Trienzyme Treatment for Food Folate Analysis:
Optimal pH and Incubation Time for
α-Amylase and Protease Treatments

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Summary Recent reports have indicated that trienzyme treatment
before folate determination is essential to obtain the proper folate content
in foods. Trienzyme treatment is performed by using α-amylase and
protease for folate extraction from carbohydrate and protein matrices,
and folate conjugase for the hydrolysis of polyglutamyl folates. We
evaluated the conditions of pH and incubation time for the treatment with
α-amylase and protease. Four food items, including fresh beef, white bread,
cow’s milk, and fresh spinach, were selected for this investigation. We
found that optimal pHs for α-amylase treatment of beef and cow’s milk
were 7.0 and 5.0, respectively, whereas those for white bread and spinach
were not distinctive at pHs from 2.0 to 7.0. The optimal incubation time
for α-amylase was 4 h for fresh beef and cow’s milk, whereas no distinctive
optimal incubation period was found for white bread and fresh spinach.
Our data indicate that the conditions for enzyme treatments vary de-
pending on food items. Trienzyme treatment resulted in an increase of
more than 50% in the mean folate content over folate conjugase treatment
alone. It is necessary to treat food samples with not only traditional folate
conjugase, but also with α-amylase and protease before folate deter-
mination to obtain the actual folate content.

Key Words folate, microbiological assay, protease, α-amylase, folate
conjugase

The results from three recent independent studies indicated that trienzyme
treatment is essential for obtaining the possible maximum values of food folate
content, when analyzed by either microbiological assay or an HPLC method (1–3).
Trienzyme treatment includes the use of α-amylase (EC 3.2.1.1) and protease (EC
3.4.24.31), besides the traditional treatment that uses pteroylpoly-γ-glutamyl
hydrolase (folate conjugase, EC 3.4.19.9) alone. The role of α-amylase and protease

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treatments is an extraction process believed to be the digestion of carbohydrate and protein matrices where food folates are possibly trapped (2). Folate conjugase hydrolyzes polyglutamyl folates, the major forms in foods, to mono- or diglutamyl forms that can be utilized by the organism that is used for microbiological assay (4, 5).

The commercial preparations of α-amylase and protease are known to contain various types of isoenzymes, of which pH optima can vary depending on the substrates present in the foods (6, 7). Therefore it has been difficult to generalize which pH should be used for the treatment using these two enzymes before folate conjugase treatment and folate determination. Furthermore, no consensus has been reached on how long these enzyme treatments should be carried out for obtaining the possible highest values of folate content in different food items, considering the labile nature of reduced forms of food folate. We undertook the study presented here to identify optimal pH and duration of enzyme treatments by using four food items commonly found in a regular diet.

METHODS

Food samples. All food samples, fresh boneless stewing beef, white bread, cow’s milk and fresh spinach, used in this study were purchased from a local supermarket from July to September 1997. Approximately 5 g of each solid food was minced, and then homogenized in five volumes of 0.1M potassium phosphate buffer containing 114 mM ascorbic acid (final of pH 4.1), using a Polytron at maximum speed for 30–120 s on ice. Cow’s milk was mixed with an equal volume of the same buffer. These homogenates and the mixture were heated in boiling water for 10 min. After cooling, the samples were kept at −70°C until further treatments.

Enzymes. α-Amylase prepared from Aspergillus oryzae, protease prepared from Streptomyces griseus, and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The α-amylase and protease were dissolved in distilled-deionized water at concentrations of 10 and 20 mg/mL, respectively. These solutions were filter-sterilized with a microfilter (0.22 μm, Corning Glass Works, Corning, NY, USA) immediately before use (2). The endogenous folate in each enzyme was determined after folate conjugase treatment. Although the protease did not contain any measurable folate by microbiological assay using Lactobacillus casei (L. casei, ATCC 7469), the α-amylase contained approximately 1.54 ng of folate per mg of solid. This endogenous folate in α-amylase was subtracted for the final calculation of food folate content. The rat serum was obtained from Harlan Bioproducts for Science (Madison, WI, USA) and was used as a source of folate conjugase (8). Endogenous folate in rat serum was removed by mixing the serum with one-tenth volume of charcoal for 60 min on ice and filtering it through a microfilter. Aliquots of filtrate were stored at −70°C until used.

