Food Structure

Volume 11 | Number 4 Article 1

1992

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FOOD STRUCTURE, Vol. 12 (1993), pp. 289-299 1046-705X/93$3.00+.00
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IDENTIFICATION OF PROTEINS AND COMPLEX CARBOHYDRATES IN SOME COMMERCIAL LOW-FAT DAIRY PRODUCTS BY MEANS OF IMMUNOLOCALIZATION TECHNIQUES

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Abstract

Macromolecular assemblies of proteins and hydrocolloids in low-fat dairy products contribute to the structure and texture of these foods. Immunolocalization techniques were used to identify ß-lactoglobulin, casein, bovine whey proteins, and egg albumin in low-fat frozen desserts, reduced fat process cheese, and salad dressings. Simplesse® 100 protein particles were examined and characterized by these methods and compared to naturally occurring protein structures in the low-fat foods. Hydrocolloid identification in low-fat salad dressing was accomplished by complexing cellulase and hemicellulase with colloidal gold and applying the probe as a pre-embedding step, on sections of embedded specimens or on material dried on grids. This enzyme-gold method may be generally applied for identification of cellulose and hemicellulose in complex food systems. Immunolocalization techniques employed in this study should prove useful in probing the fundamental structure-texture relationship in foods.

Key Words: Immunolocalization, Simplesse® 100, transmission electron microscopy, milk proteins, structure, casein, ß-lactoglobulin, microcrystalline cellulose, immunogold.

Initial paper received June 16, 1992
Manuscript received December 1, 1992
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Introduction

Over the past ten years, the food industry has developed innovative protein- and carbohydrate-based products to respond to consumer demands for healthy alternatives to fat, sugar, and cholesterol. In the United States, approximately 37% of caloric intake is derived from fat; recommended levels are 30% or less of total calories derived from fat and no more than 10% from saturated fat (U.S. Department of Health and Human Services, 1988).

Natural dairy products include foods with low fat content, such as skim milk, as well as high-fat products such as super premium ice cream, cheese, and dairy-based salad dressings (Table 1). The food industry has developed a broad range of natural and synthetic fat substitutes with textural attributes of fat and oil as replacements in foods for individuals who wish to reduce dietary fat and calories (Table 2). Acceptance and success in the marketplace require a safe substitute closely matching the traditional item in sensory attributes such as appearance, flavor, and taste, as well as imparting the structural components of texture. In the development of reduced-fat or fat-free food products, the important structural contributions of fat have to be replaced by fat substitutes. Visualization of the structure of the fat substitute in low-fat food systems and the effect of processing on the structure will allow for a better understanding in the selection of the fat substitutes and creation of low-fat products to closely match the texture of the full-fat counterpart.

At the microscopic level, the texture and stability of food products is related to the supramolecular organization of the food components. In most cases, the identification of the composition of components in a complex mixture by simple morphology and size is not possible. The processing steps necessary to produce a stable, wholesome product alter the structure and dimensions of components in a complex food system such as cheese, frozen desserts, and other dairy products. Examples of processing-induced changes on the structure of food components may be drawn from the literature on casein

TABLE 1. Fat content of some common dairy foods*

Food description	% Fat
Milk, whole	3.5
Milk, skim	0.1
Yogurt made from partially skimmed milk	1.7
Yogurt made from whole milk	3.4
Cream, half-and-half	11.7
Light whipping cream	31.3
Heavy whipping cream	37.6
Ice cream, regular	10.6
Ice cream, premium	16.1
Ice milk	2.4
Natural cheeses:	
Cheddar	32.2
Swiss	28.0
Cottage, creamed	4.2
Pasteurized processed cheese	30.0

*Data compiled from the U.S. Dept. Agriculture's Handbook of the Nutritional Content of Foods (1975).

micelles: in raw uncooled milk, casein forms approximately spherical particles of 0.02-0.3 μm in diameter (Walstra and Jenness, 1984; Holt, 1985). Normal processing procedures such as acidification (Calapaj, 1968) and ultra high temperature sterilization (Freeman and Mangino, 1981; Harwalkar *et al.*, 1989) led to the aggregation and enlargement of the micelles as well as the production of a class of particles smaller than casein micelles.

General identification of food proteins can be accomplished by means of fluorescent dyes such as Acridine Orange (Yiu, 1985; Modler *et al.*, 1989) or Eosin Y (Heathcock, 1985). Few authors have identified specific proteins in food as components of unique structures: Mottar *et al.* (1989) illustrated the heat-dependent precipitation of ß-lactoglobulin in the form of filaments on the surfaces of casein micelles in yogurt; Schmidt and Buchheim (1992) localized κ-casein on the surface of casein micelles in milk cryosectioned according to the procedure of Tokuyasu (1980).

