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The Effects of pH and Heating on the Surface Activity of Muscle Proteins

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ABSTRACT

The effects of pH and thermal denaturation on the surface active properties of muscle proteins at the air–solvent interface were determined by the drop volume method. The surface pressures attained after 40 min, π_{40}, by myosin and actomyosin solutions heated at 45°C for 30 min were similar to those attained by the unheated protein solutions. However, the π_{40} values were increased when the proteins were heated at 60°C prior to determining surface activity. The rate of surface tension decay was much faster following heating of myosin at 45°C compared with the unheated sample. Myosin heated at 60°C showed an initial induction period, which was followed by a very rapid rate of surface tension decay. The rate of surface tension decay for actomyosin heated at 45°C was similar to that of the unheated protein solution; however, heating at 60°C increased the rate compared to that of the control. The surface pressures attained after 40 min (π_{40}) by myosin and F-actin solutions were not significantly affected by the pH of the solutions. However, π_{40} values for both G-actin and actomyosin were greater at pH 11·0 than at pH 5·6 or 7·0. For all the proteins studied, pH greatly affected the rates of surface tension decay, the rates being slowest at pH 5·6 and fastest at 11·0.

INTRODUCTION

The unique structural properties of proteins are largely responsible for the production of a diverse range of food systems such as foams, gels and

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emulsions. Emulsion-based foods are primarily dependent on the ability of specific protein components, probably present in relatively low levels, to rapidly reach the oil–solvent interface, effectively lower the interfacial tension and stabilize the food. Considerable research in recent times has begun to unravel the structural basis of the emulsifying properties of muscle proteins in comminuted sausage-like meat products (Jones & Mandigo, 1982; Jones, 1984; Dickinson *et al.*, 1987).

Differences in surface behaviour of proteins are, in general, manifestations of differences in their molecular properties, e.g. molecular flexibility, surface hydrophobicity, tertiary structure, etc. (Graham & Phillips, 1979*a–c*). However, environmental factors such as pH, temperature and salts are known to alter the interfacial behaviour of many proteins (Graham & Phillips, 1976; Tornberg, 1978; Waniska & Kinsella, 1985; Arnebrant *et al.*, 1987). Thus we decided to study the effects of pH and thermal denaturation on the surface active properties of muscle proteins.

MATERIALS AND METHODS

All chemicals used were of reagent grade. The water used for surface activity measurements and protein preparation was twice distilled in glass apparatus. All the glassware used was allowed to stand overnight in sulphuric acid/dichromate solution and then rinsed several times with double distilled water. Dialysis tubing was washed first in 1% acetic acid, rinsed with 1% Na_2CO_3 solution containing 1 mM EDTA and then heated at 75°C for 10 min in the latter solution. The tubing was rinsed with double-distilled water and again heated at 75°C in double-distilled water and rinsed several times with double-distilled water prior to use.

Determination of protein content

Protein content of all solutions were determined by the Kjeldahl method (AOAC, 1975) and by the Biuret method.

Preparation of protein solutions

Rabbit skeletal muscle was used in all studies. Myosin was prepared by the method outlined by Margossian and Lowey (1982). In order to remove traces of C-protein and actin, myosin was further purified by chromatography on DEAE-Sephadex using a linear KCl gradient (0–0·5M KCl in phosphate buffer, pH 7·5). The myosin-containing fractions were pooled and

exhaustively dialysed at 4°C against 0·6M KCl, pH 7·0. F-actin was prepared by the procedure outlined by Pardee and Spudich (1982). The pellets of F-actin were gently resuspended in cold 10 mM imidazole buffer, pH 7·0, containing 0·1M KCl and 0·5 mM dithiothreitol and dialysed overnight against the same buffer. The F-actin solution was then dialysed exhaustively against 0·1M KCl, 10 mM ATP, pH 7·0. G-actin was prepared by resuspending F-actin pellets in cold 2 mM Tris-HCl buffer, pH 8·5, containing 0·2 mM ATP, 0·5 mM dithiothreitol and 0·2 mM $CaCl_2$, and dialysing against 10 volumes of this buffer with two changes over a 3-day period. The depolymerized G-actin solution was centrifuged at 80 000*g* for 3 h and subsequently dialysed against distilled water. Purified actomyosin was prepared by the method of Hay *et al.* (1972) and dialysed against 0·6M KCl, pH 7·0. Purity of the preparations was determined by SDS-polyacrylamide gel electrophoresis using the method of Greaser *et al.* (1983).

