ABBREVIATIONS USED

Chloramphenicol, CAP; swab test on premises, STOP; thin-layer chromatography/bioautography, TLCB; microbial inhibition test, MIT; tryptose agar slants, TAS; trypticase soy broth, TSB; phosphate-buffered saline, PBS; plate count agar, PCA; retardation factor, R_f.

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Evaluation of Molecular Interactions in Myosin, Fibrinogen, and Myosin–Fibrinogen Gels

E. Allen Foegeding,*[1] William R. Dayton, and C. Eugene Allen

Myosin, fibrinogen, and myosin–fibrinogen gels formed by heating at a rate of 12 °C/h were disrupted with guanidine hydrochloride, urea, and 2-mercaptoethanol. The degree of disruption was quantitated by turbidity, and complexes that remained soluble were analyzed by gel filtration in 6 M guanidine hydrochloride and SDS–polyacrylamide gel electrophoresis. Myosin and fibrinogen together or individually formed gels at 70 °C that were more difficult to solubilize than gels formed at 50 °C. Myosin and fibrinogen gels formed at 70 °C were stabilized by both noncovalent and disulfide bonds. Noncovalent and disulfide bonds are formed in myosin–fibrinogen gels at 50 and 70 °C.

Thermally induced gelation of muscle proteins is important to muscle foods because it affects texture and water-holding properties of comminuted meat products (Acton et al., 1983). Thus, the gelling properties of proteins added to muscle foods, such as the blood protein fibrinogen, should be relevant to their functionality in a meat system.

Thermally formed myosin, fibrinogen, and myosin–fibrinogen gels were investigated in two previous studies (Foegeding et al., 1986a,b). Gel strength was shown to be dependent upon (1) the specific protein or proteins used to form a gel matrix, (2) protein concentration, and (3) the heating method used to form the gel. The characteristics of the protein gel matrices that caused the variations in gel strength were not determined.

Strength of myosin gels is a function of the gel matrix geometry (microstructure) and also may be affected by the chemical bonding within the matrix. The formation of a fine-stranded gel microstructure, as opposed to a coarse structure, makes a more rigid myosin gel (Ishioroshi et al., 1979; Hermansson et al., 1986). The microstructure is affected by variations in pH and ionic strength; however, the heating temperature from 55 to 65 °C has no effect (Hermansson et al., 1986). The rigidity of gels heated from 55 to 65 °C was not determined so the study by Hermansson et al. (1986) did not establish a relationship among heating temperature, microstructure, and rigidity.

Van Kleef (1986) investigated the chemical bonding within gel matrices by determining the ability of urea or urea plus 2-mercaptoethanol to dissolve ovalbumin and soybean protein gels. The combination of urea and 2-mercaptoethanol dissolved all gels, whereas urea alone dissolved soy protein but not ovalbumin gels. Thus, disruption of gel matrices with denaturants provides information on the bonding within the matrices.

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The objective of this study was to investigate the bonding within myosin, fibrinogen, and myosin–fibrinogen gels of different gel strengths by disrupting gels with guanidine hydrochloride, urea, and 2-mercaptoethanol. Furthermore, aggregates resistant to disruption were investigated by gel filtration chromatography in 6 M guanidine hydrochloride and SDS–polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Protein Preparation. Porcine myosin and bovine fibrinogen were isolated, and their homogeneity was established as previously reported (Foegeding et al., 1986a). Myosin preparations always contained some C protein and M protein, but actin was never detected by gel electrophoresis. In addition, a minor protein band that did not enter the separating gel was present. This band was probably titin (MW 1 000 000) and/or nebulin (MW 500 000) (Robson and Huiatt, 1983); however, no attempt was made to identify the contaminating proteins.

