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# Modification of Vital Wheat Gluten with Phosphoric Acid to Produce High Free Swelling Capacity

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**ABSTRACT:** Wheat gluten reacts with phosphoric acid in the presence of urea to produce natural superabsorbent gels. Fourier Transform Infra-red (FT-IR) spectroscopy and two-dimensional gel electrophoresis (2DE) reveal chemical changes from the reaction. Temperatures above 120°C and dry conditions create the opportunity for reaction. FT-IR analyses confirm the formation of esters, carbamates, and phosphoramides on the gluten samples. 2DE protein composition topographies indicate a shift in the isoelectric point (p*I*) to lower values along with extensive inter-protein linkages. A free swelling capacity (FSC) in excess of 85× the mass of the converted gluten is obtainable using a conservative vacuum-assisted method to recover and quantify the properties of the wet gel. Other methods produce FSC values nearly twice as high. FSC for acid-treated gluten is lower for solutions containing solutes than the FSC for deionized water. Native gluten produces FSC values that are about 2% of those for treated gluten, but these values are less sensitive to the presence of ionic solutes and increase slightly in the presence of aqueous ethanol up to a mole fraction of 0.25..


**KEYWORDS:** biopolymers and renewable polymers; adsorption; crosslinking; gels; proteins

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## INTRODUCTION

Most commercial superabsorbent materials are derived from synthetic monomers, such as acrylic acid and acrylamide. For instance, one of the most successful superabsorbent materials is the sodium salt of polyacrylic acid which is a completely synthetic polymer. These polymers can absorb hundreds of times their original weight in water and are widely used in the hygiene industry. However, the use of petroleum-based polymers has several disadvantages. One is that the polymers are produced from a non-renewable resource. Another disadvantage is that these synthetic polymers are not biodegradable, which leads to persistence and accumulation in the environment.

Vital wheat gluten is a complex protein matrix comprised of approximately equal portions of protein subunits that are linked covalently and subunits that are bound together by secondary forces. The subunits are themselves amino acid polymers, but are treated as the building blocks for the gluten matrix. Vital wheat gluten has a free swelling capacity (FSC) for water of 1.4–1.5 and has been modified to produce superabsorbent materials. The modification occurs when native gluten is reacted with polyhydroxy acids such as sulfuric,[1–3] citric acid,[4] or phosphoric acid.[5] The acid-modified gluten samples were reported to absorb up to hundreds of times their weight in water. The increased absorptivity is believed to be due to the formation of esters with hydroxyl groups in gluten amino acids such as serine, threonine, and tyrosine. The reported phosphoric acid method also made use of urea to reduce hydrogen bonding and, thereby, to facilitate the reactant accessibility to susceptible amino acids. Because water is produced in the esterification reaction, dry or dehydrating reaction conditions are generally desirable to ensure completeness of the reaction. The esterification reaction using phosphoric acid in conjunction with carbamide formation using urea is also a way to alter starch properties. Starch modified in this way had FSC of 50.[6]

In this study, we react vital wheat gluten with phosphoric acid to produce differing absorption properties by modifying the reaction process steps. We examined the resulting chemical

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changes to the gluten using Fourier transform infrared spectroscopy (FT-IR). We also applied two-dimensional gel electrophoresis (2DE) that is the result of (a) isoelectro-focussing (IEF) to separate proteins according to their characteristic isoelectric points followed by (b) separating according to their molecular weight using polyacrlyamide gel electrophoresis (PAGE) and (c) staining the gels with a protein specific stain. The 2DE method makes use of a detergent solution and a chemical reducing agent and produces a flat display of p*I*, $M_r$, and optical density. These were converted to three-dimensional protein subunit topographies describing the p*I*, $M_r$, and optical density distributions of the individual protein building blocks of the gluten protein matrix. We also measured FSC for both modified and unmodified gluten. The ability of the gluten-based sorbent to absorb solution containing ionic and non-ionic solutes was determined.

## EXPERIMENTAL

### Materials

Vital wheat gluten was obtained from a local supplier of flour and ingredients for the baking industry (Giusto, San Francisco, CA). Proximate analysis of this material indicated moisture free protein content of 82% based on 5.7xN%. Phosphoric acid, as an 85% solution of $H_3PO_4$ in water and urea at 99% purity, was obtained from Sigma Aldrich, St. Louis, MO. Reagent grade NaCl, $MgCl_2$, $Na_3PO_4$, and $Na_2SO_4$ were used in absorption studies.

### Chemical Reaction

The steps for the modification of vital wheat gluten are shown in Figure 1(A) and consist of mixing of the ingredients, drying, reaction induction, neutralization, and displacement drying of the solids. In the first or mixing step, vital gluten (100 g) was mixed with water (200 g) in a KitchenAid Professional Mixer (K5M50PWH) fitted with a standard mixing pad for 5 min, and then phosphoric acid (18 mL) was added to give a 0.35*M* final acid concentration and urea (30 g) was added to give a final 2.5*M* concentration. Mixing continued for 25 min. The mixture was vacuum-dried or, occasionally, oven-dried. The mixture was broken or cut into small pieces that were less than 0.6 cm to ensure complete drying. The vacuum-dried gluten, which dried rapidly and expanded into a foamy mass, was heated after milling in an impact mill with a 1 mm screen or, alternatively, left in its foamy state. Temperature and duration of the drying process was 40 or 60°C for up to 7 days. The dry gluten reaction mixture usually was milled. We also applied heat to vacuum-dried mixtures that were not milled prior to treatment. In either case, the samples were heated in a laboratory oven. Temperatures applied in the 30 min reaction were in the range from ambient to 149°C. Following this step, NaOH or $Ca(OH)_2$ was added to achieve a pH of 7.5–7.8, and the wet filtered solids were washed by suspension in 500 mL of 100% ethanol or 70–100% acetone and filtering. Washing in these solvents helped to remove residual water in the solids by physical displacement and extraction.[7] The washing procedure was repeated two more times. Residual solvent on the filtered solvent was removed by evaporation in a laboratory hood or alternatively freeze-dried at −10°C in a laboratory freeze-drier. Lastly, the sample was manually pulverized in a mortar and pestle.

