MINIREVIEW

Trienzyme Extraction in Combination with Microbiologic Assay in Food Folate Analysis: An Updated Review

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For decades, the traditional food folate extraction method involved two steps including heat treatment, to release folate from its binding proteins, and folate conjugase treatment, to hydrolyze polyglutamyl folate to monoglutamyl folate. However, a trienzyme-extraction method of food folate was developed in the mid 1990s. This method involves the use of α-amylase, protease, and folate conjugase and allows for a more complete extraction of folate trapped in carbohydrate or protein matrices in food than the traditional method. In the last several years, this extraction method became widely used. However, the method is not uniform among various investigators, and it may be difficult for a new investigator to select the most suitable method in his or her laboratory. Therefore, in the review presented here, we summarize a variety of trienzyme-extraction procedures that were used by various researchers and offer a recommended procedure for food folate extraction. It is our hope that the wide use of an appropriate procedure of the trienzyme-extraction method, in combination with a reasonable detection method, help in establishing accurate and reliable food-folate tables and that this, in turn, makes it possible to accurately assess folate intake in the general population. Exp Biol Med 230:444–454, 2005

Key words: food folate; trienzyme extraction; folate conjugase; α-amylase; protease

Introduction

Adequate folate nutritional status is essential for maintaining health and for the prevention of certain diseases (1, 2). In various parts of the world, it is often difficult to ascertain adequate folate intake through regular diet alone, although the folic acid (i.e., pteroylglutamic acid) fortification of staple foods is practiced in several countries (3–6). The method of food folate measurement has improved over the years; however, food folate tables are notoriously unreliable for estimating accurate dietary intake (7). To improve the method of food folate analysis, the trienzyme-extraction method (i.e., the use of preparations of α-amylase, protease, and folylpoly γ-glutamate carboxypeptidase II [folate conjugase]) was developed about 15 years ago by researchers in Georgia, United States (8, 9). When a marked increase in folate content in a food composite was initially reported in 1997, and the increase was far beyond the imagination at that time (10), certain resentment was seen among researchers in the folate field. In the last several years, however, many investigators have begun using the method to measure food folate content. For example, as the findings of a recent international comparison of food folate analysis indicate, this extraction method is widely used (11). Furthermore, folate values in the USDA National Nutrient Database have been updated using microbiologic assay after trienzyme extraction (12).

We now realize that, compared with folate-conjugase treatment alone, food folate values do not always increase after the trienzyme extraction (13–15). The increase in folate values depends on the food item, which is possibly due to the difference in food matrices as well as forms of folate that can be labile against longer incubation periods than a single-enzyme treatment. In 1998, Tamura (7) published a review on the trienzyme-extraction method. However, this method has been constantly modified and improved to obtain the "highest values of folate" in foods. Therefore, we present this article to review the current use of trienzyme extraction

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1535-3702/05/2307-0444$15.00

of food folate and to propose a simplified, possibly better, method for food folate extraction.

Use of the Trienzyme-Extraction Method

In 1990, Martin *et al.* (8) first reported an increase of food folate values by the trienzyme-extraction method and established a procedure of the simultaneous incubation of a heated food homogenate with folate conjugase and α-amylase followed by the incubation with protease. Using this method, De Souza and Eitenmiller (16) published folate contents in various food items. Tamura *et al.* (7, 10) proposed a slight modification of the original method by Martin *et al.* (8): the step of hydrolysis of polyglutamyl folates by folate conjugase should be done after all folates are released from the food matrix by α-amylase and protease treatments. Subsequently, however, Pfeiffer *et al.* (17) and Rader *et al.* (18, 19) used the method established by Martin *et al.* (8). In the past several years, investigators have used a variety of procedures for the trienzyme extraction of food folate, as summarized in Table 1 (8, 10, 13–45). We reviewed the literature involving the trienzyme-extraction method for food folate analysis (published before April 2005) as exhaustively as possible, and we will examine each procedure of folate extraction from foods and the methods of folate detection in the following sections.

Food Homogenization

The procedures of food folate assay generally begin with homogenization of a single food or food mixtures in a buffer with an appropriate pH containing ascorbic acid and/or 2-mercaptoethanol. The pHs of these buffers ranged widely (Table 1). It is uncertain whether the pH of the extraction buffer is important because α-amylase and protease treatments, if perfect, should result in complete folate extraction.