Gel Disruption. Myosin (6 mg/mL), fibrinogen (6 mg/mL), and myosin–fibrinogen (3–3 mg/mL) suspensions were heated in 5-mL volumes at 12 °C/h to form gels as previously described (Foegeding et al., 1986a). After being heated to 50 or 70 °C, the gels were placed in a 4 °C cold room and held for 20–24 h. Absolute-grade guanidine hydrochloride and urea (Research Plus, Bayonne, NJ) were used in gel disruption and denaturing chromatography studies. For each type of protein gel, duplicate gels were disrupted by addition of either 2.87 g of guanidine hydrochloride (two gels) or 2.40 g of urea (two gels) at room temperature. The final concentrations of guanidine hydrochloride and urea were 4.42 and 6.05 M, respectively. Gels were similarly mixed by vortexing at 1.0–0.5-min intervals to disrupt the gel and dissolve the denaturant. After the mixtures were allowed to stand at room temperature for 1 day, 12.5 μL of 2-mercaptoethanol (MCE) was added to one of each duplicate sample pair and vortexed as stated previously. Disrupted gels were held at room temperature and mixed daily for up to 6 days. After 6 days the absorbance was measured at 370 nm in a 0.5-cm path length quartz cell to determine turbidity. Gel disruption was replicated twice with myosin and fibrinogen from two separate isolations.

Denaturing Chromatography. Denaturing chromatography and sample dialysis were done at room temperature, and buffers were adjusted to the selected pH at 20 °C. Sepharose CL-2B (Pharmacia) gel filtration resin was equilibrated with 6 M guanidine hydrochloride–20 mM Tris-HCl, pH 7.5 buffer (guanidine buffer) and degassed for 1 h prior to pouring of the columns. Two 1.6 × 30 cm columns were poured and equilibrated with guanidine buffer at 11.7 mL/h for ca. 24 h. The columns were connected in tandem with buffer flowing upward through the first column and downward through the second column. Samples were applied and eluted at 5 mL/h. Fractions were collected at 36-min intervals.

Gels disrupted with guanidine hydrochloride were dialyzed against guanidine buffer, and gels disrupted with guanidine hydrochloride plus MCE were dialyzed against guanidine buffer containing 0.25% (v/v) MCE (guanidine–MCE buffer). The disrupted gels were dialyzed for ca. 24 h and clarified by centrifugation (100000*g* for 1 h at 4 °C). The volume (3 mL) of solubilized protein suspension applied to the columns was held constant among samples so that the relative concentration of soluble proteins among and within samples would be reflected in the chromatogram. After completion of the chromatography of the guanidine hydrochloride disrupted gels, the columns were equilibrated with guanidine–MCE buffer and the guanidine hydrochloride plus MCE disrupted samples were analyzed.

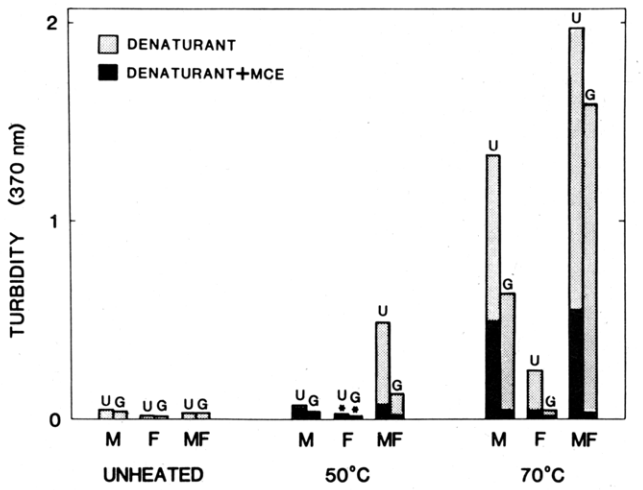

Figure 1. Turbidity of myosin (M, 6 mg/mL), fibrinogen (F, 6 mg/mL), and myosin–fibrinogen (MF, 3 mg/mL–3 mg/mL) gels disrupted with guanidine hydrochloride (G), guanidine hydrochloride plus 2-mercaptoethanol, urea (U), and urea plus 2-mercaptoethanol. Asterisk indicates that there was no difference between denaturant and denaturant plus 2-mercaptoethanol.

