Protein Coagulation Cloud in Citrus Fruit Extract. 2. Structural Characterization of Coagulates

Ilan Shomer,*,‡ Rosa Vasiliver,‡ and Rephael Salomon§

Institute for Technology and Storage of Agricultural Products and Institute of Plant Protection, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

The structure of coagulated proteins of a serum of citrus fruit aqueous peel extract (SPEX) was studied, considering protein interactions with colloidal cloud constituents. The coagulates' structural properties are affected by the coagulation conditions such as enzymatic pectin degradation (EPD), temperature, pH, concentration, and tissue origin (flavedo or albedo). The electrophoretic profiles were characteristic for each coagulation temperature but were not affected by the concentration level. The ultrastructure was highly dependent on the coagulating conditions, and different samples appeared in various patterns such as fibrils, spongelike structures, granules, amorphous patterns, and aggregates. Without EPD, the coagulation was suppressed in the SPEX, while it appeared, but delicately, in the flavedo extract. Following the EPD treatment, distinct coagulates were obtained, which appeared as aggregated granules.

INTRODUCTION

Protein coagulates and their structure are of importance for various roles in colloidal plant tissue extracts and their stability. The cloud of citrus fruit aqueous peel extract was found to be composed of several fractions of colloidal particles, each of which is characterized by structure, density, and chemical composition (Merin and Shomer, 1984; Shomer et al., 1985). Although there are differences in the suspendibility among the various types of colloidal constituents, in some cases all of them precipitate together, resulting in clarification.

Formation of pectate gels, due to enzymatic demethoxylation, was studied comprehensively in relation to cloud stability (Baker, 1980; Bruemmer, 1980). Proteins were studied with regard to cloud stability from two main aspects: (i) as enzymes which were inactivated either by heat treatment or by proteases which were supposed to decrease enzymatic activity due to enzymatic decomposition of pectinases [no positive results were obtained (Biggs et al., 1970; Krop and Pilnik, 1974)]; (ii) insoluble protein constituents which were obtained for two main reasons; protein coagulation (Shomer, 1988; Shomer et al., 1982) or protein complexes with other constituents such as phenols and tannic acid (Van Buren and Robinson, 1969; Van Buren and Way, 1978). Complexes were found to be composed of proteins associated with essential oils and pigments (Shomer, 1988), pectin with its associated neutral sugars (Klavons and Bennet, 1985, 1987; Shomer, 1991), hesperidin crystals, and unidentified components (Merin and Shomer, 1984; Shomer et al., 1985).

Although proteins were found in the juice, and insoluble proteins were found to be a component of the insoluble cloud components (Klavons and Bennet, 1985, 1987; Sinclair, 1984), no evidence was presented for protein coagulation in the juice serum. Shomer (1988) showed that encapsulation of cloud particles by coagulated biopolymers, such as pectin (as pectate gel) and proteins are involved in clarification. These findings may explain the tendency of both high- and low-density (emulsified essential oil) components to precipitate in a homogeneous multisolute liquid system.

Since coagulated proteins were found to be involved in cloud stability (Shomer, 1988, 1991), it is reasonable to

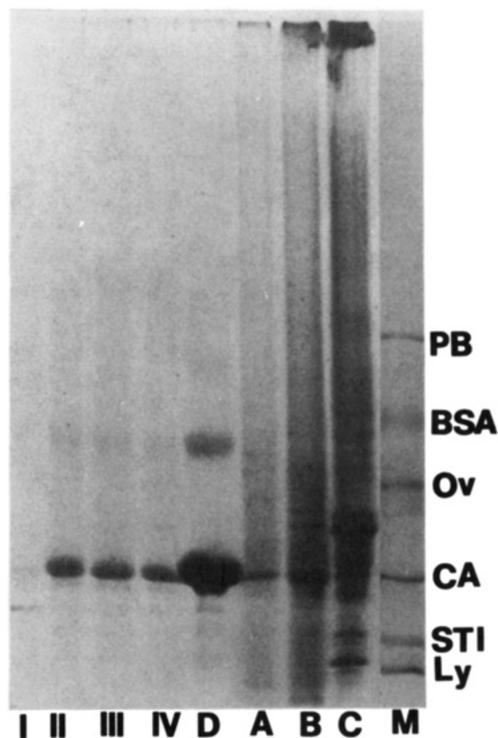

Figure 1. Electrophoretic patterns of SDS–PAGE of heat-coagulated proteins and of precipitated proteins in heat vacuum concentrates, obtained in heat-inactivated and enzymatic pectin degraded (EPD) serum of aqueous peel extract. (A–D) Coagulation temperature of 40, 60, 80, and 100 °C, respectively (each coagulation step was done after the coagulated matter obtained in the lower coagulation temperature was removed from the serum). (M) molecular weight markers: PB, phosphorylase B (97 000); BSA, bovine serum albumin (66 200); Ov, ovalbumin (45 000); CA, carbonic anhydrase (31 000); STI, soybean trypsin inhibitor (21 500); Ly, Lysozyme (14 400). The heat coagulations were done in the same serum solution, where the coagulated proteins were removed after each coagulation before the next higher temperature was applied. (I–IV) Serum solutions at concentrations of ×2.5, ×5.0, ×7.5, and ×10, respectively.