A buffer (i.e., pH, 7.85; 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES), 50 m*M*; and HEPES, 50 m*M* containing 2% ascorbic acid and 0.2 *M* 2-mercaptoethanol) that Wilson and Horne (46) developed has generally been considered best for folate extraction from biologic samples, including foods. However, when food composites were homogenized at a pH of 4.1 and treated with α-amylase and protease at the same pH, Tamura *et al.* (10) obtained higher folate values than those after the procedures were done at higher pHs, including a pH of 7.85. Rader *et al.* (18) showed that there were no significant differences among folate values for four enriched-flour products obtained using various pHs (i.e., 4.3–7.8) for food homogenization and enzyme treatments. Aiso and Tamura (20) reported that the appropriate pH for α-amylase and protease treatments varies depending on the food item. Recently, Pandrangi and LaBorde (38) reported a similar experiment using various pHs and incubation periods for α-amylase and protease. To our knowledge, there have been no further studies to identify the most suitable pH for food homogenization and subsequent enzyme treatments. Because the trienzyme-extraction method should, theoretically, provide nearly complete folate extraction, we conclude that the pH of extraction buffers may no longer be an important issue, when microbiologic assay is used. However, for chromatographic analysis, pH is an important issue because the interconversion of folate derivatives can occur at different pHs.

Heat Treatment

For decades, a heat-treatment step after food homogenization has been used to release folates from folate-binding protein(s). However, one may wonder whether this extra step is necessary for food folate extraction because protease treatment, if perfect, should result in the complete destruction of proteins and the release of folates from the binding protein. In addition, by skipping this step, an extra chance of folate degradation may be eliminated.

We postulated that heat treatment is unnecessary and tested this hypothesis using 11 foods (Table 2). We found that the mean folate content (*Lactobacillus rhamnosus* [formerly known as *Lactobacillus casei*, ATCC 7469] activity) was about 14% higher when homogenates were not heated before enzyme treatments (47). Therefore, we conclude that heat treatment is unnecessary for releasing folates from the binding protein as long as protease treatment is performed.

Order of Enzyme Treatments

As shown in Table 1, the order of the treatments using the three enzymes varied among investigators. Originally, Martin *et al.* (8) simultaneously treated food homogenates with folate conjugase and α-amylase, and then protease was used. However, Tamura *et al.* (10) proposed that folate-conjugase treatment should be carried out after all folates are released from the food matrix. Furthermore, Rader *et al.* (18) reported that when protease was added first, there were significant increases in total folates in certain foods compared with the procedure of α-amylase treatment followed by protease treatment. Ndaw *et al.* (27) performed food folate extraction by simultaneous treatment with protease and α-amylase before folate-conjugase treatment, and they concluded that the trienzyme extraction was not effective to obtain higher food folate values than folate-conjugase treatment alone. However, it is unclear whether the simultaneous use of protease and α-amylase is valid because protease may destroy α-amylase, as concluded by Martin *et al.* (8). Konings *et al.* (25) also showed no significant increases in folate values in certain foods after the trienzyme extraction compared with folate-conjugase treatment alone. According to the description of trienzyme treatment in a prior report by Konings (22), it appears that food homogenates were incubated with protease, α-amylase, and folate conjugase all together, although clarification is needed for this issue. After the trienzyme extraction, the homogenates showed an increase in folate concentrations

Table 1. Summary of Trienzyme-Extraction Methods and Findings by Various Investigators[a]

