for the residue, another indication that fractionation may be taking place. The high sulfur contents of the materials which were precipitated from the unhairing liquors directly by the addition of acetic acid are due to the presence of free sulfur. Dialysis of the liquors to the complete removal of the sulfide and alkali before acidification also removed any free sulfur.

The highest yield of product and the best recovery were obtained in Sample II. Better than 90% of the hair was accounted for. The aeration technique, as previously mentioned, caused some mechanical losses and without these may have been as good as the technique used in preparing Sample II. Eventually, a compromise may have to be made between yield and purity. This may in part be determined by the use for which the material is intended.

Some preliminary work is now in progress on spent unhairing liquor obtained from a tannery and, although additional problems have arisen, the results are promising. A product of equal quality can be obtained from these liquors with a little additional effort. We are also in the process of making a large enough quantity of the recovered protein on which to obtain some information regarding its physical properties as well as its effectiveness in animal feeding studies.

There is considerable interest in research along these lines in the tanning industry because of the pressures on it to eliminate pollution. The research reported here and the proposals made may be a step in the right direction to help relieve some of this pressure.

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Preparation and Isolation of Acid-Catalyzed Hydrolysates from Wheat Gluten

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The rate of acid-catalyzed hydrolysis of gluten to polypeptides was investigated using hydrolytic agents that form single-phase systems with gluten. Controlled digestion on commercial gluten preparations and on laboratory-extracted samples could be achieved with similar end effects. The rate of partial hydrolysis of gluten was considerably improved by disrupting its disulfide linkages before digestion. Hydrolysis was followed by measuring the number of cleaved peptide bonds, the number of degraded amide groups, and the changes in relative viscosity. The hydrolysates were purified by fractionation on Sephadex gel. Preparations from unoxidized gluten could be obtained in acetic acid-soluble or water-soluble forms and showed similar characteristics when examined by analytical test, viscosity and sedimentation measurements, and by gel filtration. Hydrolysates originating from the oxidatively cleaved gluten samples were soluble in water or sodium hydroxide and showed increased fragmentation of the protein by all experimental methods applied.

Since wheat gluten protein can be readily produced commercially and has unique amino acid distribution, efforts were directed to prepare from it novel products with potential for industrial application. Peptides from gluten with appropriate chemical modification could be suitable for uses such as films, adhesives, coatings, and surfactants. The specific objective of the work reported here was to develop a controlled acid-catalyzed hydrolysis method and to isolate and purify the polypeptide mixture obtained.

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MATERIALS AND METHODS

Hydrolysis experiments were conducted on gluten extracted from wheat flour in our laboratories, on Vital Gluten, a product of the Hercules Powder Company, and on a laboratory-extracted gluten sample that had been exposed to performic oxidation to cleave its disulfide bonds.

Protein samples obtained in the laboratory from flour were extracted by the process of Jones *et al.* (1959).

The commercially available Vital Gluten is extracted in an industrial process and contains 72.5% protein; the remainder is mainly residual starch and some lipid.

The performic acid oxidation method of Hirs (1956), as adapted for gluten by Nielsen *et al.* (1962), was used for preparing samples in which the disulfide linkages in the protein were disrupted. The reaction was performed on 10-g batches of laboratory-extracted gluten dissolved in 200 ml of 99% formic acid. The performic acid was prepared by mixing 190 ml of 99% formic acid with 10 ml of 30% hydrogen peroxide. Oxidation was conducted at 0°C for 30 min.

Since the hydrolytic studies were confined to single-phase aqueous systems, the limited solubility of gluten considerably narrowed the choice of hydrolytic agents. Because it was anticipated that amide groups in gluten participate in the hydrogen bonding of the molecular structure, hydrolytic agents of high hydrogen bonding strength were used to assure easy solubilization of the protein. Those examined were formic acid, di- and trichloroacetic acid, and dilute hydrochloric acid in the presence of acetic acid. Although these agents proved to be about equally effective under the conditions tested, the combination of 0.1 *N* hydrochloric acid as the digesting substance plus 4 *N* acetic acid as the solubilizing substance was found most suitable because the analytical tests and the posthydrolytic preparative techniques applied were most compatible with this digesting system.