Electrophoresis. The proteins present in various peaks separated by denaturing chromatography were identified by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Separating gels were 10% (w/v) polyacrylamide and 0.267% (w/v) bisacrylamide; stacking gels were 3.5% (w/v) polyacrylamide and 0.093% (w/v) bisacrylamide. Prior to electrophoresis, samples were dialyzed into a 50 mM sodium phosphate, pH 6.0–0.5 M NaCl buffer. The SDS–PAGE procedure was detailed previously (Foegeding et al., 1986a).

RESULTS AND DISCUSSION

Gel Disruption. Figure 1 shows the turbidity of gels disrupted with either urea or guanidine hydrochloride each in the presence or in the absence of MCE. Gels formed at higher temperatures, which were heated for longer periods, had greater turbidities after suspension in urea or guanidine hydrochloride. Thus, the 70 °C gels were disrupted less extensively by the denaturants, suggesting that increased bonding could contribute to the greater strength of gels formed by heating to 70 °C (Foegeding et al., 1986a,b). Comparison of protein compositions of gels at both temperatures indicated that the myosin–fibrinogen gels were disrupted to the least extent. This supports previous data for 50 °C gels (Foegeding et al., 1986a,b), which indicated myosin and fibrinogen interacted to form a gel matrix that was stronger than the gel matrix of either individual protein. At 70 °C the relationship between gel strength and resistance to disruption did not hold true. Myosin–fibrinogen gels heated to 70 °C were more difficult to disrupt than 70 °C myosin gels; however myosin gels had a greater gel strength (Foegeding et al., 1986a,b). Thus, the extent of disruption appears to be associated with one component of gel strength.

The addition of MCE to guanidine hydrochloride disrupted gels that were formed at either temperature decreased the turbidity to values corresponding to those of unheated proteins (Figure 1). This was true for gels of each protein composition. The data suggest that intermolecular disulfide bonds were formed in myosin–fibrinogen gels at 50 °C and in all gels at 70 °C. Fibrinogen has no sulfhydryl groups and 29 disulfides (Henschen, 1979). Myosin has

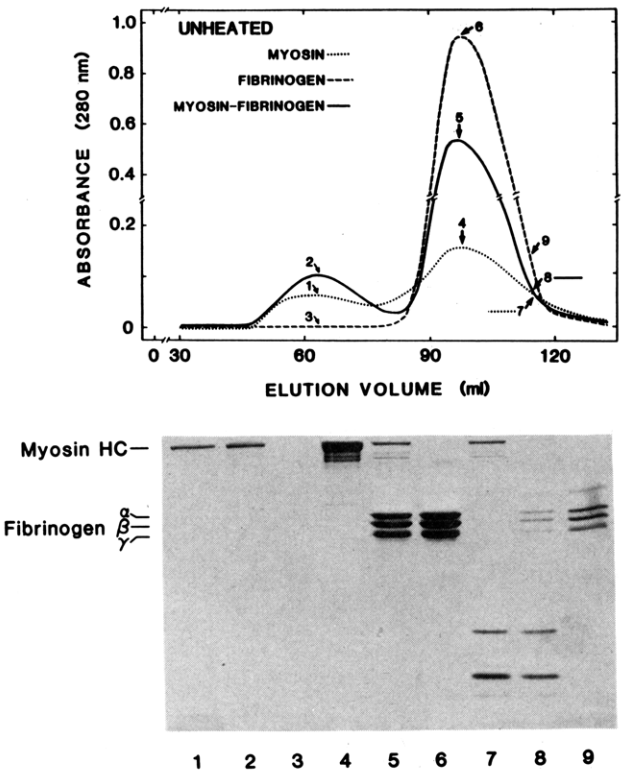

Figure 2. Elution profiles of guanidine hydrochloride denatured proteins (unheated) and SDS–polyacrylamide gel of selected fractions. The proteins eluting at various points in the profiles can be seen in the SDS–polyacrylamide gel lane of identical number. Thirty-three microliters of protein solution was placed in each well.

