The Relation of a "Globulin" Component of Wheat Flour to Purothionin[1]

C. C. NIMMO, MARY T. O'SULLIVAN, and **J. E. BERNARDIN,**
Western Regional Research Laboratory, Albany, California

ABSTRACT

The most rapidly migrating protein component of wheat flour is apparent on gel electrophoresis in aluminum lactate buffer, pH 3.1, as a pair of bands having the same position as purothionin, the low-molecular-weight, high-sulfur protein first reported crystalline in 1942 by Balls, Hale, and Harris. Classified among the globulins in previous reports, this component has been separated from crude globulins by preparative electrophoresis and gel filtration (Sephadex G-50, 0.05*M* acetic acid, 0.01*M* KCl) to yield a water-soluble material ("globulin") resembling purothionin in elution volume, UV absorption spectrum, and amino acid composition. Both purothionin and the "globulin" have unusually low fluorescence, and purothionin apparently lacks tryptophan. The "globulin" which resembles purothionin apparently is produced by exposure of a globulin component to low pH, as in aluminum lactate gel electrophoresis, and does not exist as an individual component in the crude globulins.

Electrophoretic analysis of wheat proteins in starch or polyacrylamide gel, with the use of aluminum lactate-lactic acid buffer, pH 3.1, has consistently shown a relatively isolated, closely spaced pair of bands leading the rest toward the cathode. This pair has been found to occur among the components precipitated by dialysis against water of proteins extractable by salt solutions at near neutrality, and has been assumed to be in the globulin group of wheat proteins (1,2). It was observed in this laboratory that a sample of purothionin, the low-molecular-weight, high-sulfur, basic protein reported crystalline in 1942 (3,4), showed the same band pair as major components. Because of the interesting chemical and physiological properties of purothionin (3–7), we have studied further the relation between the globulin preparations and purothionin.

At a point where the principal conclusions of this work had been made, we learned that Nathan Fisher, of the Flour Milling and Baking Research Association, Chorleywood, Herts., England, was preparing to report on the fractionation and characterization of purothionin.[2] Our work has been primarily concerned with the purothionin-"globulin" relationship, but has inevitably involved further definition of purothionin.

MATERIALS AND METHODS

Purothionin

The sample used for reference, and for the first observations, was prepared by the Balls, Hale, and Harris method (3) from a commercially milled, unbleached soft wheat flour. Designated Purothionin Ivory 2X, it was prepared and twice crystallized by Walter S. Hale in 1956. (This is PI-1 of Figs. 2 and 3.) For PI-2, PI-1, was fractionated on Sephadex G-50, 2.5 × 90 cm., in 0.05*M* acetic acid, 0.01*M* KCl. Three zones were eluted from the

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[2] Editor's Note: See Fisher *et al.*, page 48 in this issue.

column, as will be shown later, and the material from the third zone was dried from the frozen state, dialyzed against water to remove salt, and redried. A third preparation, PI-3, used only for the ultraviolet absorption spectrum, was prepared as a center cut from the third zone of a similar fractionation.

Another sample was prepared from a commercial hard spring wheat flour (spring wheat long patent 4-64), unbleached and unmalted, in order to have material from the same flour source as the "globulin" preparation. It was prepared by extraction of 2.0 kg. of flour with about 3 liters of Skellysolve B, taken through the described procedure (3), and twice crystallized to yield about 150 mg. This material was fractionated on Sephadex G-50, as for the PI-2 above, to give about 70 mg. of white powder, designated Purothionin SWLP.