### Gel Electrophoresis

For each substrate, 50 mg of sample was suspended in 1 mL of sodium dodecylsulfate (SDS) buffer containing 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 40 mM tris-(hydroxymethyl) aminomethane–hydrochloride (Tris–HCl), at pH 8.5 and incubated for 1 h at room temperature on a rocker platform (Stovall Life Science, Greensboro, NC). Following incubation, insoluble material was removed from leachates by centrifugation at 16,000 × *g* for 15 min at room temperature (Eppendorf 5415C; Brinkman Instruments, Westbury, NY). Aliquots were removed from the SDS extracts, protein precipitated with four volumes acetone to one of aliquot, and protein amount determined calorimetrically using a standard curve based on a protein-sensitive Folin phenol reagent.[8,9] 2DE was performed by the method described by Hurkman.[8] In particular, proteins were precipitated from the SDS extracts by the addition of four volumes of acetone to one of extract and incubation overnight at −20°C. Protein was recovered by centrifugation at 16,000 × *g* for 10 min, dissolved in urea buffer (9*M* urea, 4% Nonidet P-40, 2% 3–10 ampholytes, and 1% DTT) and 18 μg applied to the first dimension IEF gel. Proteins were separated in the second dimension by SDS PAGE and visualized by staining with Coomassie Brilliant Blue G-250 (Novex, LC6025).

The original two-dimensional gels were digitized by scanning and converted to three-dimensional representations or protein subunit topographies by the digital image processing functions of the software application Mathematica v.7 (Wolfram Research, Champagne, IL). We used the 2DE digital data to compute a one-dimensional band pattern that is displayed along the $M_r$ scale. This computed pattern simulates a conventional one-dimensional SDS–PAGE gel (1DE) and succinctly summarizes the molecular weight distribution of the proteins on the 2DE gel. To emphasize the derivation, the computed pattern is labeled s1DE. The digital processing included background subtraction using optical density in a zone of the gel between the end of the isoelectric focusing gel and the molecular weight standards. Optical densities were converted to mass fractions by numerically dividing pixel optical density value by the total optical density of the gel. Finally, the mass fractions were normalized to the maximum mass fraction in the 2DE for the native protein to ensure that the Mathematica color assignment function consistently represented the data (Figures 2 and 3).

### Fourier Transform Infrared Spectroscopy

FT-IR spectra were obtained in transmission on 7 mm potassium bromide (KBr) pellets containing the sample, which were made using the Perkin Elmer Quick Press. Sample concentration in the KBr varied from 1.9% to 2.1%. A KBr pellet spectrum was used as background for each sample. Spectra were scanned over the range of 400–4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ using the Perkin Elmer FT-IR System 2000 equipped with a deuterated triglycine sulfate (DTGS) detector. Fifty scans were collected for each sample.

Normalized spectra were obtained by dividing spectral absorbance values by the tested sample mass. Each spectrum was

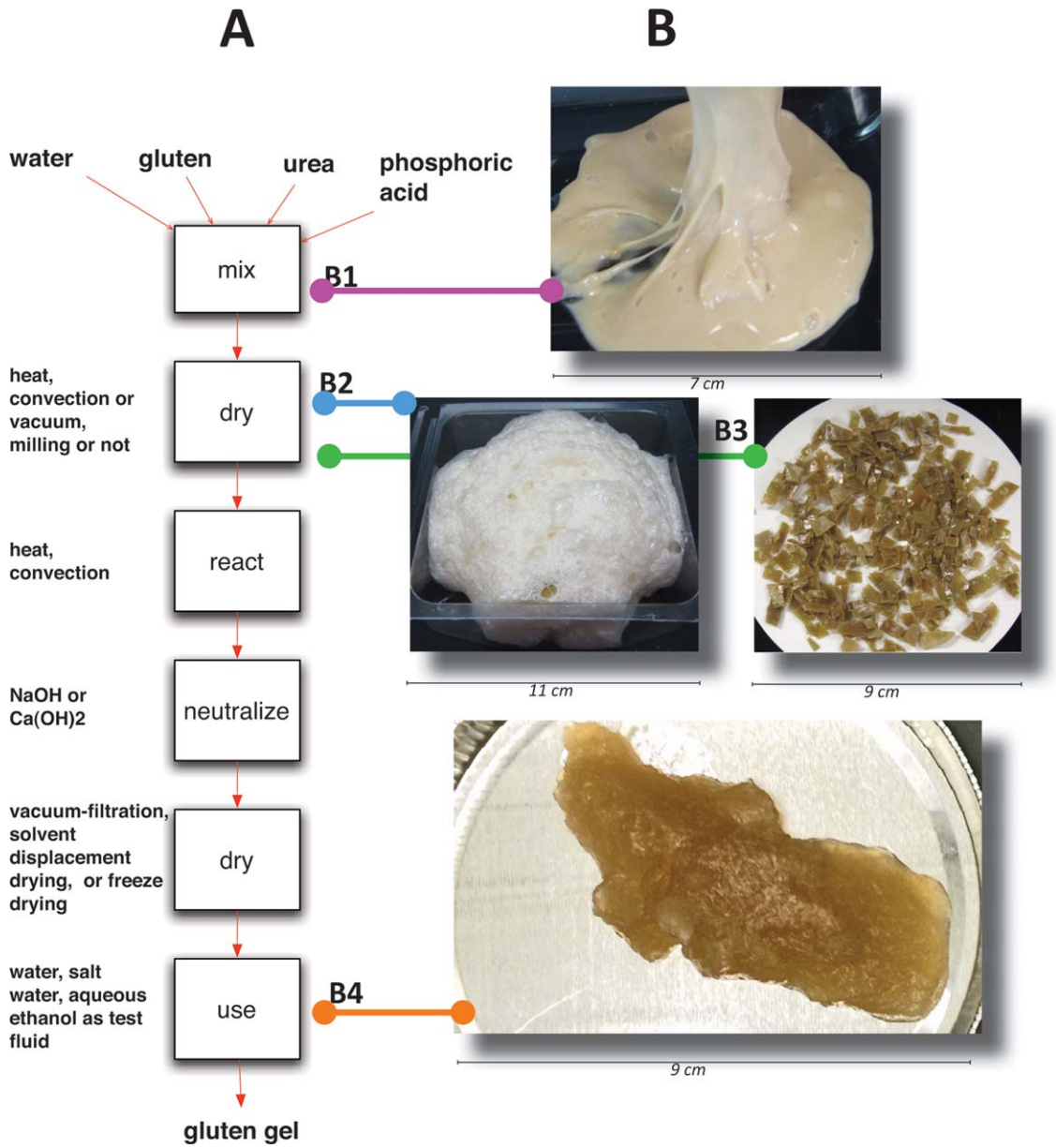


**Figure 1.** Annotated process schematic (A) for phosphoric acid conversion of wheat gluten to form a gel with high capacity for absorbing water. Example photographs (B) illustrating the physical forms of the gluten at selected stages of processing including after mixing (B1), vacuum drying (B2), heated convection drying (B3), and the final product in use as an absorbent with absorbed water (B4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

smoothed using the block average smoothing algorithm. By this method, spectra were smoothed using nine points in each block. The method averages points in a block around the target point to eliminate sharp noise peaks. The difference spectrum was obtained by subtracting the spectrum of un-reacted gluten.