In the present study, specific proteins in several food products were identified using immunolocalization techniques. The foods were embedded at low-temperature in a resin and thin sections were then reacted with gold-labeled antibodies. Developed over the last ten years, low-temperature embedding techniques have proven invaluable in the study of protein interactions in numerous biological systems (Armbruster and Kellenberger, 1986; Carlemalm and Villiger, 1989). The polar resin Lowicryl K4M is normally used for immunolocalization based on the theory that a polar embedding medium would preserve native structure as compared to apolar, hydrophobic resins. As a case in point, the adverse effects of embedment in non-polar resins on casein submicelle structure have been documented by Schmidt (1982). Horisberger and Vauthey (1984a) demonstrated the application of low-temperature embedding methods to investigate the distribution of κ-casein in casein micelles. Our paper constitutes the first comprehensive application of low-temperature embedding and immunolocalization techniques to the study of food ultrastructure.

The colloidal gold probes employed in this study are universally accepted as superior, electron-dense markers for identification of proteins and sugar residues. In related techniques using gold, lectin-gold probes have been used to identify κ-casein by means of a galactose-specific *Ricinus communis* lectin probe either in a bridging network between casein micelles (Horisberger and Vonlanthen, 1980) or uniformly distributed throughout most micelles (Horisberger and Rouvet-Vauthey, 1984). In the study of Schmidt and Both (1982), α-, β-, or κ-caseins labeled with gold particles were allowed to reassemble as micelles to determine the distribution of the three forms of casein in the micelle. The adsorption isotherm of ß-lactoglobulin onto gold particles and the stability of the particles as a function of the number of molecules adsorbed per particle was investigated by Horisberger and Vauthey (1984b). In addition to immunolocalization procedures used in this study, enzyme-gold probes were also employed to differentiate complex carbohydrate hydrocolloids in food products.

The demand for foods with low or no fats, new foods with unique textures, and foods containing no additives has created a renewed interest in understanding the relationship of structure and texture of foods and the effect of processing-induced changes on these relationships. The results of this study show that these methods are extremely useful in the identification and organization of components in complex food systems and can contribute to the understanding of food structure and texture relationships.

Materials and Methods

Food products

The samples of skim milk, low-fat Ranch and full-fat Thousand Island salad dressings, and two low-fat frozen dessert products examined here were obtained from commercial sources. Compositions and ingredients of the commercial products include the following: dehydrated egg protein, alginate, pickles, soybean oil, sugar,

Table 2. Classification of fat substitutes based on their possible fat replacement mechanisms in foods.

Fat Replacement Mechanism	Fat Substitutes
FAT EXTENDERS: *Hydrocolloids which bind water or form gels and develop a slippery-feel. Emulsifiers which extend perception of fats.*	Natural and modified starches, fibers, maltodextrins, carboxy methyl cellulose, cellulose ethers, carrageenans, guar gum, xanthan gum, pectin and gelatin. N-Lite® and Stellar® (starch based), Slendid® (pectin based), Paselli SA2, Maltrin® M-040 (maltodextrin), Oattrim (maltodextrin and beta glucan) are some trade names of products in this category. Food grade emulsifiers - mono- and di-glycerides, esters of fatty acids and polyvalent alcohols are used in combination with other ingredients as fat extenders.
FAT MIMETICS: *Discrete protein/carbohydrate particles which "mimic" the properties of fats.*	Simplesse® - Microparticulated protein; Avicel® - Microcrystalline cellulose and CMC; Lita® - Microparticulated zein; Trailblazer® - Particulated protein/xanthan complex and Rice*Complete® - Amylase treated rice starch and protein complex; Raftiline® - Inulin; Fibercel® - Yeast beta glucan.
SYNTHETICS: *Chemically constructed molecules which simulate the properties of natural fats and oils*[1].	Medium chain triglycerides - Caprenin[2]; Jojoba oil derivatives; Polyol fatty acid polyesters - Olestra[3]; Esterified propoxylated glycerol; Dialkyl dihexadecyl malonate; Polyorganosiloxanes (silicones); Trialkoxycitrate; Trialkoxytricarballyate

[1]Need FDA approval; [2]Petitioned FDA for GRAS; [3]FDA approval pending for use as a frying oil.

vinegar, and microcrystalline cellulose in the full-fat Thousand Island salad dressing; skim milk, sour cream, cultured skim milk, and cellulose gel in the low-fat Ranch salad dressing; skim milk, milk, cream, egg yolk, cellulose gum, carob bean gum, guar gum, and carrageenan in the two low-fat frozen desserts. Simplesse® 100 and process cheese with Simplesse® 100 were produced at our facility per standard protocols (Singer *et al.*, 1988; Singer and Dunn, 1990).