Globulin Preparations

One kilogram of the spring wheat flour was extracted successively with 2.0 and 1.0 liter of 1*M* NaCl, and the extract was dialyzed against water at 2°C. to remove salt. The collected precipitate was re-extracted with 300 ml. 1*M* NaCl and the soluble portion dialyzed as before. The precipitate from this dialysis was suspended in 25 ml. water, adjusted to pH 3.2 by dropwise addition of 0.1*N* HCl with stirring, and centrifuged at $12{,}000 \times g$ for about 1 hr. to remove a persistent turbidity. This step is the first exposure of the *crude globulin* to a pH below about 5.6; conversion to a form existing at the lower pH is important to subsequent fractionation. The supernatant solution was made 0.15*M* in acetic acid and fractionated on a free-film preparative electrophoresis apparatus (Elphor) in 0.15*M* acetic acid, at about 38 volts/cm., 50 ma., 10°C. As the desired material is among the constituents moving most rapidly to the cathode at this pH, the electrophoresis separation served to remove much unwanted carbohydrate and protein material. Solutions containing the desired fractions were lyophilized. The solids were dissolved in 4.0 ml. 0.05*M* acetic acid, 0.01*M* KCl, and twice fractionated through the same Sephadex G-50 column used to prepare the Purothionin SWLP. The final fractionation was made to correspond as nearly as possible to the principal component of the purothionin preparation. After dialysis to remove salt, and lyophilization, the yield was 25 mg. of white solid. Both this material and the purothionin preparations were highly soluble in distilled water. For lack of a better name, this preparation from the globulin fraction is referred to as "globulin," to distinguish it from the crude, unacidified, water-insoluble globulins.

Electrophoretic Analysis

Polyacrylamide gels, 6%, 0.6 × 12 × 27 cm., were used in a water-cooled apparatus. Gels were prepared with the use of tetramethylethylenediamine and ammonium persulfate catalysts, and were held at least 1 day in 4 liters distilled water, then at least 24 hr. in 4 liters of the buffer to be used. After electrophoresis, the gels were stained with 1.0% amido black in methanol:water:acetic acid 5:5:1, and destained electrophoretically.

Ultraviolet Spectra

A Cary Model 15 spectrophotometer was used, with samples in 0.001*M* HCl, 0.01*M* KCl.

Gel Filtration

The preparative column already described was used at flow rates of 4–5 ml./cm.^{2} hr. In first experiments, no salt was used. Comparisons between eluants of $0.05M$ acetic acid alone and $0.05M$ acetic acid plus $0.01M$ KCl showed earlier elution in the latter. Because of the possibility of ion exchange in salt-free solvent on Sephadex, use of the salt was continued. The detector was a Gilson ultraviolet absorption monitor peaked at 280 mμ (Gilson Medical Electronics). For tandem absorption-fluorescence monitoring, the fluorescence monitor was a Turner Model 111 (G. K. Turner Associates, used in the line at a known volume interval from the absorption monitor, as described in a separate publication (8). For these latter experiments, the G-50 column was 1 × 100 cm., in $0.001M$ HCl, $0.02M$ KCl, flow rate 12 ml./cm.^{2}/hr.

Amino Acid Determinations

Protein samples were dried at least 6 hr. over P_2O_5 at < 0.1 mm. Hg, in a drying pistol heated by boiling methanol. Dry protein (25 mg.) was weighed into a 10-ml. volumetric flask and dissolved in water to volume. Two milliliters for each hydrolysis time was transferred to an ampule, 2 ml. fresh concentrated HCl was added, and the solution was frozen. The samples were thawed under vacuum, nitrogen-flushed and re-evacuated twice (9), and sealed under vacuum. Hydrolysis was at 110 ± 1°C. in an aluminum block, for 24, 48, and 72 hr. for the "globulin" and 23, 46, and 70 hr. for the purothionin preparation. The completely clear, pale-yellow hydrolysates were rotary-vacuum-evaporated in 50-ml. flasks and re-evaporated 3 to 4 times from water solution. The dry material was dissolved in 5 ml. of analysis buffer and sampled for analysis on a Phoenix automatic amino acid analyzer. Tabulated values for serine and threonine were determined by extrapolation to zero time of the three time values for each protein preparation. All other amino acid values are averages of the time series results.

