"Ferripolyphosphate" as a Whey Protein Precipitant

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"Ferripolyphosphate," a liquid complex of ferric ion with a long-chain polyphosphate, precipitates virtually all the protein in commercial acid whey at pH 3.2–4.0. A typical precipitate contains 22% protein, 12% iron, 39% P_2O_5, and 5% calcium. The lyophilized product is white, fluffy, and has a mildly acidic flavor. This precipitation technique is of potential value for recovering high quality protein from industrial discharges of whey in a form useful for enrichment of foods with iron.

The precipitation of whey proteins at acid pH by the polyphosphate anion (Gordon, 1945) and by heavy metal cations, including Fe(III) (Block and Bolling, 1955) has been described. This paper describes the preparation of an iron polyphosphate–protein powder from cheese whey by means of a precipitation technique using "ferripolyphosphate" (Hazel *et al.*, 1968), a soluble complex of ferric ions with a long-chain polyphosphate. The powders, containing up to 15% iron, are white, fluffy, mild tasting, and uniform in texture. A preliminary report of these has been made (Jones *et al.*, 1971).

EXPERIMENTAL

Preparation of "Ferripolyphosphate." Soluble "ferripolyphosphate" complex was prepared from ferric chloride and the sodium polyphosphate glass, Calgon (Calgon Corporation, Pittsburgh, Pa.). Ferric chloride solution, 0.5 *M*, was freshly made at room temperature from reagent grade solid iron(III) chloride hexahydrate and distilled water. A solution which was 3 *M* in sodium phosphate monomer was prepared at room temperature by dissolving 30.6 g of unadjusted powdered Calgon in distilled water to give 100 ml of solution. Calgon was easily dissolved in a Waring Blendor. Potentiometric titrations were used to determine average phosphate chain lengths (Van Wazer *et al.*, 1954) in solutions prepared both with and without the blender, and no difference in chain length was found. The phosphate and iron solutions were cooled to 10°C and mixed quickly in a blender. Subsequent experiments indicated that neither the cooling of the solutions nor the order of mixing was essential to prepare satisfactory soluble complexes.

Three forms of "ferripolyphosphate" were prepared which differed from each other in their relative proportions of iron and phosphate. These forms were a sparingly soluble solid gel, a liquid system 0.25 *M* in iron, and a liquid system 0.167 *M* in iron. Combining one volume 0.5 *M* ferric chloride with one-half volume 3 *M* Calgon produced a gel with an Fe/P mole ratio of approximately 1/3. Although poorly soluble in water, the gel dissolved readily in excess sodium polyphosphate solution. Thus, a liquid system having a pH of 1.2 and an Fe/P mole ratio of approximately 1/6 was produced by mixing equal volumes of ferric chloride and Calgon. The liquid complex system, 0.25 *M* iron, was clear and light orange in color. After standing at 25°C for about 3 days, or at 4°C for about a week, a large amount of white precipitate accumulated in the container. The liquid remaining was pink in color and was an effective protein precipitant.

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Mixing one volume of 0.5 *M* ferric chloride with two volumes of 3 *M* Calgon produced a liquid system having a pH of 2.2 and an Fe/P mole ratio of approximately 1/12 (0.167 *M* in Fe). After standing at room temperature for about 4 days, the initial pale orange color changed to pink. Some preparations contained suspended solid material which settled in 24 hr. In these same preparations, precipitation within the system commenced in about 10 days at room temperature. Precipitation could be retarded by storage at 4°C. Some other 1/12 preparations contained no suspended solid and remained clear indefinitely at room temperature. Such variations in stability were apparently related to differences in quality of the reagent grade ferric chloride from different suppliers. In the experiments described here, the "ferripolyphosphate" soluble complex of mole ratio 1/12 was used extensively because of its demonstrated stability, as compared to the 1/6 complex, at low temperature over a period of months. Polyphosphate complexes of ferrous chloride were prepared, but did not cause precipitation of protein. Ferric nitrate complexes behaved like ferric chloride complexes.

