Electrophoretic Analysis, Redox Activity, and Other Characteristics of Proteins Similar to Purothionins from Tomato (*Lycopersicum esculenta*), Mango (*Mangifera indica*), Papaya (*Carica papaya*), and Walnut (*Juglans regia*)[1]

Larry S. Daley* and L. J. Theriot

Low molecular weight proteins stable to heat, acid, and organic solvents were extracted from tomato leaves and from mango, papaya, and walnut seed. These proteins were further purified by a new method utilizing triacylglycerol lipase (EC 3.1.1.3) and (diethylamino)ethyl-Sephadex. Electrophoretic analysis and assays for redox activity and interaction with crude nonreduced papain (which contains EC 3.4.22.2. and EC 3.4.22.3) and the specific sulfhydryl reagent ammonium 4-chloro-7-sulfobenzofuranozan showed that these proteins have low molecular weights, net positive charges, and electrophoretic migration characteristics similar to thionin; abundant sulfhydryl groups; redox properties; and interactions with crude papain proteolytic activity. The possibility that these proteins may be thionins is discussed, and the pseudo-first-order kinetics of their reaction with insulin is demonstrated.

Over 40 years ago, an unusual protein from unbleached wheat flour was isolated (Balls and Hale, 1940) and named purothionin (Balls et al., 1942a,b), from puro (Greek for wheat) and thio (Greek for sulfur). Purothionin is one of a group of homologous small molecular weight lipid-binding proteins called thionins that can be extracted by dilute acids, are resistant to heat and very low pH, and are frequently associated with lipids; when lipids are removed, these proteins have a strong net positive charge [reviewed briefly in Daley et al. (1983), Jones et al. (1982), and Lecomte et al. (1982)]. Purothionins are physiologically active (Coulson et al., 1942) and toxic to bacteria (Fernandez de Caleya et al., 1972), brewers' yeast (Okada et al., 1970), and insect larvae (Kramer et al., 1979). Additional properties of some thionins: modification of membrane permeability (Okada and Yoshizumi, 1973; Kashimoto et al., 1979; Carrasco et al., 1981), DNA synthesis (Nakanishi et al., 1979), and DNA binding (Woynarowski and Konopa, 1980); thioredoxin-like activities (Wada and Buchanan, 1981a,b); modulation of papain (Balls et al., 1942b) and amylase (Jones and Meridith, 1982).

Thionin homologues are common in cereal seeds, and thionin amino acid sequence is correlated with genetic divergence (Jones et al., 1982). The strongly conserved amino acid sequences common to thionins, viscotoxins, phoratoxins, crambin, and related proteins (Hedrickson and Teeter, 1981; Lecomte et al., 1982) and the varied physiological effects described above suggest that thionins are important regulatory proteins. Viscotoxins are toxic proteins from leaves and stems of the European mistletoe (*Viscuum alba*) (Winterfeld and Leiner, 1956; Samuelsson, 1961). Phoratoxin is found in the leaves of other mistletoes (e.g., *Phoradendron tomentosum* G. = *Phoradendron serotinum*) (Melstrand and Samuelsson, 1973) and crambin is found in seeds of *Crambe abyssinica* (Van Etten et al., 1965).

The search for other thionins is a unifying theme in genetic and physiological studies. Characteristics of low molecular weight (~5 kDa), heat and solvent stability, redox activity, strong positive charge, sulfhydryl reactivity, and frequently, but not always, toxicity and modulation of papaya protease activity draw attention to candidate proteins (Daley et al., 1983). Seeds are a traditional choice of material (Balls and Hale, 1940) because they are easy to collect and store and are relatively uniform. Proteins from tropical tree crop seeds may provide additional information on adaptation of enzyme function to different temperature ranges through changes in amino acid sequence. Until recently, thionin enzymic functions were investigated infrequently. Possible reasons for this include (a) explanations of the importance of small redox proteins in plants are relatively recent (Wada and Buchanan, 1981a,b; Jacquot et al., 1983); (b) detection of thionins by methods used commonly to extract and detect proteins is difficult; and (c) many enzymologists are unfamiliar with thionins (Jones, B. L., personal communication, 1986).

Thionin electrophoretic methods (Fernandez de Caleya et al., 1976) have evolved separately from methods used in most enzymological laboratories (Ornstein, 1964; Davis, 1964). A probable reason for this is that standard enzymological electrophoretic techniques are much less suitable for this use than those developed specifically for thionins. This paper presents methods of detection and assay of proteins similar to thionins (PSPs) in various dicotyledonous plant materials; PSPs is a term adapted from Jones and Cooper (1980) who used it to refer to a corn protein with amino acid composition similar to that of rye thionin but was nontoxic and generated more chymotryptic peptides. It is probable that this protein is the same as that reported by Wada and Buchanan (1981b). Our long-term interest is in the physiological function related to redox activity of PSPs (Wada and Buchanan, 1981a,b). Thus, for our purposes we define PSPs in this paper as small molecular weight lipid-binding proteins rich in sulfhydryl groups able to catalyze the reduction of proteins by sulfhydryl reagents even after contact with strong acids and organic solvents, with electrophoretic migration similar to that of wheat purothionin.

MATERIALS AND METHODS

Materials. Papaya (*Carica papaya* L. cv. Blue Solo) and mango (*Mangifera indica* L. cvs. Haden, Tommy Atkins, and Kent) fruit were imported from Mexico by Produce Center (Berkeley, CA). Walnuts (*Juglans regia* L. cv. Payne) were donated by J. Barton (Plant Pathology, University of California—Berkeley). Tomato leaves (*Lycopersicum esculentum* Mill.) were collected from field plots. Wheat flour (unbleached whole-wheat, Stone Buhr Milling Co., Seattle, WA; lot 23/83 4, 23 83 42) was pur-

Department of Horticulture, National Clonal Germplasm Repository, Oregon State University, Corvallis, Oregon 97331 (L.S.D.), and Department of Chemistry, North Texas State University, Denton, Texas 76203 (L.J.T.).

[1]Oregon Agricultural Experiment Station Technical Paper No. 8153.

0021-8561/87/1435-0680$01.50/0

chased. Viscotoxin and wheat purothionin were prepared by K. Wada (Wada and Buchanan, 1981a,b). Spectrapore 3 dialysis tubing (Pierce Chemical Co., Rockford, IL) was used since it retains proteins as small as 3.5 kDa. All chemicals were reagent grade, except some "certified grade" solvents.

General Methods. Protein was determined with bovine serum albumin standards (Bradford, 1976). Amino acid analysis of purothionin after Bradford assay determined that this reagent's response to both proteins was equivalent. When seeds were ground (Waring commercial blender, CB-6 Model 31BL79), the temperature was allowed to rise to about 40 °C, since it favored purification by denaturation of proteins less heat stable than thionins. Evaporation of solvent extracts was done under reduced pressure and mild heat in a rotary evaporator. Lyophilization was performed after freezing with liquid nitrogen.

