Whey Protein Edible Film Structures Determined by Atomic Force Microscope

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ABSTRACT

Atomic force microscopy was used to study edible films produced from whey proteins. The films were imaged under ambient conditions with no special sample preparation. Low resolution imaging of areas from 10 μm to 150 μm on a side was performed in the contact mode. Higher resolution scans of 350 nm to 2,700 nm required use of the noncontact imaging mode. Features about the same size as the primary protein in whey, beta-lactoglobulin (7 nm), were identified in the film samples. Molecular aggregates in the range of 1 μm, reported in other studies using transmission electron microscopy of whey protein gels, were combined in results from atomic force microscopy.

INTRODUCTION

AN EDIBLE FILM IS A THIN, CONTINUOUS LAYER OF EDIBLE MAterial coating or a film placed between food components to provide a barrier to mass transfer (Mahmoud et al., 1993, Guilbert, 1986) (e.g., moisture, oxygen, lipids, solutes). Food spoilage from oxidation and respiration reactions could potentially be controlled by covering with edible films (McHugh and Krochta, 1994). They can function as a carrier of food ingredients and additives, and/or improve food handling. In addition, edible coatings and films have the potential to reduce package complexity, waste, and cost of packaging systems (McHugh et al., 1993; McHugh et al., 1994).

Film formulations consist of three primary components: a high molecular weight polymer [lipids, polysaccharides and proteins (Krochta, 1992)], a plasticizer, and a solvent. Formulation directly affects the nature and properties of the film. The plasticizer must be miscible with the polymer and both plasticizer and polymer must be soluble in the solvent (Mahmoud and Savello, 1992). A primary raw material being evaluated for use in production of edible films is whey protein.

Whey is the soluble fraction of milk separated from the casein curd during cheese manufacturing (Aguilera, 1995). It contains about 20% of the total protein in milk (Maynes and Krochta, 1994). About 40% of liquid whey produced is not utilized, generating large amounts of waste (Guilbert, 1986). There isconsiderable interest in applications for whey proteins (McHugh et al., 1994). Production of coatings and films from whey would allow its use in a new class of end products (Mahmoud and Savello, 1993).

β-Lactoglobulin is the most abundant protein in whey (57%). At room temperature (~23°C) and at pH 5 to 7 β-lactoglobulin exists as a dimer consisting of two identical subunits, each of molecular weight 18,400 g/mole. Above 40°C they dissociate into monomers, which is the most important species of β-lactoglobulin during heat denaturation. β-Lactoglobulin contains two disulfide bridges and one free thiol group Which can interact to form new disulfide bonds. α-Lactalbumin, the second most important protein in whey (19%), is the smallest and most heat resistant (deWit and Klarenbeck, 1984). Other notable proteins in whey are bovine serum albumin (7%), immunoglobulins (13%), and specific polypeptides (4%).

Transglutaminase has been used to catalyze the covalent polymerization of whey proteins. Crosslinking polymerization of concentrated whey protein solutions induced gelation in the protein, plasticizer, and solvent mixture. Films have been produced by casting the gelled whey protein solutions, followed by air drying (Mahmoud and Savello, 1992). The high cost of transglutaminase has limited its use in edible films (Mc Hugh *et al*., 1994).

In the absence of a catalyst, heat treatment is required for the formation of edible films based on whey proteins. The heat facilitates formation of intermolecular disulfide bonds by thiol-disulfide interchange and thiol oxidation reactions, which results in water insoluble films. The minimum heat treatment required for formation of intact whey protein films is 75°C for 30 min. This is very near the denaturation temperature (78°C) for β-lactoglobulin, the major component (McHugh et al., 1994).

Properties of whey protein films, such as plasticity or permeability, can vary with small variations in chemical content. This sensitivity can be applied to provide optimum properties. It is generally desirable that films exhibit sufficient mechanical strength and plasticity to maintain integrity under normal food packaging and handling conditions. They should also provide sufficient resistance to oxygen diffusion to slow oxidation. Some applications require that the film prevent moisture transport. Achieving optimum properties may involve compromising between desirable and undesirable characteristics. For example, plasticizers reduce brittleness and increase flexibility by decreasing attractive intermolecular forces. However, plasticizers can also alter characteristics such as permeability, toughness, strength, and tear resistance (Mahmoud and Savello, 1992).