Investigator (reference)	pH	Procedure of trienzyme extraction	Folate assay	Number of foods assayed	Overall results
Martin *et al.*, 1990 (8)	H: 6.8	Homogenize → heat → C (chicken pancreas)/A: 4 hrs → P: overnight → heat → filter	M	12 (e.g., cereals, formula, vegetables)	TE increased measurable folate levels in 12 foods by an average of 19 % compared with CT alone
De Souza and Eitenmiller, 1990 (16)	H: 6.8	Homogenize → heat → C (chicken pancreas)/A: overnight → P: 4 hrs → autoclave → centrifuge or filter	M	16 (e.g., infant formula, baby foods)	TE is necessary to quantitate starch and protein-bound folate in foods
Tamura *et al.*, 1997 (10)	H: 6.3 P: 4.3 A: 4.3 C: 7.0	Homogenize → heat → A: 4 hrs → P: 16 hrs → heat → centrifuge → C (chicken pancreas): 3 hrs	M	4 (food composites)	1. The mean folate content in four food composites after TE increased to 271% of those obtained after CT 2. Protease and α-amylase treatments should be carried out prior to CT 3. Treatments with α-amylase and protease at pH 4.1 gave the highest values compared with higher pHs
Pfeiffer *et al.*, 1997 (17)	E: 7.85 C/A: 7.85 P: 7.85	Freeze dry → grind → add buffer → heat → homogenize → C (rat plasma)/A: 4 hrs → P: 1 hr → heat → centrifuge	M and HPLC	9 (unfortified and fortified cereals)	1. TE is necessary to determine folate levels in cereal-grain products 2. Folate content using M and HPLC was comparable 3. Long-term incubation (i.e., 16 hrs) with protease caused decrease of folate
Rader *et al.*, 1998 (18)	H: 7.8	1. Homogenize → autoclave → C (chicken pancreas)/A: 4 hrs → P: overnight → autoclave 2. Homogenize → autoclave → A: 2 hrs → P: 3 hrs → heat → C (chicken pancreas) overnight → autoclave 3. Homogenize → autoclave → P: 3 hrs → heat → A: 2 hrs → C (chicken pancreas) overnight → autoclave	M	56 (enriched foods)	1. For about 30% of the foods examined, folate content was higher after TE than CT alone 2. No significant differences were found in values obtained at various pHs used for extraction 3. Higher folate values were obtained when protease was added prior to α-amylase and conjugase; the order of enzyme treatments more significantly affected the values than the extraction pH
Aiso and Tamura, 1998 (20)	H: 4.1 A: 2.0 –7.0 P: 2.0 –7.0 C: 7.0	Homogenize → heat → A: 0–12 hrs → P: 0–12 hrs → heat → centrifuge → C (rat plasma): 3 hrs	M	4 (beef, white bread, milk, spinach)	1. Mean folate content increased more than 50% in TE compared with CT 2. Optimal pH and incubation time for enzyme treatments vary depending on food items

Table 1. *(Continued)*

Investigator (reference)	pH	Procedure of trienzyme extraction	Folate assay	Number of foods assayed	Overall results
Lim *et al.*, 1998 (21)	E: 4.1 A: 4.1 P: 4.1 C: 7.0	Add buffer → heat → A: 4 hrs → P: 8 hrs → heat → C (rat serum): 3 hrs	M	84 (human milk)	1. Measurable folate in human milk was increased by an average of 85% after TE 2. Neither the pH of the buffer for treatments with α-amylase and protease nor the source of folate conjugase had a significant effect on folate values
Konings, 1999 (22)	H: 7.85 A/P/C: 7.0	Homogenize → heat → homogenize → A/P/C (rat plasma): 4 hrs → heat → centrifuge	HPLC	4 (lyophilized mixed vegetables, liver, milk powder, flour)	Total folates analyzed by HPLC were 20%–35% lower than those by M
Ruggeri *et al.*, 1999 (23)	E: 7.85 C/A: 4.9 P: 7.0	Freeze dry → add buffer → heat → homogenize → C (hog kidney)/A: 3 hrs → P: 1 hr → heat → centrifuge	HPLC	5 (legumes, processed meats, mixed diet)	HPLC results were 24%–52% lower than the microbiologic assay findings
Rader *et al.*, 2000 (19)	H: 7.8	Homogenize → autoclave → C (chicken pancreas)/A: 4 hrs → P: overnight → autoclave → filter	M	162 (enriched cereal-grain products)	There were significant differences between the amount of folate found and the amount mandated by law
Shrestha *et al.*, 2000 (24)	H: 6.1 P: 4.5 C: 7.2 or 4.5	Homogenize → autoclave → centrifuge → P: 16 hrs → heat → A: 4 hrs → C (chicken pancreas, human plasma): 3 hrs → heat → centrifuge	M	4 (spinach, fortified bread, ready-to-eat breakfast cereals)	1. TE was not necessarily better than a 3-hr CT alone 2. Treatment with chicken-pancreas conjugase showed higher folate values than treatment with human plasma
Konings *et al.*, 2001 (25)	H: 7.8	Homogenize → heat → A/P/C (rat plasma): 4 hrs → heat → centrifuge	HPLC	35	Folate values obtained by actual analysis were lower than those in food tables
Whittaker *et al.*, 2001 (26)	H: 7.8	Homogenize → C (chicken pancreas)/A: 4 hrs → P: overnight → autoclave	M	28 (ready-to-eat breakfast cereals)	Folate values obtained by actual analysis in cereals were higher than values on labels
Ndaw *et al.*, 2001 (27)	P/A: 6.0 C: 7.0	Freeze → grind → P/A: 4 hrs → heat → centrifuge → C (chicken pancreas, 1 hr; rat plasma, 2 hrs)	HPLC	10 (e.g., peas, spinach, wheat flour, apples)	1. TE was not essential 2. Chicken-pancreas conjugase was more effective in hydrolyzing polyglutamyl folates than rat plasma conjugase
Johnston *et al.*, 2002 (13)	E: 4.2 A: 6.3 P: 6.3 C: 7.0	Add buffer → heat → A: 3 hrs → P: 3 hrs → heat → C (rat plasma): 2 hrs	M	210 (dairy products)	Folate content was increased approximately 45% after TE than with CT alone
Johnston *et al.*, 2002 (14)	H: 6.3 A: 6.3 P: 6.3 C: 7.0	Homogenize → heat → A: 4 hrs → P: 4 hrs → heat → C (rat plasma): 2 hrs	M	56 (fast foods)	Folate values were higher than those in the literature due to folic acid fortification and the use of TE

