Organic Acids in Selected Foods Inhibit Intestinal Brush Border Pteroylpolyglutamate Hydrolase in Vitro: Potential Mechanism Affecting the Bioavailability of Dietary Polyglutamyl Folate

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Dietary folates exist largely as polyglutamates that require deconjugation prior to absorption. This process is catalyzed by intestinal pteroylpolyglutamate hydrolase (PPH) mainly associated with the jejunal brush border membrane. Previous studies have shown that citrate ion as well as the soluble fraction of many foods can inhibit PPH in vitro. This study was conducted to characterize further the in vitro inhibition of porcine PPH by fractions of selected foods (orange juice, tomatoes, and lima beans) and by organic anions to evaluate potential effects on bioavailability. Organic anions tested were competitive inhibitors with respect to the polyglutamyl folate substrate, with the following K_i values: citrate, 6.42 mmol/L; malate, 10.1 mmol/L; phytate, 6.48 mmol/L; ascorbate, 19.6 mmol/L. Neutralized orange juice in the reaction mixture strongly inhibited PPH activity, while neutralized tomato homogenate caused weaker inhibition and lima bean homogenate inhibited much more weakly, all with kinetics indicating competitive inhibition. Fractionation of food extracts indicated that the inhibitors were anions of low molecular mass (<6−8 kDa). Chromatographic separation followed by in vitro assay indicated that citrate was the major inhibitor, with lesser inhibition by malate and phytate. These results indicate the potential for dietary constituents to retard the action of intestinal brush border PPH, with possible inhibitory effects on the bioavailability of polyglutamyl folates. The in vivo significance of this effect would depend on the extent of inhibition relative to the total intestinal PPH activity.

Keywords: *Folate; pteroylpolyglutamate; pteroylpolyglutamate hydrolase; bioavailability*

The adequacy of folate nutrition depends on the quantity and bioavailability of ingested folate relative to the requirement for this vitamin. A large fraction of dietary folate exists as pteroylpolyglutamates ($PteGlu_n$) that must undergo deconjugation to pteroylmonoglutamates ($PteGlu_1$) by a pteroylpolyglutamate hydrolase (PPH; EC 3.4.12.10) prior to intestinal absorption. In humans and pigs, the form of PPH primarily responsible for the deconjugation of dietary $PteGlu_n$ is a zinc-dependent enzyme associated with the jejunal brush border membrane (Chandler et al., 1986; Gregory et al., 1987; Reisenauer et al., 1986).

The bioavailability of naturally occurring dietary folate has not been determined fully but is known to be incomplete [see reviews by Gregory (1995, 1997)]. Perhaps the most conclusive estimate was provided by Sauberlich et al. (1987), who reported that the overall bioavailability of naturally occurring dietary folate was no more than 50% relative to a folic acid supplement. Further evidence of low bioavailability of naturally occurring folate in humans has been reported by Cuskelly et al. (1996). Studies with human subjects have indicated that foods differ widely in folate bioavailability (Retief, 1969; Tamura and Stokstad, 1973; Babu and Srikantia, 1976). Although many studies of folate bioavailability have been conducted using animal models (rat and chick), the usefulness of animal bioassays in predicting folate bioavailability for humans is uncertain (Gregory, 1995, 1997). Marked differences exist in the mechanism of deconjugation of dietary polyglutamyl folates among rats, chicks, and humans (Chandler et al., 1986; Kesavan and Noronha, 1992; Rosenberg and Neumann, 1974). Consequently, findings regarding the bioavailability of dietary polyglutamyl folates derived from studies using rats or chicks may have little relevance to factors governing folate utilization in humans (Gregory, 1995, 1997).

In the in vitro studies described here and in in vivo studies reported separately (Wei et al., 1996), we have examined factors affecting the bioavailability of folate in three foods of plant origin: orange juice, tomatoes, and lima beans. We selected these foods because they represent classes of foods that are important sources of dietary folate and because conflicting data have been reported regarding the bioavailability of folate in these types of foods.

Orange juice is a major source of dietary folate in the United States (Subar et al., 1989). Tamura and Stokstad (1973) reported that the bioavailability of naturally occurring folate in orange juice was ≈35% when administered as a large single dose to folate-saturated human subjects. These authors suggested that the low pH and large quantity of juice administered in the previous study may have impaired the deconjugation of polyglutamyl folates in this protocol (Tamura et al., 1976). In contrast, Rhode et al. (1983) administered a single daily dose of either orange juice providing 100 μg of total folate/day (in two-150 mL servings) or folic acid tablets (100 μg/day) to folate-restricted women for a 9-week period. Although the serum folate concentration of the

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orange juice group was lower than that of the folic acid group, the difference was not statistically significant. This finding indicated similar bioavailabilities of endogenous orange juice folate and supplemental folic acid. Animal bioassays have indicated effective utilization of folate from dried orange juice added to rat diets (Abad and Gregory, 1987; Clifford et al., 1990, 1991).

Incomplete bioavailability of folate from a wide variety of legumes has been reported (Babu and Srikantia, 1976; Devadas et al., 1979; Tamura and Stokstad, 1973). However, Keagy et al. (1988) reported that California white beans had no effect on the bioavailability of exogenous polyglutamyl folate in human subjects.