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‡ Institute for Technology and Storage of Agricultural Products.

§ Institute of Plant Protection.

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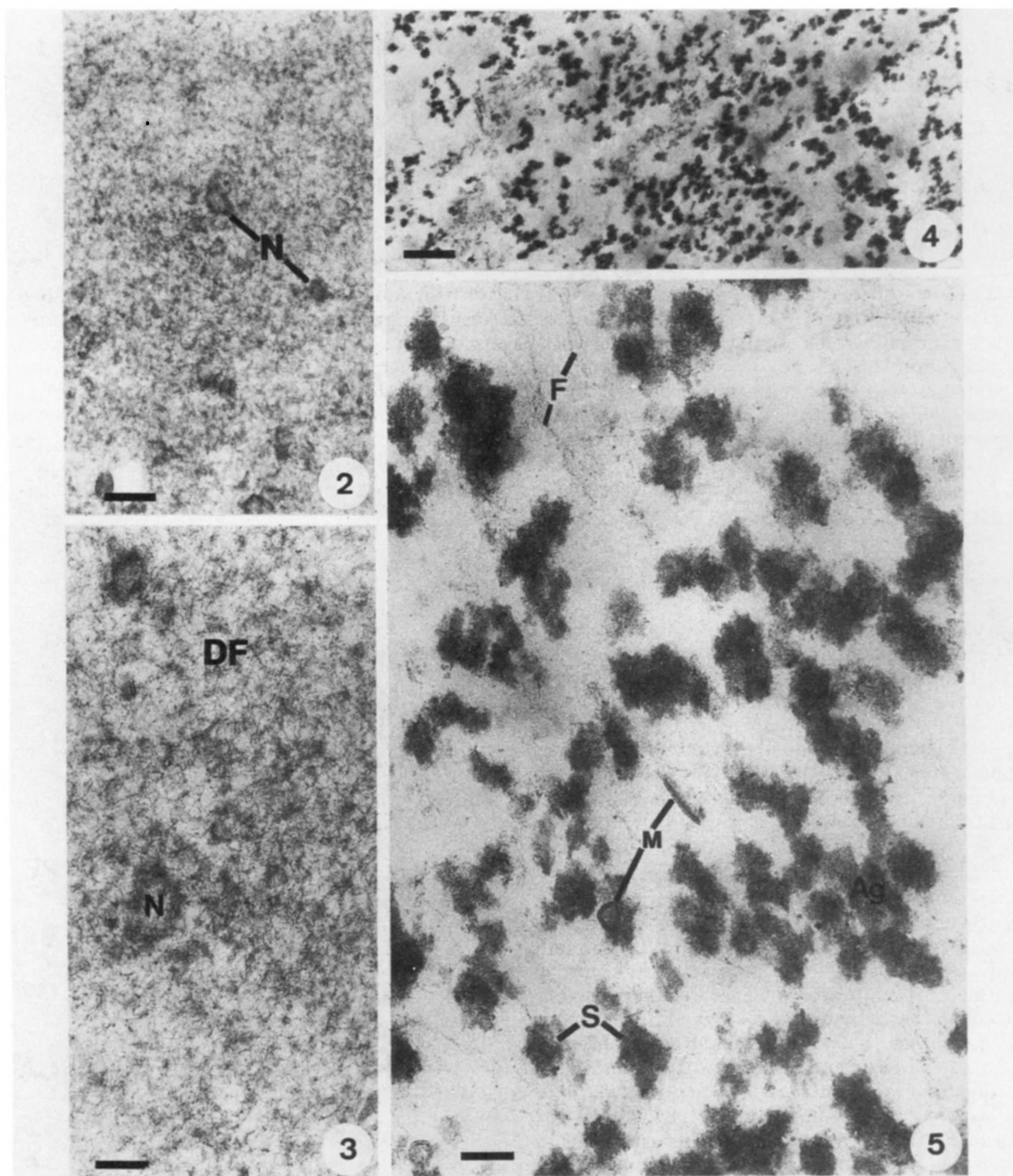

Figure 2. Ultrastructural patterns of coagulated proteins obtained in whole peel SPEX after EPD. (Panels 2 and 3) After incubation at 40 °C for 30 min (bars = 0.7 and 0.1 μm, respectively). (Panels 4 and 5) After removal of the coagulated proteins from the incubating solution and heat coagulation at 60 °C (bars = 0.7 and 0.1 μm, respectively). DF, dense fibrillar lattice; N, coagulation nuclei; M, double membranelike structure; F, fibrillike pattern; S, separated granules.

assume that also in the juice some portion of the protein is involved in cloud stability, and this aspect deserves further elucidation. Ultrastructural characterization of coagulated proteins from SPEX may contribute to the identification of proteinaceous components and to an understanding of the latter's role in the cloud stability of natural juice.