All samples were hydrolyzed at 60°C for 24 hr. The protein content of the system was adjusted to 2%, a concentration at which the laboratory prepared gluten is soluble in the digesting mixtures at room temperature before as well as after completion of hydrolysis.

In the case of commercial Vital Gluten samples it was found that the hydrochloric acid–acetic acid method could also be used to hydrolyze the protein in the presence of the residual starch and lipid contamination. These compounds did not interfere with hydrolysis, were insoluble in the digesting mixture, and could be removed after hydrolysis by centrifugation. The starch was eliminated as a sediment, and the lipid accumulated at the top of the centrifuge tube when the sample was centrifuged for 1 hr at 40,000 × *g* in a Spinco model L preparative ultracentrifuge. The clear hydrolysate solution was separated by using a syringe.

To evaluate the effect of the digesting agents, the relative viscosity change in the system during progressive hydrolysis was determined. Approximately 2% solutions of gluten were dissolved at room temperature in the hydrolytic agents tested and filtered through a coarse sintered-glass filter. A 2-ml aliquot of the filtrate was placed in a semimicro-Ubbelohde capillary viscometer and maintained at 60°C. Viscosity readings were taken at regular intervals of 15 to 20 min in the first 9 hr and less frequently in the following 3 days.

Since it was not established to which extent the viscosity transition reflected changes in configuration, aggregation, or peptide cleavage in the gluten molecule, the rate of gluten hydrolysis was also followed by chemicals analysis.

The ninhydrin reaction was used for quantitative determination of the increase in primary amino groups during hydrolysis. Krull *et al.* (1961) adapted the original method of Moore and Stein (1954) for gluten by using dimethylsulfoxide in the diluent to keep the analyzed protein hydrolysate in solution and reading absorbancy at 580 mμ. The digesting mixture of 0.1 *N* hydrochloric acid and 4 *N* acetic acid could be applied best to this analytical test. Another agent, formic acid, interfered

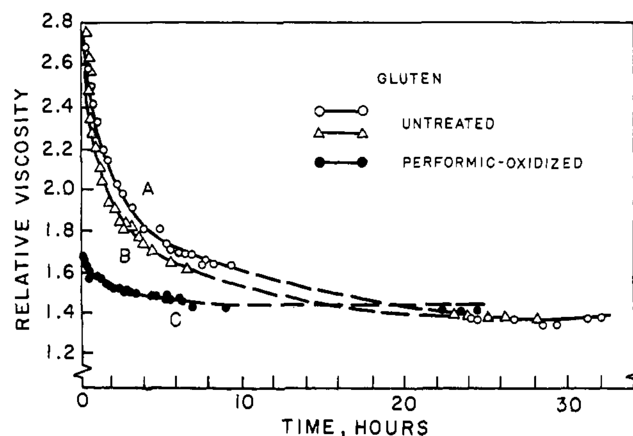

Figure 1. Hydrolysis of gluten at 60°C before and after oxidative cleavage as determined by the change in relative viscosity in 6 *N* formic acid (A and C) and in a mixture of 0.1 *N* hydrochloric acid and 4 *N* acetic acid (B)

with the colorimetric determination in the presence of dimethylsulfoxide.

Because of the high amide content in gluten, ammonia release and peptide cleavage occur simultaneously during acid hydrolysis. Since the ninhydrin test is also sensitive to ammonia it was necessary to measure the amount of the amide ammonia independently and determine its contribution to the ninhydrin reaction. The Nessler colorimetric method of Johnson (1941) was adapted for this purpose. In the original process the Nessler reagent is first mixed with the sample, then the ammonia is liberated by addition of 2 *N* sodium hydroxide, and the characteristic yellow color produced is read in a spectrophotometer at 490 mμ. With gluten, however, the partially hydrolyzed protein precipitated upon addition of the reagents and interfered with the reading of optical density. This difficulty was overcome as follows. Since gluten is soluble only at very low ionic strength, exposing hydrolyzed aliquots to a 1 *M* sodium chloride solution precipitated the partially hydrolyzed protein, which was then removed by centrifugation. Aliquots of the supernatant that contained the ammonia were analyzed without any difficulty.