### Solution Uptake

Each gluten sample (0.5 g) was mixed with 50 mL of deionized water and rested for 0.5 h before measurement. Three methods ere applied to recover the swollen gel: (1) filtration using a Buchner flask with a 10 $\mu$m nylon SpectraMesh filter (Spectrum Medical) with vacuum, (2) gravity filtration using a Buchner flask with a 10 $\mu$m nylon SpectraMesh filter with no applied vacuum, and (3) centrifugation at 1000 $\times$ $g$ followed by decantation of the unincorporated fluid. The gel sample was dried in a Denver Instrument IR-200 moisture analyzer (Bohemia, NY) heated to 115°C to constant sample weight. Free Swelling Capacity, FSC was calculated as

$$\mathrm{FSC} = \frac{m_H - m_D}{m_D} \quad (1)$$

FSC is the ratio of the mass of absorbed fluid to the mass of dry phosphorylated gluten. This is equivalent to water retention value, WRV, and FSC as described elsewhere.[6,10] In eq. (1) $m_H$ is total mass of the wet gel and $m_D$ is mass of the dry sample. An alternative form for reporting FSC incorporates the original unreacted mass of gluten as the basis. This value or $\mathrm{FSC}_r$ was calculated as

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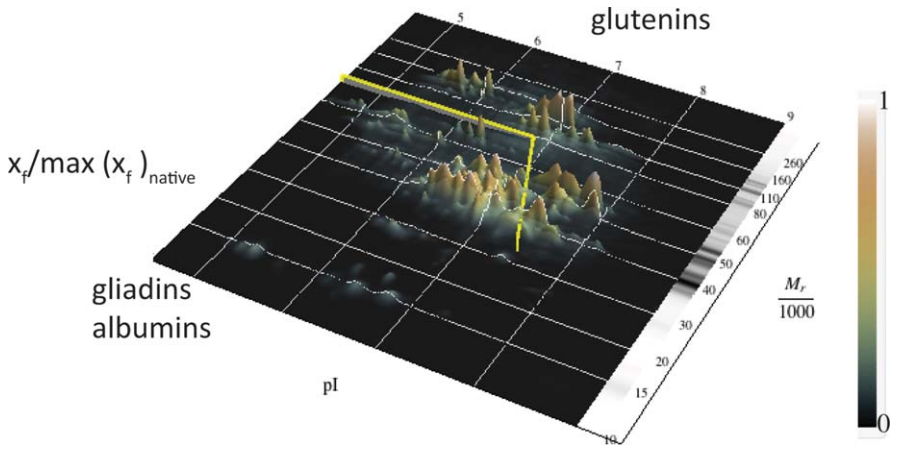


**Figure 2.** 2DE protein subunit topography of unmodified, vital gluten employed in these studies. Ordinate is mass fraction calculated from optical density values. A gray-scale virtual 1DE gel or s1DE (constructed by summing the densities in each row of the gel scan after background removal) is shown at the edge of this gel. Yellow line approximately locates the division between glutenin subunits and all other protein types. High molecular weight glutenin subunits, or HMW-GS, are those above $M_r$ of 80 and low molecular weight glutenin subunits, or LMW-GS, are those below $M_r$ of 80 and with p*I* values to the right of the yellow line. HMW-GS and LMW-GS are capable of chain extension by disulphide linkages while all the other proteins interact only by non-covalent interactions in native gluten. Ordinate values are scaled to produce a maximum value of 1 on this plot (see "Methods" section). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$$FSC_r = FSC \cdot Y \quad (2)$$

In eq. (2), $Y$ is the fractional yield or mass product per mass of gluten.[6]

## RESULTS

### 2DE and FT-IR spectroscopy

The protein subunit compositional topography for native or vital gluten is shown in Figure 2 and the topography for phosphorylated gluten is shown in Figure 3. These topographies show molecular weight ($M_r$) on the *y*-axis, isoelectric point (p*I*) on the *x*-axis, and mass fraction on the *z*-axis. Each of these topographies is derived from digital scans of the original stained gels using custom notebooks written in the Mathematica application. The protein peaks in Figure 2 correspond to the separated protein building blocks for the gluten polymer. The 2DE method used to generate these topographies eliminates covalent disulphide linkages by chemical reduction and the chaotropic or solubilizing action of the detergent SDS minimizes non-covalent interactions such as hydrogen bonds, hydrophobic interactions, and van der Waals interactions. Hence, for the most part, each spot on the original gel and each peak in the protein subunit topography was a single protein type. The p*I* values are the pH values at which the net charges on the protein are balanced, but both positively and negatively charged regions remain on each protein. We note reported exceptions to the peak uniqueness, since at high $M_r$ some individual proteins may appear as charge chains that include a primary central peak and several subordinate peaks at the same $M_r$, but at higher and lower p*I*.[11,12]

The native gluten protein subunit topography in Figure 2 exhibits a pattern of protein isoelectric point (p*I*) versus $M_r$ very

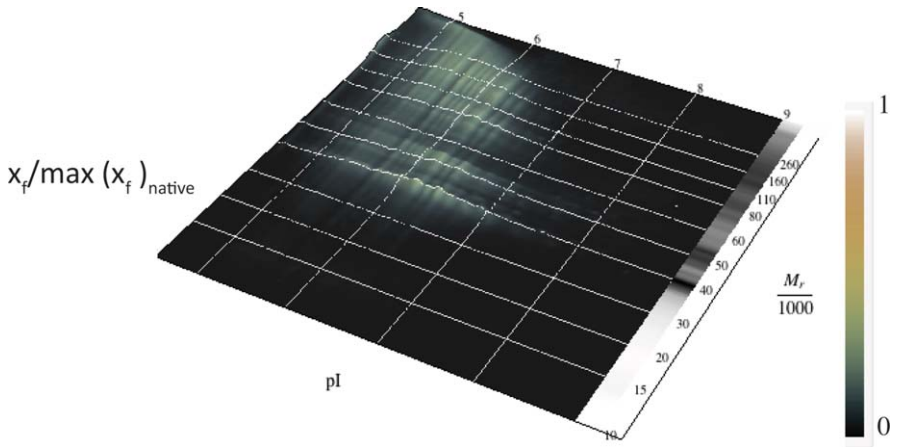


**Figure 3.** 2DE protein subunit topography of phosphorylated vital gluten with s1DE result as in Figure 2. Ordinate is scaled using the same factor as in Figure 2 so that the color scale is consistent between these figures. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

similar to that reported for proteins from wheat flour. This result was expected since the process by which gluten is concentrated from flour conserves the water insoluble proteins that are displayed in 2DE analysis of flour.[13] Therefore, we tentatively identified protein classes based on a "key" previously reported for a "bread" wheat variety, Butte 86.[11] In Figure 2, the characteristic high-molecular-weight glutenin subunits (HMW-GS) occupy the region in which $M_r$ is greater than 70 k and p*I* values are between 4.5–9, and the low molecular weight glutenin subunits (LMW-GS) may be found at the high p*I* side of the gel where the p*I* is greater than 7.5 and $M_r$ is between 35 and 70 k. Some HMW-GS may form branches and both HMW-GS and LMW-GS subunits have the capacity to propagate chains by forming disulphide linkages. Other proteins outside of the regions defined above include gliadins and albumins that do not participate in either chain-propagating linkages or branching interactions but do contribute to gluten properties via non-covalent interaction.[13]

