of table eggs (1.0 vs. 0.7 mg/kg). This means that 3-HBA development starts *before* day 6 of incubation but not *after* 6 days as stated by Littmann et al. (1983).

Some Final Conclusions. The development of 3-HBA, which strongly depends on the moment of embryo growth termination, makes it impossible to calculate the amount of incubated eggs in egg product based on the 3-HBA content. Finally, in the present EEC legislation there is talk of incubated eggs. This work has shown that 3-HBA is not so much an indicator for "incubation" as (a) unfertilized incubated eggs do not show an increase in the 3-HBA content, not even after 18 days of incubation and (b) fertilized eggs stored at tempertures higher than 25 °C, which is a form of incubation, show an increase of the 3-HBA content. Especially for the latter it should be advisable not to refer to incubation alone, but also to embryo development or to storage at temperatures not exceeding 25 °C. However, the 3-HBA method as a control method for the misuse of longer than 6-days-incubated clears in egg products has been confirmed. In practice it is not possible to candle the incubator clears in such a way that only unfertilized eggs are selected.

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Functional Properties of Phosphorylated Yeast Protein: Solubility, Water-Holding Capacity, and Viscosity

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Phosphorylation of 30% of the lysine groups of yeast nucleoprotein facilitated the preparation of phosphoprotein (72%) with only 2.7% nucleic acid. Phosphorylation improved solubility slightly between pH 6.0 and 7.0 and greatly enhanced the water-holding capacity from 10 to 25 g of water/g of protein between pH 6 and 7.5. Viscosity increased with protein concentration and with pH, especially above pH 6.5 reflecting increasing electrostatic repulsion of the modified proteins. The phosphoproteins showed shear thinning at low shear rates, and viscosity decreased with increasing temperatures. The data indicated that phosphorylated yeast proteins might be useful as thickening agents in certain foods.

INTRODUCTION

To fully exploit their desirable functional properties, it is necessary to separate yeast proteins from the cell biomass (Kinsella and Shetty, 1979). However much of the isolated proteins exist as ribosomal, i.e. ribonucleic acid–protein, complexes and ribosomes (Shetty and Kinsella, 1979, 1980, 1982a,b; Damodaran and Kinsella, 1984). On the basis of the concept that electrostatic interactions are important for the integrity of these nucleoprotein complexes, we developed methods based on chemical derivatization for the separation of proteins from contaminant nucleic acids (Kinsella and Shetty, 1979; Shetty and Kinsella, 1982a, 1982b) and recently we showed that chemical phosphorylation of yeast proteins facilitated the recovery of protein concentrates (75–80% protein) from yeast with approximately 2% contaminant RNA (Damodaran and Kinsella, 1984).

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The successful adoption and use of proteins isolated from microbial biomass depend on their physicochemical properties, which determine their usefulness as functional food ingredients (Kinsella, 1976). There is limited information available concerning the functional properties of isolated yeast proteins (Vananuvat and Kinsella, 1975; Huang and Rha, 1971; Cooney et al., 1980), and more information is required to assess their potential in food systems (Litchfield, 1983; Batt and Sinskey, 1984).

Many of the important functional properties of food proteins are related to their interactions with water. Solubility provides an index of native structure and is a desirable prerequisite of proteins in beverages, in liquid foods, and for emulsion and foam formation (Kinsella, 1982). Water-holding capacity, a quantitative indication of the amount of water retained within a protein matrix under a defined condition, is important in doughs, batters, and comminuted meat systems. Knowledge of the viscosity of protein dispersions is of practical significance in relation to processing, process design, mouthfeel of viscous fluid products, and new product development (Hermansson,

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1975).

In order to assess the potential of phosphorylated yeast proteins in food products, we studied their hydration and viscosity behavior.