Actin, myosin and actomyosin solutions (10^{-1}% w/v protein) were adjusted to pH 5·6, 7·0 and 11·0 using 0·2M HCl or 0·2M NaOH, allowed to equilibrate overnight, and the pH checked and readjusted where necessary. Actomyosin and myosin solutions (10^{-1}% w/v, protein), pH 7·0, were heated at 45 or 60°C for 30 min and cooled to 20°C. The pH was readjusted to pH 7·0 using 0·2M HCl or 0·2M NaOH where necessary.

To remove any protein particles that could sediment during surface tension measurements, all protein solutions were centrifuged at 20 000*g* for 30 min and filtered through Whatman No. 1 filter paper prior to surface activity measurements. The protein contents of the filtrates were determined by the Biuret method and adjusted to 10^{-2}% (w/v) using the relevant solvents. Surface tension measurements were made immediately after protein preparation.

Surface tension measurements

The surface activity of the protein solutions at the air–solvent interface at 25°C was determined by the drop volume principle as described by Tornberg (1977). Calibration of the drop volume apparatus and calculation of surface tension were carried out as described by Tornberg (1978) and Arnebrant and Nylander (1985). For measurements of time-dependent surface tensions, a drop of a certain volume, corresponding to certain surface tension value, was expelled rapidly, and the time necessary for the surface tension to fall to such a value that the drop becomes detached was measured. This procedure was repeated for differing drop sizes, i.e. for different values of surface tension. Protein solutions were prepared in triplicate and measurements of surface tension at each drop size were performed in quadruplicate. The mean surface tensions were plotted as a function of time.

RESULTS AND DISCUSSION

Effect of heating

The time dependence of surface tensions (γ) of unheated and heated myosin and actomyosin solutions (10^{-2}% w/v in 0·6M KCl, pH 7·0) at the air–solvent interface at 25°C are shown in Figs 1 and 2. The initial surface tension (γ_0), which corresponds to the surface tension of the solvent, was 73·27 mNm^{-1}. The surface pressures attained after 40 min (π_{40}), i.e. the reduction in surface tension after 40 min, were not significantly affected when the protein solutions were heated at 45°C prior to determining surface activity. However, the π_{40} values were significantly increased following heating at 60°C. The π_{40} values for unheated myosin, myosin heated at 60°C, unheated actomyosin and actomyosin heated at 60°C were 21·02, 23·02, 19·27 and 21·27 mNm^{-1}, respectively. Previous studies have shown that heating increases the surface hydrophobicity of myosin and actomyosin (Lim & Botts, 1967; Niwa, 1975; Wicker *et al.*, 1986). Since surface hydrophobicity is reported to correlate well with surface activity (Kato & Nakai, 1980; Kato *et al.*, 1981), the increase in π_{40} values may be attributable to increased surface exposure of hydrophobic groups as denaturation proceeded.

Heating at either 45 or 60°C had a marked effect on the rate of surface tension decay of the myosin solution (Fig. 1). The rate was much faster following heating at 45°C compared with the unheated sample. Following

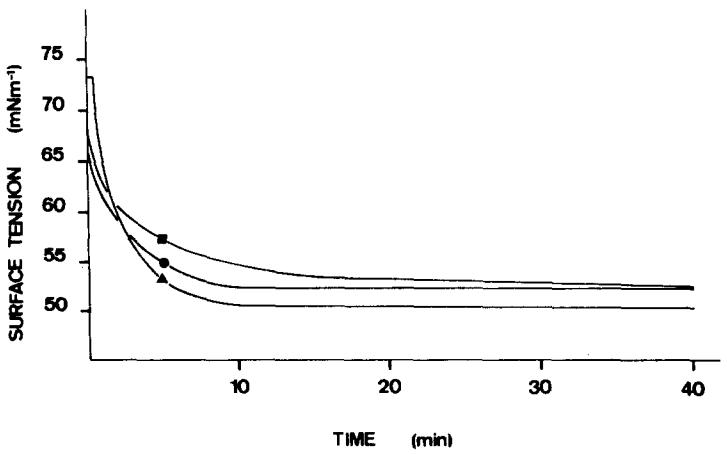

Fig. 1. Time dependence of surface tension at the air–solvent interface of unheated and heated myosin at a bulk phase concentration of 10^{-2}% w/v protein: unheated myosin (■), myosin heated at 45°C for 30 min (●) and myosin heated at 60°C for 30 min (▲).