Precipitation of Whey Proteins. Sufficient "ferripolyphosphate" was added at room temperature to whey at pH 4.6 to bring the concentration of iron to a desired level. Routinely, 250 ml of mole ratio 1/12 "ferripolyphosphate" was added for each liter of whey to give an iron concentration of 0.041 *M* or 2.29 g/l. A white floc was generated with the addition of even small amounts of "ferripolyphosphate." The pH of the system normally fell into the range 3.5–3.8, or it was adjusted to that pH. Precise control of pH within the range 3.2–4.0 was not necessary to obtain satisfactory powders. In 4 to 6 hr the precipitate had settled sufficiently so that the supernatant liquid could be siphoned from the vessel. The remaining liquid was removed by centrifugation. The precipitate was washed with distilled water, dialyzed exhaustively against distilled water at 4°C, and lyophilized.

All experiments noted here were conducted with acid whey obtained as a cottage cheese by-product from Sealtest Dairies, Chambersburg, Pa. The whey as obtained contained relatively large amounts of calcium ions and a low protein level which varied between 0.2 and 0.3% (w/v). (Acid whey prepared in the laboratory by acidifying raw skim milk to pH 4.6 and removing the precipitated casein contained about 0.8% protein.)

Analytical Methods. Iron analyses were performed by atomic absorption spectrophotometry. Phosphorus analyses were by the method of Meun and Smith (1968). Nitrogen determinations were by the Kjeldahl method, using a conversion factor of 6.25. Zone electrophoresis of some "ferripolyphosphate"–protein powders (hereafter referred to as FIP–protein powders) was performed in 8% polyacrylamide gel in pH 9.2 TRIS (trihydroxymethylaminomethane) buffer,

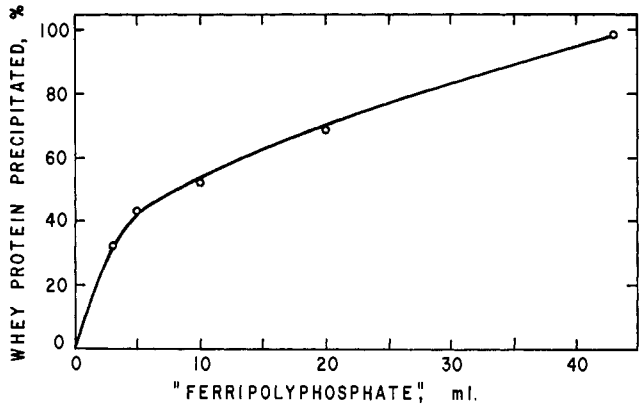

Figure 1. Percent of whey protein precipitated from 100 ml of whey at various levels of "ferripolyphosphate," mole ratio 1/12. The powders thus produced are described in Table III

Table I. Compositions of Two FIP–Protein Powders Prepared with "Ferripolyphosphates" of Different Fe/P Mole Ratios[a]

	Fe/P mole ratio[b] of "ferripolyphosphate"	
	1/6	1/12
Material yield from 50 ml of whey, mg	600	459
Protein, mg	119	78.5
Protein, %	19.9	17.1
Fe, %	16.4	10.0
P_2O_5, %	48.5	44.4
Percentages of Protein, Fe, and P_2O_5 Recovered in the FIP–Protein Precipitates		
Protein[c]	95.2	62.8
Fe	23.5	16.5
P_2O_5	9.07	4.78

[a] Age of complexes: 8 weeks. [b] As described in text. [c] Using a protein value of 0.25% (w/v) for the commercial whey.

with and without 4.5 *M* urea (Peterson, 1963). The proteins were visualized by amido black dye.

RESULTS AND DISCUSSION

From 10 l. of commercial whey plus 2.5 l. of "ferripolyphosphate" (mole ratio 1/12) the yield was 110–120 g of FIP–protein powder. A typical powder contained 22% protein, 12% iron, 39% P_2O_5, and 5% calcium. Total ash was 65%. The product was insoluble but easily dispersible in water. It was soluble in dilute base with some decomposition indicated by the formation of orange iron oxide.

Table I is a comparison of FIP–protein powders prepared with "ferripolyphosphate" complexes of different Fe/P mole ratios, each 8 weeks old. The complex having Fe/P mole ratio equal to 1/6 produced the greater quantity of precipitate, containing higher levels of iron and protein. In Table I are the percentages of added iron and phosphate which appear finally in the precipitate. For the system prepared with "ferripolyphosphate," mole ratio 1/6, it will be noted that about 95% of the protein is removed from the whey, while approximately 76% of the added iron and 91% of the added phosphate remain soluble.