To establish the extent of the ninhydrin color due to ammonia, the ninhydrin calibration curve was determined not only with the usual leucine standard but also with a known ammonium chloride standard. Combining the two calibration curves in correlating those leucine and ammonium chloride concentrations that had identical optical densities demonstrated that 1 μ*M* of ammonia gave the same optical density with ninhydrin as 0.6 μ*M* of leucine. By expressing the amounts of ammonia in leucine equivalents and making the appropriate correction, it was possible to determine the number of primary amino groups in the hydrolyzed sample.

Because of the relatively large amounts of ammonia present and the one-order-of-magnitude difference in the concentration range in which the ninhydrin and the Nessler colorimetric reactions respond to Beer's Law, the method in this form is suitable only for following the rate of acid hydrolysis of gluten.

To obtain pure protein hydrolysate preparations, the hydrolyzing agents as well as the byproducts of the hydrolytic process had to be removed from the reaction mixture. Fractionation by gel filtration on Sephadex was used for this purpose. Sephadex G25, coarse grade in bead form, with an exclusion limit of 5000 was utilized.

To swell and equilibrate the Sephadex and to elute the columns, 4 *N* acetic acid was used. The samples were applied

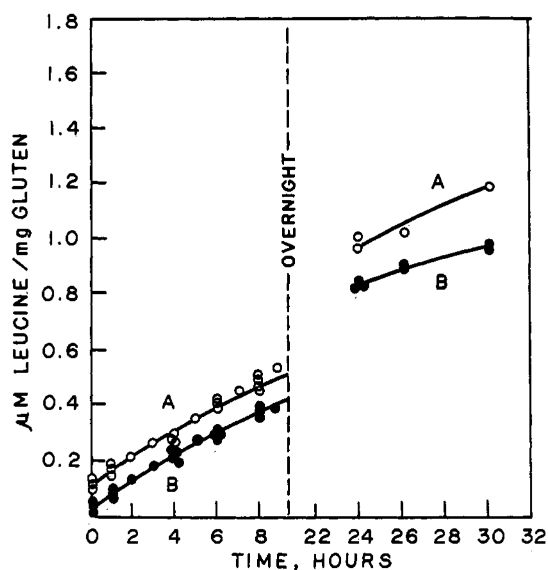

Figure 2. Hydrolysis of gluten in a mixture of 0.1 N hydrochloric acid and 4 N acetic acid at 60°C as determined by the (A) ninhydrin and (B) Nessler methods

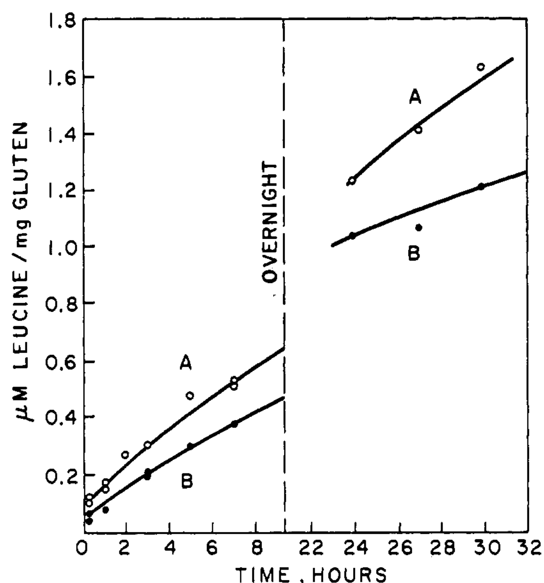

Figure 3. Hydrolysis of performic-oxidized gluten in a mixture of 0.1 N hydrochloric acid and 4 N acetic acid at 60°C as determined by the (A) ninhydrin and (B) Nessler methods

directly in the digestion mixture of 0.1 N hydrochloric acid and 4 N acetic acid and were eluted with 4 N acetic acid. The effluent fractions were collected automatically by the constant volume method using a Gilson fraction collector. Peaks were located by measuring absorbancy at 280 mμ for the protein and by testing aliquots of the collected fractions with Nessler reagent at 490 mμ.