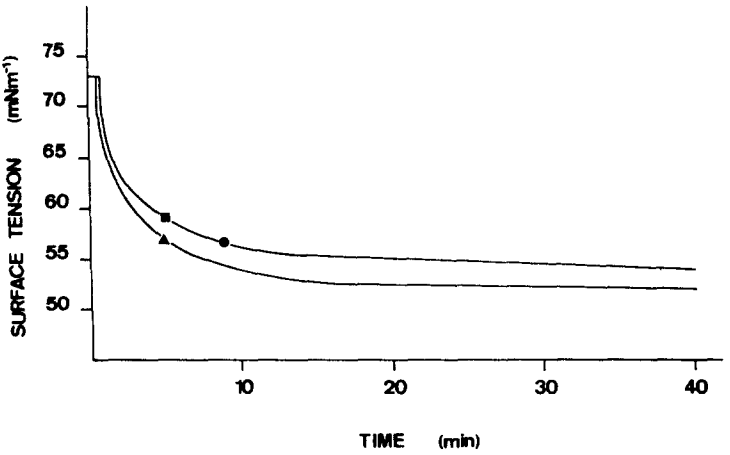

Fig. 2. Time dependence of surface tension at the air–solvent interface of unheated and heated actomyosin at a bulk phase concentration of 10^{-2}% w/v protein: unheated actomyosin (■), actomyosin heated at 45°C for 30 min (●) and actomyosin heated at 60°C for 30 min (▲).

heating at 60°C an initial induction period occurred, and this was followed by a very rapid rate of surface tension decay which was faster than that of either the unheated myosin solution or that heated at 45°C.

Heating at 45°C did not affect the rate of surface tension decay exhibited by the actomyosin solution. However, when actomyosin was heated at 60°C, the rate of surface tension decay was more rapid than for unheated actomyosin (Fig. 2).

The differences in surface behaviour of proteins at interfaces are generally regarded as manifestations of differences in their compositional and conformational characteristics (Graham & Phillips, 1979*a–c*; Castles *et al.*, 1987). In the present study, the observed differences in the surface active properties of unheated and heated myosin and actomyosin solutions can solely be attributed to conformational differences in the proteins. For the purpose of this discussion, the conformational changes occurring in myosin and actomyosin during heating may be considered together, since thermally induced conformational changes in the actomyosin complex are dominated by changes in the myosin moiety (Wright *et al.*, 1977; Acton *et al.*, 1981).

The thermal denaturation of myosin is complex and involves a series of independent co-operative processes which are associated with discrete regions of the molecule (Burke *et al.*, 1973; Samejima *et al.*, 1981; Ishioroshi *et al.*, 1982; Wright & Wilding, 1984). In general, two major heat-induced transitional changes occur in myosin; the first transition is assigned mainly to the globular heads of myosin and occurs at $\sim$43°C (T_{m_1}) whereas the

second is associated with major conformational changes occurring in the helical myosin tail and occurs at ~55°C (T_{m_2}) (Acton & Dick, 1984). Thus the initial rapid rate of surface tension decay observed in the present study when myosin was heated at 45°C (Fig. 1) probably reflects conformational changes in the head region of the molecule. While conformational changes in the myosin heads dominate the first transition (Samejima *et al.*, 1981; Ishioroshi *et al.*, 1982), there is also evidence for an apparent early disruption of α-helix structure in localized regions of the myosin tail (Wright & Wilding, 1984; Walker & Trinick, 1986), which may also contribute to the increased rate of surface tension decay.

It is of interest to note that the surface pressure attained by the myosin solution after 40 min (π_{40}) was not affected by heating at 45°C (Fig. 1). This finding was unexpected since the surface active properties of partially disordered proteins are likely to be superior to those of the parent undenatured molecules (Graham & Phillips, 1976). We suggest that certain structural domains of the undenatured myosin, in particular the highly flexible globular heads (Lowey *et al.*, 1969), can readily rearrange at the interface once adsorbed. Thus the overall unfolding at the interface is similar for both unheated and heated myosin. However, the thermally disrupted molecule could, at least in the initial stages, cause a more rapid change in the surface tension. The data presented in Fig. 1 support this conclusion.