Previous in vitro studies in this laboratory have indicated that a wide variety of foods inhibited PPH activity in jejunal brush border membrane vesicles isolated from human and pig intestine (Bhandari and Gregory, 1990). In particular, orange juice and tomato juice had a marked inhibitory effect on PPH activity. Although citrate was found to be an inhibitor of PPH activity, its mechanism of inhibition and the potential roles of other anions were not determined. Butterworth et al. (1974) reported that legumes contain an inhibitor of certain types of PPH, but the PPH of brush border membrane had not been identified at the time of their investigation.

The in vitro study reported here was conducted in conjunction with a stable isotopic evaluation of the effect of orange juice, lima beans, tomatoes, and a citrate buffer solution on the relative bioavailability of monoglutamyl and polyglutamyl folate in humans (Wei et al., 1996) intended to determine the extent and mechanism by which components of human diets influence the bioavailability of ingested folates. The objectives of these in vitro investigations were (1) to determine the relative effect of several foods (orange juice, lima beans, and tomatoes) on intestinal brush border PPH activity, (2) to separate and identify the inhibitors of PPH activity from these foods and to further evaluate organic anions as PPH inhibitors, and (3) to determine the mode of inhibition of porcine PPH activity by organic anions and food extracts. Brush border membrane vesicles were used as a source of PPH activity in all experiments to mimic the natural intestinal environment of brush border PPH.

MATERIALS AND METHODS

Materials. Folic acid, *p*-hydroxymercuribenzoate (pHMB), bovine intestinal alkaline phosphatase, citric acid, malic acid, phytic acid, and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Pteroyltriglutamate ($PteGlu_3$) was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). Frozen concentrated orange juice, canned diced tomatoes, and canned lima beans were obtained from a local grocery store.

Isolation of Porcine Jejunal Brush Border Membrane. Several studies (Chandler et al., 1986; Day et al., 1984; Gregory et al., 1987; Reisenauer et al., 1986) indicated that PPHs of brush border membrane from human and pig jejunal mucosa have similar pH optima, zinc requirement, K_m values for $PteGlu_n$, mode of action, and physiological function. As porcine intestine is more readily available than human tissue, we chose to work with porcine intestinal brush border PPH for these studies. The procurement was approved by the University of Florida Animal Care and Use Committee and followed the National Research Council guidelines.

Segments of porcine jejunum were obtained immediately following slaughter from healthy, normal adult pigs at the University of Florida Animal Science Department and stored frozen at −20 °C. Prior comparisons of brush border membrane preparations from frozen and fresh tissues indicated no adverse effects of freezing on PPH activity (Gregory et al., 1987). Jejunal brush border membrane was isolated following the procedures of Selhub and Rosenberg (1981). Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as standard. Alkaline phosphatase activity, a brush border marker, was determined using the method of Bessey et al. (1946). The isolated brush border fraction was suspended as membrane vesicles in 30 mmol/L tris-HCl buffer (pH 7), containing 100 mmol/L NaCl and 0.1 mmol/L zinc acetate, and stored at −20 °C until used.

Assay of PPH Activity. The standard enzyme assay mixture (pH 7.0) contained 15 μg of porcine jejunal brush border membrane protein, 30 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1 mmol/L zinc acetate, and 10 μmol/L $PteGlu_3$ in a total reaction volume of 500 μL at 37 °C for 60 min (Gregory et al., 1987). $PteGlu_3$ has been shown to be as effective a substrate for this enzyme as the long-chain polyglutamyl folates (Chandler et al., 1986). The assay mixture also contained 0.2 mmol/L pHMB to inhibit the activity of any intracellular PPH that may contaminate the brush border fraction (Reisenauer et al., 1977). Enzymatic reactions were stopped by addition of 50 μL of 500 g/L trichloroacetic acid, followed by centrifugation at 1000*g* for 10 min. In vitro effects of various food extracts and pure food components were evaluated by including them in the standard enzyme assay mixture after neutralization.

Reaction rates were expressed as the rate of conversion of $PteGlu_3$ to products ($PteGlu_2$ and $PteGlu_1$). In these assays, the concentration of $PteGlu_3$ following incubation was determined by HPLC analysis as follows:

$$[PteGlu_3]_{remaining} = [PteGlu_3]_{initial} \times (\text{area of } PteGlu_3 \text{ peak})/(\text{sum of areas of } PteGlu_3, PteGlu_2, \text{ and } PteGlu_1 \text{ peaks})$$

For routine assays, initial $PteGlu_3$ concentration was 10 μmol/L. Initial rates were evaluated for all assays at <20% conversion of $PteGlu_3$ to products $PteGlu_2$ and $PteGlu_1$. The rate of reaction was constant up to ~30% hydrolysis of $PteGlu_3$ under these conditions and was linearly related to enzyme concentration. The concentration of $PteGlu_3$, $PteGlu_2$, and $PteGlu_1$ was determined after termination of the reaction by an HPLC procedure with postcolumn fluorogenic derivatization (Gregory et al., 1984, 1987). The isocratic mobile phase, 0.05 mol/L potassium acetate buffer (pH 4.9) containing 20 mL/L acetonitrile, was pumped at a constant flow rate of 1.5 mL/min through a Perkin-Elmer 3 × 3 C_{18} column (3-μm octadecylsilyl, 4.6 mm i.d. × 3 cm; Norwalk, CT). A postcolumn reagent containing 0.1 mol/L K_2HPO_4, 0.2 mol/L NaCl, and 0.8 mmol/L NaOCl was metered at 0.075 mL/min into the column effluent. The mixture was passed through a 3-m Teflon (0.8 mm i.d.) delay coil at ambient temperature and then into the flow cell of the fluorescence detector. The dual monochromator fluorescence detector (model FD-300, Spectrovision, Inc., Chelmsford, MA) was set with excitation wavelength at 285 nm and emission wavelength at 435 nm. Quantification of peaks was performed by electronic integration. Molar fluorescence responses (peak area/mole) were equivalent for $PteGlu_3$, $PteGlu_2$, and $PteGlu_1$ in this procedure. $PteGlu_3$ concentrations from 0.2 to 5 μmol/L were employed in enzyme reaction mixtures to determine the K_m and V_{max}; $PteGlu_3$ concentrations from 0.4 to 8 μmol/L were used in the evaluation of organic anions for mode of inhibition. All kinetic calculations were performed by nonlinear regression using EZ-FIT software (Perrella, 1988).