Table 1. (Continued)

Investigator (reference)	pH	Procedure of trienzyme extraction	Folate assay	Number of foods assayed	Overall results
Arcot *et al.*, 2002 (28)	—	AOAC/AACC collaborative study	M	3 (soybean, tempeh, soymilk)	Soaking and boiling the soybean caused a significant loss of folate during tempeh and soymilk preparation
Pentieva *et al.*, 2002 (29)	H: 7.85 A: 7.85 P: 7.85 C: 7.85	Homogenize → heat → trienzyme treatment (precise protocol was not written in this abstract)	M	6 (composite meals)	TE increased folate content 1.8 times higher than CT alone and 4.6 times higher than the estimated values based on food tables
McKillop *et al.*, 2002 (30)	H: 7.85	Homogenize → heat → A: 2 hrs → heat → C (rat serum): 2 hrs → heat → P: 2 hrs → heat → centrifuge	M	4 (spinach, broccoli, potato, beef)	Folate content is dependent on food items and cooking methods
Johansson *et al.*, 2002 (31)	E: 6.1	Freeze dry → grind → add buffer → A: 10 mins (boil) → P: 3 hrs → heat → C (hog kidney): 3 hrs → heat → filter	HPLC and M	2 (breakfast rolls fortified with folic acid)	The lower level of fortification (i.e., 200 μg folic acid/day) seems to be sufficient to improve the folate status of healthy women
Doherty and Beecher, 2003 (32)	H: 6.0 A: 6.0 P: 6.0 C: 6.0	Homogenize → A: 1 hr → P: 3 hrs → heat → C (rat plasma): overnight → heat → centrifuge	HPLC	4 (orange juice, flour, spinach, milk)	Values by an HPLC method are slightly lower than those obtained by M
Iwatani *et al.*, 2003 (33)	H: 6.1	Homogenize → autoclave → centrifuge → P → A → C (chicken pancreas) based on AACC method	M	2 (spinach, Chinese broccoli)	TE was not effective in increasing food folate content
Freisleben *et al.*, 2003 (34)	H: 7.85 A: 7.85 P: 7.85 C: 7.85	Homogenize → heat → A: 4 hrs → P: 2 hrs → heat → C (rat serum): overnight → heat → centrifuge	SIDA with HPLC/MS	10 (vegetables, orange juices, cereals, meat)	Total folate content measured by a new method (SIDA) was compared with literature data based on HPLC or M
Yon and Hyun, 2003 (15)	H: 4.1 C: 7.0	Homogenize → heat → P: 2 hrs → heat → A: 2 hrs → centrifuge → C (rat serum): 2 hrs	M	111	Folate values by TE were higher than CT alone in nearly all foods
McKillop *et al.*, 2003 (35)	—	Procedure of Tamura (7)	M	3 (egg, spinach, yeast)	A protocol was developed to produce concentrated extracts of food folates for use in human bioavailability studies
Han and Tyler, 2003 (36)	H: 6.0	Homogenize → heat → A: 4 hrs → P: 1 hr → C (chicken pancreas): 4 hrs → heat → filter	M	9 (pulses)	Mean folate content in dry beans was generally higher than those previously reported
Pentieva *et al.*, 2004 (37)	—	Procedure of Tamura (7)	M	4 (low-folate diet)	The total folate content of the low-folate diet was determined after TE
Pandrangi and LaBorde, 2004 (38)	H: 4.1 A: 3.0 P: 4.0 C: 7.0	Homogenize → heat → A: 6 hrs → heat → P: 8 hrs → heat → C (rat serum): 3 hrs	M	1 (spinach)	A dual-enzyme treatment (i.e., protease and conjugase) resulted in higher folate values than trienzyme treatment