Major structural disruption in the tail region of myosin occurs when the protein is heated at ~55°C (Samejima *et al.*, 1981; Wright & Wilding, 1984) and results in exposure of hydrophobic residues normally masked by the α-helix (McLachlan & Karn, 1982). These conformational changes may account for the observed increase in surface activity of myosin heated at 60°C (Fig. 1). The induction phase and the slower initial rate of surface tension decay for myosin heated at 60°C, compared with the unheated control, may be due to some protein–protein interaction occurring in the bulk phase as a result of heating, resulting in a slower rate of mass transport of the larger molecular complexes to the interface. From the results it appears that once the partially denatured myosin molecules reached the interface they exerted a greater surface pressure than the native protein molecules and may, as suggested by Dickinson *et al.* (1987), have begun to form a viscoelastic film.

Thermally induced changes in the actomyosin complex at 60°C resulted in a faster rate of surface tension decay compared with the unheated protein (Fig. 2). This effect is attributed to dissociation of the actomyosin complex (Jacobson & Henderson, 1973) and heat-induced conformational changes in the helical portion of myosin. The F-actin moiety also shows increased flexibility when heated to this temperature (Jacobson & Henderson, 1973; Ziegler & Acton, 1984).

The present results show that moderate heat treatment of myosin and actomyosin caused an increase in surface activity and an overall faster rate of surface tension decay. Partial heat denaturation has previously been reported to enhance the surface activity of other food proteins (Mitchell *et al.*, 1970; Graham & Phillips, 1976; Kato *et al.*, 1981; Arnebrant *et al.*, 1987).

The stability of meat emulsions is considered to be dependent on both interfacial protein load and on the viscoelastic characteristics of the continuous phase (Jones & Mandigo, 1982). Heating may increase the hydrophobicity of myosin molecules already at the interface, thereby enhancing emulsion stability. However, protein–protein interactions which are likely to occur in the bulk phase during the thermal processing of meat emulsions (Morrissey *et al.*, 1987) may greatly inhibit mass transport of protein to the interface and also result in loss of molecular flexibility at or near the interface. It is likely that the improvement in emulsifying properties due to increased exposure of hydrophobic groups on heating may be more than counterbalanced when the interfacial protein layer becomes somewhat inflexible due to heating.

Effects of pH

The time dependence of surface tensions of G-actin (in water), F-actin (in 0·1M KCl containing 10mM ATP), myosin and actomyosin (both in 0·6M KCl) at the air–solvent interface (at 25°C and at a protein bulk phase concentration of 10^{-2}% w/v) at different pH values are shown in Figs 3–6.

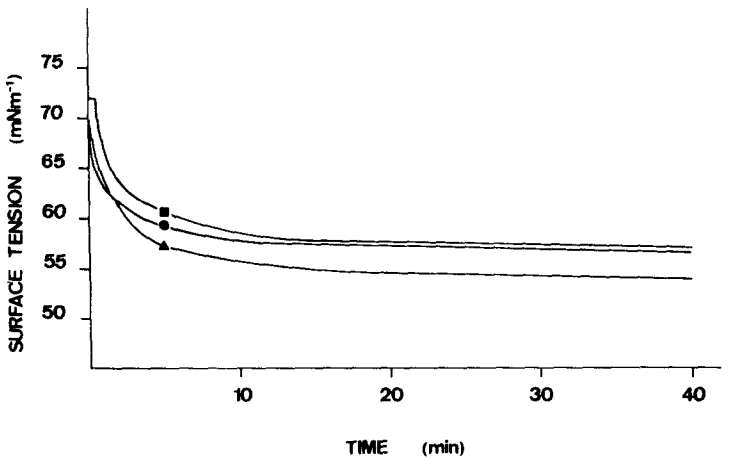

Fig. 3. Time dependence of surface tension at the air–solvent interface of G-actin at pH 5·6 (■), pH 7·0 (●) and pH 11·0 (▲), at a bulk phase concentration of 10^{-2}% w/v protein and at 25°C.