Reported studies on whey protein edible films have mostly been directed at their fabrication and measurement of bulk properties. Our objectives was to conduct a detailed structural examinationof such films using atomic force microscopy (AFM). Food product samples have traditionally been examined using electron microscopy, which has two primary limitations. To prevent sample charging effects the nonconductive specimen must be coated with a conductive material prior to placement in the path of an electron beam. Also, to avoid problems with moisture evaporation during examination of a sample it must be dehydrated or frozen prior to placement in an electron microscope vacuum chamber. Both of these preparation techniques can considerably alter the molecular structure.

AFM imaging modes can potentially provide structural information for a sample in its more natural state (without dehydration or coatings). Rather than employing an electron beam for imaging, a light mechanical force (e.g. 0.1 nN) is applied to a probe that is scanned across the sample. An image of the sample surface is created by monitoring motion of the probe tip across the surface. Other researchers have reported on the use of AFM for imaging biological systems (Dietz et al. 1992; Centoni et al, 1996).

MATERIALS & METHODS

Sample preparation

Whey protein edible film samples were provided by the Dept. of Food Science (Univ. of California, Davis). In addition to whey protein isolate, the films also contained 40% glycerol (dry basis) that

was added as a plasticizer. The samples were cast and dried for a minimum of 72h before they were examined by AFM. Film preparation was described by Matte and Krochta (1996).

Atomic force microscopy

A Topometrix(Explorer atomic force microscope system (Santa Clara, CA) was used for all edible film examinations. Two types of AFM scanners were used for surface examination of the films. Imaging of areas from 150 µm square to 10 µm square was performed with a tripod, piezoelectric scanner. The highest quality images in this range were obtained in the contact mode. Higher resolution, small area scanning was done using a tube-type scanner with a cylindrical piezoelectric configuration. Areas from 2,700 nm square to 350 nm square were imaged with this scanner. In scanning to obtain high resolution images the noncontact mode is preferred. Contact mode scanning in this range produced streaked images that resulted from either probe tip deformation of the sample surface or capillary forces between tip and sample.

All imaging was done under ambient (in air) conditions. Noncontact mode scanning was primarily performed using low resonant frequency tips. These tips were found to provide better quality images of the whey protein films than high frequency tips. We also determined that viewing the films from a 3-dimensional perspective provided a topographic detail that could not be seen in the top views. Most images are presented from that perspective.

X-ray diffraction

In their natural state whey proteins exhibit crystallinity, which may be lost during denaturation in the gelation step of edible film fabrication. In gelation of whey proteins the amino acid chains were reported to uncoil upon heating, exposing intrachain disulfide bonds. These bonds are broken and new cross-linked disulfide bonds are formed with other protein chains (Aguilera, 1995). During cooling and drying following gelation, as the protein chains rearrange from a gel structure to a solid film, some recrystallization may occur. In an attempt to determine whether crystallinity was present in the film, samples were analyzed using a Siemens D5000 diffractometer with a copper K_α X-ray source. A position sensitive detector was scanned through the 2è range 20 to 50 degrees. Under these conditions no peaks that would indicate crystallinity were observed. No further X-ray diffraction evaluations were performed.

RESULTS & DISCUSSION

Interpreting images

Because AFM imaging applied to food systems is a relatively new technique, interpreting the images requires the microscopist to apply supplemental knowledge of the system being studied. As shown in the images we developed, the features tend to lose sharpness as they approach the size of the probe tip (~100 nm). At that size the acquired image can represent a mathematical convolution of the geometry of the probe tip and the true topography of the surface. A review of this as well as other potential artifacts and precautions in AFM imaging has been published (Sheiko et al., 1994)

Macrostructure examination

Initial examination of the whey protein edible film samples was performed using a tripod scanner to provide large area, general views of the surface. Although the first examinations were performed in the noncontact scanning mode, contact mode imaging was attempted to reduce noise from mechanical vibration of the microscope. We determined that high quality images could be obtained when the contact mode was employed using a tripod scanner.