Table 1. *(Continued)*

Investigator (reference)	pH	Procedure of trienzyme extraction	Folate assay	Number of foods assayed	Overall results
Rychlik, 2004 (39)	H: 7.85	Homogenize → P: 6 hrs → A: 2 hrs → heat → C (rat serum): overnight → filter	SIDA with HPLC/MS	9 (vegetables, orange juice, meat, liver, bread, rice)	Total folate content by SIDA was compared with literature data based on HPLC or M
Johnston and Tamura, 2004 (40)	H: 4.2 A: 6.0 P: 6.0 C: 7.5	Homogenize → heat → A: 3 hrs → P: 3 hrs → heat → C (rat plasma): 2 hrs	M	92 (sandwich breads)	Folic acid in breads made of enriched flour declined after 2001
Hannon-Fletcher *et al.*, 2004 (41)	—	Procedure of Tamura (7)	M	4 (meals), 2 (spinach, yeast)	The polyglutamate to monoglutamate ratio in spinach and yeast was 50:50 and 100:0, respectively
Picciano *et al.*, 2004 (42)	H: 7.0	Procedure of Tamura (7)	M	24 (whole-day meals)	Mean folate contents of the meal homogenates obtained by TE were 48%–56% higher than those measured after CT alone
Ginting and Arcot, 2004 (43)	E: 7.85 C/A: 6.0	Freeze dry → mill → add buffer → heat → P: 2 hrs → heat → C (human plasma)/A: 4 hrs → heat → centrifuge	HPLC and M	6 (soybean seeds, tempeh)	HPLC results were 38%–55% lower than the values obtained from M
DeVries *et al.*, 2005 (44)	H: 7.8 C: 7.8	Homogenize → autoclave → P: 3 hrs → A: 2 hrs → C (chicken pancreas): 16 hrs → autoclave → filter	M	20 (cereal-grain products)	Based on the results of the collaborative study of thirteen laboratories, M with TE was recommended for adoption as official First Action
Han *et al.*, 2005 (45)	H: 4.1 C: 7.0	Homogenize → heat → P: 2 hrs → heat → A: 2 hrs → centrifuge → C (rat serum): 2 hrs	M	32 (cooked foods)	Mean folate loss in 32 foods caused by cooking was 29%

[a] H, homogenization buffer; C, folate-conjugase treatment at 37°C; A, α-amylase treatment at 37°C; P, protease treatment at 37°C; M, microbiologic assay; TE, trienzyme extraction; CT, folate-conjugase treatment; E, extraction buffer; AOAC, Association of Analytical Chemists; AACC, American Association of Cereal Chemists; SIDA, stable isotope dilution assay; MS, mass spectrometry.

Table 2. Effect of Initial Heat Treatment of Homogenates Before Enzyme Treatments on Folate Values (47)[a]

Food	Folate value (μg/100 g)		% increase[b]
	With heat treatment	Without heat treatment	
Broccoli	282.0	326.3	15.7
Garlic	180.5	203.0	12.5
Mushroom	38.0	54.7	43.9
Black-eyed pea, raw	662.7	729.3	10.1
Cow's milk	6.5	6.8	4.6
Beef, raw	4.4	4.8	9.1
Eggs, raw	104.7	116.8	11.6
Hamburger (with bun)	35.5	38.3	7.9
Sausage	12.5 ± 1.7 (3)[c]	13.0 ± 0.6 (3)	4.0
Ham	10.7 ± 5.3 (3)	13.2 ± 6.9 (3)	23.4
Fish cake	11.6 ± 2.1 (2)	12.4 ± 3.2 (2)	6.9
Mean	—	—	13.6 ± 11.4

[a] Folate content was determined by microbiologic assay after trienzyme treatment. The precise protocol is illustrated in Figure 1.
[b] Percent increase = (folate value of no heat treatment – folate value of heat treatment) ÷ folate value of heat treatment × 100.
[c] Mean ± SD (number of food items from different manufacturer).

only in a few foods, compared with folate conjugase treatment alone (22, 25). Furthermore, when the values were compared with those obtained by separate treatments (13–15), the extent of the increases in folate values in these foods by the simultaneous treatment with three enzymes was relatively small (22, 25).