MATERIALS AND METHODS

Materials. Acrylamide, bis(acrylamide), Coomassie Brilliant Blue, 2-mercaptoethanol, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ribonuclease A, ovalbumin, human transferrin, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Sodium dodecylsulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Bromophenol blue was purchased from Fisher (Fair Lawn, NJ). All other chemicals used in this study were reagent grade. Deionized distilled water was used for making reagent solutions.

Methods. Yeast nucleoprotein and phosphorylated yeast protein were prepared by the methods described previously (Damodaran and Kinsella, 1984a,b; Huang and Kinsella, 1986).

Electrophoresis. The molecular sizes of yeast nucleoproteins and phosphorylated protein were examined by electrophoresis. A modification of the method of Laemmli (1970) was used for sodium dodecylsulfate–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE). The separating gel was prepared with 10% acrylamide–bis(acrylamide) solution containing 0.38 M Tris-HCl, 0.1% $NaDodSO_4$, 0.025% (w/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 0.046% ammonium persulfate (added immediately before gel formation). The stacking gel was composed of 4.0% acrylamide with 0.106% bis(acrylamide) with 0.125 M Tris-HCl, 0.1% $NaDodSO_4$, 0.025% (w/v) TEMED, and 0.046% ammonium persulfate. The yeast nucleoprotein and phosphorylated protein (3 mg/mL) were made up in 62.5 mM Tris-HCl, 1.0% $NaDodSO_4$, 2.0% 2-mercaptoethanol, 8 M urea, 0.025% bromophenol blue, and pH 6.8 dissociating buffer and incubated at 25 °C for 6 h before loading 15 μL of samples in the wells of the gel. Ribonuclease A (M_r 13 700), ovalbumin (M_r 45 000), bovine serum albumin (M_r 68 000), and human transferrin (M_r 90 000) were used as the molecular weight markers.

All samples were electrophoresed at 15 mA for 5–6 h with 25 mM Tris-HCl, 0.192 M glycine, and 1.0% $NaDodSO_4$, pH 8.3, as running buffer. The gel was fixed in 10% acetic acid and 50% methanol, stained in 0.05% Coomassie Brilliant Blue R-250, 10% acetic acid, and 50% methanol, and destained in 10% acetic acid with 10% methanol.

Solubility. Dispersions of phosphorylated yeast protein were made in distilled water (1% by weight); the pH of the suspensions was adjusted to the designated value by the addition of 0.1 N HCl or NaOH and stirred for 1 h at 25 °C. The suspension was centrifuged at 10000*g* for 20 min at 5 °C, and the soluble protein in the supernatant was determined by the micro Kjeldahl method (Morr et al., 1985).

Water-Holding Capacity. The water-holding capacity of phosphorylated yeast protein was determined according to the method of Regenstein et al. (1979). Dispersions of phosphorylated yeast protein were made in water pH 7.0 (1% by weight), stirred for 1 h at 25 °C, and transferred quantitatively to 50 mL of polycarbonate centrifuge tubes. The suspensions were centrifuged at 30000*g* for 15 min at 2–4 °C. The supernatant was decanted, and the centrifuge tube above the protein pellet was dried with filter paper wrapped around a glass rod. The tube with the hydrated pellet was reweighed. The protein concentration of the original solution, of the supernatant, and of the pellet was determined. The moisture content of the pellet was determined by vacuum oven drying at 100 °C for 20 h, and the water-holding capacity was calculated.