The largest possible sample area examined with a tripod scanner is a square measuring 150 µm on each si ee interesting features are seen in the image. At this resolution a majority of the surface appears relatively flat. This confirmed visual examination of the sample, which indicated the shiny top surface characteristic of low optical scattering. AFM examination of the bottom surface was of limited value because an imprint of the casting surface obscured any film structural details. Also in this image are large features varying in size from ~1 µm ∞1 µm to 15 µm ∞ 15 µm. Their origin is unknown. It is clear that these "particles" were embedded in the surface of the film, as attempts to remove them with compressed nitrogen were unsuccessful. Also, particles that were not fixed to the surface would be moved by the scanning probe tip, causing streaking patterns in the image. Particle density averaged 1,000 per cm^2, an unreasonably high number for dust in the class 1,000 laboratory environment where the films were cast. Follow-up examination of additional samples confirmed that they were part of the film. Some may be dust particles embedded in the film surface. Also, possibly due to the mixture of proteins in the isolate some of them may cross-link and agglomerate faster than others, yielding somewhat larger particles. The third feature in this image was surface porosity. The porosity was probably due to dissolved gases escaping from the film during casting and drying (McHugh et al., 1994).

To reveal additional details of surface features the microscope was zoomed in to examine a smaller portion of the are µm on each side. Some of the larger areas of surface porosity were seen as well as a large protein globule. The larger, almost circular pores in this image had diameters of 13.4 and 15.7 µm. A large protein globule located at the bottom of the field of view was 14.9 µm long and 8.8 µm along the minor axis. It has been reported, from transmission

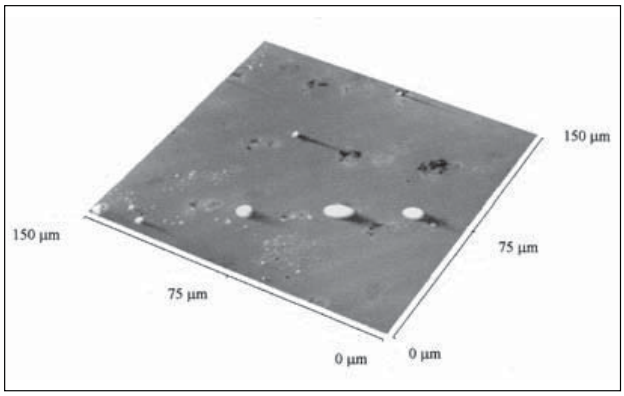

Fig 1—150 mm ∞ 150 mm image of whey protein edible film. Three dimensional view of a typical area from sample number 1, acquired in the contact mode. Several areas of surface porosity are observed, as well as three large protein globules protruding above the relatively smooth surface. Near the lower left corner are several smaller proteins extending above the surface.

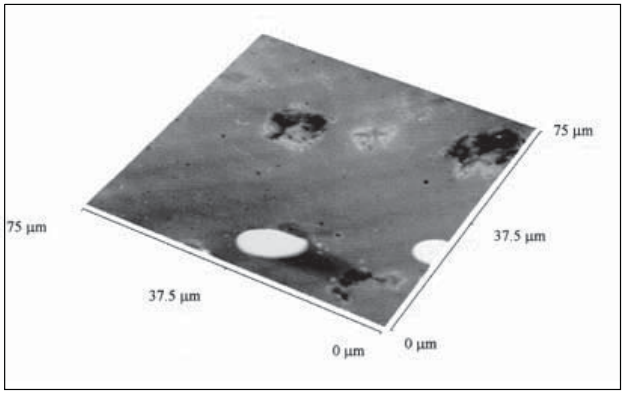

Fig. 2—75 µm ∞ 75 µm image of whey protein edible film. Three dimensional view of sample number 1 zoomed in to reveal detail from the Fig. 1. The protein globule seen at bottom is 14.9 µm in the long direction and 8.8 µm along the minor axis. Measurement of the two larger pores indicated diameters of 15.7 and 13.4 µm. This image was also acquired in the contact mode.