Electron microscopy

Several of the samples were stabilized for embedment according to the procedure of Heathcock (1985): skim milk, two frozen dessert samples, Simplesse® 100, and two salad dressings were mixed 1:1 with 2% molten agar and allowed to solidify. The agar-encased samples as well as cheese samples were cut into 0.5 mm cubes and fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for one hour at room temperature. An alternative method for the liquid samples was to mix equal volumes of sample and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for one hour at room temperature, then centrifuge the preparations in an International Equipment Company clinical centrifuge at 7100 rpm for 5-10 minutes. The samples were washed twice for 10 minutes in phosphate buffer, dehydrated in a graded series of ethanol (30%, 50%, 70%, 95%, 100%, 1 hour for each step) followed by infiltration with Lowicryl K4M at -20 °C in a freezer, then embedded and polymerized in Lowicryl K4M at -35 °C for 12 hours according to a protocol described in greater detail by Armbruster and Kellenberger (1986). The blocks were further hardened under ultraviolet (UV) light for two days at room temperature. Thin sections were cut with a diamond knife on a Leica Ultracut E ultramicrotome, left unstained or were stained 20 minutes with saturated aqueous uranyl acetate, and examined in a Hitachi H-600 transmission electron microscope operated at 50 kV.

Cellulase and hemicellulase gold probes

Cellulase from *Trichoderma viride* and hemicellulase from *Aspergillus niger* were purchased from Sigma Chemical Company and colloidal gold suspensions of 5 nm and 30 nm Aurobeads were purchased from Amersham International. Several excellent references on colloidal gold preparation and coating procedures are available, most notably those contained in the volumes edited by Hayat (1989).

The enzymes were complexed to colloidal gold by published techniques (Bendayan, 1985; Berg *et al.*, 1988): the pH of 10 ml of the 5 nm gold suspension was adjusted to 4.5 for the cellulase probe and 10 ml of the 30 nm gold suspension was adjusted to 5.5 for the hemicellulase probe. To the appropriate gold suspensions was added 1 mg of enzyme dissolved in 0.5 ml distilled water. After stirring for 5 minutes, the enzyme-gold complexes were stabilized by the addition of 1 ml of 0.5 mg/ml polyethylene glycol (Carbowax, molecular mass 20,000 daltons, i.e., 20 kDa). The hemicellulase-gold was pelleted at 18,000 xg for 30 minutes and the

cellulase-gold was pelleted at 30,000 xg for 1 hour. The supernatant was removed from the tube and the gold pellet resuspended in 10 ml of buffer at the appropriate pH for each enzyme. After a second centrifugation at the same speed, the supernatants were removed and the pellets suspended in 1 ml of buffer containing 0.5 mg polyethylene glycol per ml. The enzyme-gold probes were applied as a dilute, buffered suspension to thin sections of the embedded samples and also to hemicellulose dried on grids; as a demonstration of pre-embedment labelling, the probes were mixed with the low-fat Ranch salad dressing sample prior to embedding. Section surfaces were exposed to the enzyme-gold probes for 15-30 minutes. The low-fat Ranch salad dressing was incubated for 4 hours with the probe, then centrifuged three times for 15 minutes at 7100 rpm in a clinical centrifuge, each run being followed by a buffer rinse before further processing for microscopy. As a labelling control, sections of the low-fat Ranch salad dressing were incubated with the hemicellulase-gold probes.

Antibody preparation

A polyclonal antibody to ß-lactoglobulin used in this study was purchased from Nordic Immunological Laboratories. The antibody was affinity-purified in our laboratory using an Avid AL resin from BioProbe International. The Avid AL resin contains a synthetic mimic of protein A and is selective for IgG immunoglobulins. The specificity of the antibody to β-lactoglobulin was analyzed by means of Western blots according to the method of Laemmli as reported by O'Farrell (1975). Isolated milk caseins and whey proteins were loaded in wells of a stacking gel on top of a 12.5% acrylamide slab gel. Electrophoresis of proteins was carried out for 4 hours. Protein bands were transferred from the slab gel and covalently bound to nitrocellulose paper for Western blotting. The β-lactoglobulin antibody reacted with protein bands specific to β-lactoglobulin on the blots. No cross-reaction of antibody with any other bovine milk proteins was observed. A comparable antibody examined by Monti *et al.* (1989) using a competitive radioimmunoassay showed cross-reactivity only with a 20 kDa fragment from the N-terminus of human lactoferrin.