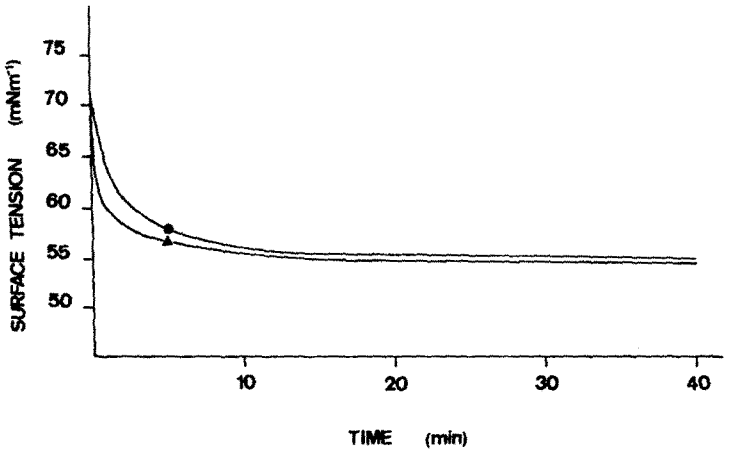

Fig. 4. Time dependence of surface tension at the air–solvent interface of F-actin at pH 7·0 (●) and pH 11·0 (▲), at a bulk phase concentration of 10^{-2}% w/v protein and at 25°C.

The initial surface tensions (γ_0) were 71·91, 72·25 and 73·27 mNm^{-1} for distilled water, 0·1M KCl containing 10 mM ATP and 0·6M KCl, respectively. The results show that the surface pressures attained after 40 min (π_{40}) by myosin and F-actin solutions were not significantly affected by the pH of the solutions. However, the π_{40} values for both G-actin and actomyosin solutions at pH 11·0 were greater than at pH 5·6 or 7·0. We propose that the enhanced surface activity of G-actin at high pH ($\pi_{40} \sim 15$ and 18 mNm^{-1} at pH 7 and 11·0, respectively) is due to pH-induced conformational changes in

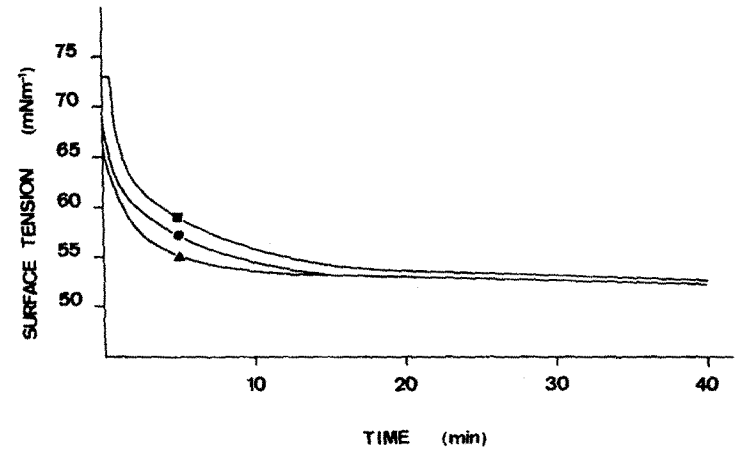

Fig. 5. Time dependence of surface tension at the air–solvent interface of myosin at pH 5·6 (■), pH 7·0 (●) and pH 11·0 (▲), at a bulk phase concentration of 10^{-2}% w/v protein and at 25°C.

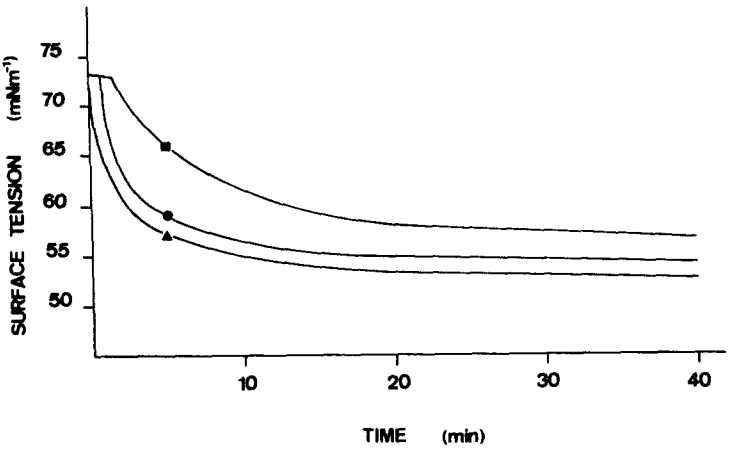

Fig. 6. Time dependence of surface tension at the air–solvent interface of actomyosin at pH 5·6 (■), pH 7·0 (●) and pH 11·0 (▲), at a bulk phase concentration of 10^{-2}% w/v protein and at 25°C.