Lipase–(Diethylamino)ethyl-Sephadex (Lipase-DEAE) Treatment. The solutions were adjusted to pH 6.0 with solid 3-*N*-morpholinopropanesulfonic acid (MOPS), solid tris(hydroxymethyl)aminomethane (Tris) base and/or HCl as required. Lipase (triacylglycerol lipase, E.C. 3.1.1.3, from *Rizopus arrhizus*, Sigma type XI, 1000 U/mL) was incubated 1 h at room temperature with the protein solution. A plastic syringe of the appropriate size to contain the extract (3–50 mL) was chosen. The needle end of a plastic syringe barrel was covered with glass wool; enough dry DEAE-Sephadex to absorb the amount of solution to be used was placed inside the barrel; the lipase-treated solution was added through the plunger end. After the solution was allowed to stand for about 20 min, the moist DEAE-Sephadex was squeezed dry with the syringe plunger. The expressed liquid was collected. Distilled water was added to the squeezed DEAE-Sephadex, and the process was repeated. This continued until a volume of fluid about half of the original lipase-treated solution was collected. When this procedure was treated once more with new dry DEAE-Sephadex, the sample was considered lipid free, since the protein found in this expressed fluid migrated toward the negative electrode on electrophoresis at pH 8.0.

The insulin assay for redox activity measures the reduction of a saturated solution of insulin by following the increase in turbidity caused by insoluble insulin subunits (Holmgren, 1979a,b). The assay, as modified in this paper, detects the acceleration of this process, by thionins or PLPs, in the presence of dithiothreitol (DTT). The amount of DTT used is indicated in each figure legend. The reaction has a lag period dependent on DTT concentration. Rates are given as mk from

$$mk = (\ln \Delta A)10^3/\Delta t$$

where Δt is elapsed time (in hours) since t_0 and ΔA is the change in A_{650} since t_0. At high levels of activity a more readily obtained measure of rate is the reciprocal of the initial time of precipitation; the initial time is calculated graphically as the intercept of the turbidity-generated trace with the linear base line trace of the lag period. The rationale for the $1/t$ measurement is based on previous data of Holmgren [Table II, column 4, from Holmgren (1979b)], generation of acceptable enzyme kinetics (see substrate and enzyme concentration plots in Results and Discussion), and standard kinetic procedures in similar systems (Daley and Strobel, 1983). Conditions for the reaction: 0.1 mM insulin, 20 mM ethylenediaminetetraacetic acid (EDTA), in 0.1 M phosphate buffer, pH 7.0, 25 °C.

Fluorescent labeling of thiols was adapted from Andrews et al. (1982). The sample (100 μL of 0.5 mg/mL protein or less) was treated with 12 μL of 1 M pH 8 borate (counterion Tris base), 2 μL of 0.1 M EDTA, and 2 μL of 0.1 M DTT and incubated for 30 min at room temperature. Then excess DTT was removed with 20 μL of 10 mM sodium arsenite. After 10 min, excess ammonium 4-chloro-7-sulfobenzofuranozan (SBD) (2 μg; Pierce Chemical Co., Rockford, IL) was added. The reaction mix was left overnight (room temperature) before use to assure complete labeling of reduced –SH groups.

Proteinase Assays. Azocoll (20 mg; Sigma Chemical Co., Saint Louis, MO) was placed in a microcentrifuge tube. The PSP was added to a final volume of 1 mL in a solution containing 0.4 M MOPS (counterion Tris), pH 7.4, 0.4 mM DTT, 20% glycerol, and 20 mg of crude papaya protease. The reaction was stopped after 24 h at 25 °C by filtration of diluted sample through glass wool in a Pasteur pipet. Rates are expressed as change in A_{520}/h (Daley and Vines, 1978).

Preparations of PSPs and purothionin (lipase-reactive form) were extracted with 0.05 M sulfuric acid (Fernandez de Caleya et al., 1976). Yields are in milligrams of protein (Bradford, 1976) after lipase–DEAE treatment. *Purothionin and viscotoxin* purified as in Wada and Buchanan (1981b) were a gift from K. Wada. For preparation of the *lipase-reactive form of wheat purothionin*, wheat flour (2.3 kg) was eluted with dilute H_2SO_4 (50 mM, 3 L), and the filtrate was allowed to drip overnight into alcoholic HCl (344 mL of concentrated HCl in 4 L of 95% ethanol). By the next day, a protein-positive precipitate formed. The precipitate was collected by centrifugation (6000*g*, 0 °C, 10 min) and water added to a volume of 50 mL. The solution (pH 0.8) was neutralized (to pH 7.0) with 1 M KPO_4 and nonsoluble material removed by centrifugation (1000*g*, 10 min, 0 °C). This preparation was stored in 50% glycerol (–20 °C) until use. The yield was 68 mg/kg with a insulin-reducing activity of 10.8 mmol/mg of protein 27 °C by the method of Holmgren et al. (1979a,b). *Walnut PSP* was eluted from shelled walnuts by grinding and washing in 50 mM H_2SO_4 (about 2 L/kg) and letting the mixture drip through filter paper overnight at room temperature into 5 L/kg of seed of 95% ethanol. No precipitate was observed, even after acidifying with HCl. Thus, the eluted material was collected, by evaporation with repeated pH correction (NH_4OH). Protein yield was 2.8 mg/kg of shelled seed. Samples were stored in 50% glycerol at –20 °C until treatment with DEAE. *Papaya seed PSP* was eluted from air-dried seed by grinding and washing in 50 mM H_2SO_4 (about 2 L/kg) and letting the mixture drip through filter paper overnight at room temperature into 3 volumes of acetone. Acetone concentration was adjusted to 75% and the precipitate collected on filter paper. The precipitate was air-dried overnight and dialyzed against water (5 L/kg of seed, 4×, room temperature) and 71% ethanol (5 L/kg of seed, 4×, room temperature). The dialyzed material was freeze-dried and washed with ethanol by filtration. The precipitate was air-dried and stored in 50% glycerol at –20 °C until treatment with DEAE. After lipase–DEAE treatment, protein yield was 5.4 mg/kg seed. *Mango seed PSP* was prepared as papaya seed PSP and stored in 50% glycerol at –20 °C until treated with DEAE. After lipase–DEAE treatment protein yield was 0.8 mg/kg of seed. *Tomato leaf PSP* was prepared as follows: Tomato leaves were extracted (Wildner and Criddle, 1969). The extract (about 10 mL) was dialyzed against water (2 L, 0–4 °C), lyophilized, and stored in 50% glycerol at –20 °C until treatment with DEAE. After lipase–DEAE treatment protein yield was 66.7 mg/kg of leaf.

Crude papaya protease was prepared as follows: Green papayas were washed with distilled water and placed stem end down in a beaker. Longitudinal shallow cuts were made in the epidermis of the fruit with a freshly broken glass slide. After 0.5 h, the white exudate was washed with 20 mL of water/fruit into the beaker. The wash was immediately frozen in liquid nitrogen, lyophilized, and stored at 0–4 °C, above $CaCl_2$ until use. Protein content was 84 mg/g DW (dry weight). To avoid activation of proteolytic activity (Kierstan et al., 1982) no sulfhydryl reagents were added.

Electrophoresis. Gels were vertical polyacrylamide slabs.