The present study characterized the structural nature of coagulated proteins in SPEX as affected by enzymatic pectin degradation, denaturation temperature, concentration, pH, and tissue origin (flavedo and albedo).

MATERIALS AND METHODS

Preparation of Peel Extracts and Coagulates. Preparation of SPEX, enzymatic pectin degradation, protein coagulation, heat vacuum concentration, and coagulate separations were as described in part 1 (Shomer, 1991).

Protein Characterization. To know whether any serum constituent (other then pectin) affects the structure of the coagulates and their electrophoretic properties, coagulates obtained directly from the serum following EPD were compared with proteins salted out from the serum and redissolved in distilled water. The separation of the proteins from the SPEX was done by precipitation with ammonium sulfate at 90% saturation. The precipitated proteins were collected by centrifugation and dialyzed against distilled water for 48 h at 4 °C. The soluble proteins of the ammonium sulfate extraction were heat-coagulated and then examined as the nonseparated serum proteins. The ultrastructural and electrophoretic properties of both the salted out and the EPD heat-coagulated proteins were found to be similar. Hence, for the structural study and for the electrophoresis, the proteins and their coagulates were separated directly from the SPEX.

Electrophoretic resolution of the coagulated proteins was performed after the proteins were boiled in Laemmli buffer,

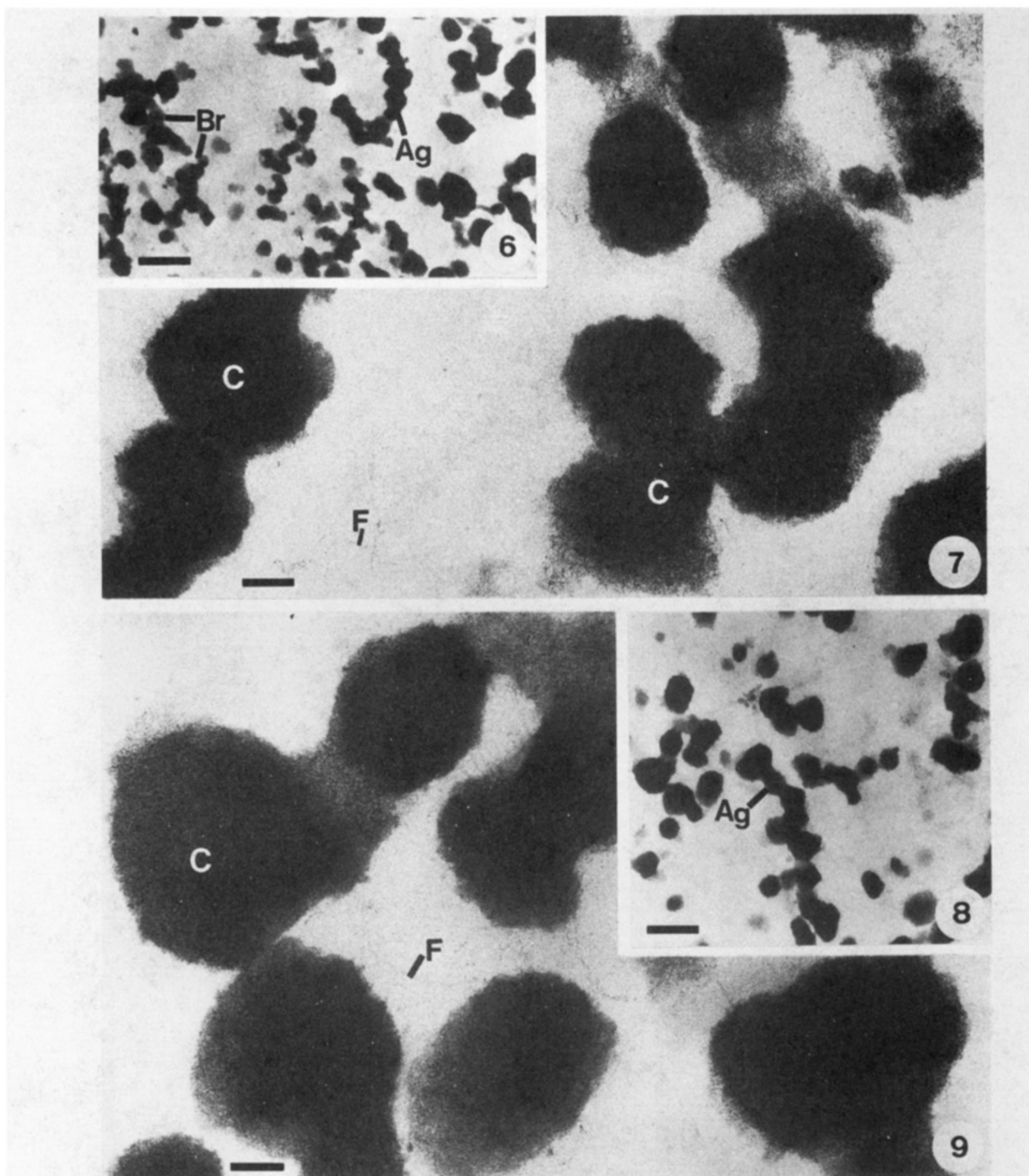

Figure 3. Ultrastructural patterns of heat-coagulated proteins in a serum of whole peel extract after EPD. (Panels 6 and 7) After removal of coagulate obtained at 60 °C (panels 4 and 5 of Figure 2) and coagulation at 80 °C (bars = 0.7 and 0.1 m, respectively). (Panels 8 and 9) After removal of coagulate obtained at 80 °C (panels 6, 7) and coagulation at 100 °C (bars = 0.7 and 0.1 μm, respectively). Ag, aggregate; Br, bright sites; F, fibrillike pattern; C, conglomerate.

consisting of 0.1 M Tris-HCl buffer, pH 6.8, containing 3% sodium lauryl sulfate (SDS), 5% 2-mercaptoethanol, and 10% glycerol. Samples containing 50–100 g of protein were loaded on top of a polyacrylamide 5–15% gradient gel containing 0.1% SDS (PAGE SDS).

Electrophoresis at 100 V was carried out for 16 h, after which time the gel was fixed and stained with a solution of 0.2% Coomassie brilliant blue in 50% ethanol/45% acetic acid, followed by excess dye washing in 25% methanol/7% acetic acid (Alper et al., 1984).

Electron Microscopy. The preparation of the coagulated material for transmission electron microscopy was carried out according to the method of Shomer et al. (1982) with some modifications. Coagulation-treated SPEX was cooled in an ice bath, and the coagulated material, precipitated overnight at 4 °C, was rinsed with distilled water and the supernatant decanted. The coagulate was solidified at once by the addition of 2–3 drops of 2% agar (Difco agar) solution cooled to ~50 °C. Previous experiments showed that the agar does not affect the specific structure of the examined material (Shomer and Mingelgrin, 1978; Shomer et al., 1982). The solidified agarose including the coagulate was cut into small cubes of about 2 mm³. The cubes were then fixed by 3.5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 6.0 at 4 °C, rinsed several times with 0.1 M phosphate buffer, pH 6.0, and postfixed by 2% OsO_4 for 2 h in the phosphate buffer at room temperature. The fixed material was rinsed several times with water, dehydrated with ethanol and acetone, and embedded in epoxy resin (Agar 100 Agar Aids England). The ultrastructure of the coagulated proteins was studied on ultrathin sections stained with uranyl acetate and lead citrate by transmission electron microscopy (TEM) on a JEOL 100CX at 80 kV.

RESULTS AND DISCUSSION

The presence of colloidal insoluble constituents of the cloud masks the visual ultrastructural and electrophoretic identifications of coagulated constituents obtained from soluble biopolymers. Hence, the protein coagulates in the present study were obtained from the SPEX. The flavedo SPEX became turbid during incubation with EPD, due to the appearance of suspended insoluble protein. The SPEX of both the whole peel and the albedo was not found