electron microscope studies of whey protein gels, that proteins can form spherical aggregates of ~1 micron (Otte et al., 1996). In that same study, spheres were also shown to form aggregates with other spheres in the gel. The solutions in that study were pH 5.2, near the isoelectric point of the proteins, and had been heated for 30 min at 80°C (Otte et al., 1996). The presence of such large globules in our samples indicated that the addition of 10% whey protein isolate to water may lower the pH to a value near the isoelectric point of the proteins. Smaller proteins extending above the flat surface and pores not easily identified before (in Fig. 1) were visible as well.

Zooming in on the flat areas revealed much surface detail not readily apparent in the previous images. An area from the center owed many small proteins and surface pores not clearly visible in the earlier images. The apparently smooth and relatively featureless surface (Fig. 1) is now seen to contain many features that lend a slight topography to the surface—on the order of 100 nm.

The highest resolution images acquired with the tripod scanner were of areas 10 µm on each side, 15 times greater than shown in Fig. 1. Features (taken from the center of Fig. 3) were again viewed at this sca n globules in this field of view had a maximum length of 1.3 µm and the pores ranged from 0.8 µm dia ~1.2 µm in length. Another area of this sam his same resolution showed a higher concentration of surface proteins about the same size as those seen in the previous image (Fig. 4).

Microstructure examination

A tube scanner was used to reveal fine structural details of the whey protein edible films with the AFM. Two film samples were examined using this scanner in the non-contact mode. An area of the first sample, 2,700 nm on each side, was examined underthese conditi revealed surface details at a scale not achievable with the tripod scanner. The Topometrix (software enabled analysis of topographical features by looking at pseudo-cross sections (a "cross-section" of the surface topography). The largest proteins had lengths of 420 and 460 nm. The smallest features, not clearly visible in the image but seen in pseudo-cross section were in the range 50 to 80 nm. At a much higher resolution, it was possible to identify features in the 6 to 10 nm range (Fig. 4) with a pseudo-cross section. This size range compared quite favorably with the 7 nm dimension reported for -lactoglobulin dimers (Aguilera, 1995).

To determine whether there was a consistent structure throughout the whey protein edible films a second sample was examined. Sample 2 was taken from a different location on the same film. A non-contact mode image of this sample (Fig. 8) was obtained using a scan length of 2700 nm. These images are typical of the surface of sample 2. The largest protein globule observed in this view had a major axis length of 380 nm and a minor axis of 240 nm, within 18% of the size of the largest proteins observed in sample 1 at this scale smallest proteins, measured in pseudo-cross section, were in the 37

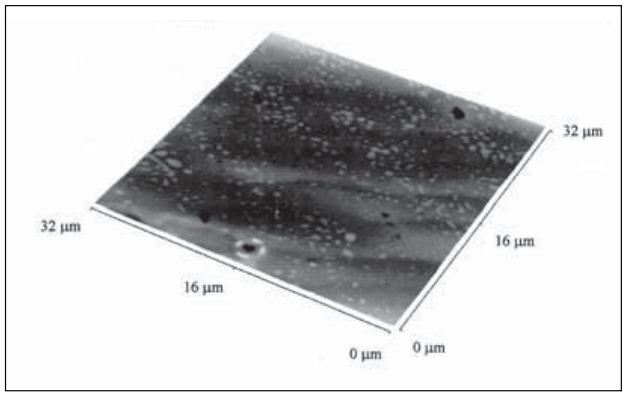

Fig. 3—32 mm ∞ 32 mm image of whey protein edible film. Higher resolution contact mode AFM image of sample number 1, taken from the center of the image shown in Fig. 2, which reveals both small pores and protein globules at the surface in greater detail. Both the pores and proteins have dimensions around 1 micron.