A number of differences were noted between the phosphorylated gluten topography (Figure 3) relative to that of the native gluten (Figure 2). One difference was a shift to lower or more acid p*I* values. Since all of the data falls within the pH range of the isoelectric focusing gel, we calculated that the mean p*I* decreased from 6.7 to 5.8. Another change was a shift in subunit $M_r$ to larger values reflecting both the reduction of protein in the low $M_r$ range and the appearance of altered protein subunits in the region above a $M_r$ of 160. For unmodified native gluten there is no protein indicated above $M_r$ of 160. We computed a median $M_r$ of 48 for the native gluten and 77 for the chemically modified gluten. However, in spite of the noted changes in $M_r$, there were "before and after" distribution similarities as seen by comparing the computed s1DE molecular weight distributions in Figures 2 and 3 for bands in the 35–90 $M_r$ range. There was also an overall loss of 2DE spot intensity and resolution with the protein distributed as a broad poorly defined region, and a loss of s1DE band resolution or sharpness. These observations are compatible with both hydrophilic substitution and the establishment of non-disulphide cross-links between protein subunits to create higher overall $M_r$.

Mass-normalized FTIR spectra for native or unprocessed gluten or phosphorylated gluten are summarized in Figure 4 with

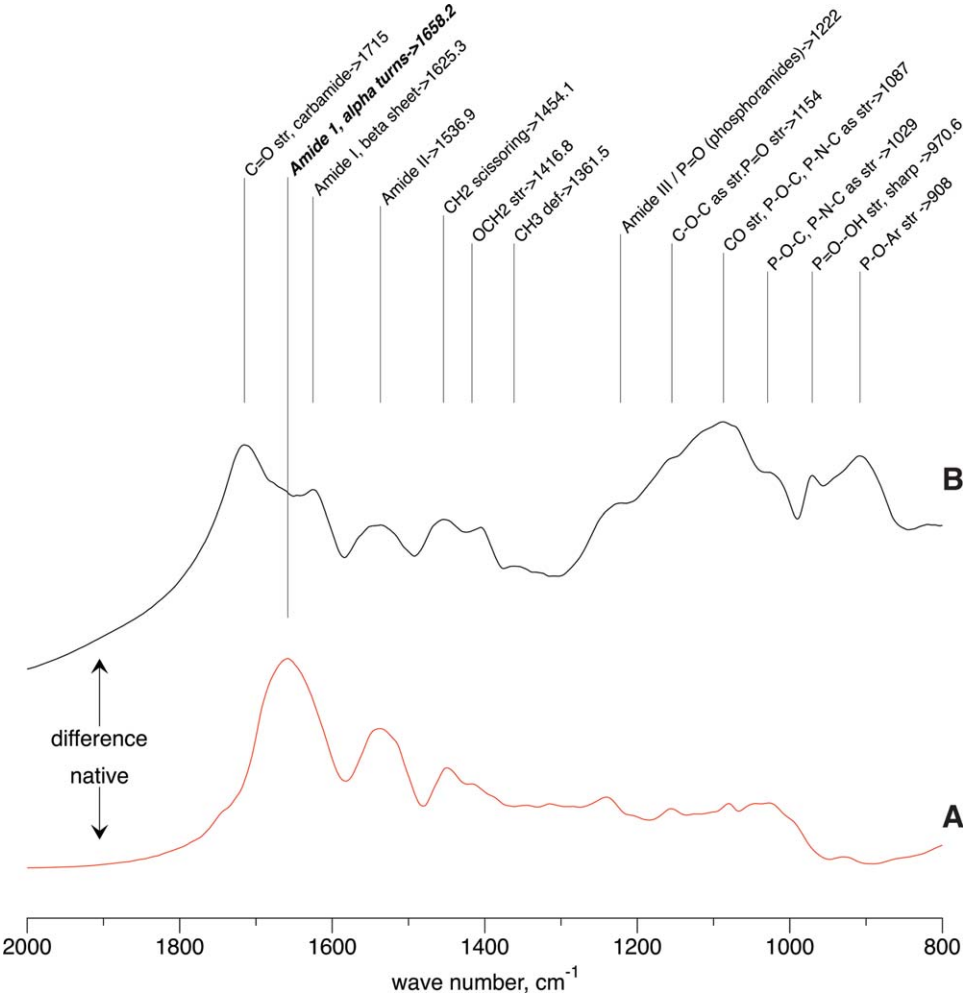


**Figure 4.** IR spectroscopy of gluten in its native or unprocessed form (A) and the difference spectra (B) between spectra of modified and native gluten. Known IR absorption bands are labeled according to the associated functional group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Table I.** Free Swelling Capacity (FSC): Methods of Measurement

| | Methods | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Vacuum filtration | | | Gravity filtration | | | Centrifugation | | |
| Group | FSC | SD as % of mean | Range | FSC | SD | Range | FSC | SD as % of mean | Range |
| 1 | 58 | 10.2% | 50-64 | 90 | 15 or 16.8% | 81-106 | 115 | 17.9% | 79-129 |
| 2 | 65 | 2.3% | 64-65 | 85 | 3 or 3.3% | 81-88 | 104 | 7.2% | 98-115 |

Group 1: four conversion batches, each measured by three methods.