Polyclonal antibodies to bovine whey protein and egg albumin were purchased from Sigma Immunochemicals, casein antibodies were provided by R. Goldman's laboratory (Northwestern University Medical School, Chicago, IL); these antibodies were used without further purification at concentrations determined by test labelling studies (bovine whey protein, 1/50; egg albumin, 1/25; casein, 1/25; ß-lactoglobulin, 1/100). A suspension of 15 nm diameter protein A-gold was purchased from Amersham International.

Figure 1. Microparticulated protein in process cheese assumed a spheroidal form with a distinctive core structure appearing in one particle. Bar = 1 μm.

Figure 2. Shown at the same magnification, casein micelles in skim milk were clearly distinct in size from microparticulated protein particles. Bar = 1 μm.

Figure 3. ß-Lactoglobulin was identified as a component of Simplesse® 100 in this labelling study. The protein A-gold probes are 15 nm in diameter. Bar = 1 μm.

Figure 4. In a control labelling experiment, no labelling reaction occurred when the sections of microparticulated protein were exposed to the casein antibody followed by the suspension of 15 nm diameter protein A-gold particles. Bar = 1 μm.

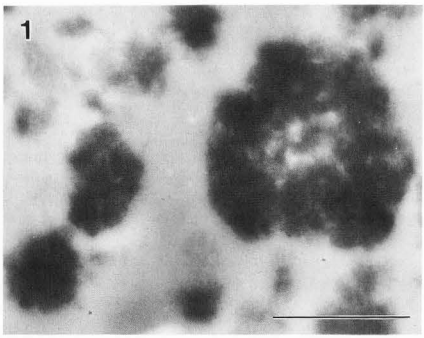
Figure 5. ß-Lactoglobulin was also identified as a component of extended protein gels and isolated dense aggregates in process cheese (arrowhead). The protein A-gold probes are 15 nm in diameter. Bar = 1 μm.

Immunolocalization

A standard protocol for antigen localization on thin sections of resin-embedded tissue developed from Roth (1983) was followed for all sectioned samples:

1. Preincubate grids in drops of 1% fish gelatin (Sigma Chemical Co.) in phosphate-buffered saline (PBS) for 5 minutes at room temperature to block nonspecific binding sites.
2. Transfer grids to drops of antibody solution at the appropriate dilution and incubate for 1-12 hours at room temperature.
3. Wash sections with a stream of PBS from a spray bottle and place on drops of PBS for 5 minutes.
4. Incubate grids on drops of protein A-gold suspension diluted 1/25-1/50 for 1 hour at room temperature in a humid environment.
5. Spray-wash grids with PBS for 30 seconds, then wash with distilled water. Dry grids with filter paper, then view stained or unstained as above.

To test the specificity of the immunolabelling protocol, two controls were run: **1)** sections of Simplesse®, which did not contain casein, were reacted with casein antibody followed with protein A-gold as a negative control (as shown later, Figure 4), and **2)** the primary antibody step was deleted (as shown later in Figure 9) to demonstrate the absence of reaction between the gold probes and the sectioned samples.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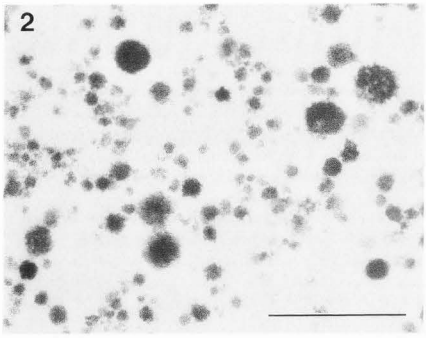
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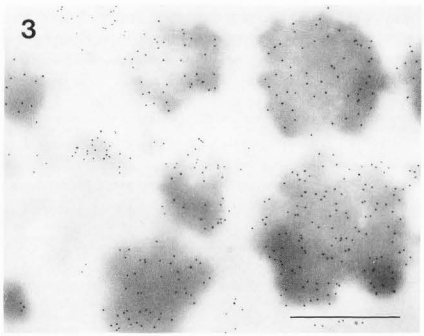
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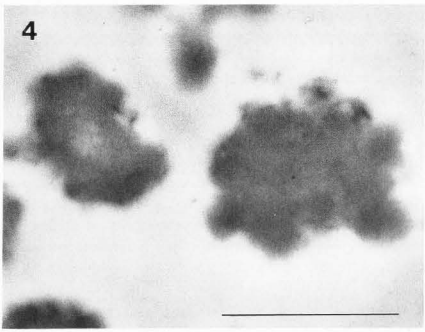
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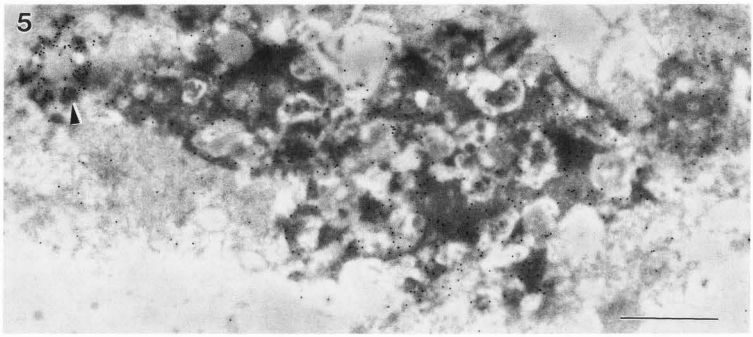
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Results