Cystine and methionine were separately determined by the performic acid oxidation method (10). Aliquots of the original 10 ml. estimated to contain 0.1 mg. cystine were put in hydrolysis ampules, rotary-evaporated under vacuum to dryness, oxidized 4 hr. at 0°C. with 2 ml. performic acid reagent, and treated with 0.3 ml. 48% HBr. The samples were carefully rotary-vacuum-evaporated to near-dryness, then dried under high vacuum. Three milliliters of $6N$ HCl was added and hydrolysis carried out as described above, but for 18 hr. only.

Nitrogen analyses were made by micro-Kjeldahl method on samples of the original 10 ml.

RESULTS AND DISCUSSION

Gel Filtration

Comparison (Fig. 1) of the gel filtration elution diagrams from Sephadex G-50 for the "globulin" and the purothionin shows similarity in the volume and profile of each third fraction, peaking at about 1.8 times void volume. This fraction contains the electrophoretically fastest-moving band pair for both purothionin and "globulin," with at least one other component, as will be shown later. Generally similar results were obtained with $0.1M$

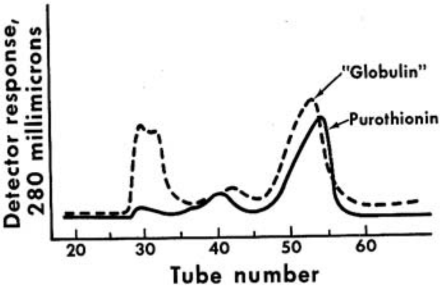

Fig. 1. Gel filtration elution diagram for "globulin" and purothionin preparations. Sephadex G-50, 2.5 × 90 cm., 0.05*M* acetic acid, 0.01*M* KCl, tube volume 5.5 ml., flow rate 4–5 ml./cm.^{2}hr.

acetic acid as eluant for G-50 and G-75 columns, except that the elution was somewhat later in the absence of salt. As mentioned earlier, inclusions of 0.01*M* KCl decreased the elution volume of the fraction significantly. To avoid possible adsorption, the salt was used in elution of all components discussed. G-50 gave the best elution volume position between void and elution volumes. G-25 was not satisfactory, passing the components of interest near void volume.

Gel-Electrophoretic Analysis

The migration of the three purothionin samples at pH 3.1 in aluminum lactate-lactic acid was coincident with that of the fastest-moving component pair in the "total" protein preparation T (Fig. 2). The twice-crystallized

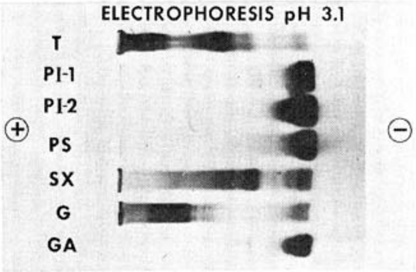

Fig. 2. Electrophoresis, 6% polyacrylamide gel (0.6 × 12 × 27 cm.), aluminum lactate-lactic acid, pH 3.1 Γ/2 0.05 12 v./cm., 60 ma. T="total" protein; PI-1= purothionin Ivory 2x; PI-2=PI-1 purified by gel filtration; PS=purothionin SWLP from spring wheat; SX=salt-soluble proteins from petroleum ether extract solids; G=crude globulin; GA="globulin" from low pH fractionation. (See text for details.)

PI-1 has a few faintly visible bands not seen in the further fractionated PI-2. PS (Purothionin SWLP) is the purothionin from the spring wheat flour used for the "globulin" preparation. All of the purothionins shown have two major bands, but the quantity used was too great to make this clear. These components stain very heavily with amido black[3], presumably because of the high content of basic amino acids. As a result, detection of impurities

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[3] Lawrence, J. M. Unpublished observation.

in these components, with the use of gel electrophoresis and amido black as a stain, is sometimes unreliable.