Anionic Gels. The standard anionic gel system (Ornstein, 1964; Davis, 1964) was modified following Parkinson et al. (1981) by substituting β-alanine for glycine in the upper buffer. In this system, once the proteins have entered the separating gel, the trailing constituent β-alanine [p*K*(β-amino group) 10.2] becomes the leading anion (behind chloride) and the running pH is about 10 (Parkinson et al., 1981). PSPs preparations before lipase–DEAE treatment moved into the stacking gel when β-alanine, but not when glycine, was used. The upper buffer was prepared at 10× concentration (6.3 g Tris base, 4.67 g of β-alanine, pH 9.3) and diluted just prior to use. Gels (0.75 mm thick, 14 cm wide) were run at a constant 45 mA at room temperature. Staining (Coomassie blue) and destaining, except for the use of ethanol, follow Lara et al. (1980). The gels were dried under vacuum (Model 224 gel slab drier, Bio-Rad, Richmond, CA) and photographed with standard black and white continuous-tone film, and then image contrast was enhanced for publication by Photo-Mechanical-Transfer (Eastman Kodak, Rochester, NY) onto high-gloss paper. Ethanol was used in destaining solutions, since careful visual control of destaining would otherwise involve aspiration of a harmful methanol vapor.

Cationic Gels. The system of Thomas and Hodes (1981; MOPS buffer, leading ion K^+, trailing ion histidine) was modified by using longer stacking gels, increased percent acrylamide, persulfate instead of riboflavin, and a more alkaline (pH 8.0) lower buffer (negatively charged electrode). To standardize polymerization, the running gel was prepared from three solution: A, 30% acrylamide, 0.8% *N,N*-methylenebisacrylamide (BIS); B, 2 M MOPS/K^+, pH 6.8; C, a persulfate solution to yield a final concentration in poured gel of 1 mg/mL. Final concentrations were 14–18% acrylamide, as required, and 0.2 M buffer. Solutions A and B were mixed in appropriate proportions to achieve desired final poured gel concentrations, and sufficient *N,N,N′,N′*-tetramethylethylenediamide (TEMED) was added to obtain final concentration of 0.7% in the poured gel. Solution C was then added. All solutions (A + B, C) were chilled, degassed, then mixed, and poured rapidly. The stacking gels contained 8–10% acrylamide as required [thionins and PSPs have low molecular weights (Jones and Cooper, 1980)] and 0.057 M MOPs/K^+, pH 8.0. Solutions A–C were mixed to the required proportions and degassed as above, except a final concentration of 0.5% TEMED was used and the mixture was degassed again before pouring. The upper buffer (anode) contained 0.1 M histidine, 0.02 M MOPS titrated to pH 6.8 with KOH. The lower buffer had the same compositions but was titrated to pH 8.0. The slab gels were 14 cm wide, about 6.5 cm long (14 cm including wells and stacking gel), and 0.75 mm thick. The most satisfactory stacking gel length was about 4.5 cm. Crystalline jack bean urease was used to calibrate the stacking gel, since it has two impurities appearing as two discrete bands in stacking gel. Whale myoglobin and cytochrome *c* were used as standards. Since Pyronine Y runs slightly behind the front, electrophoresis was terminated when this dye was almost 2 cm from the end of the gel. Gels were run at a constant 45 mA, stained with Coomassie blue, destained, dried under vacuum, and photographed as described above.

RESULTS AND DISCUSSION

Electrophoretic Analysis. Proteins similar to thionins (PSPs) and wheat purothionin were prepared as described in Material and Methods. Lipid-sensitive wheat purothionin was used for comparison with PSPs before lipase–DEAE treatment and the donated purified wheat thionin for comparison after this treatment. Dilute sulfuric acid was used in extraction (Fernandez de Caleya et al. (1976), Method c, p 689) even if not all interfering substances may be removed, because it is one of the mildest methods in use and we wanted to preserve as much as possible any enzymic properties that may exist in the extracted proteins. Fernandez de Caleya et al. (1976) and Hernandez-Lucas et al. (1978) removed these interfering substances during two-dimensional electrophoresis [see streaking in first dimension in Figure 2b,c of Fernandez de Caleya et al. (1976) and in Figure 3a,c of Hernandez-Lucas et al. (1978)]. These streaks are not seen when thionins are prepared by petroleum ether extraction, which involves precipitation from petroleum ether with three volumes of alcoholic 1 N HCl [Figure 2a of Fernandez de Caleya et al. (1976); Figure 3b of Hernandez-Lucas et al. (1978)]. Although it is known that complete lipid removal is assured by use of anhydrous alcohols/ethyl ether mixtures containing 1.1 N HCl (Bekes and Smied, 1980), methods requiring strong acid (e.g., 1 N HCl) were not considered appropriate for the investigation of redox properties of the PSPs. The petroleum ether method was also not used because addition of lipids may be required to extract purothionin homologues in species other than wheat (Hernandez-Lucas et al., 1978). Before lipase–DEAE treatment the preparations were examined by anionic (Figure 1A) and cationic electrophoresis (Figure 1B). Protein staining was only observed with anionic electrophoresis (Figure 1A). The PSPs all showed either broad bands or two closely placed bands with very similar migration to purothionin. Tomato PSP is not shown on this gel but gave the same migration pattern on anionic gels and lack of staining on cationic gels. This migration pattern was also obtained with wheat extracts. Thus, in Figure 1A, lane E shows two bands for wheat purothionin, presumably α and β bands of thionin (Jones et al., 1982). The enhanced separation of lane E over that of lane D is attributed to inhomogeneities of the electric field (smiling). Deliberate induction of smiling with wedge-shaped strips is used in clinical electrophoresis to enhance separations (Cellogel RS electrophoresis, Chemetron, Milan, Italy). This migration toward the positive electrode is considered unusual since it implies that lipids are still bound after acid treatment. Two possible explanations are immediately apparent: (a) Some negatively charged lipid is still not removed from the PSPs acid treatment. (b) Charged lipids are acquired after extraction. Explanation (a) is not favored by experienced scientists in the field (B. L. Jones, personal communication), but this possibility should not be discarded entirely since the dilute sulfuric acid extraction method used although much more efficient (Fernandez de Caleya et al., 1976) is much less acidic than that used by others and thus may be more readily neutralized by the materials being extracted. The second explanation (b) is that a negatively charged lipid may be

