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Optimization of microbiological assay of folic acid and determination of folate content in spinach

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Summary The microbiological method for the determination of folate in plant foods uses the growth response of folate-dependent *Lactobacillus rhamnosus* in extracts that have been enzymatically treated to release the bound vitamin. However, the use of cryoprotected cultures is hampered by low recovery of the microorganism after extended frozen storage times. In this study, growth of *L. rhamnosus* was enhanced using a microaerophilic enrichment procedure and optimal pH conditions and enzyme reaction times were determined for the release of bound folate in spinach. Optimum pH values for the release of bound folate in spinach samples treated with α-amylase or protease were 3.0 and 4.0, respectively. Although treatment with α-amylase had no significant ($P > 0.05$) effect on measured folate, addition of protease at pH 4.0 significantly ($P \leqslant 0.05$) increased the release of folate at an optimum incubation time of 8 h. Therefore, a dual-enzyme treatment (protease and conjugase) is sufficient to determine folate content in spinach.

Keywords Folate, *Lactobacillus rhamnosus*, microbiological assay, spinach.

Introduction

Folates are a group of water-soluble vitamers that function in biological reactions to transfer one-carbon units from one compound to another. Deficiency states lead to impaired cell division manifested as megaloblastic anaemia and foetal development defects in humans. Folate deficiency has also been linked to elevated levels of serum homocysteine, a condition implicated as an independent risk factor for coronary artery disease and stroke (Green & Jacobsen, 1995).

Total folate in foods is determined after extraction by homogenization, deconjugation of polyglutamate forms to monoglutamates using conjugase (γ-glutamyl hydrolase) and microbiological assay or HPLC determination of individual vitamins. More complete extraction of folates trapped in or bound to polysaccharides or proteins is achieved by treating samples with α-amylase or protease, respectively. Yamada (1979) reported an increase in total measurable folate of human milk, pig liver and cod muscle when a protease digestion step preceded treatment with conjugase. Pederson (1988) similarly demonstrated release of bound folates in starch-based foods by treating the sample matrix with α-amylase. Martin *et al.* (1990) reviewed the effects of different enzyme treatments and developed a trienzyme procedure that consisted of sequentially treating the food matrix with protease and α-amylase before addition of conjugase.

Further improvements to the trienzyme method have been made by modifying pH conditions and changing the order of addition of enzymes (Tamura *et al.*, 1997). Several researchers have suggested that trienzyme treatment may be essential to release bound folate from the food matrix (Pffeifer *et al.*, 1997; Tamura *et al.*, 1997; Aiso & Tamura, 1998; Rader *et al.*, 1998). However, after studying trienzyme treatment of milk, bread, spinach and

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beef, Aiso & Tamura (1998) concluded that the optimal combination of enzymes and reaction conditions varies depending on the composition of the food. The influence of food composition on the optimal method for release of bound folate was demonstrated by Shrestha *et al.* (2000) and Iwatani *et al.* (2003), who determined that a dual-enzyme treatment (α-amylase followed by conjugase) or even a single-enzyme treatment (conjugase alone) led to significantly higher values of folate in breakfast cereals and Chinese broccoli compared to the trienzyme treatment (α-amylase, protease and then conjugase). These studies demonstrate that optimal conditions for folate analysis need to be determined for individual foods and that suboptimal conditions can result in under estimation of the vitamin.

The microbiological assay for folate in foods measures the growth response of *Lactobacillus rhamnosus* (ATCC 7469) inoculum in sample extracts utilizing a ninety-six-well microplate technique (Newman & Tsai, 1986). Bacterial growth is compared by measuring the turbidity of samples after a suitable incubation period. An important improvement in folate analysis is the use of glycerol-cryoprotected cultures because they can be grown in large quantities and frozen for later use and require less time and labour to maintain than serial cultures (Wilson & Horne, 1982). Despite advances in the use of cryoprotected cultures, the assay is not very convenient because the organism remains difficult to grow. Furthermore, cryoprotected cultures lose viability in 2–3 months making it difficult to maintain cultures that will yield adequate inoculum levels.

Lactobacilli are Gram-positive, facultatively anaerobic bacteria that preferentially grow in a microaerophilic environment; i.e. low oxygen and high carbon dioxide (5–10% CO_2). They are also nutritionally fastidious organisms that grow only in complex media containing carbohydrates as energy and carbon sources in addition to essential nucleotides, amino acids and vitamins (Kandler & Weiss, 2000). It is hypothesized that microbiological assay for folate may be improved by recovery of *L. rhamnosus* in cryoprotected cultures using a microaerophilic enrichment procedure. The objectives of this study are, therefore, to (i) optimize the folic acid assay by improving growth conditions of cryoprotected *L. rhamnosus* and (ii) determine the optimum conditions for trienzyme treatment of spinach using the improved method for preparing inoculum.