In analytical gel filtration experiments, the purified hydrolysates were examined on Sephadex G25, G50, G75, and G100.

Sedimentation velocities of the purified hydrolysate preparations were measured in a Spinco model E analytical ultracentrifuge. The experiments were conducted at 59,780 rpm and 20°C using synthetic boundary cells.

RESULTS AND DISCUSSION

Figure 1 shows the changes observed in relative viscosity with progressing hydrolysis time at 60°C in two hydrolytic

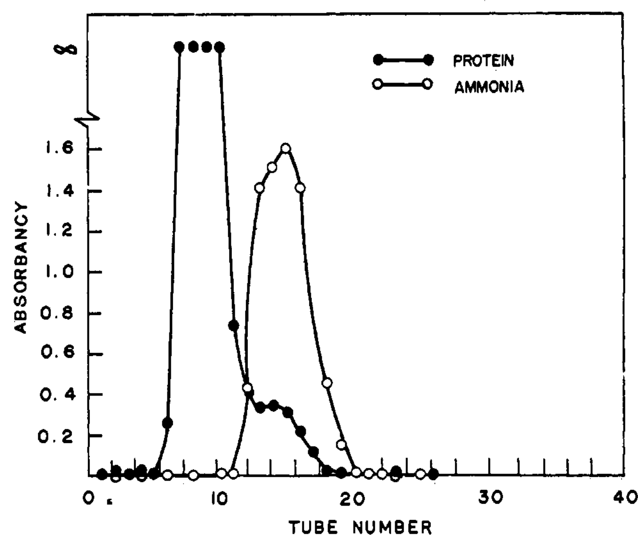

Figure 4. Fractionation of laboratory-extracted hydrolyzed gluten on Sephadex G25 in 4 N acetic acid. Sample: 240 mg, V_0; 20 ml, Fractions: 4 ml

agents. Curves A and B of Figure 1 show that the effects of 6 N formic acid and the mixture of 0.1 N hydrochloric acid and 4 N acetic acid are quite similar. The rapid initial relative viscosity decrease in the first 5 hr is followed by a decrease in the rate of change, so that a final relative viscosity equilibrium value is approached asymptotically in about 24 hr. A similar effect, with only slight deviations, was observed by using other hydrolytic agents. In the case of 6 N trichloroacetic acid, the initial relative viscosity decrease was faster, and the final equilibrium value was lower than in the first two agents examined.

To compare the behavior of performic-oxidized gluten with that of untreated gluten, a sample of the oxidized compound was examined in 6 N formic acid under the same conditions. The relative viscosity values measured with progressing hydrolysis time for this experiment are plotted in Figure 1C for easy comparison with similar data on untreated gluten. These curves show that, as a result of the disulfide cleavage, the initial relative viscosity of this preparation is significantly lower and the final equilibrium value is reached in a shorter time than in the case of the untreated sample. The equilibrium relative viscosities of the two preparations are similar.

The hydrolysis rate of both untreated and performic-oxidized gluten was followed by the double analytical test. Samples were digested in a mixture of 0.1 N hydrochloric acid and 4 N acetic acid at 60°C. Two aliquots were taken for analysis at regular time intervals, one for the Nessler and the other for the ninhydrin colorimetric test.

The results, calculated in μM leucine equivalents per mg protein, are plotted as a function of hydrolysis time in Figures 2 and 3 for the digestion of untreated gluten and performic-oxidized gluten samples, respectively. The results indicate that peptide bond cleavage was more pronounced in the performic-oxidized samples. In these samples the ninhydrin curve increased more steeply from the start, and therefore increasing peptide bond cleavage was observed from the beginning of hydrolysis. In the untreated samples, the rates of peptide and amide hydrolysis were similar; an increase appeared only after 24 hr.