GA of Fig. 2 shows the "globulin"; it resembles purothionin by this criterion. SX and G preparations were, in contrast to others displayed here, not exposed to low pH conditions before being put in the aluminum lactate buffer. The former represents the components extracted by $1M$ NaCl from the solid residue produced by evaporating the Skellysolve extract of flour to near dryness. The latter represents a crude globulin preparation after completion of the repeated solution and dialysis procedure described earlier, but before acidification as in the preparation of "globulin." In the low pH electrophoretic analysis, the fastest-moving bands, coincident with purothionin, appear in both preparations.

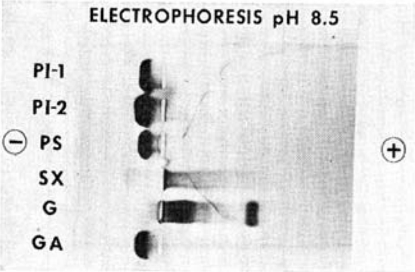

Fig. 3. Electrophoresis, 6% polyacrylamide gel (0.6 × 12 × 27 cm.), *tris*-citric acid, pH 8.5, $\Gamma/2$ 0.05, 5 v./cm., 75 ma. Samples as for Fig. 2 (no T).

In Fig. 3 are the diagrams from electrophoresis of the same preparations (lacking T) at pH 8.5 in a *tris*-citric acid buffer. All purothionins moved slightly to the cathode. The coincidence of GA to these supports the idea of similarity of "globulin" to purothionin. The lack of purothionin bands in SX and G leads to the conclusion that the purothionin-like "globulin" is developed only under low pH conditions, the precursor of GA being contained in the components visible in G, in the slot or in the gel.

Ultraviolet Absorption

The purothionin and "globulin," at this stage of purification, have similar, though not entirely identical absorption spectra in $0.001M$ HCl, $0.01M$ KCl (Fig. 4). All three spectra are very different from the spectra (not shown) of the first two elution zones of the fractionation (Fig. 1) of the crystalline purothionin, and are characteristic of a protein in which tryptophan is absent or very low. Evidence from absorption spectra in $0.1M$ NaOH indicated that tryosine:tryptophan ratio was at least 6:1, and preliminary fluorescence spectra on the highly purified PI-3 showed no evidence of tryptophan.

Concentrations were not exactly known, so that the spectra are for qualitative comparison only.

Amino Acids

Purothionin and "globulin" from the spring wheat flour showed marked resemblance in pattern of amino acid content (Table I). Calculated as percent by weight of the total amino acid content in the purothionin sample,

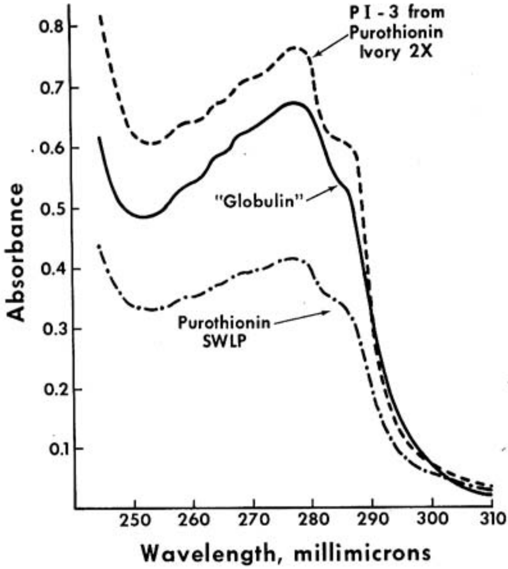

Fig. 4. Ultraviolet spectra for "globulin" and purothionin.

cystine equivalents were 15.9%, sulfur 4.99%, arginine 15.2%, and tyrosine 3.2%. These are of the order of those reported previously on the hydrochloride basis (3), at 15.7, 4.42, 20.4, and 3.0%, respectively. The trace values for histidine and methionine, particularly in a protein of this molecular size, suggest their absence. The absence of methionine would support the tentative conclusion of the earlier work (3). We have concluded above that tryptophan is probably absent in purothionin, and this conclusion was supported by a complete lack of color development in the method of Spies and Chambers (11) re approximately 5 mg. of the protein preparation was used. With these assumptions, and with the analysis figures for ammonia, recovery of nitrogen in the separate hydrolyses was 99–101%. The amount of ammonia recovered, taking into account that formed by destruction of