the protein as exposure of G-actin to pH > 8·5 is reported to result in loss of ability to polymerize, to bind ATP and to activate myosin ATPase, and also leads to changes in optical rotation and increased reactivity of SH groups (Martonosi, 1968). It is, therefore, reasonable to conclude that the increased surface activity observed at pH 11·0 was due to loss of tertiary structure and unfolding of monomeric actin, leading to increased surface exposure of hydrophobic regions of the molecule, i.e. residues 120 to 153, 292 to 309 and 336 to 357 (Collins & Elzinga, 1975). Unfolding of the molecules at high pH may also be aided by lateral electrostatic repulsion at the interface, which tends to obstruct a high packing density of adsorbing molecules (Liedberg *et al.*, 1986). Thus it is likely that at neutral pH G-actin forms a condensed interfacial film, similar to lysozyme, with only limited denaturation of the protein at the interface (Graham & Phillips, 1979*c*). However, at high pH a transition to a more unfolded interfacial configuration occurs.

For all of the proteins studied pH greatly affected the rates of surface tension decay (Figs 3–6). The rates were slow at pH 5·6, which is near the isoelectric point of the proteins (Hamm, 1960). An induction period was observed for myosin and G-actin at pH 5·6 and the induction period for actomyosin at pH 5·6 was greater than that at pH 7·0. The rates of surface tension decay, particularly during the first 5 min, were faster, for all the proteins, at pH 11·0 than at pH 7·0.

The present findings are at variance with those of MacRitchie (1978), Kim and Kinsella (1985) and Waniska and Kinsella (1985), who observed that the rates of adsorption of proteins at interfaces were increased near the isoelectric point due to minimum electrostatic repulsion and close packing

of molecules at the interface. Our data suggest that, at pH 5·6, some protein–protein interactions occurred in the bulk phase which slowed the rate of mass transport to the interface. The occurrence of protein–protein interactions in solutions of myosin and actomyosin at low pH has previously been reported by Godfrey and Harrington (1970*a*,*b*) and Ziegler and Acton (1984). The formation of large molecular complexes may also be responsible for the reduced surface activity of actomyosin at pH 5·6. It is interesting to note that myosin, actin and actomyosin are also reported to have poor emulsifying properties near their isoelectric point (Hegarty *et al.*, 1963).

Since molecular conformation is an important determinant of the interfacial behaviour of proteins (Graham & Phillips, 1979*a–c*; Song & Damodaran, 1987), it is likely that the increased rate of surface tension decay observed at pH 11·0 (Figs 3–6) reflects pH-induced conformational changes of the proteins studied. At high pH, F-actin depolymerizes (Kasai *et al.*, 1962; Nagy & Jencks, 1965) and the monomers probably undergo alkaline denaturation in a similar manner to that previously proposed for G-actin. The light chains of myosin dissociate from the main core at pH >10, causing loss of ATPase activity and actin-binding properties (Dreizen *et al.*, 1967; Gershman *et al.*, 1969), and the effective hydrophobicity of myosin increases at high pH (Cheung, 1969). Alkaline pH dissociates the actomyosin complex and the individual components, i.e. actin and myosin, undergo alkaline denaturation as discussed above. These conformational changes may account for the rapid decrease in surface tension at pH 11·0 exerted by the proteins. Thus, at pH 11·0, it appears that there are two opposing phenomena: (i) intermolecular electrostatic repulsions which tend to slow down the rate of protein adsorption and (ii) alkaline destabilization of the native conformation of the proteins which may increase their effective hydrophobicity and molecular flexibility, thus favouring rapid adsorption at the interface. Our data suggest that for muscle proteins the latter may be dominant, resulting in a very rapid decrease in surface tension at pH 11·0.

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