Preparation and Separation of Food Extracts. Extracts of orange juice, tomatoes, and lima beans were prepared for evaluation of their effects on brush border PPH activity. Fifty milliliters of frozen, concentrated orange juice was diluted with 50 mL of the homogenization buffer (30 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1 mmol/L zinc acetate, pH 7.0). Canned diced tomatoes and canned lima beans were drained,

and then 50 g of each was finely chopped and mixed with 50-mL portions of homogenization buffer. These mixtures were homogenized in a Waring blender at high speed for 60 s and then further homogenized using a Potter-Elvehjem glass−Teflon homogenizer for 60 s, both processes at 4 °C. Each mixture (orange juice, tomato homogenate, and lima bean homogenate) was then centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was passed through several layers of cheesecloth to remove any large particles and then adjusted to pH 7.0 with Tris-base before testing. These fractions were designated the food extracts used in studies of PPH inhibition.

The food extracts were fractionated further using centrifugal ultrafiltration membranes of nominal molecular mass cutoff of 3, 10, 30, and 100 kDa (Centricon Microconcentrators, Amicon Corp., Danvers, MA). A portion of each filtrate was then dialyzed with standard cellulose dialysis tubing (molecular mass cutoff between 6 and 8 kDa; Spectrum Medical Industries, Inc., Los Angeles, CA) against the homogenization buffer (30 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1 mmol/L zinc acetate, pH 7) at 4 °C for 24 h to collect the retentate fractions.

The neutralized whole extracts, various filtrates, and retentates, from 0 to 400 μL, were incorporated into the standard enzyme assay mixture (15 μg of porcine jejunal brush border membrane protein, 30 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1 mmol/L zinc acetate, and 10 mol/L $PteGlu_3$ as substrate in a total reaction volume of 500 μL) to evaluate inhibitory effects on porcine PPH activity, as described below.

The food extracts were also fractionated into ionic and nonionic fractions using columns containing cation- or anion-exchange resins (0.7 cm × 10 cm). Each extract was adjusted to pH 4.0 with 0.1 mol/L HCl and was applied to the cation exchanger (Bio-Rad AG 50W-X8 resin, 100 mesh, H^+ form, Bio-Rad Laboratories, Richmond, CA; or CM-Sephadex A-25, Sigma Chemical Co., St. Louis, MO). After the noncationic fraction flowed through and was collected, distilled water was added to elute the remaining noncationic material from the column. NaOH (1 mol/L) was used to elute the cationic fraction from the resin and this fraction (10 mL) was collected. Noncationic and cationic fractions were adjusted to pH 7.0 with Tris-base and evaluated in the standard enzyme assay mixture to determine their effects on porcine PPH activity. The same procedures were performed with anion-exchange resins (Bio-Rad AG 2-X8 resin, Bio-Rad Laboratories; or DEAE-Sephadex A-25, Sigma) except that the sample was applied at pH 7.0 instead of 4.0 and 1 mol/L HCl was used to elute the anionic fraction. The two types of each ionic sorbent (i.e. the hydrophobic styrene−divinylbenzene copolymers of the Bio-Rad resins and the hydrophilic dextran-based Sephadex beads) were used as a way of evaluating whether fractionations were based on charge and not related to other characteristics of the stationary phase.

The ion chromatography method of Bouzas et al. (1991) was used for preparative separation of the inhibitors in food extracts. Separations were performed using a Bio-Rad Aminex HPX-87H ion exclusion column (9-μm spherical, sulfonated polystyrene−divinylbenzene beads with 8% cross-linking, 7.8 mm i.d. × 300 mm; Bio-Rad Laboratories). The isocratic mobile phase was 7 mmol/L H_2SO_4 at a flow rate of 0.5 mL/min. The injection volume was 100 μL, and detection was by UV absorbance at 210 nm using a Perkin-Elmer LC-235 diode array detector. Each peak was manually collected, adjusted to pH 7.0 with Tris-base, and evaporated to dryness with a freeze-dryer. After 100 μL of distilled water was added to reconstitute these fractions to a concentration equivalent to that in the 100-μL injection solution, the effects of these fractions on brush border PPH activity were evaluated as described below. Tentative identification was performed by comparing the retention time of each peak to the organic acid standards and by fortifying each extract with individual standards to confirm retention times.

Effects of Food Extracts on Porcine PPH Activity. The effects of neutralized food extracts and various isolated fractions on porcine PPH activity were evaluated. Portions (0−250 μL) of each food extract and isolated fraction were incorporated into the standard enzyme assay mixture (15 μg of porcine jejunal brush border membrane protein, 30 mmol/L Tris-HCl, pH 7.0, 100 mmol/L NaCl, 0.1 mmol/L zinc acetate, and 10 mol/L $PteGlu_3$ in a total volume of 500 μL). All reactions were conducted at 37 °C and stopped with 50 μL of 500 g/L trichloroacetic acid. The effect of different concentrations of various extracts and separated fractions on porcine PPH activity was determined by comparing the rate of hydrolysis of $PteGlu_3$ in each test sample to a control reaction conducted under standard assay conditions (i.e. without food components added).