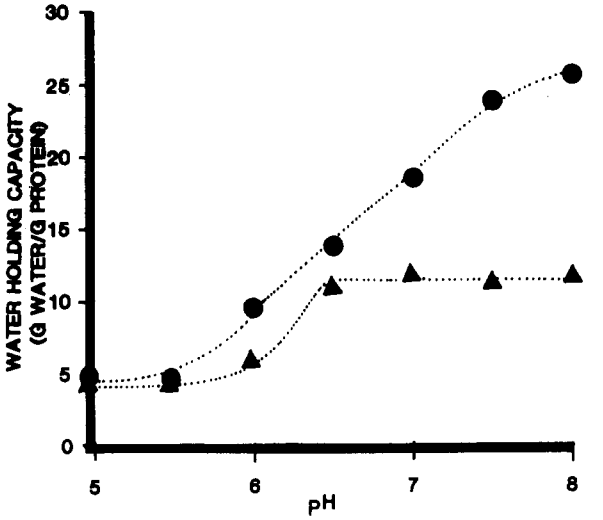


Figure 1. Effect of pH on the water-holding capacity of phosphorylated yeast protein: yeast nucleoprotein (▲); phosphorylated yeast protein (●).

Determination of Viscosity. A Haake viscometer (Haake, Karlsruhe, W. Germany) equipped with a NV narrow-gapped concentric cylinder spindle and cup was used to measure the shear stress of samples (10 mL) within 1 min after equilibration. Temperature was controlled in a thermostated circulating water bath.

The viscosity was calculated by $\eta = KUS$, where η = apparent viscosity (cP), K = calibration constant (0.053), U = speed factor (583.2/rpm), and S = scale reading $\gamma = B/U$, where γ = shear rate (s^{-1}) and B = rate of shear factor (3140).

RESULTS AND DISCUSSION

Under the reaction conditions employed using phosphorus oxychloride, approximately 30% of the ε-lysine groups were phosphorylated. The compositions of the isolated material were 72, 2.7, 3.1, and 5.7% phosphorylated protein, nucleic acid, carbohydrate, and lipids, respectively (Huang and Kinsella, 1986). The original nucleoprotein contained 64, 20, 6, and 5% protein, nucleic acid, carbohydrate, and lipid materials, respectively. The phosphorylated yeast was light creamy colored and possessed a bland acidic flavor.

Water-Holding Capacity. Phosphorylated yeast proteins were very wettable and hydrated easily. At all pHs studied, the water-holding capacity (WHC) of phosphorylated yeast protein was enhanced following phosphorylation (Figure 1). This could be attributed to the increased hydration of the added phosphoryl group, which can bind five to six water molecules per group (Falk et al., 1963; Saenger, 1984), and the loosening of protein structure resulting from the repulsion between the negatively charged phosphoryl groups and anionic carboxylic groups (Shetty and Kinsella, 1982a). The WHC increased with increasing pH from 5.0 to 8.0. Since ionized groups bind considerably more water than nonionized groups (Kuntz, 1971), the increase in WHC could be partly due to the ionization of the phosphoryl groups. The pK_a of the second acidic group in the phosphorylated derivative is between 5.5 and 7.0 (Jordan, 1960; Corbridge, 1980).

Above pH 6.0 a semitransparent gellike pellet was observed when the phosphorylated yeast protein dispersions were centrifuged. Possibly, the gellike network structure was formed during or following phosphorylation of yeast

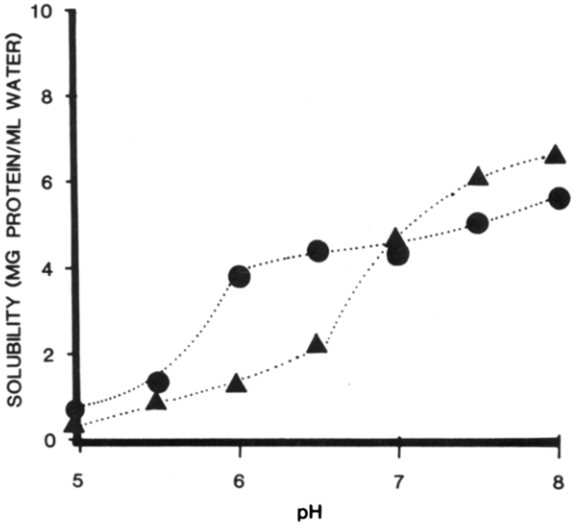


Figure 2. Solubility–pH profile: phosphorylated yeast protein (●); yeast nucleoprotein (▲).