Freshly-made "ferripolyphosphate" was a comparatively poor protein precipitant in commercial whey at pH 3.5–3.8, but after the liquid systems had aged for 2 weeks, their addition to commercial whey caused abundant precipitate to form. When the precipitate was removed and TCA (trichloroacetic acid) was added to the supernatant to a final concentration of 5%, light turbidity developed and some additional protein

Table II. Compositions of Two FIP–Protein Powders Prepared with "Ferripolyphosphates" of Different Ages[a]

	Age of "ferripolyphosphate"	
	24 hr	8 weeks
Material yield from 50 ml of whey, mg	186	600
Protein, mg	83.9	119
Protein, %	45.1	19.9
Fe, %	8.73	16.4
P_2O_5, %	31.4	48.5
Percentages of Protein, Fe, and P_2O_5 Recovered in the FIP–Protein Precipitates		
Protein[b]	67.1	95.2
Fe	3.88	23.5
P_2O_5	1.79	9.07

[a] Mole ratio Fe/P of both complexes: 1/6. [b] Based on a protein level in the whey of 0.25% (w/v).

Table III. Compositions of Five FIP–Protein Powders Produced from 100 ml of Commercial Whey by Increasing Additions of "Ferripolyphosphate"[a]

FIP added, ml	Yield, g	Protein, mg	Percentage composition			
			Protein	Fe	P_2O_5	Ash
3	0.222	79.3	35.7	8.29	24.3	54.4
5	0.378	108	28.7	10.6	30.0	64.6
10	0.677	131	19.3	11.8	42.6	66.5
20	1.12	169	15.0	14.2	40.5	77.2
43	1.73	247	14.3	16.5	39.6	87.5

[a] Age of FIP: 5 weeks; molar ratio Fe/P 1/12.

settled overnight. Table II compares powders prepared from aged and fresh "ferripolyphosphates" at pH 2.3. "Ferripolyphosphate" which had aged for 8 weeks yielded more product with more protein and greater percentages of iron and phosphate. Table II indicates the percentage recovery of protein, as well as the percentages of added iron and phosphate in appearing in the precipitate.

Figure 1 indicates the ability of "ferripolyphosphate" to precipitate protein from commercial whey. To 100-ml portions of whey were added increasing volumes of "ferripolyphosphate" (mole ratio 1/12), aged 5 weeks. The average protein content for several lots of whey was 250 mg per 100 ml. The addition of 43 ml of "ferripolyphosphate" precipitated 98–99% of the protein present, at pH 4.0. Table III lists the compositions of the powders produced from this series of experiments.

The number average chain length in solution of the Calgon starting material was determined to be 11.2 by potentiometric titration (Van Wazer *et al.*, 1954). No orthophosphate was detected in freshly prepared Calgon solutions or in the freshly made "ferripolyphosphate" liquid systems by thin-layer chromatography on polyethyleneimine-impregnated cellulose (Tanzer *et al.*, 1968). However, orthophosphate was present in FIP liquid systems several weeks old, indicating hydrolysis of the chain. Evidence for pyrophosphate and tripolyphosphate was also present. However, most of the phosphate present was polymers, chain length greater than 4. It is known that polyvalent cations accelerate hydrolysis of the polyphosphate chain (Thilo and Wieker, 1961) and this may be presumed to be the primary activity within the system during the aging period.

Figure 2 is a gel electrophoresis pattern of FIP–protein powders in the presence of 4.5 *M* urea. The standards are α-lactalbumin (slot 1) and β-lactoglobulin (slot 6), displaying