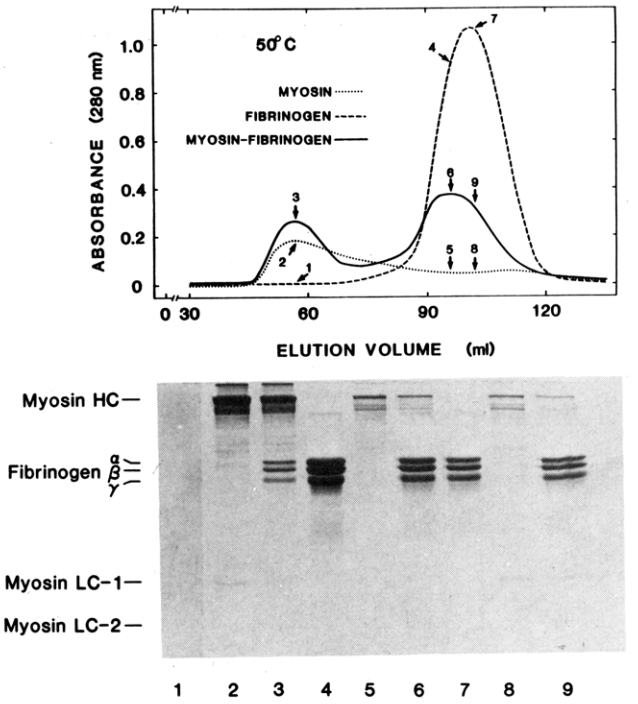

Figure 3. Elution profiles of guanidine hydrochloride solubilized gels and SDS–polyacrylamide gel of selected fractions. Gels were formed by heating to 50 °C. The proteins eluting at various points in the profiles can be seen in the SDS–polyacrylamide gel lane of identical number. Thirty-three microliters of protein solution was placed in each well.

ca. 42 sulfhydryl groups and no disulfides (Buttkus, 1971; Hofmann and Hamm, 1978). Formation of intermolecular disulfides in the myosin–fibrinogen gels indicated that the breaking of fibrinogen disulfides must have occurred at 50 °C.

MCE did not decrease the turbidity of 50 °C fibrinogen gels below that achieved by addition of urea or guanidine hydrochloride. Thus, either the gel structure formed by fibrinogen at 50 °C did not involve any intermolecular disulfide bonds or disulfide-linked aggregates were below the sensitivity of the turbidity measurement. This indicated that the gel structure formed by fibrinogen at 50 °C was held together mainly by noncovalent bonding.

Urea was much less effective than guanidine hydrochloride in disrupting the gels (Figure 1). Guanidine hydrochloride is generally considered to be a more effective denaturant than urea (Lapanje, 1978), and this is consistent with our results. The addition of MCE to urea-disrupted myosin and fibrinogen gels formed at 70 °C and myosin–fibrinogen gels formed at 50 and 70 °C did not decrease the turbidity to that of unheated proteins. This appeared to be related to the inability of urea, at the concentration and pH used in this study, to completely disrupt all noncovalent bonding.

Denaturing Chromatography. Figure 2 shows the eluction profiles of unheated myosin, fibrinogen, and the myosin–fibrinogen mixture and a SDS–PAGE electropherogram of select fractions. The initial myosin peak at 63 mL was myosin heavy chain. This was an associated form of myosin heavy chain because a second peak containing mainly myosin heavy chain was seen at 99 mL. Our data support that of Gershman et al. (1969) who showed by sedimentation velocity experiments that myosin heavy chain aggregation occurred in 5 M guanidine. The presence of two myosin peaks shows that two hydrodynamically dissimilar forms of myosin were present and that intermolecular disulfide bonding occurred in the unheated myosin. Furthermore, polyacrylamide gels (not shown) indicated that myosin–fibrinogen fractions that eluted between 54 and 72 mL contained only myosin heavy chain. The presence of disulfide-linked aggregates in the unheated myosin indicated that intermolecular disulfide bonds were present in the isolated myosin or that they were formed during unfolding in guanidine hydrochloride. Since there is an equal probability of formation of disulfide bonds due to unfolding in guanidine hydrochloride for unheated and 70 °C heated myosin, the contribution of unfolding in guanidine hydrochloride to disulfide bond formation appeared to be much less than that due to heating. This is based on the observation that when the unheated myosin and 70 °C myosin gel were unfolded in guanidine hydrochloride, the heated myosin remained more turbid. The addition of MCE was required to decrease the turbidity to that of unheated myosin (Figure 1). However, a quantitative relationship between the two pathways for myosin disulfide bond formation was not established.