Purification of the crude hydrolysate was accomplished by gel filtration on Sephadex G25. The acid hydrolysate mixture was added to a column of 1.2 × 50 cm and eluted with 4

Table I. Analytical Data on Purified Gluten Hydrolysates

Hydrolysate	Preparation	% N	μM leucine equivalents per mg total nitrogen
Laboratory-extracted gluten[a]	1	14.6	1.44
Commercial Vital Gluten[a]	2	12.8	1.31
	3	14.3	1.21
	4	14.4	1.42
	5	14.5	1.47
Commercial Vital Gluten[b]	6	11.2	1.40
Laboratory-extracted performic-oxidized gluten[b]	7	13.3	2.20
	8	12.2	2.86
	9	14.2	2.64
	10	10.0	2.58
	11	12.6	2.46

[a] Solubilized in acetic acid. [b] Solubilized in water.

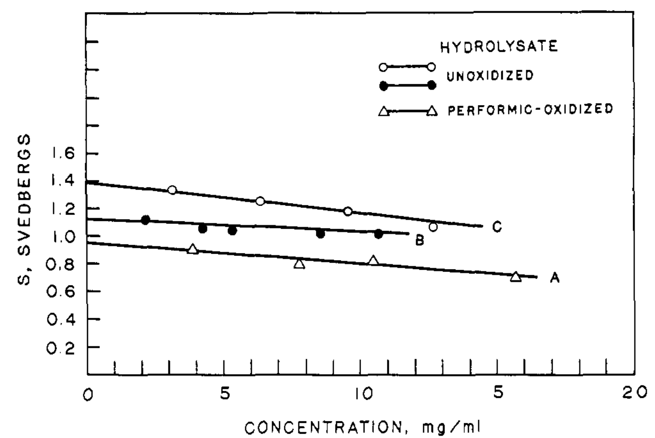

Figure 5. Sedimentation velocities as a function of concentration for gluten hydrolysates determined at 59,780 rpm and 20°C in pH 8.0 sodium phosphate buffer (A and C) and 4 *N* acetic acid (B)

N acetic acid. A typical elution profile on a sample of laboratory-extracted gluten hydrolyzed in 0.1 *N* hydrochloric acid and 4 *N* acetic acid is shown in Figure 4. The greater portion of the hydrolysate was excluded from the gel matrix in the void volume of G25, as indicated by the first large peak obtained in the ultraviolet tracing. Void volume was determined by use of Blue Dextran solution. A second much smaller peak followed, often represented only by a shoulder attached to the tail end of the main peak. The first peak, corresponding to the exclusion limits of Sephadex G25, represents larger peptides that are not able to penetrate the gel pores and, therefore, percolate through the void volume of the column. The second peak, also absorbing in the ultraviolet, indicates the presence of smaller-sized peptides that, entering the gel matrix, are delayed in their progress through the column and appear in the effluent with retardation.

Tracing the collected fractions for ammonia with the Nessler colorimetric reaction shows one large peak corresponding in place to the elution of the smaller-sized peptides. The ammonium salts present in the reaction mixture are formed as a result of the acid digestion of the numerous amide groups in the glutamine residues of the gluten protein. Monitoring the ammonia by Nesslerization thus localizes the fraction containing the inorganic byproducts of the hydrolytic process.

After the fractionation process had been established in small scale experiments, the method was adapted to larger columns capable of handling more material with better efficiency. Columns with total bed volumes (V_t) of about 1000 ml were applied.

The collected effluent was separated into ammonia-free and ammonia-containing protein fractions and lyophilized. The ammonia-free portion, which contained the large peptides, was pure white after drying, had a total nitrogen content of about 14%, and was completely soluble in 4 *N* acetic acid. The recovery of hydrolyzed protein from the main fraction varied between 80 and 90%. The ammonia-containing fraction was discarded.

Fractionation experiments conducted on hydrolyzed commercial Vital Gluten showed similar elution patterns and gave the same end product as laboratory-extracted hydrolyzed gluten.

In an attempt to obtain a water-soluble preparation, the purified, dried acetic acid-soluble gluten hydrolysates were dispersed at 5% solids content in distilled water. With vigorous stirring, 1 *N* sodium hydroxide solution was added to the dispersion, which started to clear up about pH 6.8 and was completely solubilized at pH 8.0. The free carboxyl groups created by the hydrolysis of peptide bonds and amide groups were neutralized by the sodium hydroxide. The dry product obtained after lyophilization was a white fluffy powder easily soluble in water, which could also be solubilized in 4 *N* acetic acid but less readily than the starting material. Hydrolysate preparations obtained from laboratory-extracted or commercial gluten samples could be equally well transformed into the water-soluble form.