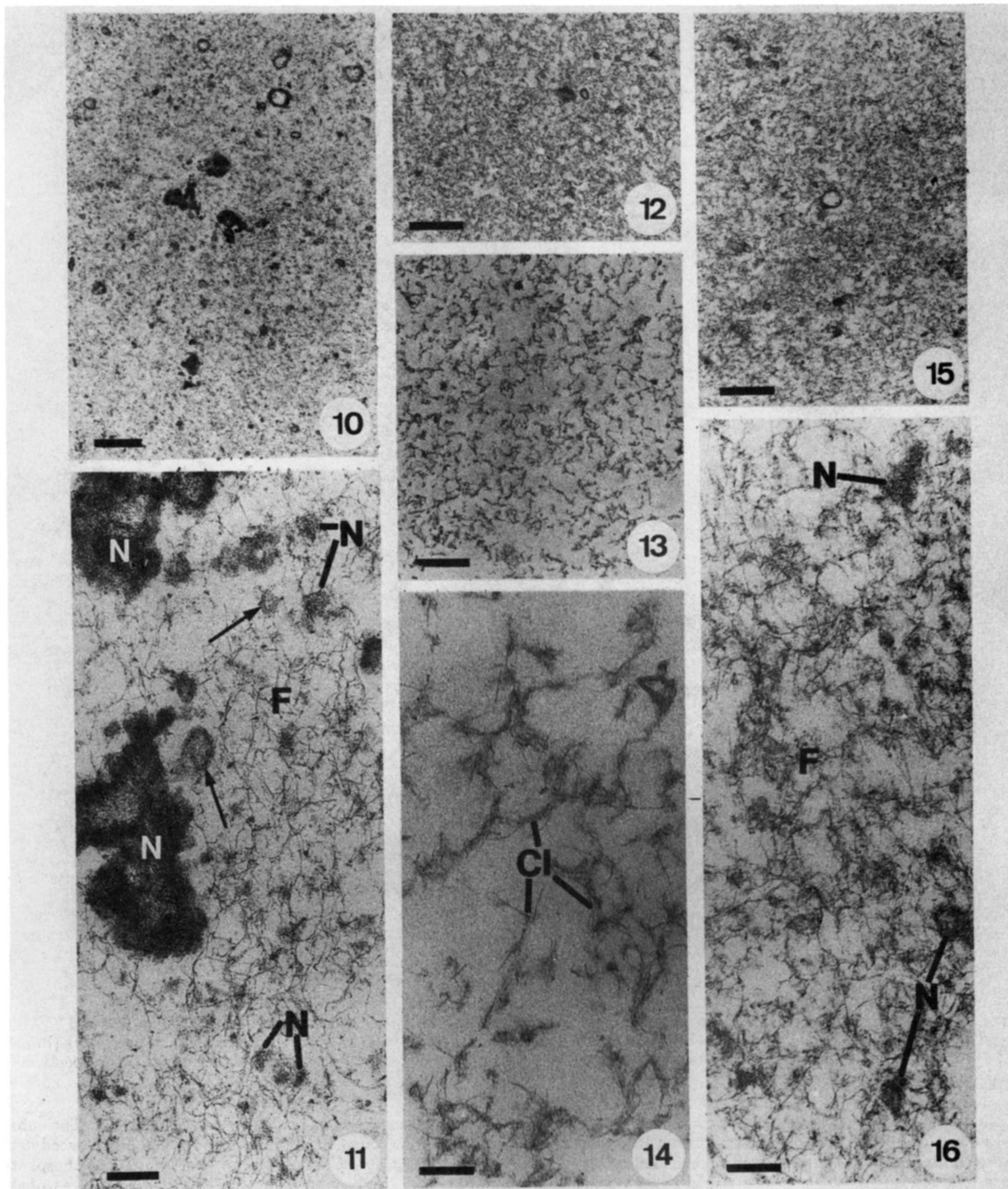

Figure 4. Ultrastructural patterns of coagulated proteins in a serum of aqueous flavedo extract. (Panels 10 and 11) Coagulate obtained as a result of enzymatic pectin degradation (EPD), at pH 4; arrow indicates double membranelike pattern (bars = 0.7 and 0.1 m, respectively). (Panels 12–14) Coagulate obtained as a result of heat coagulation at 90 °C without EPD (bars = 0.7, 0.5 and 0.1 m, respectively). (Panels 15 and 16) As panel 12, but at pH 6 (bars = 0.7 and 0.1 m, respectively). Cl, clusters of fibrillike patterns; F, fibrillike pattern; N, coagulation nuclei.

to develop turbidity during the EPD incubation. Furthermore, in all of the treatments, the albedo SPEX did not result in protein coagulation.

Electrophoresis of Proteins. The PAGE SDS patterns of coagulated proteins, obtained from various denaturation temperatures and different concentration levels, are shown in Figure 1. The coagulate that was obtained during the EPD incubation at 40 °C included a dominant protein band of 31 000 (Figure 1A). Removal of the 40 °C coagulate made it possible to obtain proteins that coagulated in the range 40–60 °C. This fraction was characterized by a pattern similar to that obtained at 40 °C coagulation, but the obtained band was denser, and additional bands of molecular weights ranging from ~14 000 to ~43 000 (Figure 1B) were seen. Removal of the 60 °C coagulate enabled the further obtaining of coagulation in the temperature range 60–80 °C, which resulted in a different profile, with a major band at about 35 000 and a relatively wide range of molecular weights between ~14 000 and 67 000 (Figure 1C). Removal of the