Group 2: a single conversion batch measured by three methods with four replicates.

absorption bands and the relevant infrared wavelengths noted. The difference between the phosphorylated and native spectra revealed increased absorption at 1087 and 908 $cm^{-1}$ suggesting formation of phosphoresters. The difference spectra also included increases that suggest the formation of phosphoramides (1222 $cm^{-1}$) and possibly carbamides (1715 $cm^{-1}$). Spectral changes in the region of the amide peaks (1500–1800 $cm^{-1}$) suggest an apparent conformational shift from α-helical forms to β-sheet forms of the protein. The attenuation due to C=O stretch at 1715 $cm^{-1}$ may include a contribution from urea either as un-reacted or reacted with OH groups on side-chains. An absorption peak at 971 $cm^{-1}$ for POOH suggests that not all of the potential ester-formation capacity of the phosphoric acid was used but the absence of an absorption band near 1006 $cm^{-1}$, for $PO_4^{3-}$ or $H_3PO_4$ for free or unincorporated acid suggests that no free acid remained in the phosphorylated gluten.[6,14,15]

### Free-Swelling Capacity

Values of FSC determined for five different batches and three different methods (group 1) as well as those for a single batch, and four replicates of each method (group 2) are reported in Table I. The data for the single batch indicate variation in the FSC method; whereas, the multiple batch data indicate variation in the FSC method compounded by the preceding mixing, drying, and reaction steps. In both cases, the vacuum filtration method produced the lowest FSC values and the lowest deviation, and the centrifugation method produced the highest FSC values and the greatest deviation. The relative rank of FSC values for the multiple batches was 1 : 1.6 : 2 for group 1 and 1 : 1.2 : 1.6 for group 2 when considering only the FSC determination. The relative rank of the % standard deviation when comparing the group 1 to group 2 was 4.4 for vacuum filtration, 5.5 for gravity filtration, and 2.5 for centrifugation.

### Reaction Conditions and Free-Swelling Capacity

In Table II, unprocessed wheat gluten has a FSC of 1.4. When dried at 40°C, but not heated, the FSC increases slightly to 2.0 and the yield recovery drops to 0.41. In the 121–149°C temperature range FSC values of 70–88 were obtained for vacuum-dried and milled samples. There was an apparent inverse relationship between FSC and yield for similarly dried samples between 121 and 149°C featuring increasing yield and decreasing absorption capacity. However, the product of these factors ($FSC_r$) increased from 17 to 26 in proportion to temperature.

When the vacuum-dried material was not milled, FSC was 43, yield was 0.44, and $FSC_r$ was 19. Vacuum drying was adopted for superior material handling issues because conventional oven drying had required frequent intervention to breakup the material to facilitate drying. The use of milled vacuum-dried material eliminated or reduced the need for this intervention. Further, the use of the intact foamy material reduced or eliminated the need for milling and filtration steps later in the process.

Some of the intermediate forms of the gluten observed during the process are shown in Figure 1(A,B). Gluten mixed with phosphoric acid and urea is shown in Figure 1(B1). Although the color of this pasty mixture was similar to that of wet gluten dough, it was far less cohesive and was not dough-like. After

**Table II.** Water Uptake as Free Swelling Capacity (FSC) by Native and Modified Wheat Gluten

| | Vacuum drying temperature/oven reaction temperature (°C) | | | | | |
|---|---|---|---|---|---|---|
| | None/none[a] | 40/none[b] | 40/121[c] | 40/121[b] | 40/135[b] | 40/149[b] |
| Particle size | <1 mm | <1 mm | 20 cm | <1 mm | <1 mm | <1 mm |
| FSC | 1.4 | 2 | 43 | 88 | 80 | 70 |
| Fractional yield, *Y* | 1.0 | 0.41 | 0.44 | 0.19 | 0.26 | 0.37 |
| $FSC_r = FSC \times Y$ | 1.4 | 0.8 | 19 | 17 | 21 | 26 |

[a] Unprocessed gluten.

[b] Milling before reaction phase, mortar, and pestle breakup before test.

[c] No milling before reaction phase, mortar, and pestle breakup before test.

drying with intermittent subdivision, the mixture browns as shown in Figure 1(B3). If the fresh reaction mixture was vacuum dried, its color was white at this stage and was characteristically foamy [Figure 1(B2)], but turned brown after drying. The physical form of the reacted and dried phosphorylated gluten is shown in application as a fully hydrated gel in Figure 1(B4).

Microscopic images produced by bright-field illumination are shown in Figure 5 after the hydrated gel was stained with Coomassie Brilliant Blue. This soft, cold set gel was not homogeneous but retained some of the particulate character of the dried and milled solids from which it was formed [Figure 5(A)]. For reference, an image of hydrated, native gluten from a bread wheat is shown in Figure 5(B) at the same magnification. In this flour, the particulate structures of protein deposits that would have been observed in the wheat flour are lost as the insoluble protein aggregates into dense membrane and web-like fibril structures. The spaces between the protein structures in the bread dough sample are not dyed suggesting the absence of protein or very low concentration of protein in these regions.

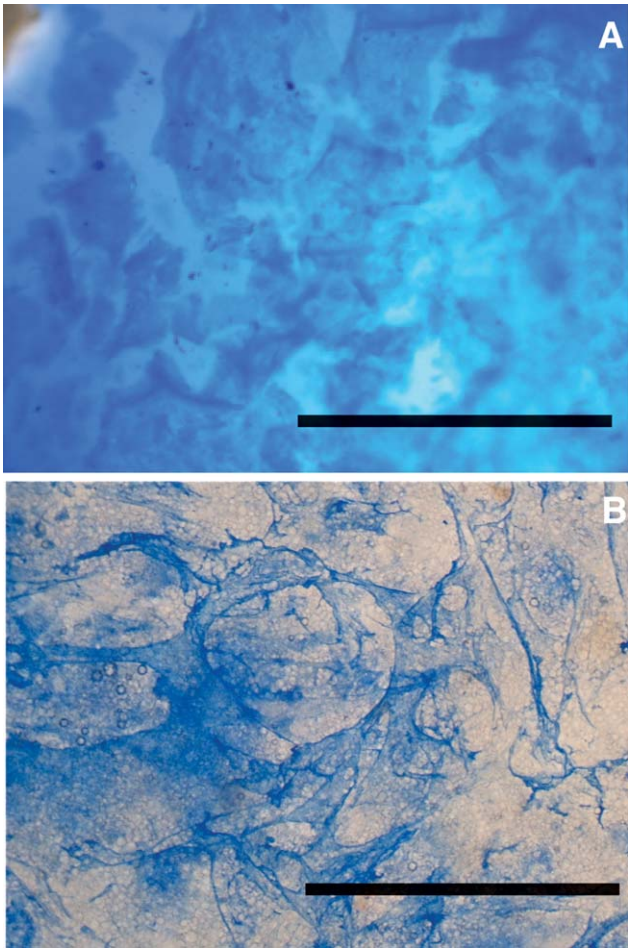


**Figure 5.** Microscopy of forms of hydrated wheat gluten. "A" is phosphorylated, hydrated, and stained gel (1mm bar). "B" is hydrated and stained native gluten (1.5 min mix, 1 mm bar). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