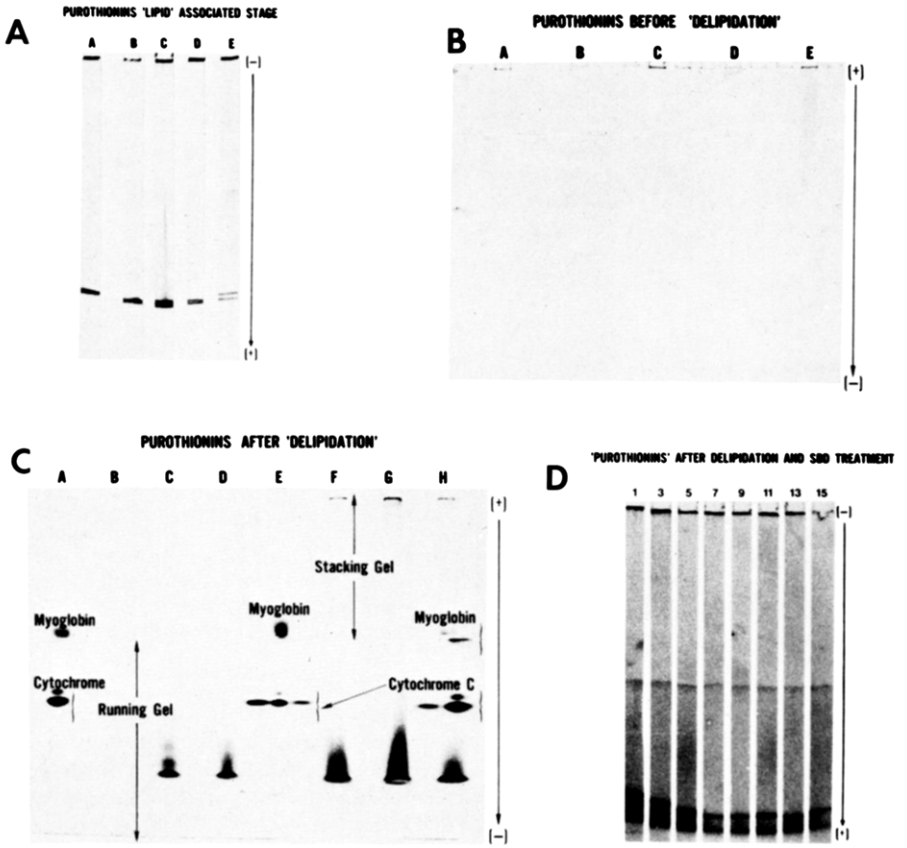

Figure 1. Electrophoresis of proteins similar to purothionin (PSPs). (A) Anionic electrophoresis of PSPs before lipase–DEAE treatment. The stacking and running gels were 4.5 and 6 cm long and contained 8% and 14% acrylamide, respectively. Lanes: A, 0.35 μg of mango PSP; B, 0.7 μg of papaya PSP; C, 0.3 μg of walnut PSP; D, 2.6 μg of wheat purothionin; E, 2.6 μg of wheat purothionin. The variability between lanes D and E is attributed to "smiling" of the gel (see Results and Discussion). (B) Cationic electrophoresis of PSPs before lipase–DEAE treatment. The stacking and running gels were 4.8 and 6 cm long and contained 9.9% and 18% acrylamide. Additions as in Figure 1A except wells D and E which had 0.5 μg of wheat purothionin. (C) Cationic electrophoresis of PSPs after lipase–DEAE treatment. Gels were run as in Figure 1B. Stacking and running gels were 4.4 and 6.5 cm long and contained 11% and 18% acrylamide. Lanes: A, 5 μg each of whale myoglobin and cytochrome *c*; B, 3.8 μg of wheat purothionin; C, 3.8 μg of wheat purothionin plus 2.1 μg of papaya PSP; D, 2.1 μg of papaya PSP; E, as A; F, 2.6 μg of mango PSP; G, 4 μg of tomato PSP; H, 2.6 μg of mango PSP plus 5 μg each of whale myoglobin and cytochrome *c*. (D) Electrophoresis of lipase–DEAE- and SBD-treated PSPs. Electrophoretic conditions as in Figure 1A. Lanes: 1, 8 μg of SBD tomato PSP; 3, 6.5 μg of SBD mango PSP; 5, 5.6 μg of SBD PSP; 7, 2.8 μg of SBD papaya PSP; 9, 7 μg of SBD walnut PSP; 11, 10 μg of SBD wheat purothionin; 13; 4 μg of SBD tomato PSP; 15; 12 μg of SBD tomato PSP.

added from glycerol (for instance reagent-grade glycerol contains about 0.05% butyric acid residues, manufacturers specifications). Addition of glycerol is a standard procedure that permits low-temperature storage and stabilizes enzymic activities (Daley et al., 1979). Thus, these negatively charged lipids from glycerol may have reconstituted a lipid–PSP complex (Hernandez-Lucas et al., 1978). Since before lipase–DEAE treatment the PSPs showed lateral migration, each was run first on separate gels without other PSPs. Then, for the purposes of illustrating the equivalent migration of each PSP band, mango (lane A), papaya (lane B), walnut (lane C), and wheat thionin (lane D) were run on the same gel but widely separated. In figure preparation the lateral distance between bands in Figure 1 was reduced, and lateral migration is not apparent. Cationic electrophoresis of glycerol-stored PSPs does not show any proteins (Figure 1B). However, when the PSP preparations are submitted to the lipase–DEAE-Sephadex procedure, Coomasie blue positive bands appear on cationic electrophoretic analysis (Figure 1C). Apparent microheterogeneity of the protein or traces of lipid remain since Figure 1C shows "trailing" stains, while other proteins (myoglobin and cytochrome *c*) do not. Lane B loaded with wheat purothionin is very faint (see discussion of Figure 3). The lateral migration of cytochrome *c* (Figure 1C) between lanes D and E and E and F (and to lesser extent between lanes G and H) may be caused by lateral diffusion of high concentrations of the small molecular weight protein in the stacking gel. We note without comment the similarity of this effect to the lateral diffusion of glycerol stored PSPs before lipase–DEAE treatment. However, despite this, the PSPs are well resolved from myoglobin, cytochrome *c*, and associated proteins (lane H). Reaction of PSPs with the thiol reagent SBD results in the reappearance of the now modified PSPs (PSPs–SBD) on the anionic gels (Figure 1D). Figure 2 shows the fluorescent spectrum of a purothionin–SBD adduct. Adduct formation is demonstrated by the maximum at about 525 nm, since the reagent SBD-Cl has a maximum at 415 nm (Andrews et al., 1982). The resulting change in the migration of the PSP–SBD adduct (Figure 1D) is more negative than would be expected for first-approximation calculations of net charge. However, the gel runs at pH ~10 while the isoelectric point of purothionin is about 10 (Ohtani et al., 1977). In addition purothionin even in gel buffered to pH 8.5 before the run migrates toward the cathode at a rate lower than expected (Nimmo et al., 1968). This effect is common and usually attributed to the increase in pH of the gel when the electric field is applied (Ornstein, 1964) and to temperature effects on pH. In addition some SBD adducts might occur with other moieties in the protein and the possible contribution from binding of the PSPs to small amounts of glycerol contaminants remaining after the DEAE step. For these reasons, in the case of wheat

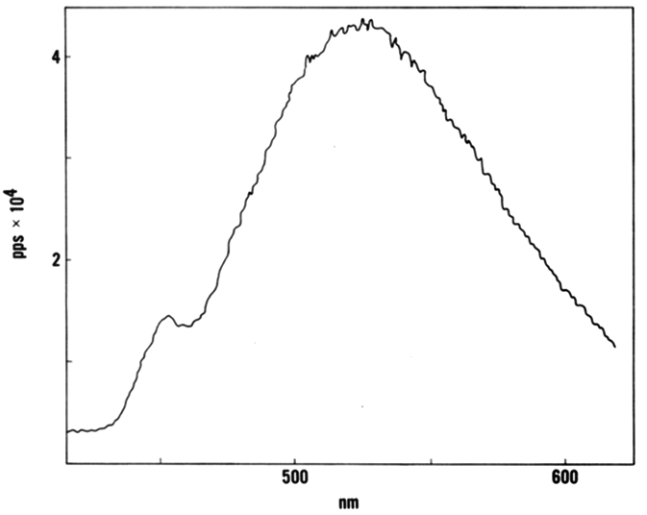

Figure 2. Fluorescent spectrum of SBD wheat purothionin. Spectra in water with trace of pH 8 borate buffer (dilution $^1/_{600}$). SBD purothionin concentration 0.8 μg/mL. Excitation at 390 nm and 10 nm slit pass. Detection of fluorescent emission done with a photon counter (calibrated by reference to the Raman spectra of water), the vertical axis representing photons per second (pps).