Fibrinogen never appeared to dissociate into subunits since only one peak with a leading edge at 81 mL was observed. The lack of subunit dissociation is in agreement with sedimentation velocity experiments (Johnson and Mihalyi, 1965), which showed that fibrinogen did not dissociate into subunits in 5 M guanidine hydrochloride.

Figure 3 shows the elution profiles of guanidine hydrochloride solubilized myosin, fibrinogen, and myosin–fibrinogen gels that were heated to 50 °C and a SDS–PAGE electropherogram of selected fractions. The initial peaks from myosin and myosin–fibrinogen gels occurred earlier (at 57 mL) than did peaks from unheated proteins. The polyacrylamide gel revealed that fibrinogen was present in the initial myosin–fibrinogen peak. Since the elution profile and polyacrylamide gel showed no fibrinogen eluting at 57 mL when fibrinogen was heated singly, there

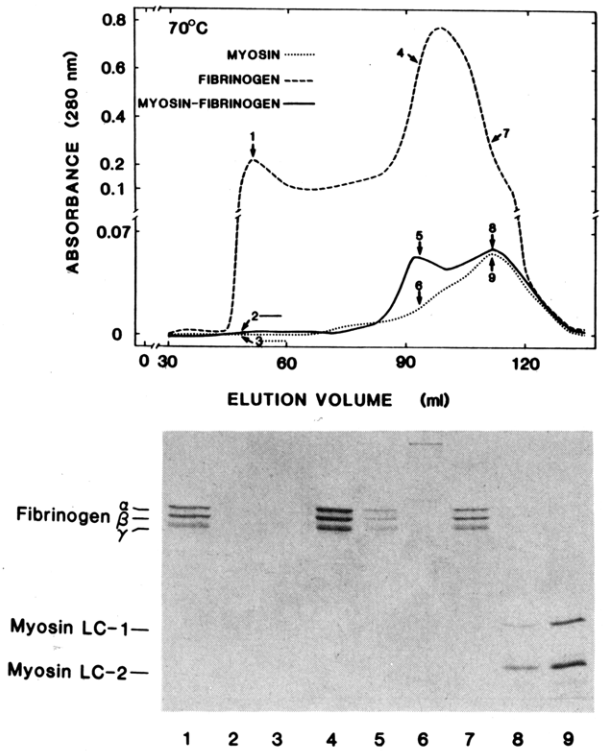

Figure 4. Elution profiles of guanidine hydrochloride solubilized gels and SDS–polyacrylamide gel of selected fractions. Gels were formed by heating to 70 °C. The proteins eluting at various points in the profiles can be seen in the SDS–polyacrylamide gel lane of identical number. Thirty-three microliters of protein solution was placed in each well.