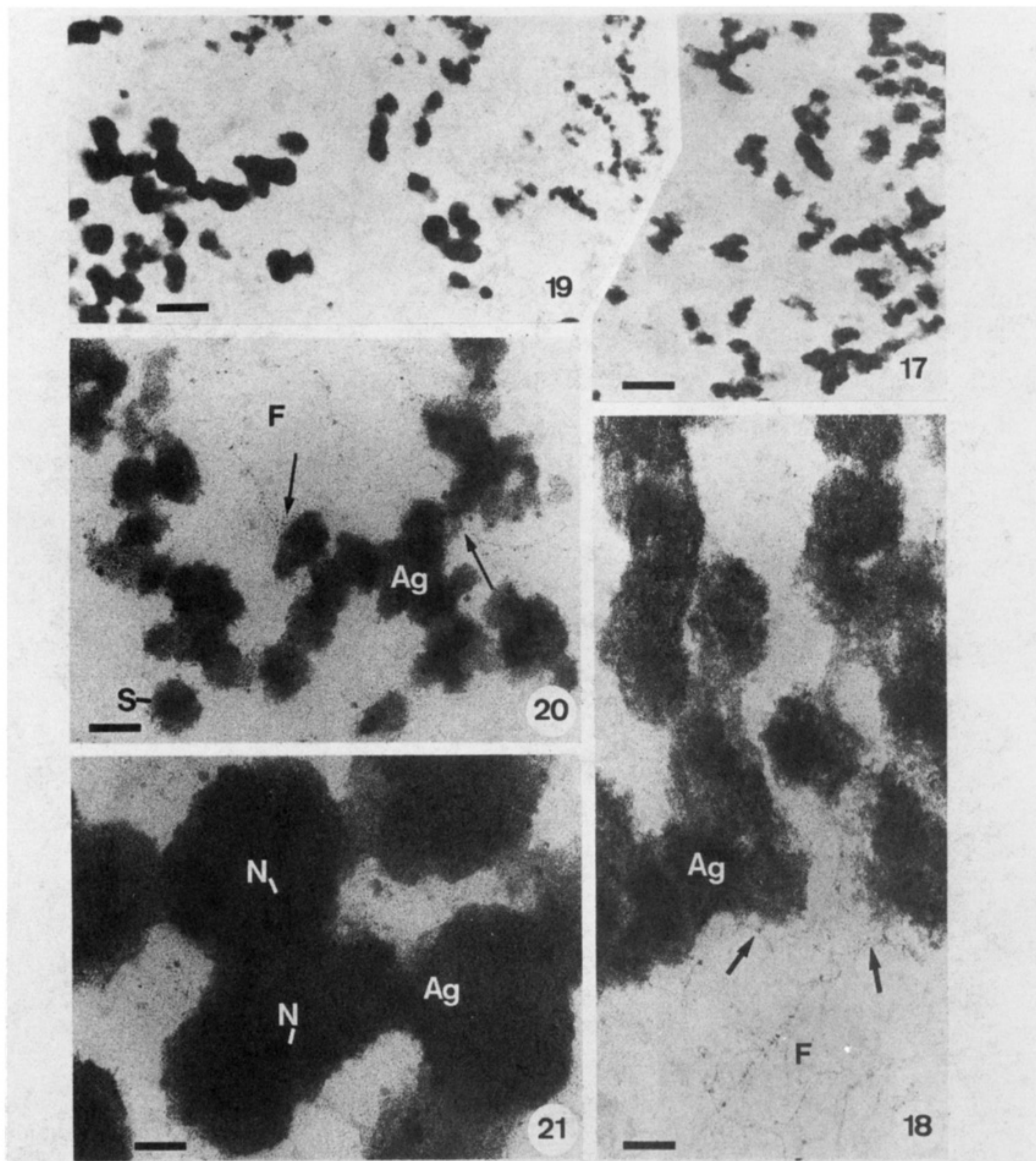

Figure 5. Ultrastructural patterns of coagulated proteins in a serum of aqueous flavedo extract. (Panels 17 and 18) Coagulate obtained as a result of enzymatic pectin degradation and heating at 90 °C at pH 4 (bars = 0.7 and 0.1 m, respectively). (Panels 19–21) As presented in panel 17, but obtained at pH 6. (Panels 20 and 21) Aggregates of granules of two sizes (bars = 0.7, 0.1 and 0.1 m, respectively). Ag, aggregate; F, fibrillike pattern; N, coagulation nuclei; S, spheroidal granule.

80 °C coagulate facilitated the obtaining of coagulation in the temperature range 80–100 °C, which was characterized by a major band at 31 000 with another strong band at ~50 000 and many minor bands (Figure 1D). These results show that it is possible with the above methods of stepwise heat coagulation to discover the presence of various proteins, each of which is sensitive to a different coagulation temperature, within the range 40–100 °C.

The coagulates obtained from all of the heat vacuum concentration levels resulted in the same electrophoretic profiles (Figure 1I–IV).