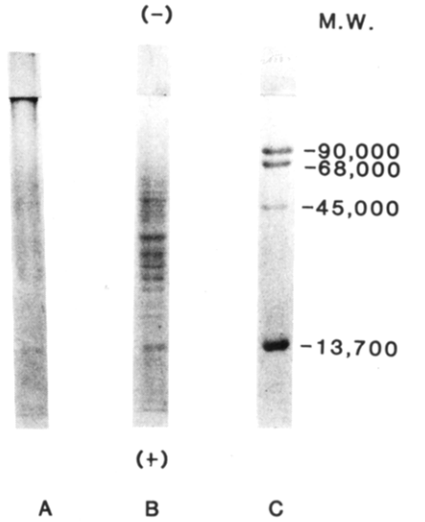


Figure 3. Gel electrophoretic patterns of phosphorylated yeast protein and yeast nucleoprotein: A, phosphorylated yeast protein; B, yeast nucleoprotein; C, molecular weight marker containing ribonuclease A (M_r 13700), ovalbumin (M_r 45000), bovine serum albumin (M_r 68000), and human transferrin (M_r 90000). Gel electrophoresis was performed according to the procedure described in the Methods section.

proteins, and water was entrapped in the network, resulting in a substantial increase in WHC. The addition of salt (0.1 M) caused a marked decrease in the amount of bound water from 18 to 5 g of water/g of protein. The decline in WHC was probably due to the reduction in electrostatic repulsion between the phosphate groups and a commensurate decrease in hydration of the protein (Eagland and Franks, 1975).

Proteins from plant sources such as soybean, sunflower, and cottonseed have WHC around 2.5–6.0 g of H_2O/g of protein (Fleming et al., 1974; Childs and Park, 1976; Rahma and Narasinga Rao, 1983). On the other hand, the WHC of actomyosin is much higher, i.e. to 17–20 g of H_2O/g of protein (Regenstein et al., 1979). Actomyosin has a high WHC, presumably because of its network structure (Hamm, 1975).

The solubility–pH profile of phosphorylated yeast protein (Figure 2) showed that between pH 5.5 and 7.0 solubility was improved by phosphorylation. The phosphorylated yeast protein dispersed very rapidly and formed a clear stable suspension. The greater solubility of phosphorylated protein compared to the yeast nucleoprotein may be attributed to the increased hydration of the added charged phosphoryl groups and the loosened structure of the derivatized protein (Shetty and Kinsella, 1982a,b). Upon centrifugation much of the phosphorylated protein sedimented to form a gellike pellet in the centrifuge tube, and only a fraction (45%) of the protein remained in true solution.

Calcium in excess of 10 mM precipitated more than 90% of the soluble proteins (data not shown) probably due to the formation of protein–calcium complexes, i.e. calcium binding reduced the electrostatic repulsion between the protein molecules, resulting in aggregation. A similar phenomenon was also observed with phosphorylated β-lactoglobulin (Woo et al., 1982). The possible formation of a network structure by cross-linking of proteins during phosphorylation was examined by polyacrylamide gel electrophoresis of phosphorylated yeast protein in the presence of $NaDodSO_4$ and 2-mercaptoethanol. Under these conditions, separation of proteins is based primarily on size (Weber and Osborn, 1975). A significant amount (>50%) of the phosphorylated yeast protein did not enter the separating gel, indicating the presence of large macromolecules in the phosphorylated yeast protein (Figure 3). This is consistent with other reports of intermolecular cross-linking of proteins, e.g. during phosphorylation of