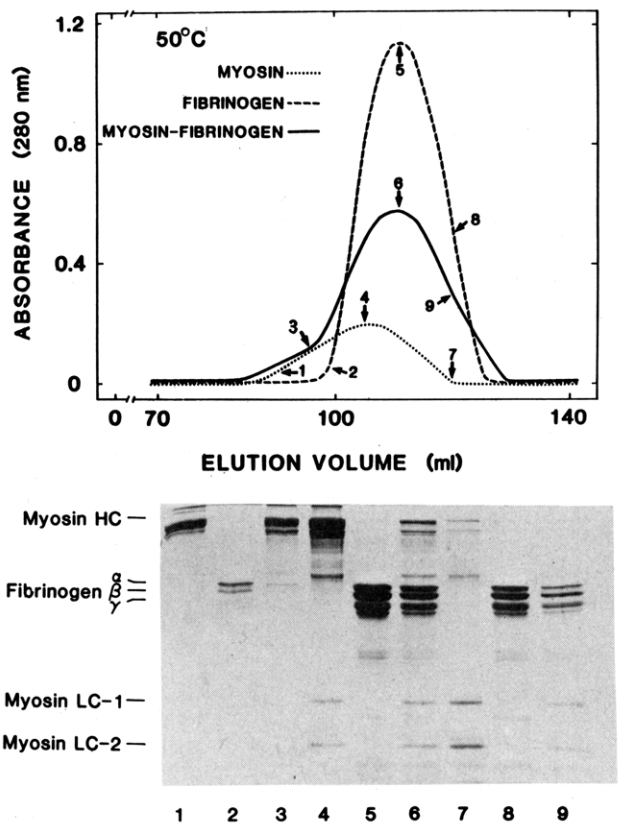

Figure 5. Elution profiles of guanidine hydrochloride plus 2-mercaptoethanol-solubilized gels and SDS–polyacrylamide gel of selected fractions. Gels were formed by heating to 50 °C. The proteins eluting at various points in the profiles can be seen in the SDS–polyacrylamide gel lane of identical number. Thirty-three microliters of protein solution was placed in each well.

appeared to be a guanidine hydrochloride resistant interaction formed between myosin and fibrinogen heated to 50 °C. Since MCE decreased the turbidity of guanidine hydrochloride and urea disrupted gels (Figure 1), and MCE-containing SDS–PAGE separated myosin and fibrinogen bands, the guanidine hydrochloride resistant myosin–fibrinogen interaction was disulfide bonding. The elution profile of myosin heated to 50 °C did not contain a second peak at 99 mL as was observed with unheated myosin. This suggests disulfide-driven aggregation of myosin increased with temperature. A protein band was seen above the myosin heavy chain band in lanes 2 and 3 of the electropherogram (Figure 3). This could indicate that non-disulfide covalent interactions occurred or that titin and nebulin were concentrated in those fractions. Thermally induced, non-disulfide, covalent interactions in myosin were reported by Peng and Nielson (1986). They showed that myosin heated at 80 °C contained a soluble peptide that remained at the top of a SDS–PAGE gel containing 7.5% acrylamide. Non-disulfide covalent interactions could contribute greatly to the strength of myosin and myosin–fibrinogen gels formed by heating to 50 °C. The identity of the large molecular weight aggregates that were resistant to SDS–MCE solubilization will be the subject of future investigations.

For myosin gels formed at 50 °C, the denaturing chromatography results indicated extensive intermolecular disulfide bonding whereas the gel disruption results showed little turbidity decrease occurred with addition of MCE. Turbidity and gel filtration chromatography show differences in particle size and shape. Thus, the results indicated that the chromatography was the more sensitive analysis under the conditions used in this study.

The elution profiles of unheated fibrinogen and fibrinogen heated to 50 °C were quite similar, indicating that guanidine hydrochloride disrupted the intermolecular bonding of the fibrinogen gel matrix formed at 50 °C. However, disulfide bonds must have been broken in this system at 50 °C since this must have occurred to permit the myosin–fibrinogen interaction at 50 °C.

Figure 4 shows the elution profiles of guanidine hydrochloride solubilized myosin, fibrinogen, and myosin–fibrinogen gels that had been heated to 70 °C and a SDS–PAGE electropherogram of selected fractions. Clarification of guanidine hydrochloride disrupted myosin and myosin–fibrinogen gels by centrifugation produced large pellets of insoluble protein. The low concentration of soluble protein in chromatographed samples is apparent from the low absorbance readings. Myosin heavy chain was not detected in the protein solubilized from the myosin–fibrinogen gels and was at a low concentration in protein solubilized from myosin gels. Furthermore, myosin light chains eluted at 111 mL and accounted for a great portion of the soluble protein from myosin and myosin–fibrinogen gels. Fibrinogen gels contained an aggregate eluting at 51 mL. This aggregate was shown by SDS–PAGE to contain all fibrinogen subunits. The protein solubilized from the myosin–fibrinogen gels lacked a fibrinogen peak at 51 mL, and fibrinogen was not detected electrophoretically at that elution volume. Thus, myosin and fibrinogen interacted at 70 °C to form a complex that was insoluble to guanidine hydrochloride.