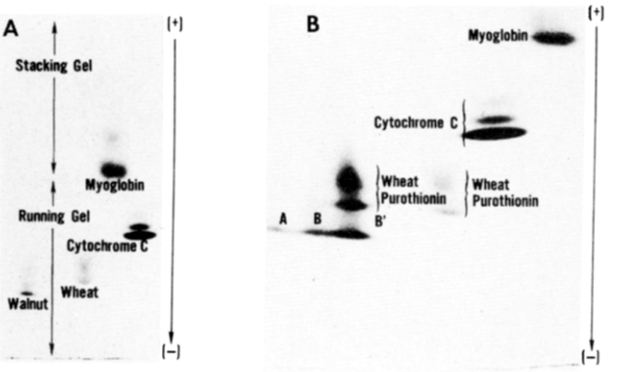

Figure 3. Electrophoretic migration of purified wheat purothionin and lipase–DEAE-treated PSPs. (A) Additions: walnut PSP, 0.3 μg; wheat purothionin, 1.25 μg; whale myoglobin, 5 μg; cytochrome *c*, 5 μg. (B) Additions: lane A, 3 μg of walnut PSP; lane B, 6 μg of walnut PSP; lane B′, 6 μg of walnut PSP plus 2.5 μg of wheat purothionin, in separate lanes as indicated, 1.25 μg of wheat purothionin, 5 μg of cytochrome *c*, and 5 μg of whale myoglobin. The stacking and running gels were 4.4 and 6.5 cm long and contained 10% and 18% acrylamide, respectively, in parts a and b of Figure 3. The stacking gel is not shown in Figure 3b.

purothionin the sum of the eight negative charges from the sulfonic acid residues (SBD attached to half-cysteine moieties) overcomes the consequences of the 9 or 10 positively charged amino acids on the protein, to yield an experimentally determined net negative charge at pH ~10. The mobilities of the PSPs investigated seem to be slightly different from those of wheat purothionin (Figure 3). The possibility that these PSPs are thioredoxins is considered remote since (a) thioredoxins have greater molecular weight and net charge (~12 kDa, isoelectric points 5.0–6.0; Schurmann et al., 1981) and thus would not be expected, at the running pH, to migrate into the 18% gel as the proteins shown in Figure 3 and (b) thioredoxin activity would not be expected to survive the harsh extraction procedures used here. References to these characteristics of thioredoxins can be found in Wada and Buchanan (1981b) and in other papers (Schurmann et al., 1981) in the same volume. If in a series of explanations the simplest is the most probable (William of Ockham, 1300–1349), given the high concentrations of acrylamide in the gels, the running pH, and similar migrations in two systems in three forms (before lipase–DEAE treatment, after treatment, and as SDS adducts), it is most probable that these proteins are similar in charge and molecular weight to thionins. In Figure 3, the relative migrations of DEAE–lipase-treated walnut–PSP, wheat purothionins, cytochrome *c*, and myoglobin are compared and are shown to be distinct. In Figure 3, as in Figure 1C, wheat purothionins stain very lightly, except when in the presence of walnut–PSP (Figure 3B, lane B′). The reason for this is not known; however, since the PSPs were loaded in glycerol that had passed through DEAE–Sephadex and that used for loading wheat purothionin was not, the negatively charged lipid contaminants from the glycerol could have re-formed lipid complexes with some of the wheat purothionin and these lipid complexes would have moved out of the gel toward the positively charged electrode in the upper buffer.

Insulin Assay for Redox Activity of PSPs. The insulin assay (Holmgren, 1979a,b) was used to measure redox activity catalyzed by thioredoxin. In this assay the proportions of insulin to reacting protein are so large that effects other than catalytic are extremely unlikely. Controls with PSP or thionins but without DTT do not cause precipitation. There are no reports known to us that measure thionin activity directly. Other enzymic assays of thionin redox activity measure activity modulation of other enzymes by thionins (Wada and Buchanan, 1981a,b). These indirect assays are not specific, since thioredoxin (Wada and Buchanan, 1981a,b) and the small subunit of ribulose diphosphate carboxylase (Jacquot et al., 1983) are also active. However, as discussed above, these two other proteins are larger and have size/charge ratios different from those of thionins (thus having different electrophoretic migration); no other proteins were detected (Figures 1 and 3). Other unspecified proteins are not expected to be active in this assay since no such proteins were found by electrophoretic analysis, and thionins have much greater activity than the dilute sulfuric acid protein extract from which they are derived (activity not detectable). Thus, no unique specificity of the insulin assay is required here. Therefore, given that there is no specific assay for thionins, this simple assay is as least appropriate as any other. Insulin's few known plant physiological effects (Csaba and Pal, 1982) do not seem directly applicable. In the insulin assay used here, DTT is the source of reduction potential. In the original assay, rate was determined by the slope of A_{650} against time. We found this less reliable than the reciprocal of the time taken for the first observed increase in turbidity. With this analysis we were able to show the catalysis of insulin reduction by wheat purothionin (Figure 4a). Linearity of rate with protein concentration is shown in Figure 4b for wheat purothionin and mistletoe viscotoxin. Rate measurement by the reciprocal of the time of initial turbidity was then replaced by the theoretically more satisfactory pseudo-first-order reaction constant mk (Aebi, 1974; Daley and Strobel, 1983). This method is illustrated in Figure 5. The recorder traces of Figure 5A are converted to mk and then plotted against protein concentration (Figure 5D). To do this, zero time t_0 is estimated by a series of successive approximations for lag time (Figure 5B). With these estimates mk is calculated along the curves of the crude data (Figure 5A). At the correct estimate of lag time, mk becomes essentially constant (Figure 5B) for all parts of the data curve. This can be checked graphically by extending the linear part of the data curve until it intercepts the base line (Figure 5C). If