Search for the optimal pH and incubation time. First, to identify optimal pH for both α-amylase and protease treatments for each food extract, the treatments
Trienzyme Treatment for Food Folate Assay

were independently carried out by using six different pHs ranging from 2.0 to 7.0. For pHs 2.0–3.0, 0.3 M malefic acid was used, and for pHs 4.0–7.0, 0.3 M potassium phosphate buffers, each containing 114 mM ascorbic acid, were used. For this experimentation, a 4-h incubation was used for each x-amylase and protease treatment (Fig. 1). The incubation mixture consisted of 250 μL of heat-treated food homogenate with an equal volume of buffers with different pHs, and 500 μL of either x-amylase or protease solution, as described above. The incubation was terminated by placing the tubes containing the enzyme-treated mixture in boiling water for 10 min. Second, to identify the optimal duration of incubation, x-amylase or protease (0–12 h) treatment was performed under the optimal pH conditions that were tentatively obtained from the first series of experiments, as described above (Fig. 2).

Trienzyme treatment. Under the optimal conditions of x-amylase and protease treatments, each food extract was first treated with x-amylase, then with protease (for an incubation period indicated in Table 1), followed by heating and a 3-h incubation with folate conjugase. All enzyme treatments were performed aseptically by using heated food extracts and presterilized enzyme solutions, reagents, and plasticware. Care was taken to avoid any possible bacterial contamination (2).

Folate conjugase treatment. For all analyses, after the centrifugation (5,000 × g for 10 min) of heated mixtures of food homogenates treated with either x-amylase or protease, the supernatant (250 μL) was mixed with 650 μL of 0.3 M potassium phosphate buffer containing 50 mM sodium ascorbate with a final pH of 7.0 and with 100 μL of rat plasma folate conjugase at pH 7.0, then incubated for 3 h at 37°C (2). The incubation was terminated by freezing the mixture at −70°C. Heat-treated food homogenates, not treated with x-amylase or protease, were centrifuged at 5,000 × g for 10 min, and the supernatants were treated with folate conjugase alone as described above.

Microbiological assay. Folate content in each enzyme-treated sample was determined by microbiological assay using L. casei, and 5-formyltetrahydrofolate (calcium salt, Sigma Chemical) was used as a standard; however, for the conversion of folate content to ng/g, the molecular weight of 441 (pterolyglutamic acid) was used. The assay was carried out by using a 96-well microplate reader (Model 450, Bio-Rad, Hercules, CA, USA), which was interfaced with a computer for data reduction. The detailed procedures of the microbiological assay methods used for this study were previously described (5).

RESULTS

As shown in Fig. 1A, the folate content in beef with x-amylase treatment (4-h incubation) varied markedly at different pHs. The optimal pH was 7.0 using potassium phosphate buffer, whereas the lowest content was observed at pH 4.0. In contrast, folate content in beef after protease treatment was similar at various pHs ranging from 2.0 to 7.0. Folate content in white bread and spinach after either
Fig. 1. Effect of pH for α-amylase (solid circles) and protease (open squares) treatments on folate contents. A, fresh beef; B, white bread; C, cow’s milk; and D, fresh spinach. Maleic acid buffer containing 2% ascorbic acid was used for pHs 2.0 and 3.0, and potassium phosphate buffer containing 2% ascorbic acid was used for pHs 4.0–7.0. Each experiment was repeated two or three times, and the most representative graph is presented.

α-amylase or protease treatment did not show appreciable changes at different pHs (Fig. 1, B and D, respectively). The optimal pH to obtain the highest folate value in cow’s milk after α-amylase was 5.0, whereas the change in pH did not affect the content obtained with protease treatment (Fig. 1C).

To obtain possible maximum folate content with α-amylase treatment, the incubation period of 2 h was most suitable for beef and cow’s milk (Fig. 2, A and C), and the peaks for the optimal incubation time were distinctive in these two food items. For white bread and spinach, the optimal duration of the incubation was 4 h and 1 h, respectively, although overall values were not markedly different throughout the 12-h incubation (Fig. 2, B and D). The optimal incubation time for beef folate analysis was 8 h for protease treatment. For bread, cow’s milk, and spinach, the optimal incubation time was 6 h, although the difference over the 12-h period was small for each food item.