The appearance of insoluble coagulate during the EPD incubation may be due to one of two possible reasons: the sensitivity of some protein fraction to denaturation at relatively low temperatures or the presence of denatured protein in the SPEX, which becomes coagulable as a result of EPD. It is possible that some of the insoluble cloud proteins in the juice are coagulates, which also play a role in the cloud stability.

Ultrastructural Characterization. The coagulated proteins that were obtained during the EPD incubation appeared as porous spongelike patterns (panels 2 and 3 of Figure 2). Removal of the insoluble protein from the serum after the EPD enabled the obtaining of protein which was coagulated at 60 °C. This coagulate appeared as separated particles, bound to fibrillar membranelike patterns (panels 4 and 5). Removal of proteins that were coagulated at 60 °C enabled the obtaining of coagulated proteins at 80 °C. This coagulate appeared as chainlike aggregates of spheric granules associated with a fibrillar pattern (panels 6 and 7 of Figure 3). Removal of the proteins that were coagulated at 80 °C enabled the obtaining of coagulated proteins at 100 °C. This coagulate appeared in aggregates of relatively large spheroids with remnants of fibrillar patterns (panels 8 and 9 of Figure 3). The differences in the ultrastructures, dimensions, and electrophoretic patterns show a diversity in the proteins fractionated by increased coagulation temperatures. Co-