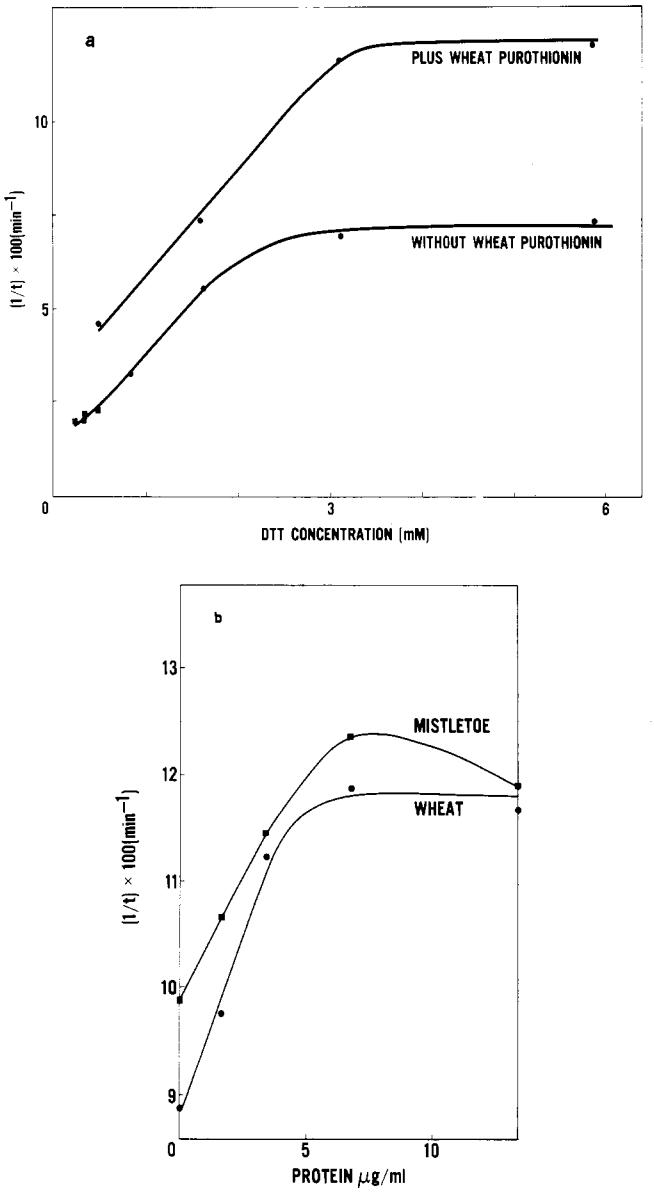

Figure 4. Substrate and protein concentration curves for the insulin reduction assay for redox activity. (A) DTT requirement for catalysis. This assay was performed with 1.7 μg/mL of purified wheat purothionin. (B) Effects of viscotoxin and purified wheat purothionin concentration on reaction rate. Rate determination is by the reciprocal of the time elapsed ($1/t$) between dithiothreitol addition and the first observed increase in turbidity.

the estimate of t_0 is correct, it should agree with the graphical determination (Figure 5C). When t_0 is calculated for each assay, mk is proportional, in the appropriate concentration ranges, to thionin or PSP concentration (Figure 5D). The reasons why zero time cannot be calculated directly from time of DTT additions are not known but are attributed to minimum particle size for turbidity detection and variable nucleation of supersaturated insulin subunits.

Relative Activities of Preparations. First estimates of PSP insulin reduction activities are presented in Table I. The optimum levels of DTT were determined for wheat purothionin (Figure 4a), other proteins were assayed at levels of DTT found best at time of assay. Since the presence of lipid may decrease the apparent amount of protein measured (Table II), lipids were removed before assay. The DEAE–lipase treatment resulted in a decrease of the treated PSPs ability to catalyze redox activity if these proteins were stored instead of being used immediately. This could be attributed to contaminating proteolytic activity in lipase preparation. Since the preparations were apparently homogeneous by electrophoretic analysis, the lower specific activities of mango and walnut PSPs are attributed to loss of catalytic ability on extraction or in storage, noting the difference in apparent DTT optima (Table II) to differences in specificity for the as yet unassigned in vivo reducing compound. Tomato leaf preparations contained proteolytic activity, had the least specific activity and the largest yield (see Materials and Methods), and lost activity most rapidly on storage. The results from the insulin assays of the PSPs are consistent with the possibility that these proteins are thionins.

Table I. Insulin Reducing Activity of Preparations

source of protein	mk/h per mg protein[a]	protein in assay,[b] mg/mL	DTT concn, mM
papaya seed[b]	119	51	0.45
walnut seed[b]	51	1–40	0.50
mango seed[b]	57	13	0.52
tomato leaf[b,c]	9	2.4	8.2
wheat flour[d]	186–160	1.7–5.1	7.0
mistletone[d]	210	1.7	7.0

[a] All rates have been corrected for nonenzymic reduction of insulin by DTT in simultaneous determination without protein. [b] Assays performed with lipase–DEAE-treated proteins. [c] Tomato leaf preparations contained proteolytic activity. [d] Purified by Wada.

Table II. Effect of Lipase–DEAE Treatment on Apparent Protein Content during Preparation of Papaya PSP

stage of extraction	vol, mL	app total protein, mg
ethanol soluble before concn	1250	8
concentrate before lipase treatment	38	1.1
lipase treatment		
1st pass through DEAE	16	1.9
2nd pass through DEAE	15	4.2

Effects on Crude Papaya Protease. Wheat purothionins were reported to interact with papain (Balls et al., 1942a,b); however, more recent investigators (B. L. Jones, personal communication) have had difficulty finding this effect. One explanation of this is that Balls et al. (1942b) may have used a crude papain prepared without sulfhydryl treatment. Since Kierstan et al. (1982) report that papain produced commercially is almost always preactivated by reduction and Balls et al. (1942b) probably did not use commercial enzyme, nonactivated crude papain was used. A second explanation is that what we call papain today is distinct from the protease preparation use in early enzymology. Crude papaya protease, about 5% papain (EC 3.4.22.2) and 20% papaya peptidase a (EC 3.4.22.3), a cystein protease (Baines and Brocklehurst, 1982), is probably closer to the protease used by Balls et al. (1942a,b) who did not have modern methods of purification. Another difference that might cause difficulties is the method of assay. For this reason we chose to assay general protease activity using Azocoll substrate. The (Azocoll) method used is more sensitive but, like the milk coagulation method used by Balls et al. (1942a,b), measures several protease activities (Daley and Vines, 1978; Ragster and Chrispeels, 1979). Moderate alteration of total proteinase activity (Figure 6) obtained prepared PSPs. These effects may have physiological significance, since alteration of activity occurred in all four cases, the response was dose dependent (suggesting a physiological effect), and only some papaya protease components are affected by sulfhydryl regulation (Baines et al., 1982; Kierstan et al., 1982). Viscotoxin and wheat purothionin were not treated since the donated supply was exhausted at this time.