Identification of Inhibitors in Food Extracts. The ion chromatography method of Bouzas et al. (1991) was also used to identify and quantify the inhibitors in food extracts. This separation was performed as described above. Quantification was relative to organic acid standards of ascorbic acid, citric acid, malic acid, and phytic acid. To obtain additional evidence of the identity of peaks tentatively designated phytic acid, portions of the food extracts were treated with alkaline phosphatase prior to ion chromatography. One hundred microliters of each extract was incubated with alkaline phosphatase (from bovine intestine, Sigma) in 100 mmol/L glycine, 1 mmol/L $ZnCl_2$, and 1 mmol/L $MgCl_2$ buffer (pH 10.3; 500-μL total reaction volume) at 37 °C for 30 min. Fifty microliters of 0.1 mol/L NaOH was added to stop the reaction. After centrifugation at 1000*g* for 10 min, the supernatant was analyzed as described above. Comparison of chromatograms before and after alkaline phosphatase treatment showed the disappearance of the phytic acid peak and provided presumptive identification.

Ion chromatography with a conductivity detector also was used to identify and quantify the organic acids in food extracts. This separation was performed using an IonPac anion-exchange column (4.0 mm i.d. × 250 mm; AS11 column; Dionex Corp., Sunnyvale, CA) with gradient elution. The mobile phase was initially 20% of 80 mmol/L NaOH blended with 80% distilled water pumped at 1 mL/min for 5 min and was linearly changed to 40% of 80 mmol/L NaOH and 60% distilled water within 20 min. Conductivity was detected by a Dionex CD20 conductivity detector. Quantification and tentative identification were relative to organic acid standards including citric acid, malic acid, and ascorbic acid.

Mode of Inhibition. The mode of inhibition was evaluated by determining reaction velocity with various concentrations of $PteGlu_3$ as substrate in the presence and absence of neutralized food extract or organic anions (citrate, malate, phytate, ascorbate). Graphical methods (Lineweaver−Burk plot, Dixon plot, and Dixon replot) were used to identify the mode of inhibition extracts (Segel, 1976). Inhibition constants (K_i) were calculated using EZ-FIT software (Perrella et al., 1988).

Effects of Added Zinc Ions. The inhibition of PPH by possible action of food components as chelators of Zn^{2+} was also investigated. It was hypothesized that if the observed inhibition of PPH by a food extract was due to chelation of the essential Zn^{2+} cofactor, then this inhibition would be lessened if additional exogenous zinc were provided in the reaction mixture. These studies were conducted in PPH assays containing food extracts by evaluation of activity at various concentrations of added zinc acetate (0.1−0.7 mmol/L) in the reaction mixture.

RESULTS

On the basis of the marker enzyme alkaline phosphatase, the enrichment of porcine jejunal brush border membrane vesicles protein following purification was >7-fold relative to the original homogenate. The specific activity of alkaline phosphatase was 2.36 μmol min^{-1} (mg of brush border membrane protein)$^{-1}$. The extent of enrichment of brush border PPH activity was not be determined in this study because the mucosal homogenate was not assayed, although our previous studies have indicated that alkaline phosphatase and brush

border undergo similar enrichment during isolation of porcine jejunal brush border (Gregory et al., 1987; Bhandari and Gregory, 1990). The rate of hydrolysis of $PteGlu_3$ did not change when assayed with or without 0.2 mM pHMB, which indicated that no intracellular PPH was associated with the brush border membrane vesicles. Michaelis−Menten plots indicated $K_m = 1.02 \pm 0.02$ μmol/L and $V_{max} = 2.0 \pm 0.2$ μmol min^{-1} (mg of brush border protein)$^{-1}$ (means ± SE) with $PteGlu_3$ as substrate, which was consistent with our previous observations (Gregory et al., 1987).

Inhibitory Properties of Organic Anions. The organic acids tested acted as competitive inhibitors and exhibited the following inhibition constants: ascorbic acid, $K_i = 19.6 \pm 3.4$ mmol/L; citric acid, $K_i = 6.42 \pm 0.07$ mmol/L; malic acid, $K_i = 10.1 \pm 0.5$ mmol/L; and phytic acid, $K_i = 6.48 \pm 0.24$ mmol/L (mean ± SE value). Kinetics were fully consistent with competitive inhibition in Lineweaver−Burk plots, Dixon plots, and Dixon replots. Representative plots for citrate are shown in Figure 1A; plots for other inhibitors were qualitatively identical. In these plots and in the study of food extracts described below, evidence of competitive inhibition was (a) a common *y*-intercept in Lineweaver−Burk plots; (b) in Dixon plots, an intersection of the family of lines at a point corresponding on the *X*-axis to $-K_i$; and (c) a linear Dixon replot with *y*-intercept at the origin (Segel, 1976).