As summarized in Table 1, folate content after folate conjugase treatment alone was lowest in each food item, and the highest values were obtained after trienzyme
Fig. 2. Changes in folate content with time of the incubation with α-amylase (solid circles) and protease (open squares). A, fresh beef; B, white bread; C, cow's milk; and D, fresh spinach. Each experiment was repeated two or three times, and the typical graph is presented.

Table 1. Comparison of folate content (ng/g wet weight or mL) with various enzyme treatments.

| Food item     | FCT Content | AT plus FCT Content | PT plus FCT Content | Trienzyme T Content |
|---------------|-------------|---------------------|---------------------|---------------------|
|               | Incubation/ | Incubation/          | Incubation/          |                     |
|               | pH          | pH                  | pH                  |                     |
| Beef (fresh)  | 92          | 173                 | 2 h/7.0             | 117                 | 8 h/6.0             | 149                 | 6.0                |
| White bread   | 693         | 697                 | 4 h/7.0             | 799                 | 6 h/7.0             | 1,090               | 7.0                |
| Cow’s milk    | 49          | 76                  | 2 h/5.0             | 61                  | 6 h/5.0             | 80                  | 5.0                |
| Spinach (fresh) | 2,240     | 2,530               | 1 h/6.0             | 2,240               | 6 h/5.0             | 3,380               | 5.0                |

FCT, value obtained after folate conjugase treatment alone (the means of AT plus FCT and PC plus FCT at 0 h); AT, highest value obtained by α-amylase treatment; PT, highest value obtained by protease treatment; Trienzyme T, values obtained after the incubation for the duration indicated for both AT and PT.
treatment, with the exception of folate content in beef. Either α-amylase or protease treatment, besides folate conjugase treatment, increased the folate content an average of 39% and 17%, respectively, compared with the values obtained by folate conjugase treatment alone. The trienzyme treatment resulted in a mean increase of 58% in the values obtained only by treatment with folate conjugase.

DISCUSSION

In her pioneering work published in 1979, Yamada (9) used protease treatment as a process of folate extraction along with the traditional folate conjugase treatment for the determination of folate content in certain foods, including breast milk, hog liver, and cod. She reported that the treatments resulted in a significant increase in folate content as compared to that obtained by using folate conjugase alone. In the 1980s, Cerna and Kas (10) and Pedersen (11) determined food folate content after α-amylase treatment and suggested that this treatment in combination with the use of folate conjugase was essential for obtaining proper values of folate in certain types of food. Furthermore, in 1990, Martin et al (1) and De Souza and Eitenmiller (12) reported an important observation indicating that trienzyme treatment (α-amylase, protease, and folate conjugase) is necessary to accurately determine folate content in foods. Recently, Tamura et al (2) and Pfeiffer et al (3) also reported the importance of trienzyme treatment for food folate analysis.

In this study, we found that food folate content measured after treatments using α-amylase or protease with folate conjugase was increased after folate conjugase treatment alone. Furthermore, as indicated in Table 1, trienzyme treatment resulted in an increase of more than 50% in the mean folate content of the foods in comparison with what it was after folate conjugase treatment alone. However, the folate content in beef after trienzyme treatment (149 ng/g) was lower than that obtained from treatments that used only α-amylase and folate conjugase (173 ng/g). Our data indicate that folates in fresh beef are likely to be labile under the conditions used for protease treatments. However, no attempt was made to identify the forms of folate in this item.

Table 2 summarizes the comparison of our values with those determined by other investigators of folate contents of four food items using folate conjugase treatment alone and trienzyme treatment. Our data after folate conjugase treatment alone are similar to those reported by others. Folate content in these food items may vary depending on such factors as seasonal effect, manufacturing handling and procedures, and cooking methods. But it is unknown whether these factors also affect the optimal conditions of enzyme treatments. After trienzyme treatment, the folate content in white bread using folic acid unfortified flour reported by Pfeiffer et al (3) was 354 ng/g. Furthermore, they reported that the content using folic acid fortified flour was 879 ng/g. Their bread was prepared based on a mandate issued by the Food and Drug Administration of the U.S. Department of Health and Human Services, indicating that starting at the beginning of 1998, cereal-grain
products should be fortified with folic acid at a level of 140 μg/g (18). This legislation was intended to increase folate intake among women of childbearing age for the possible prevention of pregnancy complicated with neural-tube defects (19). Our value of folate content of white bread was 1,090 ng/g, suggesting that the flour to make the bread used in this study had already been fortified with folic acid at the time of purchase in September 1997. Nevertheless, our findings in the study presented here confirm the importance of trienzyme treatment for food folate analysis.