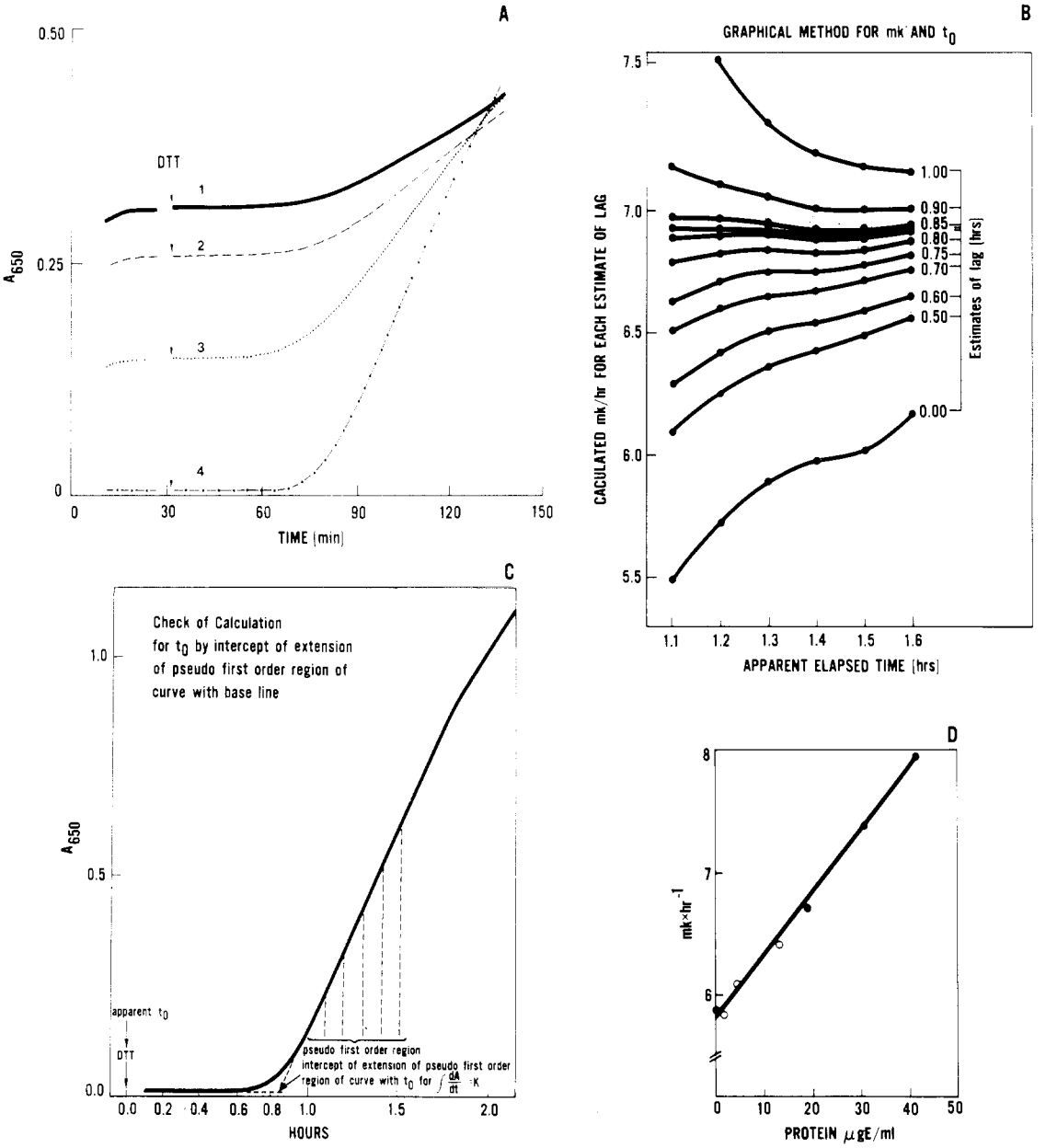

Figure 5. Determination of pseudo-first-order reaction constants for the insulin reduction assay. (A) Data points generated in the assay. Curves 1–4 were generated with 0 (endogenous rate), 19, 30, and 41 μg of delipidated walnut PSP. Cuvette path length was 3 mm and the temperature 25 °C, and the reaction was initiated by addition of 0.5 mM DTT. Parts B–D illustrate how t_0 and *mk* were calculated and verified. (B) Effect of successive approximations for t_0 lag on the calculation of *mk* for points along the curve. The values of *mk* are not yet corrected for volume. The correct value for t_0 was determined when *mk* was constant for all points along the curve (A) tested. (C) Independent check of value of t_0 by downward extension of the pseudo-first-order region of the curve until intercept with extension of the base line. (D) Volume-corrected *mk* vs. protein concentration. Closed circles represent data obtained from Figure 5A; open circles show data from other assays.

CONCLUSIONS

General techniques for thionin assay and electrophoresis have been developed to detect proteins similar to thionins in some dicots. These methods permit a general determination of the likely presence of thionins, before sequencing procedures are applied. Candidate low molecular weight proteins with the characteristics (a) stability to acid, heat, and solvents; (b) association with substances removed by lipase–DEAE; (c) net positive charge at physiological pH; (d) high reactivity with the sulfhydryl reagent SBD; and (e) ability to catalyze the reduction of insulin by DTT and modulate papain activity were found in the seeds of mango, papaya, and walnut and in alcoholic extracts of tomato leaves. The data support a rationale for further investigations of proteins similar to thionins in these species and infer a possible physiological function as redox modifiers of enzyme activity.

Note Added in Proof: Since this paper was submitted, the concept of light activation of ribulose 1,5-diphosphate carboxylase (RuBPcase) through light activation proteins has been revived (Salvucci et al., 1986). The tomato–PSP described here was prepared through the same method that was used for the original light activation factor (Wildner and Criddle, 1969, cited in text). Salvucci et al. exclude a ferredoxin–thioredoxin mechanism; however, a thionin-activated mechanism is not considered. Thus, since a thionin-activated mechanism has been proposed for RuBPCase (Jacquot et al., 1983, cited in text) and thionin-like proteins were found (this paper) in a Wildner and Criddle preparation, this matter has some interest. Recently a new toxic thionin from a species of the plant family Santalales was reported to have an amino acid se-

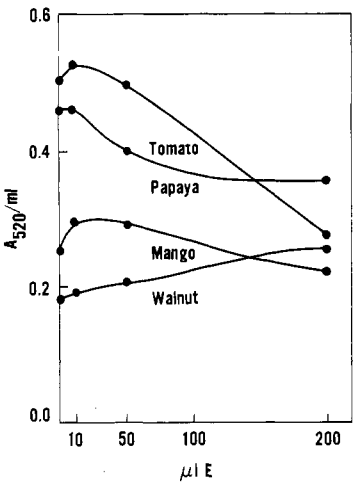

Figure 6. Effect of PSPs on crude papaya protease proteolytic activity. Assay volume is 1 mL; crude papain protein concentration was 20 mg/mL. Lipase–DEAE PSPs were added from the following stock solutions (mg/mL): tomato, 0.40; mango, 0.26; papaya, 0.28; walnut, 0.47. Volumes of stock solutions (μL of E) used are given on the abscissa of the figure.

quence even closer to Gramineae thionins than most Santalales (Vernon et al., 1985). This adds supports to the concept that thionins are important regulatory proteins in plants.

ABBREVIATIONS

BIS, *N,N'*-methylenebisacrylamide; DEAE, (diethylamino)ethyl; DTT, dithiothreitol (DL-*threo*-1,4-dimercapto-2,3-butanediol); EDTA, ethylenediaminetetraacetic acid [(ethylenedinitrilo)tetraacetic acid]; MOPS, 3-(*N*-morpholino)propanesulfonic acid; kDa, kilodaltons; *mk*, pseudo-first-order rate constant multiplied by 1000; PSPs, proteins similar to purothionins; SBD, ammonium 4-chloro-7-sulfobenzofuranozan; TEMED, *N,N,N',N'*-tetramethylethylenediamine [1,2-bis(dimethylamino)-ethane]; Tris, tris(hydroxymethyl)aminomethane.