Materials and methods

Preparation of cryoprotected cultures

Cryoprotected cultures were prepared from lyophilized *L. rhamnosus* (ATCC 7469, American Type Culture Collection, Manassas, VA, USA). The culture was reconstituted according to the manufacturer's directions and 50 μL was transferred into 5 mL of filter-sterilized folic acid casei medium (Difco, Sparks, MD, USA) containing 5 ng mL^{-1} folic acid (5-formyl tetrahydrofolate, 5-HCO-H_4PteGlu, calcium salt, Sigma–Aldrich, St Louis, MO, USA). After incubating at 37 °C for 18 h, the bacteria were sub-cultured again using the same technique. A third sub-culture was made by transferring 50 μL into 25 mL^{-1} of folic acid casei medium containing 1 ng mL folic acid and incubated as described. Following incubation, 25 mL of the culture was mixed with 25 mL of sterile 80% glycerol. Aliquots (0.5 mL) were transferred into sterile microcentrifuge tubes and stored at −70 °C until used for subsequent experiments.

Growth of frozen cryoprotected cultures using a standard method or microaerophilic enrichment method

Growth of *L. rhamnosus* after frozen storage was compared using two different methods for preparation of inoculum. In the standard method (Tamura, 1990), 0.5 mL of cryoprotected culture in a single vial was transferred to 4.5 mL of 0.1 M sterile phosphate buffer (pH 6.3) containing 50 mM sodium ascorbate. One millilitre of the diluted culture was then mixed with 14 mL of filter-sterilized folic acid casei medium. Growth that would occur in a folate-free blank was simulated by mixing 3.0 mL of the mixture with an equal amount of sterile phosphate buffer (pH 6.3) and incubating for 18 h at 37 °C.

For the microaerophilic enrichment method, 50 μL of cryoprotected culture was transferred to 10 mL of *Lactobacillus* broth AOAC (Difco, Sparks, MD, USA) and incubated for 18 h at 37 °C under microaerophilic conditions in an

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anaerobic chamber. A microaerophilic environment was achieved by using a commercially available H_2/CO_2 catalyst in an anaerobic chamber (CampyPak Plus^{TM} system, Beckton Dickinson, Sparks, MD, USA). One envelope of pre-mixed catalyst was used for each 2-L volume of the chamber. Following incubation and two successive centrifugation and re-suspension steps in 10 mL of 0.85% sterile saline to remove the enrichment medium, 50 μL of saline suspended culture was mixed with 4.95 mL of sterile phosphate buffer (0.1 M, pH 6.3) containing 50 mM sodium ascorbate. The culture was then diluted with folic acid casei medium and phosphate buffer, as previously described, to simulate a folate-free blank and incubated for 18 h at 37 °C under microaerophilic conditions. Optical density at 625 nm (OD_{625}) was used to compare microbial growth after storage at −70 °C for up to 5 months.

Folate assay

The optimal inoculum level for the folate assay was determined by preparing growth response curves using 50, 100 and 150 μL of 18-h microaerophilically enriched saline cultures diluted in 0.1 M phosphate buffer (pH 6.3) to 5 mL. One millilitre of the diluted culture was then mixed with 14 mL of filter-sterilized folic acid casei medium and the assay was conducted using the ninety-six-well microplate technique described by Tamura (1990). The slope of the growth response curve was used to determine the inoculum level that yielded the most sensitive assay condition. *Lactobacillus rhamnosus* growth was compared by measuring the optical density at 490 nm (OD_{490}) using an automated microplate reader (Model EL 340, Biotek Instruments, Winooski, VT, USA).

Fresh spinach was purchased at a local supermarket and used immediately for analysis. Damaged or bruised leaves and stems were removed and 10 g of whole leaves were homogenized in a blender with 50 mL of 0.1 M phosphate buffer containing 114 mM ascorbic acid (final pH 4.1). The homogenate was heated in a water bath at 100 °C for 10 min, cooled, and stored at −70 °C until used.

Folate content in thawed spinach homogenate was determined using microaerophilically enriched cultures of cryoprotected *L. rhamnosus* and microbiologically assayed using the ninety-six-well microplate procedure. 5-Formyl tetrahydrofolate (5-HCO-H_4PteGlu, calcium salt) was used as the folic acid standard. Pooled human blood plasma was used as an internal standard and the assay validity was confirmed by determining the folate content of triplicate samples of infant formula obtained from the National Institute of Standards and Technology (standard reference material #1846, NIST, Gaithersburg, MD, USA).

Enzymes

α-Amylase and protease enzymes were obtained from Sigma–Aldrich (St Louis, MO, USA). Rat serum (Harlan Bioproducts, Indianapolis, IN, USA) was used as the conjugase source. Endogenous folate in α-amylase and protease was measured to prevent overestimation of folate content in spinach samples. Endogenous folate in rat serum was removed by mixing with charcoal (1:10 wt./vol.) for 1 h on ice and then filter sterilizing (Aiso & Tamura, 1998). Aliquots were stored at −70 °C until use. Conjugase reaction conditions for all assays were pH 7.0 for 3 h as recommended by Tamura (1998).