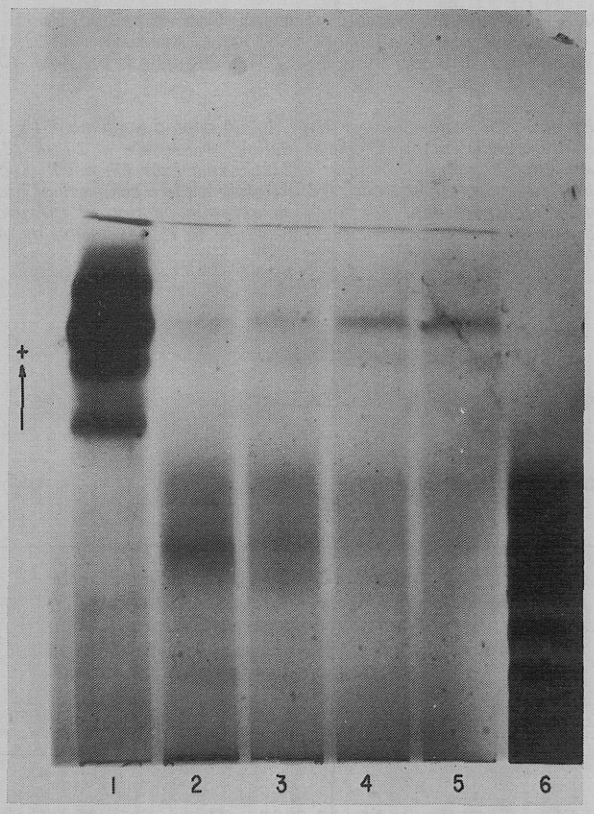

Figure 2. Polyacrylamide gel electrophoresis in 4.5 *M* urea of FIP–protein powders prepared by adding 3, 5, 10, and 20-ml volumes FIP to 100-ml portions of whey (slots 2–5). Standards are α-lactalbumin (slot 1) and β-lactoglobulin (slot 6)

Figure 3. Polyacrylamide gel electrophoresis, no urea, of FIP–protein powders (slots 2–5), α-lactalbumin (slot 1), and β-lactoglobulin (slot 6). See text for details

Table IV. Disposition of Protein in FIP–Protein Powder and Remaining Supernatant

	FIP–protein powder	Supernatant[a]
Material yield, g	70[b]	21.0[c]
Protein, %	22.2	30.2
Protein, g	15.6	6.34
Protein expected, g[d]	21.2	
Protein precipitated, %	74	
Protein left in supernatant, %		30

[a] Liquid remaining after precipitate was removed. [b] From 7300 ml of whey + 1800 ml of 1/12 "ferripolyphosphate." [c] Based on a portion of the supernatant which was dialyzed and lyophilized. [d] The protein content of this commercial whey was 290 mg/100 ml.

the multiple banding customary in urea gels. The four FIP–protein powders in slots 2–5, respectively, represent additions of 3, 5, 10, and 20 ml of "ferripolyphosphate" solution to 100 ml of commercial whey. The patterns indicate that FIP at low concentrations causes precipitation mainly of β-lactoglobulin, and that at higher levels of FIP, the precipitate contains greater amounts of α-lactalbumin.

Figure 3 is a non-urea gel containing α-lactalbumin and β-lactoglobulin in slots 1 and 6, respectively. Slots 2 and 3 contain powders prepared with "ferripolyphosphate" solution 8 weeks old and 24 hr old, respectively. Table II contains a further description of these powders. It should be noted that the FIP–protein powder obtained with the aged "ferripolyphosphate" contains more total protein (119 mg), whereas the powder obtained with the freshly prepared "ferripolyphosphate" (24 hr old) contains more protein per unit weight (about 0.45 mg/mg *vs.* 0.20 mg/mg). This difference apparently accounts for the heavier staining observed in slot 3.

Slot 4 is another FIP–protein powder prepared by the addition of 250 ml of "ferripolyphosphate" (mole ratio 1/12) per liter of commercial whey. As part of the processing of this powder, the supernatant liquid was dialyzed and lyophilized. A sample of the solids recovered in this manner is in slot 5. It is apparent that under these conditions, β-lactoglobulin and α-lactalbumin were precipitated, but that some α-lactalbumin remained in soluble form in the supernatant. Table IV gives the compositions of the powder and supernatant residues in slots 4 and 5.

"Ferripolyphosphate" appears to be a useful reagent for the recovery of highly nutritious proteins from whey. The proteins can be obtained in a form containing 12–15% iron, which is an upgrading of the nutritional value of the proteins. Thus, they could serve as important iron-enriching additives to dairy products and other commonly used foods; *e.g.*, flour, cereals, and potato flakes. This is of particular importance in light of recent nutritional surveys (ARS 62-18, 1969) indicating insufficient iron in the diets of some segments of the population.

ACKNOWLEDGMENT

We thank Annette Kravitz and Mary Gavin for the nitrogen and ash determinations.