It remains to be seen whether protease should not be used with α-amylase and/or folate conjugase because protease may inactivate the other two enzymes if incubated together. Further studies are needed to clarify whether folate conjugase should be applied after treatments that release all folates from the food matrices with protease and α-amylase. Nevertheless, to obtain the "the highest value" of food folate, the order of enzyme treatments should always be verified when the investigators intend to modify the previously established method.

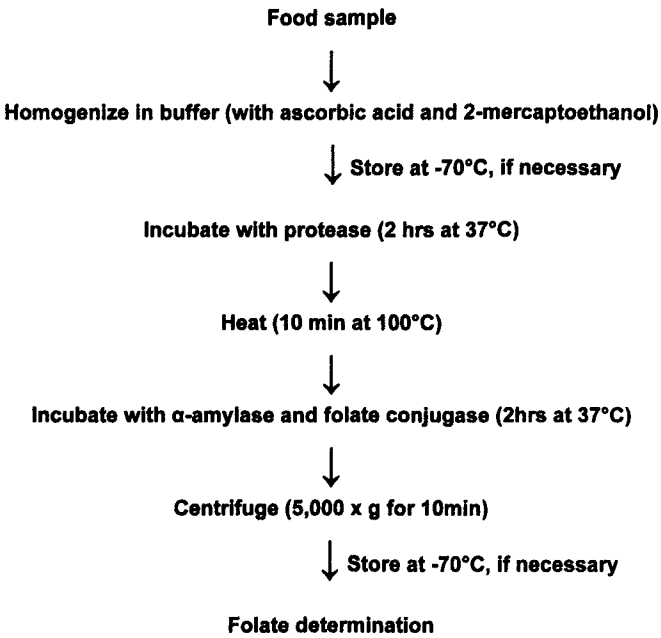

Figure 1. Flowchart of food folate assay.

Enzyme Sources

α-Amylase and Protease Preparations. For trienzyme extraction, Martin *et al.* (8) originally recommended the use of an α-amylase preparation from *Aspergillus oryzae* and a protease preparation from *Streptomyces griseus* (Type XIV), and investigators followed this recommendation. The α-amylase preparation obtained from Sigma Chemical Co. (St. Louis, MO) contains a large amount of endogenous folate; thus, it is necessary to eliminate it by charcoal treatment and filtration for the subsequent folate quantification (7). We used other preparations such as those purified from *Bacillus licheniformis* and other *Bacillus* species. However, these did not provide us with satisfactory enzyme digestion, although contents of endogenous folate in these preparations were much lower than those prepared from *A. oryzae*. Therefore, when we try to digest complex carbohydrates present in various foods, it may be necessary to use a crude α-amylase preparation such as the one originally recommended by Martin *et al.* (8).

Folate Conjugase. Naturally occurring folates exist in polyglutamyl forms (48). Prior to folate quantification by microbiologic assay or high-pressure liquid chromatography (HPLC) analysis (for the distribution of monoglutamyl folates), polyglutamyl folates must be hydrolyzed to monoglutamyl forms. Folate conjugases from several sources, including the serum of human or rat, chicken pancreas, and hog kidney, have been used for this purpose (49, 50). Since the characterization of rat-serum folate conjugase by Horne *et al.* (51), rat serum has been widely used in recent years because it is readily available, it is stable during long-term storage, and it is relatively easy to remove endogenous folate by charcoal treatment and filtration (7). Rat serum conjugase has its pH optimum

between 6.2 and 7.4 (51). Chicken-pancreas folate conjugase, with a pH optimum between 7.0 and 8.5, has also been used because it is less susceptible to the folate-conjugase inhibitors that could be present in foods (7) and is stable for long-term storage. In addition, chicken-pancreas powder has been commercially available. However, because the final product of this folate conjugase reaction is diglutamyl folate, it is not suitable for the assessment of the distribution of monoglutamyl folates using the chromatographic analysis, and it is only suitable for obtaining total food folate values using the *L. rhamnosus* microbiologic assay. Hog-kidney folate conjugase has its pH optimum of about 4.7 and yields monoglutamates (49), which is ideal for both the microbiologic assay using either *L. rhamnosus* or *Enterococcus hirae* (formerly known as *Streptococcus faecalis*, ATCC 8047) and the HPLC analysis for monoglutamyl folates. The preparation of the enzyme must be initiated from the fresh hog kidney, although purification procedures do not involve laborious steps and the preparation is stable for months at –70°C.