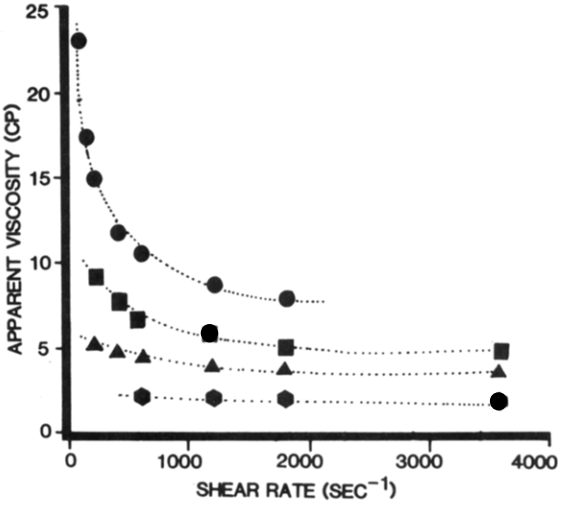
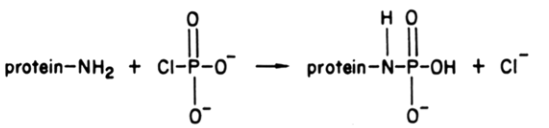


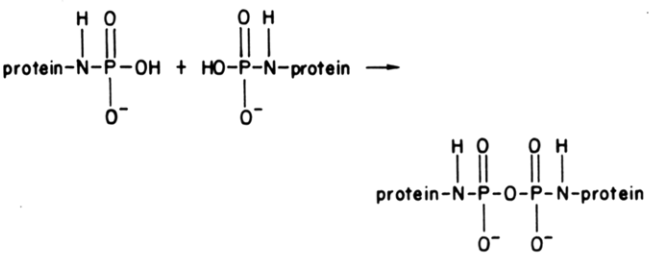
Figure 4. Effect of shear rate on the apparent viscosity of phosphorylated yeast protein at pH 7.0. Protein concentration: 1% (●); 2% (▲); 3% (■); 4% (●).

β-lactoglobulin (Woo et al., 1982), lysozome, and casein (Matheis et al., 1983).

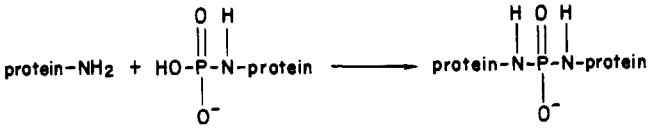
Phosphorylation of free NH_2 groups of protein is easily achieved under the conditions used.



The free OH group can further react by nucleophilic replacement with adjacent phosphorylated protein molecules and form a cross-linkage (Emsley and Hall, 1976):



Free amino groups of the protein can also react with the phosphoryl group on the phosphorylated protein by a similar mechanism:



Since there are several amino and phosphoryl groups on each protein, a cross-linked network structure could thus be formed.

Apparent Viscosity. The apparent viscosity of phosphorylated yeast protein dispersions increased progressively with increasing protein concentration (Figure 4). Similar trends have been reported for food proteins, e.g. soy proteins (Circle et al., 1964; Hermansson, 1975), sunflower proteins (Fleming et al., 1974), caseinates (Hermansson, 1975), and rapeseed protein (Gill and Tung, 1976).

In very dilute protein dispersions the apparent viscosity reflects the effects of each of the individual dispersed protein molecules. As the concentration is increased, the disturbances of flow caused by the dispersed protein molecules are no longer independent and protein–protein interactions became dominant and more water molecules are immobilized, resulting in the additional increase of apparent viscosity at high concentrations (Frisch and Sinha, 1956). This may account for the progressive increase in apparent viscosity of phosphorylated yeast protein with increasing protein concentration.