Inhibitory Properties of Food Extracts and Components. Neutralized whole orange juice, tomato, and lima bean extracts each inhibited porcine brush border PPH activity. Graphical analysis of kinetic data using Lineweaver−Burk, Dixon plots, and Dixon replots indicated that each of these extracts inhibited brush border PPH by a competitive mechanism. Representative kinetic plots (for orange juice) are presented in Figure 1B. All others were qualitatively similar. Inhibition constants (K_i) for the food extracts, expressed on a weight or volume basis in the total reaction mixture, were as follows: orange juice, $K_i = 21.4$ mL/L (for single-strength orange juice); tomatoes, $K_i = 50.0$ g/L; lima beans, $K_i = 147$ g/L. Inhibition constants for the nonfractionated extracts were expressed in this manner because these were complex mixtures that could not be defined in terms of molar concentration.

Expressed on a volume/volume basis of single-strength juice in the standard assay mixture, ≈11% (v/v) orange juice (i.e. 110 mL of orange juice/L of reaction mixture) completely inhibited porcine PPH activity under these assay conditions (i.e. 10 μmol/L $PteGlu_3$) (Figure 2). When expressed on the basis of weight of undiluted homogenized tomatoes or lima beans, this study indicated that orange juice was more inhibitory than extracts of lima beans or tomatoes (Figure 2). For each food extract tested, the various ultrafiltrates (molecular mass <3, <10, <30, and <100 kDa) exhibited inhibition equivalent to that of the unfractionated extract. The retentate fractions (6−10, 6−30, and 6−100 kDa) derived from dialysis after ultrafiltration did not significantly inhibit PPH (Figure 2). Thus, the major compounds responsible for in vitro inhibition of porcine PPH were associated with the low molecular mass fraction.

Following anion-exchange fractionation, anionic fractions of orange juice, tomato, and lima bean extracts inhibited brush border PPH activity (data not shown). Under the conditions used, in which a 30-μL portion of each eluted anionic fraction was incorporated into a 500-μL reaction mixture, the reaction with orange juice extract exhibited 24% of control PPH activity, while those with tomato and lima bean extracts showed 73% and 80% of control PPH activity, respectively. Cationic fractions of these food extracts did not significantly inhibit porcine PPH activity. Although these volumes are arbitrary, they provide a direct comparison of the relative inhibitory activity of the anionic fractions of these foods. No differences were seen between results obtained with styrene−divinylbenzene copolymer resins and beaded dextran ion exchangers, which indicates that these fractionations were based solely on ionic characteristics.

The Aminex HPX-87H ion exclusion column yielded effective separation of the major organic acids present in these food extracts for determination of their relative contributions to total PPH inhibition by direct analysis in PPH activity assays. During chromatography of the extracts, peaks corresponding to phytic, citric, and malic acids were collected (retention times of 6.7, 8.9, and 11.7 min, respectively). The neutralized and reconstituted fractions from the Aminex HPX-87H ion exclusion column were evaluated in the standard PPH assay (30 μL of reconstituted fraction per assay) to facilitate a direct comparison of these compounds in each food extract. Under conditions that yielded 75% inhibition of PPH activity with orange juice, 32% of the inhibition was due to the citric acid peak, 14% was due to the malic acid peak, none of the inhibition was due to the phytic acid peak, and none of the remaining 22% of the inhibition could be attributed to identifiable peaks in the chromatogram. Under conditions yielding 30% inhibition of PPH activity by the tomato extract, 21% of the inhibition was due to the citric acid peak, 4% was due to the malic acid peak, no inhibition was attributed to the phytic acid peak, and the remaining 5% was not attributable to identifiable peaks. Similarly, under conditions yielding 24% PPH inhibition by lima beans, 14% of the inhibition was due to the citric acid peak, 7% of the inhibition was due to the phytic acid peak, none was due to malic acid, and the remaining 3% was not attributable to identifiable peaks.

The concentration of these organic acids in orange juice, tomato, and lima bean extracts (expressed per liter or kilogram of *unfractionated* material), as determined by ion chromatography, is presented in Table 1. Single-strength orange juice contained high amounts of citric acid (11.8 g/L = 61.4 mmol/L) and malic acid (6.4 g/L = 47.7 mmol/L), while tomatoes contained moderate amounts of citric acid (6.9 g/kg = 35.9 mmol/kg) and malic acid (2.2 g/kg = 16.4 mmol/kg). Lima beans contained less citric acid (3.3 g/kg = 17.2 mmol/kg) and no malic acid, but a higher amount of phytic acid (7.64 g/kg = 11.6 mmol/kg). Single-strength orange juice and tomatoes contained low amounts of phytic acid (tomato, 2.45 g/kg = 3.71 mmol/L; orange juice, 1.21 g/L = 1.83 mmol/L). These data were based on ion chromatographic analysis using an IonPac AS-11 column with gradient and conductivity detection because this procedure yielded sharper peaks than the Aminex HPX-87H ion exclusion column with isocratic elution. The former method was more suitable for quantitative analysis, while the latter was more conducive to preparative separations because of its isocratic operation.