TABLE I

AMINO ACID COMPOSITION OF "GLOBULIN" AND PUROTHIONIN
(Millimoles amino acid per g. dry preparation)

AMINO ACID	"GLOBULIN" (18.35% N)	PUROTHIONIN SWLP (17.44% N)	AMINO ACID	"GLOBULIN" (18.35% N)	PUROTHIONIN SWLP (17.44% N)
Arg	0.89	0.84	Val	0.21	0.19
His	0.09	trace	Leu	0.77	0.67
Lys	0.92	0.94	Ile	0.11	0.06
Thr	0.38	0.43	Pro	0.36	0.40
Ser	0.80	0.84	Tyr	0.16	0.17
Asp	0.56	0.52	Phe	0.20	0.19
Glu	0.37	0.22	½ CyS[a]	1.26	1.34
Gly	0.82	0.73	Met[a]	trace	trace
Ala	0.53	0.44			
			Totals	8.43	7.98

[a] From performic acid oxidation, as cysteic acid and methionine sulfone.

threonine and serine, corresponded reasonably well with glutamic and aspartic acids being present in the amide form.

The amino acid results should be used only for comparison of the two preparations, and not as absolute figures for purothionin. Of the dry weight of sample, the amino acid residues accounted for slightly over 86% of the purothionin and 91% of the "globulin." For the purothionin only, another 3% was accounted for as KCl not removed by dialysis of the final preparation. Both samples were recovered under circumstances favoring formation of an acetate of the basic groups; assumption of an acetate form for the arginine and lysine basic groups would approximately account for the rest of the dry weight.

Gel Filtration with Fluorescence and Absorption Monitoring

This detection system was used in further studies on the gel filtration fractionation of purothionin. The Purothionin Ivory 2X was used, in a solvent of 0.001*M* HCl, 0.02*M* KCl. The third peak eluted from the G-50 was the major one for UV absorption (Fig. 5), but had a remarkably low fluorescence. This would be expected in a protein lacking tryptophan. From the monitoring curves by each method, an apparent relative quantum yield (F/A) was calculated, to be used in the detection of multicomponent systems in chromatography and gel filtration. For this, F, the relative fluorescence taken directly from the recorder chart, was divided by A, the relative absorption calculated from the recorded transmission values. From the F/A (yield) curve, the use of which is described in detail elsewhere (8), we concluded that there were five or more components in the twice-crystallized purothionin. When this method was applied on a slightly larger scale, three fractionations, rerunning the shaded areas, yielded material with very low F/A (Fig. 6). The shift in the position of the eluted material may be caused by a concentration effect. The amount of purified material finally

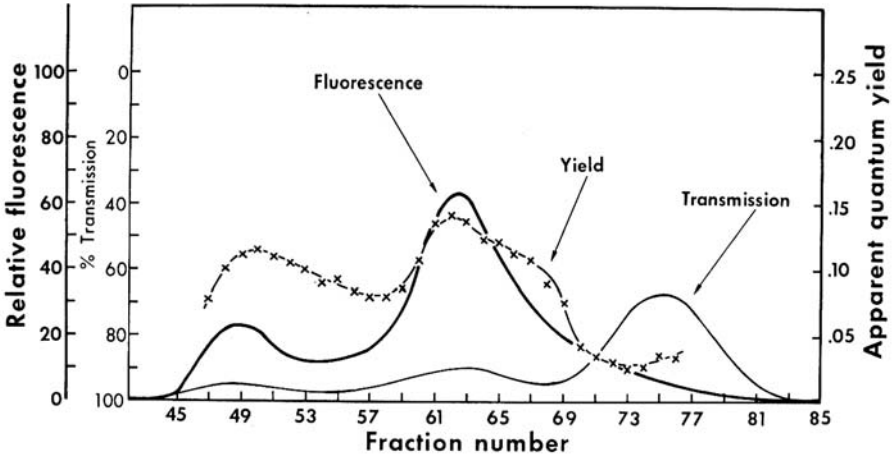

Fig. 5. Ultraviolet transmission and fluorescence recorder tracings for gel filtration elution of purothionin and apparent (F/A) calculated from the tracings (see text).