Another water-soluble hydrolysate preparation was obtained when the performic-oxidized gluten sample was digested in 0.1 *N* hydrochloric acid and 4 *N* acetic acid. In contrast to the nonoxidized gluten hydrolysates, the performic-oxidized hydrolyzed gluten was only partially soluble in the hydrolyzing medium at the end of the digestion period when cooled to room temperature. It was believed that the excessive acidity resulting from the oxidized disulfide bonds and the hydrolytically increased number of carboxyl groups were responsible. In order to purify the preparation by gel filtration, the hydrolysate was solubilized by neutralization. A solution of 10 *N* sodium hydroxide was added until a pH of 7.0 was reached. After this, total solubility could be achieved by diluting further with water. Therefore, the neutralized tobacco-colored turbid suspension of the hydrolysate was fed onto a Sephadex G25 column, and the water used as eluant also solubilized the suspension during elution. A water-clear yellow effluent was collected.

The two fractions containing the proteinaceous components and the ammonium salt byproducts were separated as in previous experiments. The lyophilized dry peptide mixture was light brown, easily soluble in water and dilute sodium hydroxide, but insoluble in acetic acid.

After these different hydrolysate preparations had been isolated, the purified peptide mixtures were examined by various methods.

Analytical tests were conducted on the purified hydrolysates to check reproducibility of the extent of hydrolysis in different experiments and to compare preparations obtained by different procedures. The total nitrogen content of the samples was determined by Kjeldahl distillation. The amount of free amino groups was measured with the ninhydrin colorimetric method and expressed in μ*M* leucine equivalents per mg total nitrogen content of the sample. Since the test was conducted on the purified fractionated peptide mixture, the need for the Nessler reaction was eliminated, and the dependability of the ninhydrin test, which was previously hampered by the presence of large amounts of ammonia, was increased.

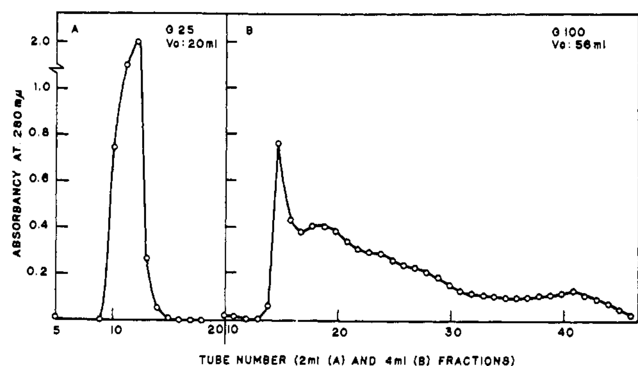

Figure 6. Gel filtration of hydrolyzed gluten on Sephadex G25 (A) and G100 (B) in 4 *N* acetic acid. Sample: 25 mg (A) and 50 mg (B)