Figure 5 shows the elution profiles of guanidine hydrochloride plus MCE-solubilized myosin, fibrinogen, and myosin-fibrinogen gels formed by heating to 50 °C and a SDS–PAGE electropherogram of selected fractions. The protein aggregates that were not disrupted by guanidine hydrochloride alone (Figure 3) were dissociated by guan-

idine hydrochloride plus MCE. To ensure that this was indeed the case, the fractions collected prior to the leading edge of the peaks (0–85 mL) were checked by SDS–PAGE. The electropherograms (not shown) had no detectable protein, confirming that large aggregates were not present. This supported our previous speculation that the aggregates were linked by disulfide interactions between proteins. The elution profiles (not shown) of unheated proteins and proteins from 70 °C gels solubilized with guanidine hydrochloride plus MCE were similar to those presented in Figure 5.

The SDS–PAGE electropherogram (Figure 5) also indicated that fibrinogen was at least partially broken into subunits since primarily α and β chains were observed in lane 2 and all three chains were observed in lane 5. The lack of well-defined subunit separation probably reflected lack of resolution by the gel filtration resin, although bonding between subunits cannot be dismissed.

Overall, the results indicated that noncovalent bonding alone or in combination with disulfide bonding accounted for the protein–protein interactions developed when myosin, fibrinogen, and myosin–fibrinogen gels were formed.

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Detection of Wheat Gluten, Whey Protein, Casein, Ovalbumin, and Soy Protein in Heated Meat Products by Electrophoresis, Blotting, and Immunoperoxidase Staining

Frederik W. Janssen,* Gerrit Voortman, and Johannes A. de Baaij

A method is reported by which it is possible to detect several nonmeat proteins in a heat-processed meat matrix. The proteins were extracted with a buffer containing sodium dodecyl sulfate, and aliquots of the extract were subjected to electrophoresis on a polyacrylamide gradient gel. The separated proteins were blotted on a nitrocellulose foil, and the nonmeat proteins were subsequently stained with a selective immunoperoxidase staining system. In model meat products, which had been heated up to 100 °C, detection of soy protein, whey protein, caseinate, egg albumin, and wheat gluten was possible down to the 0.1% level. The method provides a high level of information regarding the identity of the nonmeat protein under investigation. Screening the samples by a dot blot procedure proved to be an efficient way of sorting out samples that do not contain nonmeat proteins, thereby reducing labor costs.

Nonmeat proteins are added to meat products to enhance the emulgatory and water-binding capacity of meat proteins, especially in those cases where the emulgatory capacity of the meat proteins themselves is insufficient, as for example in low-meat-content formulations or in dietary products with a low-salt content.

Though a better product can thus be made, authorities in many countries are reluctant to give legal clearance for the use of these nonmeat proteins because, apart from the aforementioned aspect, they can also be used as meat extenders; i.e., part of the meat can be replaced by adding nonmeat protein and water.

Of the many nonmeat proteins currently in use, soy protein presumably ranks among the most frequently used ones. Of the many other proteins (wheat gluten, caseinate, whey protein, ovalbumin, peanut protein, rapeseed protein, cottonseed protein, sunflower protein) their use as meat extender has been documented (Hermansson, 1975; Hermansson and Akesson, 1975; Hand et al., 1981; Terrell et al., 1981; Wills and Kabirullah, 1981; Patana-Anake and Foegeding, 1985; Smith et al., 1973). Whether any of these proteins is actually (illegally) used remains obscure because adequate analytical methods to detect them in meat products are scarse, especially when the meat product has been heat preserved.

Of all electrophoretic methods, SDS electrophoresis is the method of choice (Lee et al., 1976; Armstrong et al., 1982; Heinert and Baumann, 1984) because even samples heated with a high-temperature/time record can be dissolved under the denaturing conditions required for this type of electrophoresis (by heating at 80–100 °C in a buffer containing ca. 2% sodium dodecyl sulfate). Quite frequently it is observed however that the electropherograms are either crowded with bands (products that have recieved

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