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Received for review August 3, 1971. Accepted September 16, 1971. A preliminary report of these findings was made at Sixth Middle Atlantic Regional Meeting of the American Chemical Society, Baltimore, Md., February 1971. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Further Chemical Studies of *N*-(2-Furacyl)glycine

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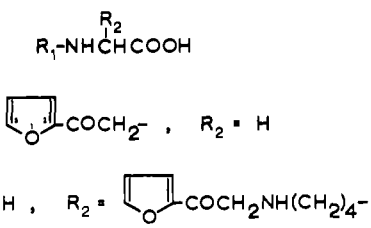
In chemical studies related to the Maillard reaction, a complex mixture of new *N*-hydroxyalkylglycine derivatives was obtained by the hydrogenation of *N*-(2-furacyl)glycine·HCl, a furan amino acid obtained by the acid-catalyzed dehydration of fructose–glycine. These stable reduction products were detectable with ninhydrin and were resolved in an amino acid analyzer. By oxidation with periodic acid, the reduction products were deduced to include *N*-mono-, di-, and trihydroxyhexylglycines. Their formation is discussed, as well as their relation to products of the glucose–glycine browning reaction.

A furan amino acid, *N*-(2-furacyl)glycine (I), derived from fructose–glycine by an acid-catalyzed dehydration, was recently identified and synthesized (Lipton *et al.*, 1971). I is structurally related to N^{ϵ}-furoylmethyl-L-lysine (II) (called furosine) which had been previously identified (Finot *et al.*, 1968; Heyns *et al.*, 1968) as the post-arginine amino acid first observed in acid-hydrolyzed dried skim milk (Erbersdobler and Zucker, 1966; Brueggemann and Erbersdobler, 1968). These *N*-furacyl amino acids appear to be



Maillard reaction intermediates which, like the fructose–amino acids from which they are derived, precede an eventual destruction of the amino acids themselves. The *N*-furacyl amino acids may have eluded earlier recognition due to their extreme lability, except under conditions of high acidity. This report describes a complex mixture of stable amino acids produced by the hydrogenation of I and a study of the oxidation of these reduction products by periodic acid. Their formation as well as their relation to glucose–glycine browning products is also discussed.

EXPERIMENTAL

Materials and Methods. Fructose–glycine and *N*-(2-furacyl)glycine·HCl (I·HCl) were prepared as previously described (Lipton *et al.*, 1971). Platinum oxide (Adam's oxide), periodic acid (H_5IO_6), and iminodiacetic acid were commercial products. Quantitative amino acid analyses were obtained on a Phoenix Biolyzer, Model 3000. The various N-substituted glycine derivatives obtained by the reduction of I were detectable and resolved in the amino acid analyzer, with no modification of the buffers and procedure previously used (Lipton *et al.*, 1971).

Hydrogenation Studies. A Parr hydrogenation apparatus was used for hydrogenation of I at room temperature. Initially the reduction was undertaken to convert I into an alcohol in order to chemically confirm the ketone function of I (efforts to obtain semicarbazone and 2,4-dinitrophenylhydrazone derivatives of I had not been successful). I·HCl was reduced (1 mg/ml of solution) in either 95% ethanol or in aqueous solution, to which was added in either case 0.01 volumes of 1 *N* HCl. The weight of platinum oxide used in the reduction was equal to that of the I·HCl. Time of hydrogenation varied from 2 min to 18 hr; pressure was either 25 or 50 lb per in.^{2} (psi). Reduction was evidenced by pressure drop and an observed decrease in absorbance at 280 nm (final value after complete reduction was approximately 0.1 that of the initial value). The catalyst was removed by centrifuging prior to evaluating the reduction products in the amino acid analyzer.

Periodate Oxidation Studies. Oxidations were carried out by the addition of an excess of solid periodic acid (25 mg/ml) to the sample either in aqueous acid or in bicarbonate-neutralized solution at either 0 or 24°C. The concentration of the sample was about 1 mg per ml. For time studies, oxidation was stopped by the addition of glycerol to consume the excess periodate. The composition of oxidized samples was determined on the amino acid analyzer. Experiments on the oxidation of hydrogenated I, I, and of fructose–glycine were carried out.

RESULTS AND DISCUSSION

Hydrogenation Products. As seen in Figure 1, a complex mixture of amino acids, which were resolved in the amino acid analyzer, was obtained upon hydrogenation of I. After 20 min at 50 psi (Figure 1A), the hydrogenation was essentially

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