Simplesse® 100 is produced by microparticulation of whey proteins; the protein mixture is simultaneously heated and sheared under controlled conditions of protein denaturation and gelation to produce microparticles of protein (Singer *et al.*, 1988). The typical structure of these microparticulated protein particles is illustrated in Figure 1. In this stained sample, the particles are spheroidal with diameters ranging from 0.1-2.0 μm. Microparticulated protein can be easily identified by size and appearance when compared to a system such as casein micelles in skim milk (Figure 2). An immunolabelling study of microparticulated protein particles illustrated in Figure 3 identified the whey protein ß-lactoglobulin as a major component of the particles. A control labelling experiment is shown in Figure 4: no labelling reaction occurs when similar sections are exposed to the casein antibody followed by protein A-gold.

Harwalkar and Kaláb (1988) and Kaláb *et al.* (1991) implicated ß-lactoglobulin or whey proteins as necessary components in the formation of protein matrices and core-and-shell structures in acid-heat-induced milk gels. In their study, the identity of whey proteins or ß-lactoglobulin in the core-and-shell structures was not confirmed. During our examination of immunolabeled process cheese samples, structures which were similar in appearance to those reported for the heat-induced gels of casein micelles in milk dialyzate were observed. ß-Lactoglobulin was found to be distributed throughout the protein network when reacted with the ß-lactoglobulin antibody (Figure 5) and associated with isolated dense aggregates (Figure 5, arrowhead).

Frozen desserts are somewhat difficult to embed due to the high water content and absence of extended structural continuity to hold the sample during processing. The use of agar in the Heathcock (1985) procedure does add stability to the samples but cannot preserve structural interactions present in the fluid state. A phenomenon common to all section-surface labelling experiments was seen in Figure 6, in which casein was localized in an unstained section of one low-fat frozen dessert. Only those regions of the particles exposed on the section surface reacted with antibody and became labeled with the electron-dense protein A-gold probes. Proteins may also form coatings around other discrete particles in contrast to the extended heat-induced gels discussed above: in the other low-fat frozen dessert, a comparison of the labelling pattern of casein in Figure 7 with that of the ß-lactoglobulin label in Figure 8 suggests that the processing method used for this frozen dessert promoted the formation of a complex of a surface component such as κ-casein with ß-lactoglobulin. A control preparation with protein A-gold alone, shown in Figure 9, illustrates the low level of background labelling with this technique.

Figure 6. Immunolocalization of casein in a frozen dessert illustrated one of the limitations of labelling on a section surface. Portions of the casein structures could be within the section and thus not available for labelling. The protein A-gold particles are 15 nm in diameter. Bar = 1 μm.

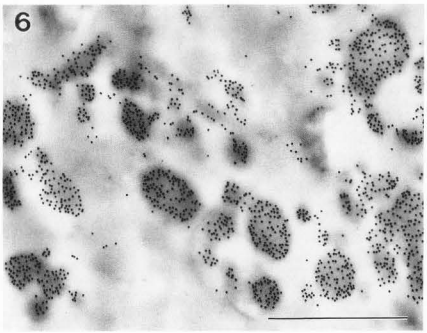
Figure 7. Casein was evenly distributed in the electron-dense protein particles in this low-fat frozen dessert. The protein A-gold particles are 15 nm in diameter. Bar = 0.5 μm.