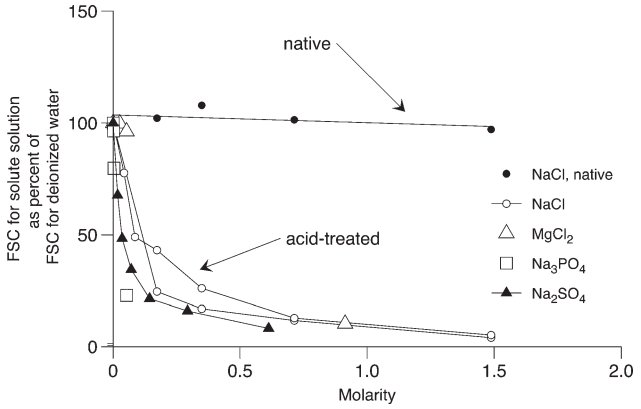


**Figure 6.** Free Swelling Capacity (FSC) of phosphorylated and native gluten when exposed to aqueous solutions containing ionic solutes relative to FSC for absorption of deionized water. Maximum solute concentration of 8% by weight applied for each solute.

The use of $Ca(OH)_2$ as the neutralizing base decreased FSC from 65 to 18 and increased yield from 0.22 to 0.45. Further, FSC decreased from 14.3 to 8.1. The use of 100% ethanol, 70–100% acetone, or freeze drying to dehydrate the final product led to FSC of 28, 40, and 19; yields of 0.48, 0.40, and 0.58; and $FSC_r$ of 13, 16, and 11, respectively.

### Solution Properties and Free-Swelling Capacity

Relative to the FSC values for deionized water absorption by acid-treated gluten, the FSC values for fluids containing ionic and nonionic solutes were lower (Figure 6). A reduction of the FSC to 10% of the solute-free value required no more than a 1*M* solution regardless of the salt, and reduction to 50% required no more than 0.25*M* solution. At concentrations below 0.25*M*, salts of $Na_2SO_4$ and $Na_3PO_4$ caused greater reductions in FSC than those of NaCl and $MgCl_2$. For solutions containing $MgCl_2$ and $Na_3PO_4$, some intermediate solute concentrations produced unusual small particles that passed through the filtration but these data are excluded from Figure 6. Similarly, the FSC values for aqueous ethanol by acid-treated gluten were lower than the FSC values for absorption of deionized water (Figure 7).

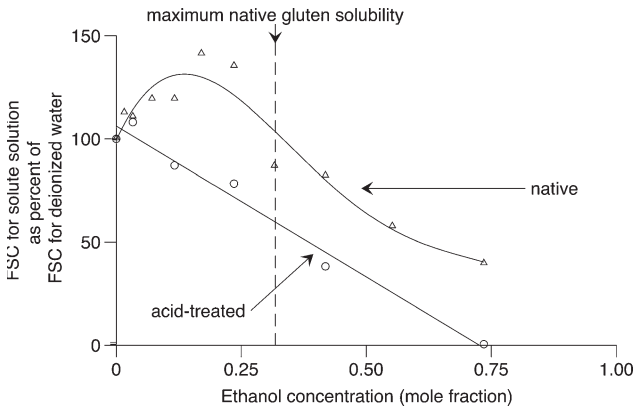


**Figure 7.** Free Swelling Capacity (FSC) of phosphorylated gluten and native gluten when exposed to aqueous solutions of ethanol relative to FSC for absorption of deionized water.

The absorption of water by native gluten was noted above (Table II) as being <2% of the absorption by the acid-treated gluten. Nevertheless, we tested the FSC by native gluten in the presence of solute solutions relative to that in the presence of pure water and found no change in the relative value of FSC when the solute was NaCl. For ethanol, there was a peak of FSC at a higher value than that for pure water at a mole fraction of 0.25.[16–18]

## DISCUSSION

### Reactions: Acid Esters, Acid Amides, and Carbamates

Hydrated native wheat gluten is an amorphous protein with a glass transition that is below room temperature. Wheat gluten is insoluble in water but forms a plastic, visco-elastic gel when mixed. The protein gel matrix is held together by the combined action of intercalating hydrogen bonds and charges and covalent disulphide bonds. The reaction with concentrated polyprotic acids at high temperature has been suggested as a means to convert uncharged side chains to charged side chains. This increases the hydrophilic character and ultimately enhances the water-holding capacity of the protein matrix. The reaction between inorganic acid and hydroxyl-containing amino acids was expected to result in an ester linkage and the formation of a water molecule, and this was confirmed by the appearance of ester linkage absorption bands in FT-IR (Figure 4).

The hydroxyl groups with which phosphoric and the other acids react are found on amino acid side chains of serine, tyrosine and threonine. These are polar, charge-neutral amino acids and have hydropathy index (HI) values of −0.7 to −1.3 in the middle of the HI scale between charged, polar, highly hydrophilic amino acids (HI = −4.5) and neutral, non-polar, highly hydrophobic ones (HI ∼ +4.5). With charge acquired as a result of the esterification, the esterified amino acids would be expected to have an HI value similar to that of unmodified charged amino acids in the range of −3.5 to −4.5.[19]

The modifications to the gluten by esterification are especially complex not only because the acid is polyprotic, but also because the substrate is polyhydroxyl with a potential for three ester links by each phosphoric acid molecule. Figure 8(A) presents the range of different linkage possibilities that range from an unmodified hydroxyl group to one that links three otherwise independent polymer strands. However, not all of the interactions would be expected to have the same enhancing effect on the hydrophilic character of the gluten polymer. For instance, reactions that involve all three acid groups would not yield ionic groups and might actually increase the hydrophobic character. Support for the assumption of linkage increase $M_r$ comes from the shift of subunit $M_r$ in the 2DE topography (Figures 2 and 3).

Urea was incorporated to help to reduce the stability of the native secondary interactions. This loss of stability improved the ease of mixing of reactants, increased homogeneity, and provided access of the acids to amino acids with hydroxyl groups. However, the FT-IR data (Figure 4) suggested that urea also may participate in carbamate formation with the same hydroxyl groups targeted by phosphoric acid. Carbamate formation replaces an OH-bearing side chain with a single NH2-bearing side chain with a more negative HI. If the reaction results in the linkage of two sites (to one protein strand or between two), there would be a loss of hydrophilic character. These possibilities are indicated in Figure 8(B).