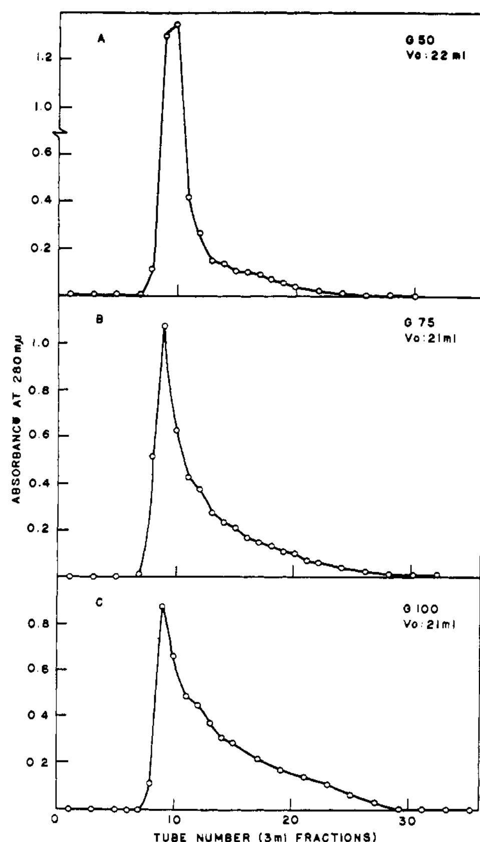

Figure 7. Gel filtration of hydrolyzed gluten (19-mg samples) on Sephadex G50 (A), G75 (B), and G100 (C) in pH 8.0 sodium phosphate buffer

Results obtained with several hydrolysate samples are compared in Table I. These data indicated that the hydrolysates of laboratory-extracted and commercial Vital Gluten samples are quite similar in extent of peptide cleavage, as demonstrated by the measured values of μM leucine equivalents per mg nitrogen. The performic-oxidized hydrolysates, on the other hand, have a much higher number of free amino groups per total nitrogen content. The increase in free amino groups could be expected, since the oxidative cleavage of the disulfide bonds should make the molecules more accessible to the attack of the hydrolytic agents.

Sedimentation velocities of the different gluten hydrolysate preparations were measured at 59,780 rpm and 20°C at various concentrations. The unoxidized hydrolysates were measured in 4 *N* acetic acid or pH 8.0 sodium phosphate buffer; the performic-oxidized hydrolysates were examined in pH 8.0 sodium phosphate buffer. The data obtained on three samples are plotted in Figure 5. Since the dependence of the sedimentation coefficient on concentration is not very strong, a direct extrapolation of these parameters rather than that of their reciprocals was used to determine S at infinite dilution (Schachman, 1959). S values obtained by extrapolation from experimental points for the three hydrolysates are listed in Table II. Viscosity corrections were not applied.

Table II. Sedimentation Coefficients of Hydrolyzed Gluten at 59,780 rpm and 20°C

Hydrolysate	Solvent	S. $10^{13,a}$
Commercial Vital Gluten	4 *N* acetic acid	1.1
Commercial Vital Gluten	pH 8.0 sodium phosphate buffer	1.4
Laboratory-extracted performic-oxidized gluten	pH 8.0 sodium phosphate buffer	0.9

[a] Values extrapolated to zero concentration.

The lower S value of the oxidized hydrolysate can be attributed to the cleaving effect of the performic oxidation on the disulfide bond and is in accord with the increased number of free amino groups observed analytically, indicating smaller average peptide size. The difference between the values for the unoxidized hydrolysates in the two solvents is probably due to charge effects in the case of the hydrolysate sample dissolved in acetic acid.

Viscosity measurements conducted at 25°C in an Ubbelohde capillary viscometer also supported the findings of the analytical and the sedimentation experiments. The unoxidized hydrolysates examined in 4 *N* acetic acid and in pH 8.0 sodium phosphate buffer gave intrinsic viscosity values between 0.13 and 0.15 dl/g. The corresponding value for the performic-oxidized hydrolysate obtained in pH 8.0 sodium phosphate buffer was 0.07 dl/g.

To obtain information on the size distribution of the purified peptide mixtures, the hydrolysates were exposed to analytical gel filtration experiments on Sephadex G25, G50, G75, and G100. Columns with diameters of 1.3 or 2.0 cm and lengths of about 50 cm for the different Sephadex bed volumes were utilized. Equilibration of the Sephadex gels and elution of the hydrolysates was accomplished with 4 *N* acetic acid and pH 8.0 sodium phosphate buffer, respectively.

The gel filtration of hydrolyzed Vital Gluten solubilized and eluted in 4 *N* acetic acid is shown on Sephadex G25 and G100 in Figures 6A and 6B. The preparation is completely excluded in the void volume on Sephadex G25. A very strong retention by the gel matrix was observed when the sample was applied onto a column of Sephadex G100.