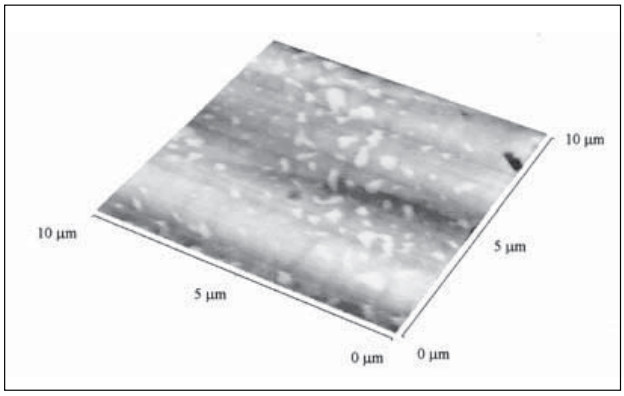

Fig. 5—10 mm ∞ 10 mm image of whey protein edible film. Contact mode image of sample number 1 at the same resolution as Fig. 4 revealing similar surface porosity and protein texture. The protein globules once again have maximum horizontal dimensions around 1 micron.

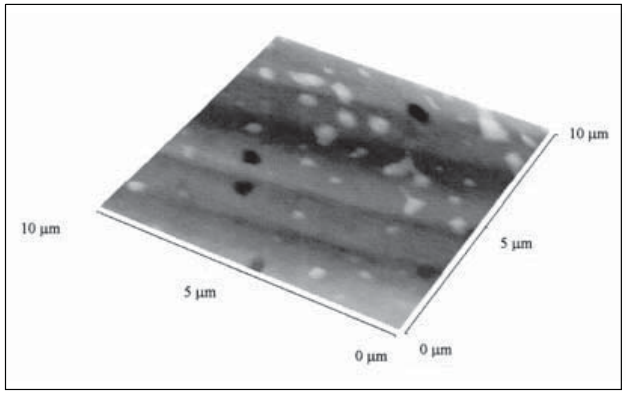

Fig. 4—10 mm ∞ 10 mm image of whey protein edible film. A zoomed contact mode image of whey protein film sample number 1, taken from center of Fig. 3, showing areas of smaller protein globules and porosity. The proteins have maximum horizontal axis of 1.3 µm and the pore major axis ranges from 0.8 to 1.2 µm.

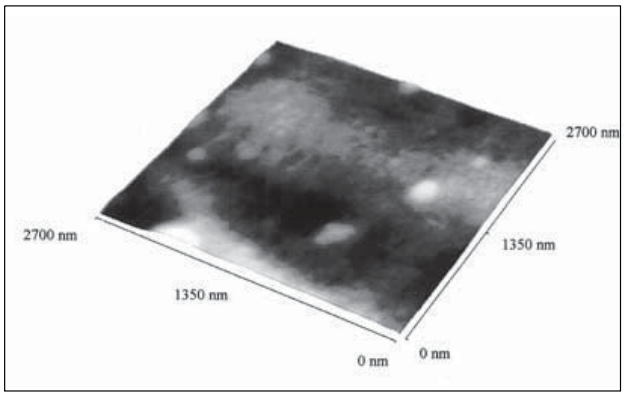

Fig. 6—2,700 nm ∞ 2,700 nm image of whey protein edible film. Noncontact mode image of sample number 1 imaged at a higher resolution than the previous images. The larger protein globules on the right side of this image have lengths of 418 and 461 nm. The smallest features found in this image are in the range of 50 to 80 nm.

to 75 nm range. This also compared quite favorably with the size of those found at this resolution in sample 1. High frequency noncontact images revealed similar features, ranging in size from 43 to 64 nm.