ACKNOWLEDGMENT

We thank R. Becker for encouragement and amino acid analysis of our preparation of wheat thionin, B. B. Buchanan for encouragement, support, use of facilities, and supplies, N. Crawford for help in technical and theoretical matters, N. Daley for help with the revised text, G. R. Dobson for aid in interpreting the kinetic data, B. L. Jones for useful interchange of ideas and other help with the manuscript, D. Koshland for use of fluorescent instrumentation, M. Nash for figure preparation, and C. W. Weiser for encouragement and motivation to complete this paper.

Registry No. Insulin, 9004-10-8.

LITERATURE CITED

Aebi, H. In *Methods of Enzymic Analysis*, 2nd ed.; Bergmeyer, H. U., Gawehn, K., Eds.; Academic: New York, London, 1974; Vol. 2. Chapter 11.
Andrews, J.; Gosh, P.; Ternai, B.; Whitehouse, M. *Arch. Biochem. Biophys.* **1982**, *214*, 386.
Baines, B.; Brocklehurst, K. *Biochem. J.* **1982**, *205*, 205.
Baines, B.; Kierstan, M.; Brocklehurst, K. *Biochem. Soc. Trans.* **1982**, *10*, 171.
Balls, A.; Hale, W. *Cereal Chem.* **1940**, *17*, 243.
Balls, A.; Hale, W.; Harris, T. *Cereal Chem.* **1942a**, *19*, 278.
Balls, A.; Hale, W.; Harris, T. *Cereal Chem.* **1942b**, *19*, 840.
Bekes, F.; Smied, I. *Acta Aliment.* **1981**, *10*, 229.
Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.
Carrasco, L.; Vasquez, D.; Hernandez-Lucas, C.; Carbonero, P.; Garcia-Olmedo, F. *Eur. J. Biochem.* **1981**, *116*, 185.
Coulson, E.; Harris, T.; Axelrod, B. *Cereal Chem.* **1942**, *19*, 301.
Csaba, G.; Pal, K. *Protoplasma* **1982**, *110*, 20.
Daley, L.; Vines, M. *Plant Sci. Lett.* **1978**, *11*, 59.
Daley, L.; Strobel, G. *Plant Sci. Lett.* **1983**, *30*, 145.
Daley, L.; Carrol, P.; Mussell, H. *Biochem. J.* **1979**, *179*, 719.
Daley, N.; Daley, L.; Theriot, L. *Hexagon* **1983**, *74*, 87.
Davis, B. J. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 404.
Fernandez de Caleya, R.; Gonzalez-Pascual, B.; Garcia-Olmedo, F.; Carbonero, P. *Appl. Microbiol.* **1972**, *23*, 998.
Fernandez de Caleya, R.; Hernandez-Lucas, C.; Carbonero, P.; Garcia-Olmedo, F. *Genetics* **1976**, *83*, 687.
Hedrickson, W.; Teeter, M. *Nature (London)* **1981**, *290*, 107.
Hernandez-Lucas, C.; Carbonero, P., Garcia-Olmedo, F. *J. Agric. Food Chem.* **1978**, *26*, 794.
Holmgren, J. *J. Biol. Chem.* **1979a**, *254*, 9113.
Holmgren, J. *J. Biol. Chem.* **1979b**, *254*, 9627.
Jacquot, J. P.; Joly, C.; Gadal, P. *Biochim. Biophys. Acta* **1983**, *745*, 97.
Jones, B. L.; Cooper, D. B. *J. Agric. Food Chem.* **1980**, *28*, 904.
Jones, B. L.; Meredith, P. *Cereal Chem.* **1982**, *59*, 321.
Jones, B. L.; Lookhart, G. L.; Mak, A.; Cooper, D. B. *J. Hered.* **1982**, *73*, 143.
Kashimoto, T.; Sakakibara, R.; Huynh, Q.; Wada, H.; Yoshizumi, H. *Res. Commun. Chem. Pathol. Pharmacol.* **1979**, *26*, 221.
Kierstan, M.; Baines, B.; Brocklehurst, K. *Biochem. Soc. Trans.* **1982**, *10*, 172.
Kramer, K.; Klassen, L.; Jones, B.; Spiers, R.; Kammer, A. *Toxicol. Appl. Pharmacol.* **1979**, *48*, 179.
Lara, C.; de la Torre, A.; Buchanan, B. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1337.
Lecomte, J.; Jones, B.; Llinas, M. *Biochemistry* **1982**, *21*, 4843.
Melstrand, S.; Samuelsson, G. *Eur. J. Biochem.* **1973**, *32*, 143.
Nakanishi, T.; Yoshisumi, H.; Tahara, S.; Hakura, A.; Toyoshima, K.; *Gann* **1979**, *70*, 323.
Nimmo, C.; O'Sullivan, M.; Berardin, J. *Cereal. Chem.* **1968**, *45*, 28.
Ohtani, S.; Okada, T.; Yoshizumi, H.; Kagamiyama, H. *J. Biochem. (Tokyo)* **1977**, *82*, 753.
Okada, T.; Yoshizumi, H. *Agric. Biol. Chem.* **1973**, *37*, 2289.
Okada, T.; Yoshizumi, H.; Terashima, Y. *Agric. Biol. Chem.* **1970**, *34*, 1084.
Ornstein, L. *Ann. N.Y. Acad. Sci.* **1964**, *121*, 321.
Parkinson, A.; Dorn, A.; Maples, A.; Brayles, R. *Anal. Biochem.* **1981**, *117*, 6.
Ragster, L.; Chrispeels, M. J. *Plant Physiol.* **1979**, *64*, 857.
Salvucci, M.; Portis, A.; Ogren, W. *Plant Physiol.* **1986**, *80*, 655.
Samuelsson, G. *Sven. Farm. Tidskr.* **1961**, *65*, 481.
Schurmann, P.; Maeda, K.; Woodland, D.; Taugita, A.; Harth, G.; Geidel, K. In *Thioredoxins, Structure and Function*; Gadal, P., Ed.; CNRS: Paris, 1981b; pp 176–182.
Thomas, J.; Hodes, M. *Anal. Biochem.* **1981**, *118*, 194.
Van Etten, C.; Nielson, H.; Peters, J. *Phytochemistry* **1965**, *4*, 467.
Vernon, L.; Evett, G.; Zeikus, R.; Gray, W. *Arch. Biochem. Biophys.* **1985**, *238*, 18.
Wada, K.; Buchanan, B. *FEBS Lett.* **1981a**, *124*, 237.
Wada, K.; Buchanan, B. In *Thioredoxins, Structure and Function*; Gadal, P., Ed.; CNRS: Paris, 1981b; pp 213–219.
Wildner, G.; Criddle, R. *Biochem. Biophys. Res. Commun.* **1969**, *37*, 952.
Winterfeld, K.; Leiner, M. *Arch. Pharmacol.* **1956**, *289*, 358.
Woynarowski, J.; Konopa, J. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 1535.

Received for review November 4, 1983. Revised manuscript received July 31, 1986. Accepted May 11, 1987. This work was supported by the Summer Visiting Professorship Program (NSF), USDA-Cooperative State Research Service at the National Germplasm Repository, Corvallis, and the Robert A. Welch Foundation.