We also found that the optimal duration for α-amylase treatment may vary depending on foods; however, there were no distinctive differences in the protease treatments for all foods used in our study. Yamada (9) used an incubation period of 3 h for the protease treatment, and Cerna and Kas (10) and Pedersen (11) digested food samples for 2–20 h with α-amylase. For trienzyme treatment, Martin et al (1), Tamura et al (2), and Pfieffer et al (3) used an incubation period of 4 h for α-amylase and overnight for protease. Although De Souza and Eitenmiller (12) examined the effects of various lengths of protease treatment, there has not been a systematic experiment to investigate the appropriate incubation time for individual foods. In consideration of the labile nature of reduced folates in food, it may be critical to protect labile folates by minimizing the duration of each enzyme treatment as much as possible. In the present study, however, we did not evaluate the appropriate amount of ascorbate or the use of other reducing agents to protect labile folates.

Although the investigators have used previously established optimal pHs for the treatment of folate conjugases obtained from various tissue sources, they have used different pHs ranging from 4.3 to 7.85 for α-amylase and protease treatments (1–3, 9–12). In the present study, we found that the optimal pHs for α-amylase treatment to obtain the maximum folate content markedly varied depending on

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**Table 2. Comparison of food folate contents (ng/g wet weight or mL) in the literature and in our study.**

| Investigators (reference No.) | Beef (fresh) | White bread | Cow's milk | Spinach (fresh) |
|------------------------------|--------------|-------------|------------|----------------|
| Folate conjugase alone       |              |             |            |                |
| Thenen (13)                  |              | 430         |            | 1,550          |
| Babu and Srikantia (14)      |              | 356         | 50         | 1,930          |
| Hoppner et al (15)           |              | 390         | 50         |                |
| Perloff and Butrum (16)      | 70           |             |            | 1,610          |
| Yamada (9)                   |              |             |            |                |
| Klein and Kuo (17)           |              |             |            |                |
| Pfieffer et al (3)           |              | 244         |            | 2,240          |
| Our data                     | 92           | 693         | 49         |                |
| Trienzyme treatment          |              |             |            |                |
| Pfieffer et al (3)           |              |             |            |                |
| Our data                     | 149          | 1,090       | 80         | 3,380          |
food items (Fig. 2). On the other hand, pH optima for protease treatment were not distinctive for all foods, since the differences in folate content by various pHs did not exceed the value of the coefficient of variation (10–12%) of L. casei assay in our laboratory (5). Therefore we suggest that the pH selected for α-amylase treatment can be used for subsequent protease treatment in individual foods.

We are now unable to explain why the optimal pHs and the optimal incubation periods for the treatment using α-amylase are different among various foods. It seems unlikely that the composition of foods in terms of protein (and/or carbohydrate) has significant influence on the selection of pH or incubation time of the enzyme treatment. Therefore we suggest that the condition of the enzyme treatment must be individually identified for each food. In particular, it may be essential to identify the optimal duration for α-amylase treatment for individual food items. In this study, we used α-amylase prepared only from a microorganism; it might be of interest, however, to use the enzyme preparations from other sources.

In recent years, the important role of folate nutriture has been stressed in terms of the possible prevention of arteriosclerosis because of increased plasma homocysteine concentrations or pregnancies with neural-tube defects (20, 21). Furthermore, a significant negative correlation between dietary folate intake and plasma homocysteine concentrations was reported in a certain segment of the population (22). Thus it is important to carefully monitor whether diets in the general population supply a sufficient amount of folate. Although these findings have drawn much attention to improve folate nutriture by increasing dietary intakes of the vitamin, it has been known that the calculated dietary folate intakes are notoriously inaccurate because of a lack of dependable data on the folate content of foods (23). Therefore it is essential, more than ever, to establish a reliable food folate table by using the procedures of trienzyme treatment, although it is acknowledged that this procedure is extremely labor intensive and time consuming. Our data suggest that it is necessary to find out the most appropriate conditions of enzyme digestion for individual food items. Although it appears that the process of completing the food folate table is overwhelmingly painstaking, and that it will take many years to achieve this goal, it is our responsibility to accomplish this task to establish dietary recommendations for folate intake for the maintenance of health and the prevention of certain diseases (20, 21).

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