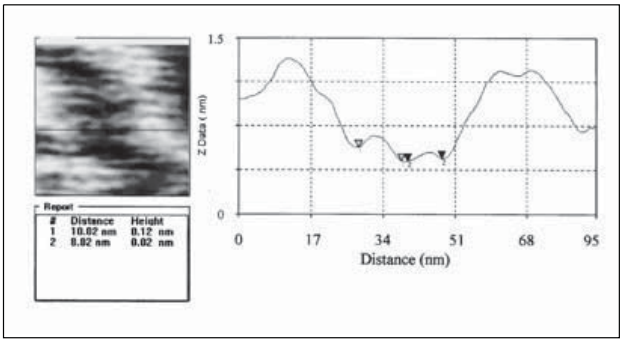

Fig. 7—Pseudo-cross section of whey protein edible film. Top view zoomed image of area from lower right side of Fig. 8 along with a scan from the line analysis software showing topography across this field of view. The small features flagged are found to be approximately 8 and 10 nm, which corresponds to the reported size of β-lactoglobulin dimers. The horizontal scanning artifacts are also present in this image, which was produced using topographic data from Fig. 8.

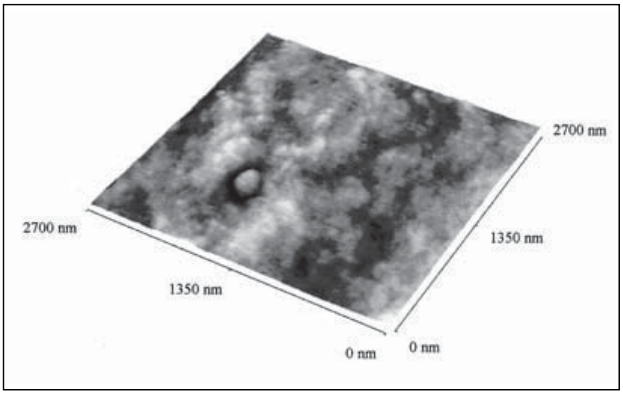

Fig. 8—2,700 nm ∞ 2,700 nm image of whey protein edible film. Noncontact mode image of whey protein film sample number 2, viewed at the same resolution as Fig. 6. The large proteinglobule near the center of this image is 382 nm along the major axis and has a minor axis length of 238 nm. Smaller proteins were found to be in the range of 37 to 75 nm.

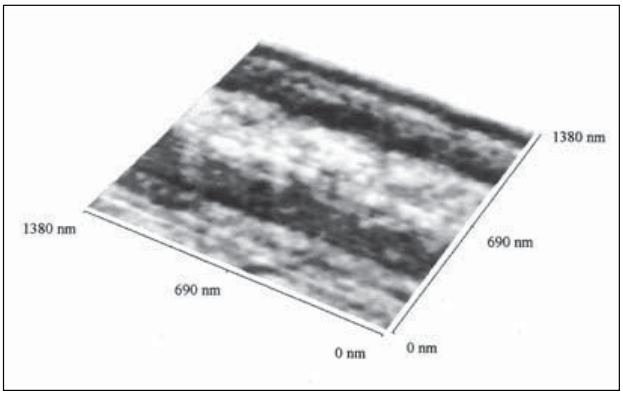

Fig. 9—1,400 nm ∞ 1,400 nm image of whey protein edible film. Noncontact mode image of sample number 2, acquired using a high frequency tip. The smallest measured proteins in this field of view are found to be in the range 44 to 93 nm. Horizontal scanning artifacts are also present in this image.

As with sample 1, zooming in on the second sample revealed smaller features than those found during initial scanning. A higher resolution image of sample obtained using a high frequency tip. The smallest features found in this image, 44 to 93 nm, were considerably larger than those seen in line analysis of images of similar resolution obtained with a low frequency tip (18 to 38 nm). No further scanning was performed using a high resonant frequency tip.

The highest resolution image of this second sample (using a low frequency tip and a scanned area of 350 nm ∞ 350 nm), revealed features that were about 10 nm in close agreement with the size observed for the smallest protein features found when sample 1 was examined at a similar resolution (Fig. 7).