Inorganic acid amides may also be formed between the acids and the plentiful amine groups on side chains. This reaction possibility was also detected in the FT-IR difference spectrum of Figure 4. A range of linkage possibilities similar to that shown for the esterification is possible, but only a single reaction is shown in Figure 8(C). However, both unreacted $NH_2$ and reacted side chains are hydrophilic so that the reactions might not be expected to alter the overall hydrophilic character of the gluten as effectively as the esterification.

### Morphology

In prior research, we applied scanning electron microscopy (SEM) to 0.15 and 0.6 mm thick thermoformed films of native wheat gluten and wheat gluten that had been treated with sulfuric acid. Although the treatment created shallow craters in a surface that was smooth and craterless without acid treatment, these craters did not appear to penetrate into the interior and may be formed by partial dissolution of the gluten.[1] Further, in recent research, we have applied SEM to dry wheat gluten in the seed and to dried samples of mixed or developed wheat dough and have found that dry gluten samples are generally amorphous and featureless and that the images reveal no information that relates to the hydrated properties of the gluten protein.

Bright field microscopy applied to dry samples similarly produces little useful information. However, hydrated samples of wheat flour of the type that would be used to produce gluten and that are altered or developed by mixing with water reveal complex microscopic features in the form of membranes and fibrils extending to orders of magnitude larger scales than the source protein particles. It is worth noting that not only all wheat varieties exhibit these structures, but also they are normal for vital wheat gluten. The membranes and fibrils are important to the formation of the gluten matrix in bread.[20]

The soft gels prepared here do not appear to form long-range continuous microstructures like the bands or tendons that are typically formed by unmodified or native gluten. However, there is a particulate or granular character evident in the gel that presumably is due to swollen fragments of the dry gel powder [Figure 5(A)]. No microscopic inter-particle attachments are evident at the scale of observation in this presentation. We suspect that cohesiveness of the material is provided at a molecular level by protein extended from particle to particle in the solution during the hydration of the gel.

### Free Swelling Capacity and Reaction Conditions

FSC values were sensitive to the method of determination as the gels were not rigid and some of the water was weakly entrapped. When the gel was not drained at the end of centrifugation and excess fluid was decanted, FSC values and the percent variation in the measurement were high. When the gel was drained with applied vacuum FSC values were a fraction of

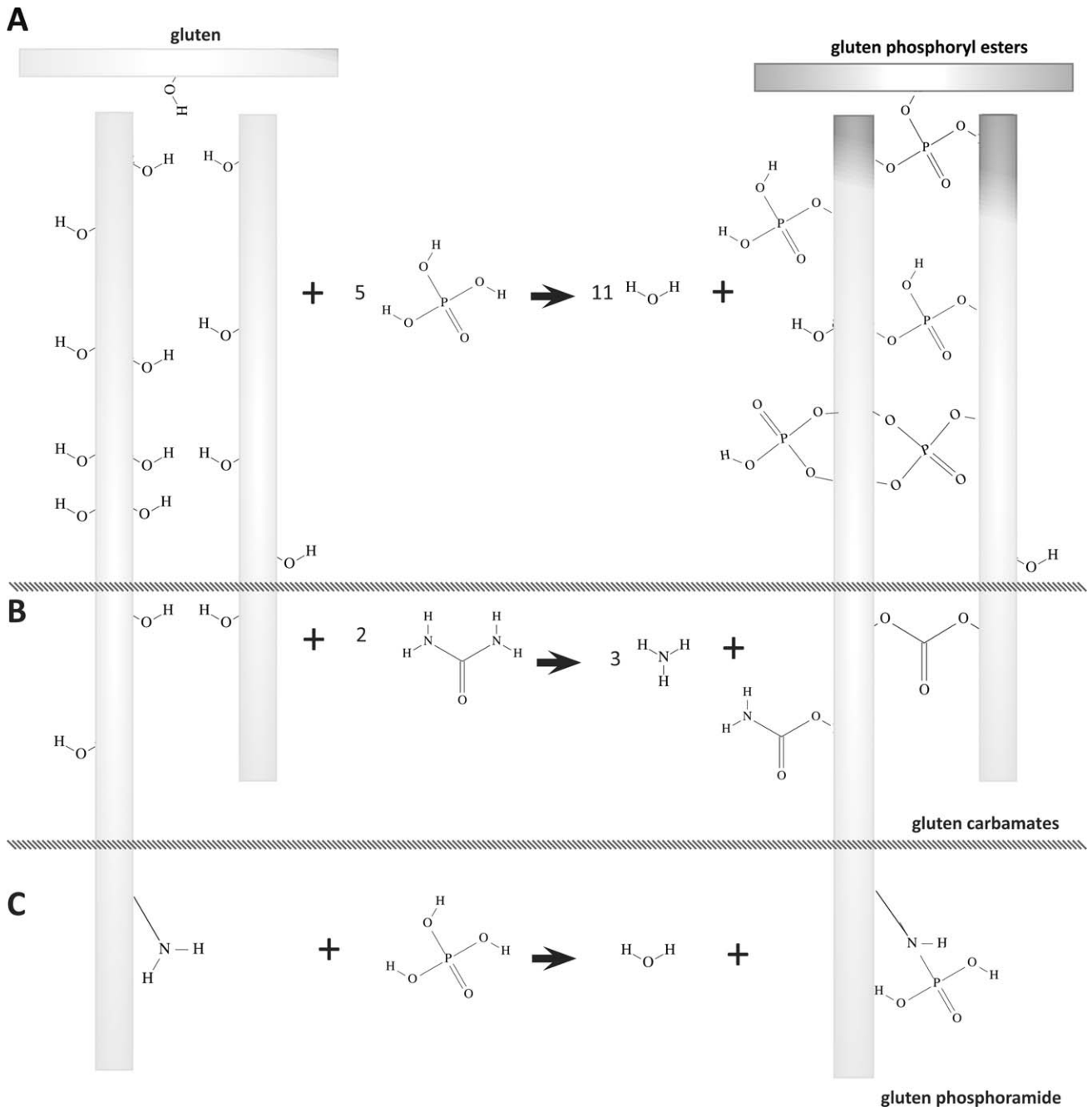


**Figure 8.** Reaction possibilities between phosphoric acid and gluten-amino acids with side chains containing –OH and –$NH_2$.

those obtained by centrifugation and decantation only. The method with gravity-driven drainage produced intermediate values. In Table I, the vacuum-filtration method determines FSC as 65 with a percent standard deviation of 2.3% while the centrifugal method estimates FSC as 104 with a percent standard deviation of 7.2%. With the exception of Table I, all FSC values in this report used the conservative, vacuum-filtration method.