Lim *et al.* (21) reported that these sources of folate conjugase had no effect on the folate content in human milk samples. In contrast, Ndaw *et al.* (27) demonstrated that hog-kidney folate conjugase was less effective in the hydrolysis of polyglutamyl folates than the other two folate conjugases. Iwatani *et al.* (33) compared the folate content of a few vegetables after hydrolysis with chicken pancreas and human plasma and showed that folate conjugase from chicken pancreas was more effective in hydrolyzing polyglutamyl folates than human plasma. However, it is unclear whether the actual comparisons of the enzyme activities were made in these studies to make such a distinction (27, 33). Engelhardt and Gregory (52) reported that hog-kidney folate conjugase was inhibited by extracts from a variety of foods. This inhibition, however, was reduced by increasing enzyme concentration or incubation time. Thus, complete hydrolysis may be achieved when the combination of the quantity of the enzyme and the incubation time is appropriate, regardless of the source of folate conjugases.

Duration of Enzyme Treatments

Martin *et al.* (8) recommended the procedure that included the 4-hr incubation of a food homogenate with folate conjugase and α-amylase simultaneously, followed by an overnight incubation with protease (Table 1). However, Pfeiffer *et al.* (17) showed that such a long-term incubation with protease caused a 20% decrease in folate content in cereals compared with a short-term incubation, suggesting that labile folates may be destroyed after a long-term incubation. Therefore, it is advisable to have a shorter incubation time using a larger quantity of enzymes.

Using a total of 65 foods, we compared values between the two incubation protocols: separate incubation (i.e., incubation with α-amylase and folate conjugase separately for 2 hrs after protease treatment) and combined incubation (i.e., incubation with α-amylase and folate conjugase together for 2 hrs after protease treatment). Folate values by combined incubation were comparable to those by separate incubation (47). These findings indicate that the final enzyme treatments (i.e., α-amylase and folate conjugase) can be combined to shorten the entire incubation.

Centrifugation of Homogenates

Tamura (7) recommended centrifugation after treatments with α-amylase and protease, followed by the incubation of the supernatant with folate conjugase before folate assay. However, the centrifugation step has not been clearly described in many articles. Shrestha *et al.* (24) and Iwatani *et al.* (33) followed the procedure that food homogenates were centrifuged before the treatments with enzymes, but they did not show a significant increase in folate values after the trienzyme extraction compared with those after folate-conjugase treatment alone.

We evaluated the effect of centrifugation before enzyme treatments. When homogenates of 110 foods were centrifuged and supernatants were subsequently treated with protease, α-amylase, and folate conjugase, the changes in folate content compared with folate-conjugase treatment alone ranged from –100%–230%, with an average of only 8% (53). In contrast, when the centrifugation step before the enzyme treatments was eliminated for these food homogenates, the changes were 0%–900%, with a mean of 79% (15). Our data indicated that the initial centrifugation of food homogenate was not only unnecessary, but also provided much lower values because this initial step eliminates residues containing folates that could have been released from food matrices by the enzyme treatments. Thus, centrifugation before trienzyme treatment is unacceptable.

Sample Storage

Food homogenates, or those after the treatment with the three enzymes, may be stored at –70°C. Folates in these samples are stable for a few months, as long as ascorbate and/or 2-mercaptoethanol were added to the buffer for homogenization or enzyme treatment. The samples should be separated into a few aliquots before storage for later use to avoid repeated freeze-thaw procedures.

Folate Determination

As previously reviewed by Tamura (50), microbiologic assay is the most widely used procedure for the determination of total food folate content. Among the 35 studies shown in Table 1, 28 were performed using the microbiologic assay method using *L. rhamnosus* and 10 were done by the HPLC method. Because it responds to most metabolically active forms of folate, *L. rhamnosus* has been the preferred test microorganism (50).

Although microbiologic assay provides only a total

folate value, the HPLC analysis makes it possible to obtain data on various forms of folates and possibly allows better prediction of their stability. A recent international, interlaboratory comparison study indicated that 20 of 26 laboratories used microbiologic assay, 4 used an HPLC-spectrometry, 1 used liquid chromatography/mass spectroscopy (LC-MS), and 1 used a radiobinding assay for food folate analysis (11). Several HPLC methods to separate and detect the individual forms of folate in foods have been developed (9, 54). Pfeiffer *et al.* (17) first used both the HPLC method and the microbiologic assay independently after the trienzyme extraction for food folate analysis. They showed that total folate contents determined by the HPLC method for several foods were comparable to those obtained by microbiologic assay. Several groups of investigators, however, reported that folate values obtained using HPLC were lower than those obtained by microbiologic assay, although the mechanism of the discrepancy is unknown. For example, Konings (22) demonstrated that total folates in several foods analyzed by HPLC were 20%–35% lower than those obtained by the microbiologic-assay method. Ruggeri *et al.* (23) also reported that the results obtained by the HPLC method were 24%–52% lower in four food items and a mixed diet than those obtained using microbiologic assay. Doherty and Beecher (32) showed that the values determined by the HPLC procedure were slightly lower than those obtained by microbiologic assay.