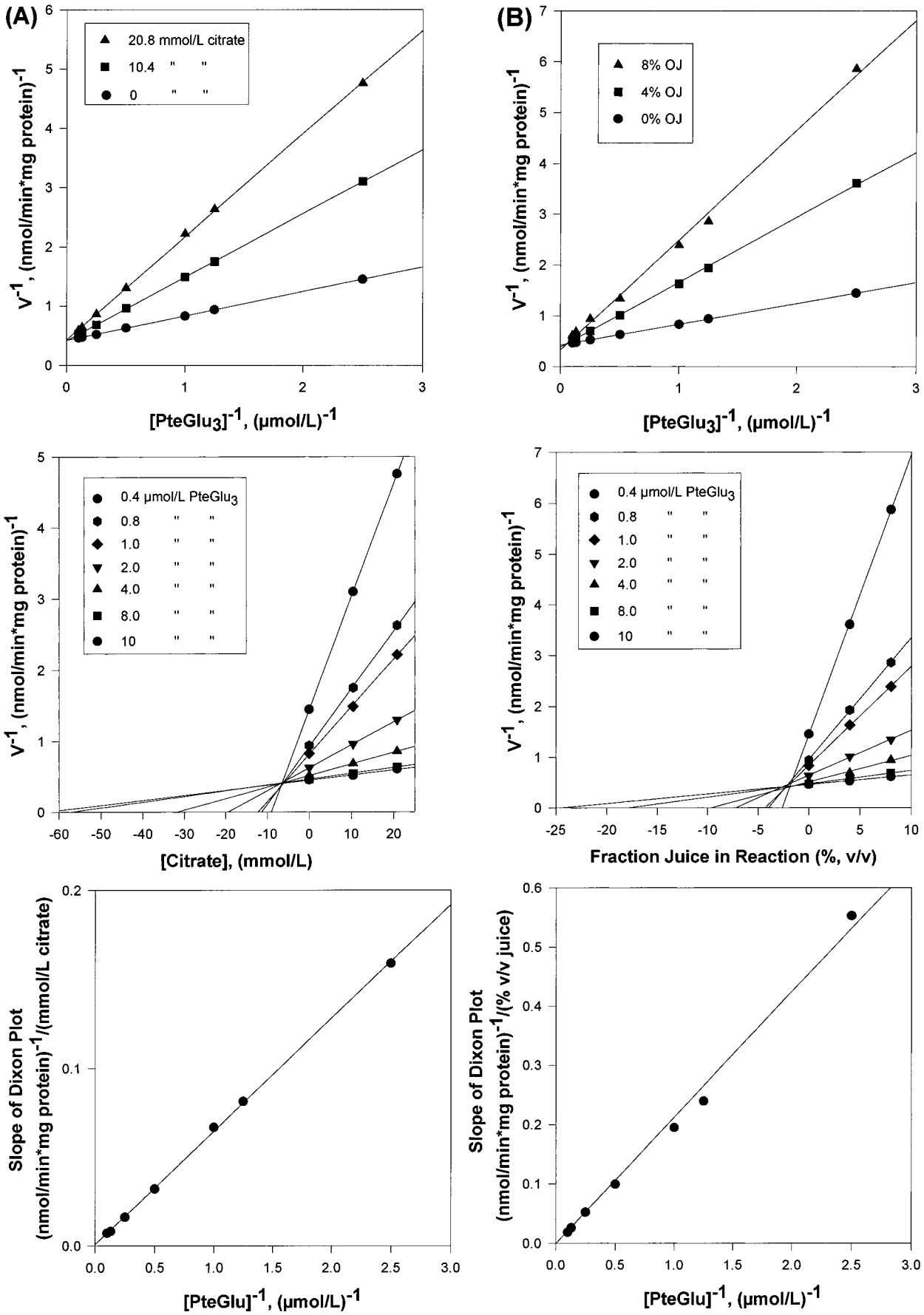

Figure 1. Lineweaver–Burk plots (upper), Dixon plots (middle), and Dixon replots (lower) illustrating competitive inhibition by (A) citrate ion and (B) orange juice. Qualitatively similar plots were obtained for malate, phytate, and ascorbate ions and extracts of tomatoes and lima beans.

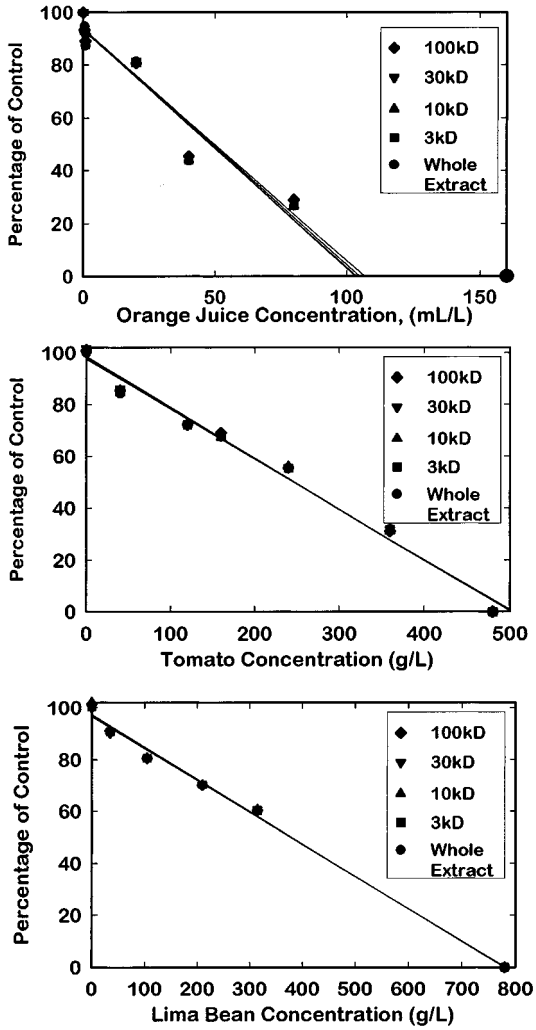

Figure 2. Effects of concentration of food extracts (orange juice, tomato, and lima bean) and their ultrafiltrates on PPH activity when added to reaction mixtures at concentrations shown (10 μmol/L $PteGlu_3$ substrate). Data points are means of triplicate experiments.