The elution diagrams obtained from Sephadex G50, G75, and G100 columns are presented in Figures 7A through 7C for hydrolyzed Vital Gluten solubilized and eluted in pH 8.0 sodium phosphate buffer. In addition to some retention, the larger portion of the hydrolysate is still excluded on G50, although the exclusion is not as complete as on G25. The more pronounced retention that becomes evident on G75 increases on G100. The elution diagrams demonstrate that, although to a varying extent, there is an excluded fraction on each of the Sephadex gels, even on G100. This indicates the presence of some high-molecular-weight components in the hydrolysates.

Performic-oxidized hydrolyzed gluten samples were also examined on Sephadex G50, G75, and G100 columns in pH 8.0 sodium phosphate buffer. These experiments were run on the same columns and with approximately the same amounts

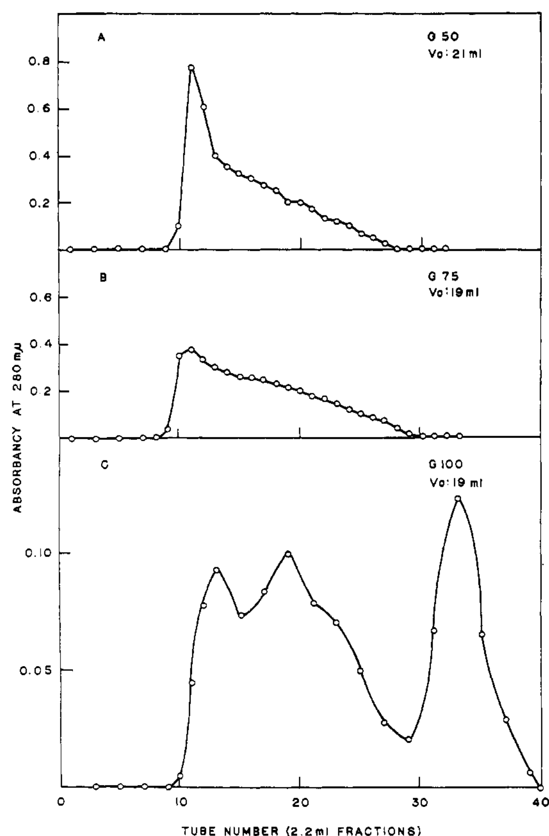

Figure 8. Gel filtration of performic-oxidized hydrolyzed gluten (14-mg samples) on Sephadex G50 (A), G75 (B), and G100 (C) in pH 8.0 sodium phosphate buffer

of sample applied as those utilized for the unoxidized hydrolysates eluted in pH 8.0 sodium phosphate buffer. The resulting elution curves are quite different from those of the unoxidized gluten hydrolysates. There is a pronounced retention on G50 that increases significantly on G75; on G100, fractionation into three peaks can be observed (Figures 8A, 8B, and 8C). This different behavior reflects the disulfide bond cleaving effect of the performic oxidation and, thereby, supports the experimental evidence already obtained in analytical, viscosity, and sedimentation measurements.

These investigations showed that controlled hydrolytic digestion on commercial gluten preparations and on laboratory-extracted samples can be achieved with similar end effects. The application of these hydrolysate preparations as starting materials for industrial nonfood uses in formulations of special pressure-sensitive adhesives, plasticizers and films, has been described in detail elsewhere (Aranyi *et al.*, 1970, 1971; Krull and Inglett, 1971). Briefly, the hydrolysate preparations were chemically modified by reactions with ethylene oxide or ethylenimine. The epoxidized gluten hydrolysates imparted pressure sensitivity to acrylic-based adhesives. The peel strength of the adhesives was a function of the concentration of the expoxidized gluten peptides in the mixture. In other applications, ethylene oxide or ethylenimine-modified gluten peptides used as plasticizers gave good flexibility to initially brittle films obtained from gluten or gluten hydrolysate preparations. Films cast from gluten hydrolysate in combination with ethylenimine-modified gluten hydrolysate were water-soluble and transparent and could be used for packaging measured amounts of laundry detergent for instant release. Other formulations for packaging films, plasticizers, and processing additives were also obtained with the use of the modified hydrolysate preparations, confirming the concept that promising areas for industrial nonfood applications of gluten have to be based on prior chemical modification of the protein.

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