Figure 8. ß-Lactoglobulin was localized as a coating or filamentous network on the surface of casein-rich protein bodies and also surrounding them in the low-fat frozen dessert shown in Figure 7. The protein A-gold particles are 15 nm in diameter. Bar = 0.5 μm.

Figure 9. A control preparation of protein A-gold reacted with the section surface of the low-fat frozen dessert without a previous antibody treatment, demonstrating a low level of non-specific labelling. The protein A-gold particles are 15 nm in diameter. Bar = 1 μm.

Figure 10. An unstained section of embedded low-fat Ranch salad dressing contained microcrystalline cellulose (C), titanium dioxide crystals (T), and some amorphous protein aggregates (arrowheads). Bar = 1 μm.

Salad dressings, like the frozen desserts, presented some sectioning difficulty (Figure 10); inorganics such as titanium dioxide and extended solids such as microcrystalline cellulose tended to tear out of the section. Both the low-fat Ranch and full-fat Thousand Island samples contained protein in the form of networks which extended several micrometers in diameter. Egg albumin was identified as a component of the network present in the full-fat Thousand Island preparation (Figure 11) and bovine whey protein was identified in the network of the low-fat Ranch salad dressing (Figure 12). The close similarity of the networks is evident from the micrographs. Such networks of protein strands were similar in appearance to matrices in process cheese using polyphosphate as the emulsifying agent (Carić *et al.*, 1985). In Figure 13, positive identification of microcrystalline cellulose in the full-fat Thousand Island salad dressing was achieved by reacting the 5 nm-diameter cellulase-gold probe on the section surface. Alternatively, the premixture of probe and sample before embedment (Figure 14) facilitated binding of the cellulase-gold to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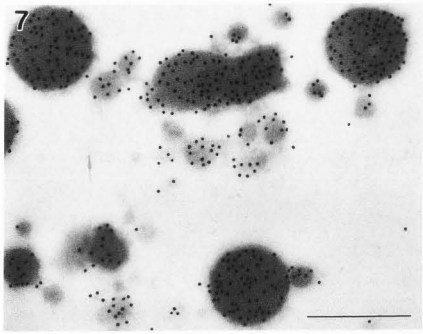
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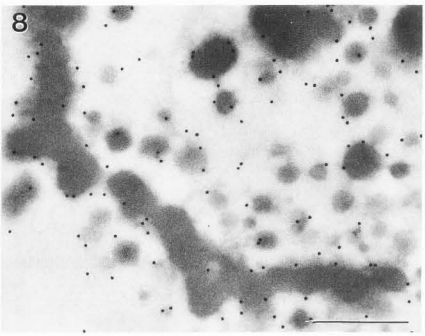
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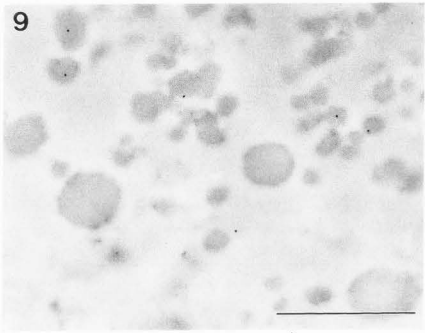
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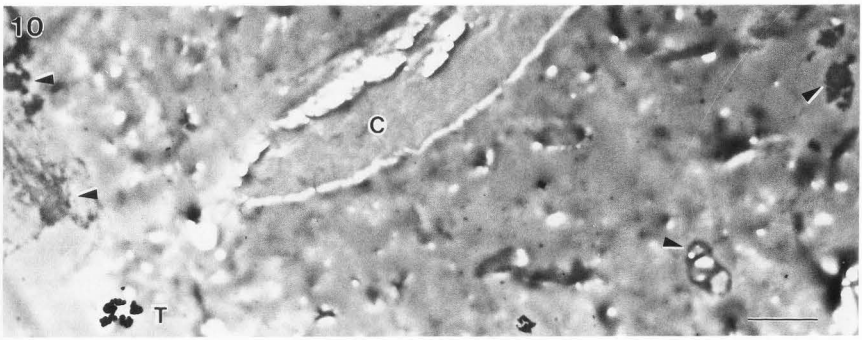
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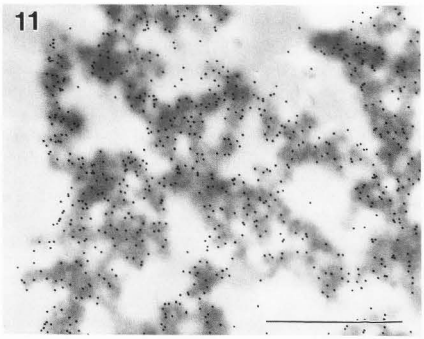