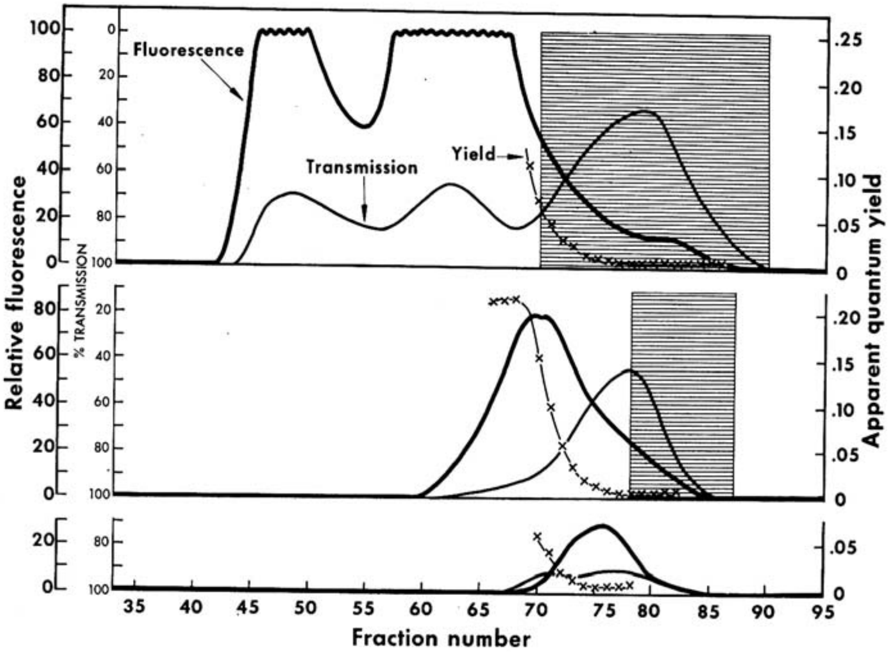

Fig. 6. Fractionation of purothionin; F/A used as guide (see text). The shaded areas were rerun to obtain the next lower result.

available was too small for analysis, and larger runs must be made. From this evidence, it seems that the low-fluorescence component, to which we have tentatively assigned identity with a pure purothionin, must be studied further as a single component.

Purification of a sample of "globulin" was attempted with this monitoring system. There was a similar trend toward lower F/A but we were not able, with the quantity available, to obtain a fraction with the low F/A of the purothionin.

The comparisons made in this work show that there were several points of similarity between the "globulin" and the purothionin as they have been prepared. It seems probable that these preparations contain one or more identical components. It also seems reasonable to conclude that the "globulin" was never from a globulin within the classic solubility definition, but from a complex of a low-molecular-weight, highly basic protein linked to some compound which conferred water-insolubility on it but allowed solubility in salt solutions. By analogy with purothionin, this other compound may be lipid in character. As the linkage is so easily broken, by simple exposure to pH 3 in the examples shown, it appears not to be covalent. Possibly ionic bonds are involved. Reversibility of this dissociation has not been investigated.

Purothionin has a chemical constitution of considerable interest. The low molecular weight and high content of basic amino acids and cystine-cysteine suggest that sequence studies would be of value. It appears that effective purification of the components of the group studied can be made with the use of the absorption-fluorescence monitoring system.

In the original work on purothionin, several interesting physiological properties were reported for the crystalline product (5,6,7). From results of this present work, these properties must be regarded as those of a multicomponent system. This adds further interest to the separation and characterization of the components of the group.

Acknowledgments

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