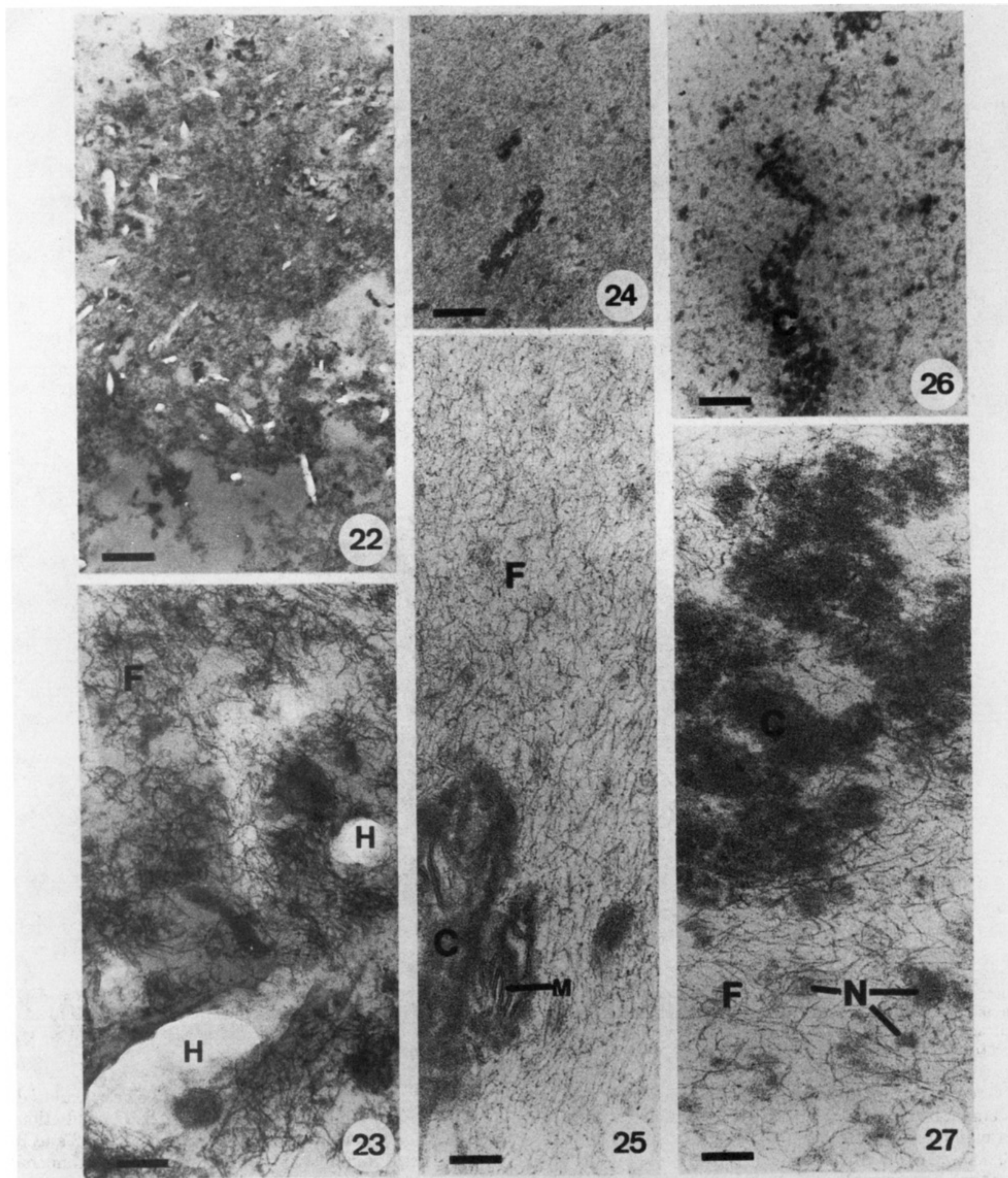

Figure 6. Ultrastructural patterns of insoluble protein obtained in a serum of aqueous peel extract as a result enzymatic pectin degradation and heat vacuum concentration. (Panels 22 and 23) Insoluble protein obtained in a 10-fold concentrate of fruit harvested in December (bars = 0.7 and 0.1 m, respectively). (Panels 24 and 25) As presented in panel 22, but of fruit harvested in March (bars = 0.7 and 0.1 m, respectively). (Panels 26 and 27) As presented in panel 24, but after heat coagulation at 90 °C for 10 min (bars = 0.7 and 0.1 m, respectively). C, conglomerate; F, fibrillike pattern; H, hesperidin crystal; M, double membranelike pattern; N, coagulation nuclei.

agulated proteins encapsulate insoluble cloud particles and reduce their stability (Shomer, 1988). It is possible that proteins of different fractions behave differently in relation to cloudiness, stability, and affinity to cloud constituents.

Coagulation in the flavedo SPEX occurred either with EPD or without EPD with heat treatment. A previous study showed that most of the heat coagulation in the SPEX of the whole peel was obtained after EPD and at pH 3.5–4.5, while above and below this pH range the coagulation decreased significantly (Shomer, 1988).

Protein coagulate in the flavedo SPEX appeared during the incubation of the EPD without heat denaturation, only at pH ~4.5 (Shomer, 1991). The structure of this coagulate was threadlike, with primary coagulating nucleations (panel 10 of Figure 4). Higher magnification

revealed a fine fibrillar lattice between the nucleations (panel 11). Heat coagulation in the flavedo SPEX was found to occur either with or without EPD at both pH 4 and 6. However, in serum without EPD, the structure of the heat coagulate at pH 4 appeared with micellar separated bodies (panels 12–14), and at pH 6 it was seen as a spongelike structure (panels 15 and 16). It appears that the soluble pectin in the flavedo SPEX did not prevent heat coagulation but resulted in a fine threadlike structure, which is supposed to represent the pectic polymer structure. Heat coagulation in the flavedo SPEX after EPD resulted in a dramatic change of the coagulate structure in comparison to the non-EPD one. At pH 4, the coagulation resulted in relatively large protein aggregates seen with remnants of fibrillar filaments (panels 17 and 18 of Figure 5). Heat coagulation of flavedo SPEX at pH 6 resulted in protein bodies of two main structural dimensions (panel 19). Higher magnification showed differences between the two structures (panels 20 and 21).