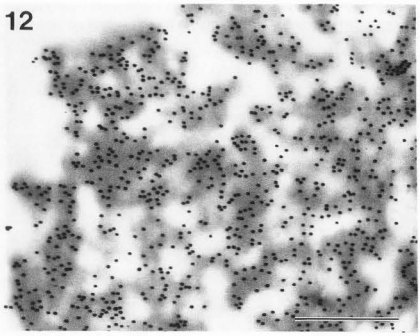

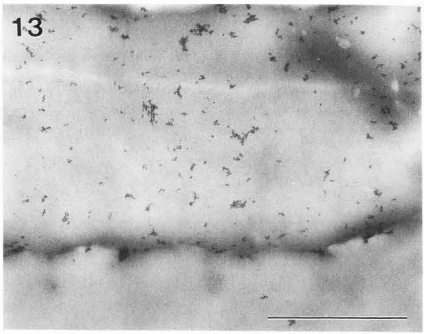

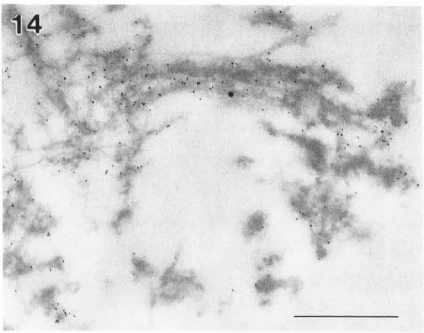

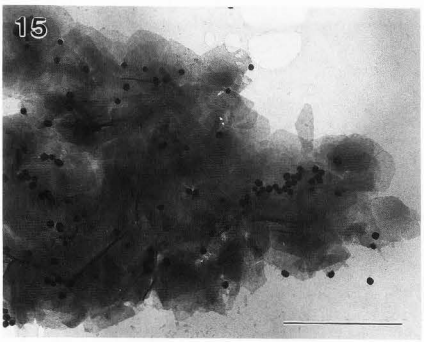

extended, diffuse cellulose networks which were present in the low-fat Ranch salad dressing. The hemicellulase-gold probes did not label the cellulose networks in this latter salad dressing. Carbohydrate gums extracted from plant sources contained high concentrations of hemicellulose which could be identified using the 30 nm-diameter hemicellulase-gold probes as shown in Figure 15.

Discussion

The immunolocalization studies presented here demonstrate that proteins and complex carbohydrates in foods form various structures and matrices having unique ultrastructures dependent on a variety of processing procedures. Application of low-temperature embedding or cryosectioning methods (Tokuyasu, 1980) in conjunction with immunolocalization could include the following: investigations of the distribution of components at

Figure 11. Egg albumin was present in the full-fat Thousand Island salad dressing in the form of an extended protein network. The protein A-gold particles are 15 nm in diameter. Bar = 1 μm.

Figure 12. Bovine whey protein formed extended protein networks in the low-fat Ranch salad dressing similar to those in the previous figure. The protein A-gold particles are 15 nm in diameter. Bar = 0.5 μm.

Figure 13. Microcrystalline cellulose was identified in the full-fat Thousand Island salad dressing using cellulase-coated, 5 nm diameter colloidal gold probes. The probes were applied to thin sections of the embedded samples. Bar = 1 μm.

Figure 14. Cellulose was also present in the form of loose bundles of filaments in the Low-fat ranch salad dressing. Cellulase-gold probes (5 nm diameter) were mixed with the salad dressing preparation in a pre-embedding step. Bar = 0.5 μm.

Figure 15. Hemicellulase-coated gold probes (30 nm diameter) reacted with carbohydrate gums dried and supported on carbon-coated Formvar film. Bar = 0.5 μm.

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various stages of food production and aging, and correlating the data with sensory and physical properties, characterization of the different sources and forms of milk proteins used as ingredients, analysis of unique structures such as core-and-shell inclusions, and the identification of proteins in dairy products which elicit allergic reactions. In addition to ultrastructural investigations, complimentary studies utilizing colloidal gold labelling systems can also be undertaken at the light microscopic level by means of silver enhancement methods (Danscher and Nõrgaard, 1983; Holgate *et al.*, 1983; Lucocq and Roth, 1985). These light-stable preparations can facilitate specific identification of proteins and hydrocolloids in a larger sampling volume without the drawbacks of fluorescence probes, namely quenching and low signal-to-noise ratios.