Phosphorylated yeast protein dispersions exhibited shear thinning over a range of shear rates (Figure 4), and this was more pronounced at higher protein concentrations and at lower shear rates. Similar pseudoplastic flow behavior has been reported for several proteins: egg albumin (Tung, 1978; Tung et al., 1971), soy protein (Circle et al., 1964; Hermansson, 1975), casein and whey protein (Hermansson, 1975), and alkali-extracted yeast protein concentrate (Huang and Rha, 1971). The shear thinning of phosphorylated yeast protein could be attributed to its high degree of hydration and/or to shear-induced alignment. At very low shear rates there was little effect on the water layers and the hydrated aggregate behaved like a large macromolecule possessing high apparent viscosity. As the shear rate was increased, the water layers were progressively removed, resulting in reduction of the size of hydrated aggregate with a concomitant decrease in the apparent viscosity (Holdsworth, 1971; Tung, 1978). Progressive alignment was another possible reason for the observed shear thinning. Thus, as the shear rate was increased, the randomly oriented and entangled asymmetric protein molecules became realigned with the shear planes so that frictional resistance was reduced (Tung, 1978). At high shear rates, the macromolecules became fully aligned along the laminar shear planes, and no further streamlining was observed beyond this point.

At constant shear rates (above 1500 s^{-1}), the time had no significant effect on the apparent viscosity of phosphorylated yeast protein dispersions. This suggests that phosphorylated yeast proteins possessed stable structures that resisted the constant shear force over a long period of time.

The apparent viscosity of phosphorylated yeast protein increased nearly fourfold when the pH was increased from 5.0 to 8.0 (Figure 5). This may be related to the increased hydration with increasing ionization of the phosphoryl groups and possibly greater electrostatic repulsion between molecules.

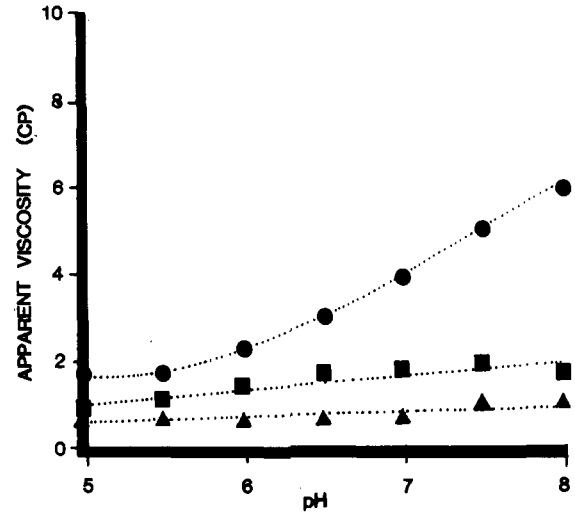


Figure 5. Effect of pH on the apparent viscosity (2% solutions): phosphorylated yeast protein (●); yeast nucleoprotein (■); BSA (▲). The apparent viscosity measurement was performed at shear rate of 1047 s^{-1}.

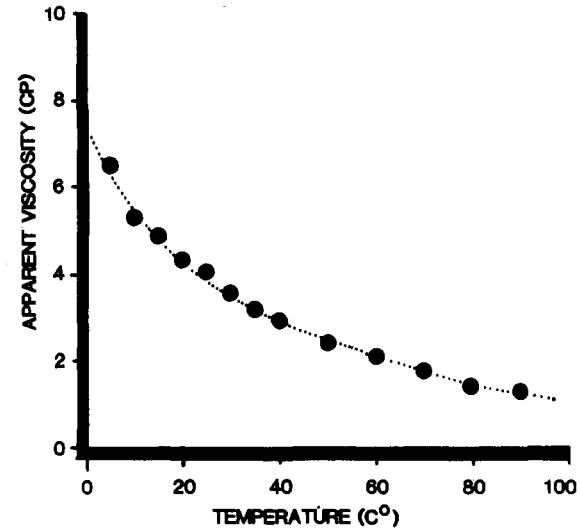


Figure 6. Effect of temperature on the apparent viscosity of phosphorylated yeast protein dispersions. The apparent viscosity measurements were performed on 2% protein dispersion at pH 7.0 and constant shear rate of 1047 s^{-1}.