Table 1. Concentration of Organic Acids in Orange Juice, Tomatoes, and Lima Beans As Determined by Ion Chromatography with Conductivity Detection[a]

	orange juice		tomatoes		lima beans	
	g/L	mmol/L	g/kg	mmol/kg	g/kg	mmol/kg
citric acid	11.8	61.4	6.9	35.9	3.3	17.2
malic acid	6.4	47.7	2.2	16.4	ND	ND
phytic acid	1.21	1.83	2.45	3.71	7.64	11.6

[a] Values are means of triplicate analyses of extracts, expressed on basis of volume (L) or mass (kg) of whole food. Values are means of triplicate analyses; relative SD values were <3%. ND, not detected.

The uncertainty of each method, as reflected by the coefficient of variability, was no greater than 3% in this study.

The inhibition of porcine PPH activity by these food extracts was not reversed by increasing the concentration of zinc acetate from 100 to 700 μmol/L in the reaction mixture. These results suggest that the inhibition of porcine PPH activity was truly competitive with respect to $PteGlu_n$ and not mediated by chelation of zinc ions essential for PPH activity.

DISCUSSION

The bioavailability of dietary folate can be affected by a variety of factors including impairment of intraluminal deconjugation, intraluminal binding or entrapment, and impairment of the transport process (Gregory, 1995). The main objective of this in vitro study was to extend the results of Bhandari and Gregory (1990) by determining which and to what extent certain components of food could lessen the enzymatic deconjugation of polyglutamyl folates by brush border PPH. Such inhibition could lessen the bioavailability of dietary polyglutamyl folates if sufficiently extensive in vivo. A secondary objective was to identify major inhibitors and kinetically characterize their mode of action.

Several studies (Day and Gregory, 1984; Gregory et al., 1987; Reisenauer et al., 1986; Wang et al., 1986) indicated that the pig would be a good animal model for studies of digestion and subsequent absorption of pteroylpolyglutamates. Because PPH from porcine jejunal brush border membrane has similar pH optimum, zinc ion requirements, $PteGlu_n$ affinities, and physiological function to human jejunal brush border PPH (Bhandari and Gregory, 1990), porcine PPH was used as a source of the enzyme for the in vitro studies described here. Other animal species, including monkey, rat, and guinea pig, lack significant jejunal brush border membrane PPH activity (Hoffbrand and Peters, 1969; Day and Gregory, 1984; Elsenhans et al., 1984; Wang et al., 1985, 1986). In this study, porcine jejunal brush border membrane PPH exhibited K_m values (1.02 $\pm$ 0.02 μmol/L) similar to human K_m values (0.9 μmol/L) (Reisenauer and Halsted, 1981; Chandler et al., 1986) and could completely hydrolyze $PteGlu_3$ to $PteGlu_1$ via an exohydrolytic process.

Extracts of many foods were previously found to have in vitro inhibitory effects on the jejunal brush border membrane PPH activity from both human and porcine intestine (Bhandari and Gregory, 1990). The observed in vitro inhibition of the jejunal brush border membrane PPH activity by the foods studied here and those examined previously (Bhandari and Gregory, 1990) was not due to pH because all extracts were neutralized before testing. In addition, the pH values of the enzyme assay system in the presence of these food extracts at the beginning and at the end of the enzyme reaction were the same. The reaction was conducted at a physiologically relevant pH. Optimum pH for the activity of jejunal brush border membrane PPH activity is neutral to slightly acidic (Chandler et al., 1986; Wang et al., 1985), whereas the pH optimum for folate transport into intestinal mucosal cells is slightly acidic (Said et al., 1987).

The results of this study indicate that low molecular mass anionic components are the major PPH inhibitors in the foods examined. This study provides the first direct identification of organic acids responsible for PPH inhibition in vitro and provides additional evidence of additional unidentified inhibitors of either weaker potency, lower concentration, or both, relative to those tested here. Among the inhibitors not directly measured in the food extracts tested in this study was ascorbic acid. Ascorbic acid was found to be a weak inhibitor of porcine PPH (K_i = 19.6 mmol/L). On the basis of expected concentrations of ascorbic acid in orange juice and tomato extracts, ascorbic acid would exert a substantially weaker inhibitory effect than that

of citric acid. The possible inhibitory role of large doses of supplemental ascorbic acid taken at meal time on the bioavailability of polyglutamyl folates should be further examined in view of this in vitro observation, however. The fact that various anions inhibited PPH by a competitive mechanism suggested that they were competing with the polyanionic folate substrates for the active site. Since ascorbic acid, which is acidic by virtue of its enediol structure rather than a carboxyl group, also inhibits PPH activity, one may conclude that inhibition of brush border PPH is not totally specific for carboxylate anions.

The analytical results reported here are in reasonable agreement with previous data for citric and malic acids in orange juice and tomato juice (Sinclair, 1961; Miladi et al., 1969; Gould, 1974) and for phytic acid in lima beans (Oberleas and Harland, 1981). The orange juice used in this study (Table 1) contained 61.4 mmol/L citric acid and 47.7 mmol/L malic acid. Both values are slightly higher than published data (Sinclair, 1961; citric acid = 52 mmol/L and malic acid = 37.3 mmol/L). Tomatoes in this study contained 35.9 mmol/kg citric acid and 16.4 mmol/kg malic acid. Citric acid in tomatoes is higher than published data, while malic acid is close to the previously reported value (Gould, 1974; citric acid = 26 mmol/kg and malic acid = 18.6 mmol/kg). The detection of citric acid in lima beans was not expected. This may have been added as an acidulant or buffer in the canning process. A high amount of phytic acid (11.6 mmol/kg) was found in the lima beans in this study, in general agreement with the results of Oberleas and Harland (1981), who reported that lima beans contain 15.3 mmol/kg. Only small amounts of phytic acid were found in orange juice (1.83 mmol/L) and tomatoes (3.71 mmol/kg).