Cheese, yogurt, and several other dairy foods can be described as filled or composite gels which consist of a network of milk proteins in which a "filler" or a dispersed phase is present within the network. The dispersed phase can be air bubbles, water or oil droplets, or crystals of ice or fat. The structure of the network formed and the size, shape, volume fraction, deformability, and filler-network interaction determine the mechanical properties and texture of these products (Morris, 1985). In the development of reduced-fat and fat-free products, the important contribution of fat as a dispersed phase or as a crystalline network has to be replaced by a fat substitute. Fat extenders and fat mimetics (Table 2) are based on proteins or polysaccharides and are capable of forming complex networks or discrete particles in food systems. The structural knowledge of these networks as well as the dispersed phases within the networks is important in the development of reduced-fat or fat-free products. There is a close similarity between the egg albumin network in a full-fat salad dressing and the whey protein network in the low-fat salad dressing as shown in Figures 11 and 12. The presence of networks in these products contribute to the flow properties and texture of the products and modify them.

Macromolecular assemblies of milk and egg proteins and complex carbohydrates in fresh and frozen dairy products directly contribute to the structural and textural properties of these food products. This contribution may be positive or negative: on the positive side, the replacement of fat with microparticulated proteins in the 0.1-2.0 μm range in frozen dairy desserts preserves the smooth texture while lowering the caloric content of the food. Negative sensory results are obtained when heat coagulation forms large protein particles which impart a rough mouthfeel. In the experiments of Mottar *et al.* (1989), the texture of yogurt was impaired when heat-denatured whey proteins precipitated onto the surface of casein micelles, resulting in a product with unacceptable consistency. Compact protein particles 10-100 μm in diameter, which formed during the pasteurization of hot-pack cheese spread made from insufficiently heated milk, imparted a gritty texture to the spread (Modler *et al.*, 1989).

General heat processing of proteins in complex food products such as process cheese or frozen desserts does not produce microparticulated proteins. Milk drying processes, such as spray and roller drying, promoted the formation of particles an order of magnitude or more larger than microparticulated protein (Carić and Kaláb, 1987); spray-dried whey powder added to control process cheese formed structureless electron-dense regions of varying size in the protein matrix of the product (Kaláb *et al.*, 1991); process cheese food subjected to hot melt conditions contained electron-dense non-spherical precipitates (Kaláb *et al.*, 1987). Our study shows that ß-lactoglobulin is a natural component of heat-induced protein gels which form as amorphous precipitates in dairy products. This result supports Harwalkar and Kaláb's (1988) hypothesis that ß-lactoglobulin is an integral component of protein gel dispersions such as core-and-shell structures as well as filamentous appendages appearing on casein micelles in

yogurt after heat treatments (Davies *et al.*, 1978). Other milk proteins, such as casein, can precipitate to form chains and clusters of micelles with varying amounts of compaction (Modler *et al.*, 1989). Heat treatment can also cause precipitation of proteins to form coated, biphasic particles as seen in this study and described by Mottar *et al.* (1989).

Although the low temperature embedding procedures employed here have been shown to preserve protein antigenicity (Armbruster and Kellenberger, 1986), food components are subject to denaturing conditions during processing, including heating and the addition of acidulants. It could be argued that only a fraction of the protein in the mixture is in some near-native conformation to permit antibody recognition. The point is illustrated in a report of Breton *et al.* (1989): polyclonal antibodies to native ovalbumin did not bind to ovalbumin-coated microtitre plates; antibodies specific for heat-denatured ovalbumin reacted with the protein which had denatured during interaction with plastic microtitre plates. In light of these findings, studies which attempt quantitative evaluations should include trials of antibodies against native and denatured antigens and a full battery of control conditions.

Avicel® and other types of microcrystalline cellulose are used to stabilize emulsions or suspensions, increase the viscosity of a product, and improve the water-binding capacity of the food. Identification of polysaccharide networks using transmission electron microscopy has been difficult due to a limitation of stains specific for polysaccharides. The enzyme-labelling technique described in this study reveals that hydrocolloids can assume several structural forms in food. The large, rod-shaped particles and fibrous networks could impart different organoleptic properties to a product. The enzyme-gold methods outlined here would be useful in tracking the stability of microcrystalline cellulose and hemicellulose gels over the shelf life of products and the interaction of these thickening agents with proteins, salts, and micronutrients. Morris (1985) highlights the need to visualize the structures of complex networks formed in food gels and the need to understand the interaction between the components so that the mechanical properties of the networks and the components can be described. In the continuing effort to engineer new foods with novel textures, the immunocytochemical techniques presented in this study should contribute to an understanding of the structure of foods and facilitate the creation of foods with a wide range of structure and texture.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers.**