongated forces.

Concerning the effect of the salt washing baths, it seemed (Figs. 8c, 8d, 9c, 9d) when the salt concentration was too weak that the protein aggregates were less individualized and the protein strands thinner. On the contrary, at higher salt concentrations the orientation of the structure was better seen. It was hypothesized that when salt concentration is high a salting out effect would provoke the protein-protein interactions; conversely, when the salt concentration is too low a salting in effect would give rise to some protein solubilization.

In conclusion, the present study has shown the important influence of dope pH and salt washing bath concentrations on spun protein structure and texture. These results allow one to determine these processing parameters in order to

get the most appropriate texture for the food fibre which, as a meat analogue, must be neither too dry nor hard and the aminoacids must not be modified.

Similar behaviour was noted with both fababean and pea spun proteins. SEM and TEM images were complementary but, although SEM showed a fibre diameter evolution, the diameters could obviously not be measured after conditioning (drying) or TEM fixation, but only on wet fibres with light microscopy.

With image analysis, the great importance of vacuole ratio on texture and fine structure has been shown.

Contrary to previous studies, structural strand orientation along the fibre axis and the double cortex-core structure were proven.

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Discussion with reviewers

K. Saio: How did you prepare pea or fababean isolates ? Did you purchase commercial products ?
Authors: Protein isolates were prepared at the pilot plant of our research Center from flours of hulled pea (var. Frimas) and fababean (var. Ascott). The proteins were solubilized at pH 7 in a slightly alkaline solution. The insoluble parts of the flours were discarded by centrifugation. Then the proteins were precipitated at pH 5.3 and spray-dried after centrifugation and washing.

K. Saio: Fat globules are observed in the TEM micrographs (Fig. 8 or 9). What percentage of fat did you have in the isolates which you used ?
Authors: The fababean and pea protein isolates contained 5.9 and 6.5 % fat, respectively, on a dry basis.

K. Saio: Did the vacuoles shown in SEM micrographs (for instance, Fig. 7c) correspond to the vacuoles in TEM micrographs ?
Authors: Yes they did. But it is noteworthy that at high pH (13.2) and 5 % NaCl content of washing bath, the protein network was more compact with very minute vacuoles (less than 0.1 µm). It was not possible to show that on the TEM micrographs owing to the magnification we used.

K. Saio: The speed of extruding through the die is higher in outer layer of fibre than the inside because of resistance to inner surface of the die whereas the one of expanding and stretching in the coagulating bath is contrary. What do you think about this point? Do you think that the effects of expansion in the coagulating bath are more important than the effects of extrusion through the die for constructing the microstructure of spun fibres?
Authors: The main factor which influences the macromolecular orientation during the flow

through the spinneret is not the velocity but the strain rate. This factor depends not only on the flow rate but also on the radius of the spinneret holes. It equals zero at the center of the spinneret hole and reaches a maximum value at the wall ($\dot{\gamma} = 4Q/\pi R^3$), with Q = flow rate ; R = radius ; $\dot{\gamma}$ = shear strain rate).

Therefore, the orientation of the macromolecules is higher at the periphery than at the center.

During the expansion of the dope filament, the macromolecules are disorganized at the spinneret exit. However, at the fibre surface, the coagulation occurs very rapidly which limits the influence of the relaxation while in the core the molecules can relax before the coagulation.

For the orientation developed in the spinneret to be effective in contributing to fibre microstructure, it must be quenched before it is relaxed. So the rate of coagulation must be greater than the reciprocal relaxation time. According to Ziabicki (1967) such is not the case in the textile fibre spinning process except for the thin surface layer. This is confirmed in the case of protein spinning by the double microstructure observed in the fibre.

W.J. Wolf: Have you examined fibers made without stretching during the spinning process ? This may give some information about the importance of stretching as it relates to orientation of protein molecules within the fibers and the diameter of the fibers.

Authors: It is not possible to get fibres without any stretching in the coagulation bath as the liquid filaments associate with each other at the exit of the spin holes and form globules. However, spinning with stretching rates lower than the one used (150 %) produces fibres with larger diameter and very low mechanical resistance.

Although we have not realized microstructure observations, these results tend to demonstrate that less orientation is obtained when stretching decreases. During stretching the free fluid jet is subjected to axial tension which: i) limits the relaxation phenomena at the spinneret exit and ii) increases molecular orientation in the elongational flow.

According to Ziabicki (1967) this mechanism can be considered as the most important one in fibre spinning.

J.M. deMan: If there is a difference in structure between cortex and core, it should be of interest to compare fibers produced with spinnerets yielding fibers of different diameter, have the authors done this ? If not, is there any information in the literature ?

Authors: Although we have used spinnerets of various diameters (from 100 to 150 µm) we have not studied the influence of this parameter on the fibre microstructure. On the other hand, we have not found any information in the literature about relationships between spinneret diameter and the cortex-core structure.

We have emphasized in the previous answers the prominent part played by the shear strain rate in the spinneret, the relaxation at the spinneret exit, the coagulation rate in the acid and salt bath and the elongation strain rate in the macromolecule orientation mechanisms.

A variation in the spinneret diameter induces modifications of these parameters, which can explain some differences in the microstructure. It is likely that spinning with increased spinneret diameter without any modification of the other processing conditions (dope flow rate, fibre take up velocity ...) produces fibres with larger diameters and with a more developed core structure.