The concentration process of SPEX resulted in a turbid concentrate which was not clarified upon reconstitution to single-strength level (Shomer, 1991). The ultrastructure of these coagulated proteins appeared with relatively fine patterns including small bodies, beside hesperidin crystals (panel 22 of Figure 6), which were identified previously (Shomer et al., 1984). Higher magnification showed threadlike structure with dense bodies (panel 23). The presence of hesperidin crystals beside the coagulated protein indicates saturation of soluble hesperidin in the concentrate. Unidentified matter was found to be in association with hesperidin crystals obtained from density gradient fractionated cloud particles (Shomer et al., 1984). The hesperidin-bound matter appeared to be coagulated proteins, since its ultrastructural pattern matches that of various coagulated-protein patterns (Shomer, 1988). Concentrated SPEX obtained later in the harvest season resulted in coagulated protein of different structure (panel 24), without hesperidin crystals. Higher magnification revealed spongelike patterns and dense bodies which include membranal structures (panel 25).

It is possible that the difference between the ultrastructure of the SPEX from two harvest dates indicates differences in the pectic polymers upon which the denaturated protein was deposited. Heat treatment of the concentrated SPEX resulted in different ultrastructure from the nonheated SPEX (panels 26 and 27). These heat coagulates were seen as relatively large bodies with an amorphous pattern.

CONCLUSIONS

The electrophoretic profiles and the ultrastructure of coagulated proteins in the SPEX are affected distinctly by the coagulation conditions. Coagulation of proteins was not found in albedo SPEX, while the flavedo SPEX appeared to include the coagulable protein. In the absence of EPD, the albedo pectin hinders the coagulation of the flavedo protein. The flavedo SPEX resulted in heat coagulation either with or without EPD. It appears that the flavedo pectin allows the protein to coagulate but it suppresses the formation of large granules and aggregates. The molecular size of the pectic polymer controls the rate and the structure of coagulated proteins in citrus fruit extracts. Mutual effects between the pectin and the protein play a role in cloud stability; however, these aspects still have to be elucidated with regard to natural juices.

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