The chromatographic fractionation study indicated that citric, malic, and phytic acids were the primary inhibitors present in the foods evaluated. A variety of other anionic compounds may have contributed to the inhibition observed here that could not be attributed to specific organic acids. As mentioned previously, ascorbic acid would be included in this group of secondary inhibitors. The various materials studied, including whole extracts, anionic fractions, and pure organic acids, all behaved kinetically as competitive inhibitors with respect to $PteGlu_3$. On the basis of the K_i values of organic acids and organic acid concentrations in food extracts, the extent of inhibition by organic acids in food extracts was estimated by assuming competitive inhibition. The extents of inhibition predicted by such calculations were less than those observed experimentally. This analysis provides additional evidence of the existence of other inhibitors of brush border PPH in these foods.

This is the first study to identify directly food components that inhibit the jejunal brush border PPH. Butterworth et al. (1974) detected and partially purified a heat-activated inhibitory factor associated with the skin of legumes. This factor inhibited several soluble forms of PPH, but brush border PPH had not yet been identified. We detected no evidence of such a PPH inhibitor in the present study. Previous studies by Gregory et al. (1987) indicated that soluble anionic polysaccharides did not significantly inhibit porcine jejunal brush border membrane PPH activity at concentrations as high as 10 g/L when in the presence of added (100 μmol/L) Zn^{2+}.

To explain the low availability of folate from some plant-derived foods, it has been suggested that certain components may bind or entrap dietary folates (De Arends, 1979). However, Bhandari and Gregory (1990) reported that soluble macromolecules in various food extracts (including legumes, tomatoes, and orange juice) did not bind $PteGlu_3$. Since the peparation of these foods was the same as that used in the present study, it was assumed that such binding was not a factor. In the direct quantification of the total $PteGlu_n$ concentration in reaction mixtures, relative to the response of folate standards, measured values were always essentially equivalent to the concentration of $PteGlu_3$ initially added. The nearly complete recovery of $PteGlu_n$ observed here indicates that such binding was insignificant.

On the basis of the in vitro observations of this study and those of Bhandari and Gregory (1990), it appears that low molecular mass organic acids in foods constitute a significant factor affecting the efficiency of utilization of dietary polyglutamyl folates. Such in vitro observations must be viewed with caution, however, because the significance of in vivo inhibition of intestinal brush border PPH would depend on several additional factors: (1) the extent to which PPH is rate limiting or becomes rate limiting in folate absorption, (2) the degree to which PPH activity throughout the intestine exceeds that needed for digestion of dietary polyglutamyl folates, and (3) the significance of folate deconjugation by alternate, less active, sources of PPH, e.g. pancreatic juice (Bhandari et al., 1990). In this regard, our recent in vivo study of the relative bioavailability of deuterium-labeled forms of $PteGlu_1$ and $PteGlu_6$ (Wei et al., 1996) permits analysis of the nutritional significance of these in vitro findings. When labeled mono- and polyglutamyl folates blended in a single portion of lima beans or tomatoes were administered, both forms of folate exhibited equivalent bioavailabilities. In contrast, the labeled polyglutamyl folate exhibited ~33% less availability when consumed in a single serving of orange juice, although mono- and polyglutamyl folates exhibited equivalent bioavailabilities when consumed in a serving of citrate buffer of equal concentration and pH to those in orange juice. These findings illustrate that results obtained in vitro do not fully allow prediction of in vivo behavior even though they provide insight into food components involved.

On the basis of the observations of Rhode et al. (1983) and the known high concentration of folate in orange juice (Subar et al., 1989), orange juice is an excellent source of dietary folate, although of possibly less than complete bioavailability. Tomato and legume products are sources of dietary folate, although the bioavailability of their folates may also be incomplete. Despite the difficulty in predicting the magnitude of in vivo bioavailability from in vitro studies of PPH inhibitors, the data of this paper and from our in vivo study (Wei et al., 1996) provide evidence that such PPH inhibition is an important factor affecting the bioavailability of dietary polyglutamyl folates in humans. Our recent in vivo study also indicated that dietary constituents can affect the intestinal uptake of monoglutamyl folates following deconjugation, potentially enhancing or inhibiting their net folate absorption (Wei et al., 1996). The mechanism of such effects and its significance in human nutrition require further investigation.

In summary, this in vitro study provides evidence that

many common constituents of foods can inhibit brush border PPH and, if sufficiently extensive, reduce the bioavailability of polyglutamyl folates. The extent of such inhibition in vivo would depend on food selection and quantity consumed and, thus, is difficult to predict. Taken together, the results of this paper and our in vivo study (Wei et al., 1996) indicate that inhibition of brush border PPH can significantly reduce the bioavailability of dietary polyglutamyl folate under certain circumstances.

ABBREVIATIONS USED

PPH, pteroylpolyglutamate hydrolase; $PteGlu_1$, pteroylmonoglutamate; $PteGlu_3$, pteroyltriglutamate; $PteGlu_n$, pteroylpolyglutamate; pHMB, *p*-hydroxymercuribenzoate; Tris, tris(hydroxymethyl)aminomethane.

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