The initial mixing step in the conversion [Figure 1(A,B1)] was designed to distribute and thoroughly mix reagents and substrate to ensure completeness and uniformity of the reaction. Hence, gluten was hydrated and acidified in the presence of urea. Although urea reduces hydrogen bonding and helps expand the protein matrix [Figure 1(B1)], it did not produce a solution with fully dispersed protein. Further this mixture needs to be dry to ensure reaction completeness (Figure 8) and the drying produces rigid solids with particle size determined by the method of drying and milling. The completeness and uniformity of the solid phase reaction is then subject to heat and mass transfer limitations with small particles allowing for

relative more rapid heating to the threshold for reaction (see below) and relatively more rapid removal of water produced in the reaction. For example, when a sample of vacuum-dried foamy mass [Figure 1(B2)] was heated to effect reaction, the FSC obtained was 43; but, when this sample was milled prior to reaction to produce smaller particles, the FSC was 88.

Note however, the foamy, low density structure of vacuum dried solids provides several process-related advantages relative to the dense solids produced by hot air convection drying [Figure 1(B3)]. In particular, these dry rapidly after mixing because of the high surface area and they are easily handled during neutralization and recovery.

With no applied heat, the FSC is essentially the same as that of the unmodified gluten. If heated to 121°C the FSC increases to 88 but declines with further heating (Table II). A similar optimum was reported by us in related research.[4] Although FSC declines as the applied temperature rises above 121°C, the yield increases. These effects may be due to increased cross-linking and concomitant loss of initial charge enhancement as the reactions are driven to greater completion (Figure 8). Nevertheless there is a net improvement (increase) in the value of $FSC_r$, a measure of the raw material "cost" of the water absorption, in that it is the amount of water absorption per unit of gluten consumed by the reaction.

The use of $Ca(OH)_2$ as the neutralizing agent lead to an FSC, yield and $FSC_r$ of 18, 0.45, and 8.1, respectively, which compares unfavorably with 65, 0.22, and 14.3 for the use of NaOH. This may be due in part to bridging by $Ca^{2+}$ at negatively charged sites created by the esterification and the consequent compaction of the gel matrix.

### Yield and Secondary Interactions

Whether originating from amide or ester formation, the expected result of the reaction is an acid modified gluten with greater hydrophilic character. Because there would be charged groups replacing side chains with strong hydrogen bond capabilities, protein–protein interactions would be weaker and charge-repulsion stronger. Proteins therefore are less likely to self-associate and become increasingly soluble. Fortunately, the gluten components are not fully soluble since a gel state is observed. However, in the case of protein subunits like the low molecular weight gliadins that lack the ability to covalently interact by disulphide linkages, there may be a greater tendency towards full solubility so that these components are unrecovered by the current process method. The formation of di-carbamide or di- and tri-ester or di- and tri-amide links is apparently inadequate to prevent full dissolution of these components of the gluten matrix. In the native gluten that we have observed previously, HMW-GS and LMW-GS are not always completely incorporated into the gluten matrix by disulphide linkages[21] and could therefore join the gliadins as part of the unrecovered acid-treated mass.

### Solute Interactions

As part of the evaluation of the properties of materials produced here, we tested the absorption of solutions containing solutes that might be encountered in practical applications. Further, we tested vital and acid-modified gluten even though the FSC values for the former substrate are typically less than 2% of the acid treated substrate. Solutions containing chloride ions at concentrations up to 1.5 molar were tested on vital gluten with no effect on FSC values relative to absorption of de-ionized water.

Chloride at 1.5*M* diminished the absorption by the acid-treated gluten to less than 5% of the solute-free value. Chloride ion concentration of 0.1*M,* or at the mid-point of the normal range for human urine, lowered the capacity for absorption to about 50% of that of de-ionized water. Chloride concentration of 0.55*M,* or about the chloride concentration of sea water, reduced FSC to approximately 20% of FSC of de-ionized water.[22–24]

Proteins are known to be especially sensitive to interactions with anionic salts that are chaotropic (increase solubility by lowering protein-protein interactions) or kosmotropic (decrease solubility by increasing protein-protein interactions). Since gels are a state that is intermediate between fully soluble and fully insoluble, a chaotropic agent ought to be enhance the capacity of a particular solid to form a gel with aqueous solutions and a kosmotropic agent ought to diminish it. The lowered FSC for acid-treated gluten gels in the presence of chloride was therefore an expected result since chloride is known to be a kosmotrope that reduces protein solubility by increasing protein-protein interactions at the expense of protein-solvent interactions that are important to gel formation. Phosphate and sulfate ions produced even greater reduction of FSC than that produced by chlorides at the same molar concentration and this is consistent with their relatively stronger kosmotropic capabilities as predicted by their position in the Hofmeister series 22. Similarly, the action of ethanol on the FSC values of acid-treated gluten was as a kosmotrope that produced lower FSC values than obtained for distilled water.

By contrast, when tested against native wheat gluten, ethanol acted as a chaotrope or kosmotrope depending on its concentration. We note that the shape of relative FSC versus ethanol concentration data is similar to the shape of native gluten solubility versus ethanol concentration data reported in the literature. Both FSC and solubility increase up to an ethanol mole fraction of 0.3, and then both decrease progressively as the ethanol concentration increases. Ethanol weakens protein-protein interactions at low concentrations, thereby increasing hydrophilic behavior and ultimately leading to higher solubility for the gliadin range of proteins where Mr<80 (Figure 2). Above a mole fraction of 0.3 (60%v/v), ethanol acts to enhance protein-protein interactions, and all components including gliadin, become increasingly insoluble and the FSC values are lowered. We suggest that the absence of an ethanol chaotropic effect in acid-treated gluten was because the tested matrix has been stabilized against ethanol by new ester linkages. Further, ethanol-susceptible native components such as those in the gliadin range may have been dissolved completely by the processes of conversion and recovery, and therefore were not a part of the final matrix.[16–18]

## CONCLUSIONS

We modified wheat gluten with phosphoric acid and produced natural superabsorbent materials that absorbed up to 88 times

their weight in deionized water. 2DE and FTIR analyses showed that reaction with phosphoric acid and subsequent neutralization with sodium hydroxide resulted in attachment of charged phosphate groups by esterification or amide formation. Negatively charged phosphate groups probably cause protein polymer chains to repel each other, resulting in increased water uptake. However, since the acid is polyprotic, linkage reactions may limit charge enhancement and the FSC of the gels, but also increase molecular weight. The acid linkages may be augmented by carbamide formation. Up to half of the initial mass was lost during conversion. Losses occur because a large proportion of the gluten lacks native cross-links and becomes so highly soluble that it is not stabilized by the links potentially provided by the reactions. The presence of solutes diminishes the FSC when compared to that for absorption of deionized water and would have to be considered in a potential application.

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