CONCLUSIONS

ATOMIC FORCE MICROSCOPY WAS AN EFFECTIVE TOOL FOR EXamination of whey protein edible films. Contact mode imaging, used for large area scans with a tripod scanner, provided high quality images of the surface for areas of 10,000 nm ∞ 10,000 nm to 150,000 nm ∞ 150,000 nm. Higher resolution imaging in the noncontact mode with a tube scanner could be performed on areas as small as 85 nm ∞ 85 nm or as large as 2,700 nm ∞ 2,700 nm.. Film features were observed in the size range 6.4 nm to 15 μm long. The smallest measurable features in the protein films were nearly the same size as that reported for b-lactoglobulin. Larger features provided evidence of protein agglomeration, during heating to induce gelation, occurring in a solution with a pH near the isoelectric point. AFM examination of more than one whey protein film sample provided evidence of a consistent structure in the material. X-ray diffraction indicated that the whey proteins had been irreversibly denatured in the gelation process. This was consistent with the formation of disulfide bonds between protein molecules as they were heated in film processing.

REFERENCES

Aguilera, J.M. 1995. Gelation of whey proteins. Food Technol. 49(10): 83-89.
Centoni, S.A., Vanasupa, L.S., and Tong, P.S. 1997. Atomic force microsocpy for ultrafiltration membrane imaging. Scanning 19(4): 281-285.
Dietz, P.K., Hansma, P., Inacker, O., Lehmann, H.D., and Herrmann, K.H. 1992. Ultra filtratron membrane imaging using atomic force microscope. J. Membr. Sci. 65: 101-105.
deWit, J.N. and Klarenbeek, G. 1984. Effects of various heat treatments on structure and solubility of whey proteins. J. Dairy Sci. 67(11): 2701-2710.
Fairley, P., Monahan, F.J., German, J.B., and Krochta, J.M. 1996. Mechanical properties and water vapor permeability of edible films from whey protein isolate and sodium dodecyl sulfate. J. Agric. Food Chem. 44(2): 438-443.
Guilbert, S. 1986. Technology and application of edible protective films, Ch. 19, in *Food Packaging and Preservation, Theory and Practice*, M. Mathlouthi (Ed.), p. 371-394. Elsevier Applied Science Publishers Ltd., Essex, England.
Kinsella, J.E. and Whitehead, D.M. 1989. Proteins in whey: chemical, physical, and functional properties. Advances in Food & Nutr. Res. 33: 343-348.
Krochta, J.M. 1992. Control of mass transfer in foods with edible-coatings and films, Ch. 39, in *Advances in Food Engineering*, R.P. Singh and M.A. Wirakartakusumah (Ed.), p. 517-538. CRC Press, Inc., Boca Raton, FL.
Mahmoud, R. and Savello, P.A. 1993. Solubility and hydrolyzability of films produced by transglutaminase catalytic crosslinking of whey protein. J. Dairy Sci. 76(1): 29-35.
Mahmoud, R. and Savello, P.A. 1992. Mechanical properties of and water vapor transferability through whey protein films. J. Dairy Sci. 75(4): 942-946.
Mate, J.I. and Krochta, J.M. 1996. Comparison of oxygen and water vapor permeabilities of whey protein isolate and -lactoglobulin edible films. J. Agric. Food Chem. 44(10): 3001-3004.
Maynes, J.R. and Krochta, J.M. 1994. Properties of edible films from total milk protein. J. Food Sci. 59: 909-911.
McHugh, T.H. and Krochta, J.M. 1994. Sorbitol- vs glycerol-plasticized whey protein edible films: integrated oxygen permeability and tensile property evaluation. J. Agric. Food Chem. 42: 841-845.
McHugh, T.H., Avena-Bustillos, R., and Krochta, J.M. 1993. Hydrophilic edible films: modified procedure for water vapor permeability and explanation of thickness effects. J. Food Sci. 58: 899-903.
McHugh, T.H., Aujard, J.-F, and Krochta, J.M. 1994. Plasticized whey protein edible films: water vapor permeability properties. J. Food Sci. 59: 416-419, 423.
Otte, J., Ju, Z.Y., Skriver, A., and Qvist, K.B. 1996. Effects of limited proteolysis on microstructure of heat-induced whey protein gels at varying pH. J. Dairy Sci. 79: 782-790.
Sheiko, S.S., Moller, M., Reuvekamp, E.M.C.M., and Zandbergen, H.W. 1994. Evaluation of the probing profile of scanning force microscopy tips. Ultramicroscopy 53: 371-380.
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