In intercomparison studies, Finglas *et al.* (55) concluded that microbiologic assay is more appropriate than the HPLC method because it is difficult to measure individual folates in certain foods, especially when folate content was low in food samples. The introduction of mass spectrometry in combination with HPLC may enhance the specificity of folate detection (34, 39). Although the procedures of food folate extraction require further scrutiny, the method may be most promising for its specificity.

Comparison of Folate Values Among Laboratories

To improve the accuracy of food folate data, researchers tested the agreement of folate values among laboratories (11, 55, 56). Using an HPLC technique after folate-conjugase treatment alone, Vahteristo *et al.* (56) examined the agreement of various forms of folate in a few food items among three laboratories and found that there was little agreement, with the exception of 5-methyltetrahydrofolate. Five intercomparison studies from 1990–1997 showed that the most consistent values obtained using the HPLC method were for 5-methyltetrahydrofolate, and it was suggested that more work is needed for an HPLC analysis of folate forms other than 5-methyltetrahydrofolate (55).

Puwastien *et al.* (11) reported that coefficients of variation of three food items among seven laboratories using trienzyme extraction with microbiologic assay (*L. rhamnosus* activity) ranged from 24%–34%, whereas average coefficients of variation of triplicate assays within the laboratory was 3%–6%. The wide variation between laboratories was mainly due to the different methods that were used for folate extraction and detection. Therefore, it is important to standardize the methods of folate extraction and detection, and the use of reliable reference materials should be encouraged to monitor the interassay or intraassay variations to ensure the quality of folate analysis.

Recommended Procedures

A recommended experimental procedure for food folate determination is shown in Figure 1. An appropriate amount of food, depending on folate content, is homogenized in a buffer with 57 m*M* ascorbic acid and 0.2 *M* mercaptoethanol. The homogenates can be divided into several aliquots and frozen at –70°C until further processing. The initial step after homogenization had been heating the homogenates to release folate from the binding protein; however, as previously discussed, this step can be eliminated once a protease preparation is used for folate extraction.

Protease and α-amylase are dissolved in distilled, deionized water or a buffer at concentrations of 40 mg/ml, which is equivalent to 1572 U–2240 U of α-amylase/ml and 208 U–220 U of protease/ml, depending on the batch of enzyme preparations. Based on our experience, the minimum of 3 U of α-amylase and 0.4 U of protease against 1 mg of the original food are generally sufficient for satisfactory folate extraction. The preparation of α-amylase contains a large amount of endogenous folate. However, it is possible to increase the quantity of α-amylase without creating a high folate blank for folate assay because endogenous folate in α-amylase can be removed by charcoal treatment and filtration through a 0.22-μm microfilter. Although only a minute amount of endogenous folate has been detected in the preparation of protease, it is always better to check the amount of endogenous folate for each batch of the preparation, especially when a large quantity of enzyme preparation is used. As a source of folate conjugase, rat serum appears to be most suitable due to its availability and easy preparation. Folate conjugase from chicken pancreas can be used for microbiologic assay, but it is not suitable for the HPLC method, as previously noted.

The incubation of food homogenates with each enzyme for 2–3 hrs instead of more than 12 hrs (overnight) is considered appropriate. We recommend the incubation of food homogenates with folate conjugase after the treatments with α-amylase and protease, which allows the release of polyglutamyl folates from food matrices. We also recommend the treatment of homogenates with α-amylase and folate conjugase simultaneously after the protease treatment. After trienzyme treatment, if necessary, the samples should be stored at –70°C in a few aliquots before folate determination.

It should be noted that the conditions of trienzyme treatments differ among foods (20, 38). Thus, it becomes potentially time consuming and labor intensive to measure

folate content in each individual food. Our current recommendation is a more efficient and practical method of food folate analysis than previously published (7). We hope that the method recommended here enhances the wide use of the trienzyme-extraction method and the reliability and accuracy of food folate tables.

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