Ionic strength has a marked effect on the flow properties of phosphorylated yeast protein, i.e. the apparent viscosity was reduced by 60% at 0.1 M sodium chloride, pH 7.0 (data not shown). The change in flow properties could be attributed to the partial dehydration of proteins by the salt ions resulting in lower apparent viscosity (Eagland and Franks, 1975; Hermansson, 1975). In addition, electrostatic repulsion between different protein molecules or parts of molecules were reduced by salts, and the protein molecules could thus assume a more compact conformation, resulting in decreased viscosity (Cantor and Schimmel, 1980).

Increasing temperatures resulted in decreased viscosity by destabilizing both protein–protein and protein–water interactions (Figure 6). With most proteins above a certain high temperature, thermal denaturation causes the viscosity to increase (Catsimpoolas and Meyer, 1970). Apparently the strong electrostatic repulsions between phosphorylated yeast proteins minimized noncovalent associations between the thermally altered proteins and inhibited coagulation. The progressive decrease of viscosity with increasing temperature reflected the thinning (decreased hydrodynamic volume) of the proteins in solution (Holdsworth, 1971). Therefore, no increase in viscosity,

Table I. Flow Behavior Parameters of Phosphorylated Yeast Protein Compared with Some Food Proteins

protein	concn, %	flow behavior n	consistency coeff (k)
phosphorylated yeast protein	1	0.945	2.770
	2	0.868	7.500
	3	0.770	16.900
	4	0.677	38.280
soy protein isolate[a]	4	0.910	0.110
	8	0.760	1.500
	12	0.670	11.000
caseinate[a]	4	0.920	0.040
	8	1.010	0.060
	12	0.900	1.100
whey powder concentrate[a]	4	0.940	0.027
	8	0.980	0.034
	12	0.930	0.079

[a] Values are from Hermansson (1975).

coagulation, nor gelation was observed even when the phosphorylated yeast protein was heated at high temperature.

Since phosphorylated yeast protein dispersions exhibited pseudoplastic, non-Newtonian flow behavior, the experimental data were analyzed by the power law formula (Tung, 1978) i.e. $\eta = kD^{n-1}$, where η = the apparent viscosity, D = the shear rate, k = the consistency coefficients, and n = the flow behavior index.

The consistency coefficient, k, a parameter corresponding to viscosity generally increases with increasing protein concentration (Holdsworth, 1971; Hermansson, 1975). The flow behavior index, n, reflects the extent of deviation of flow behavior from that of a Newtonian fluid where $n = 1$. This index generally decreases with increasing protein concentration because the flow behavior of a protein solution deviates from that of pure water (Holdsworth, 1971). Data summarizing the flow behavior of phosphorylated yeast proteins calculated from rheological data (Figure 4) are compared with values for other food proteins obtained under comparable conditions (Table I). The flow properties of phosphorylated yeast protein were characterized by large values of k. The apparent viscosity of phosphorylated yeast protein was thus much higher than those of soy protein isolate, caseinate, and whey protein concentrate over a broad concentration range. The unique flow characteristics of phosphorylated yeast protein could be attributed to the presence of the high molecular weight protein polymers and the highly hydrated protein network formed during phosphorylation. Both polymerization and hydration of proteins enhance the viscosity of the protein solution by increasing the hydrodynamic volume of protein molecules (Cantor and Schimmel, 1980). These data suggest that phosphorylated yeast protein would be useful thickening agents in foods.

The present results indicate that some functional properties of yeast proteins, especially water-holding capacity and apparent viscosity, were substantially improved by phosphorylation. The increases in solubility, water-holding capacity, and viscosity were presumably due to the increased hydration of the added phosphoryl group and the loosening of protein structure resulting from the electrostatic repulsions between the negatively charged groups. The surface-active properties of yeast proteins were also improved by phosphorylation (Huang and Kinsella, unpublished 1986), indicating that chemical phosphorylation while reducing nucleic acids is a feasible method for preparing functional proteins from yeast.

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