Optimization of enzyme reaction conditions

The effect of pH and incubation time on α-amylase and protease activity in spinach homogenate was determined using the method of Aiso & Tamura (1998) with modifications. Relative enzyme activities between pH 3.0 and 7.0 were compared by determining the amount of folate recovered in treated samples. Citrate buffer (0.3 M) was used for pH 3.0 and 4.0 reaction conditions and phosphate buffer (0.3 M) was used for pH 5.0, 6.0 and 7.0 conditions. Spinach homogenate (250 μL) was mixed with an equal volume of appropriate buffer and 500 μL of either 10 mg mL^{-1}α-amylase or 20 mg mL^{-1} protease and then incubated at 37 °C for 4 h. Enzyme reactions were terminated by heating the mixture in a boiling water bath (100 °C) for 5 min. After cooling, 200 μL of the sample was mixed with 950 μL of phosphate buffer (0.3 M, pH 7.0) containing 50 mM sodium ascorbate and 50 μL of conjugase. The mixture was then incubated at 37 °C for 3 h. Control samples contained 250 μL

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of spinach homogenate mixed with 750 μL of appropriate buffer and were incubated, heat-treated and treated with conjugase under the same conditions as the enzyme treated samples. Total folate content was then determined in triplicate using the microbiological assay.

Optimal incubation time for α-amylase or protease activity was determined using the optimal pH conditions obtained from the previous experiments. Spinach homogenate (250 μL) was mixed with an equal volume of appropriate buffer and 500 μL of α-amylase or protease was added. Samples were incubated at 37 °C for 3, 6, 9 or 12 h and enzyme reactions were terminated at 100 °C as previously described. Control samples containing 250 μL of spinach homogenate mixed with 750 μL of appropriate buffer were incubated and heat-treated under the same conditions as the enzyme treated samples. The samples were then treated with conjugase for determination of total folate as previously described.

Comparison of single-, dual- and trienzyme treatments

To determine the most effective combination of enzymes for extraction of bound folate in spinach, optimal pH reaction conditions and incubation times determined in the previous experiments were used. Single-enzyme (conjugase alone), dual-enzyme (protease followed by conjugase) and trienzyme (α-amylase, protease, followed by conjugase) treatments were compared. Microbiological assay of folate in sample extracts was conducted as previously described.

For single-enzyme treatment, 200 μL of spinach homogenate was mixed with 950 μL of phosphate buffer (pH 7.0) and then incubated with 50 μL of conjugase at 37 °C for 3 h. A dual-enzyme treatment was conducted by mixing 250 μL of homogenate with 250 μL of buffer (pH 4.0) and 500 μL of protease and incubated at 37 °C for 8 h. The extract was heated to terminate the reaction and 200 μL of cooled extract was mixed with 950 μL of phosphate buffer (pH 7.0). Conjugase (50 μL) was added and the mixture was again incubated at 37 °C for 3 h.

The trienzyme treatment consisted of mixing 250 μL of spinach homogenate with an equal amount of citrate buffer (pH 3.0) and 500 μL of α-amylase followed by incubation at 37 °C for 6 h. After the extract was heated to terminate the reaction, 250 μL of cooled extract was mixed with 750 μL of citrate buffer (pH 4.0) and 500 μL of protease and then incubated at 37 °C for 8 h. The extract was heated again to terminate the reaction and 200 μL of cooled extract was mixed with 950 μL of phosphate buffer (pH 7.0). Conjugase (50 μL) was added and the mixture was incubated at 37 °C for 3 h.

Statistical analysis

Differences between means for *L. rhamnosus* growth response experiments and single-, dual- and trienzyme treatment evaluations were compared using a paired *t*-test (Minitab, Inc., State College, PA, USA). Optimum enzyme pH and incubation time were determined using one-way ANOVA and regression analysis, respectively, with Tukey's *t*-test used for comparison of means.

Results and discussion

Growth of *L. rhamnosus* in frozen cryoprotected cultures

Total yield of *L. rhamnosus* from cryoprotected cultures stored for up to 5 months at −70 °C is shown in Fig. 1. Microaerophilic enrichment of cryoprotected cultures in *Lactobacillus* AOAC broth resulted in approximately three times greater yield of *L. rhamnosus* compared to the standard method. Yields obtained using both culture methods decreased ($P \leqslant 0.05$) with increasing storage time. However, after 5 months at −70 °C, the microaerophilic enrichment method yielded over ten times the amount compared to the standard method. These results show that larger inoculum levels can be obtained from frozen cryoprotected cultures when they are cultured using a microaerophilic enrichment method compared to the standard method.

Bacteria may be injured by a variety of sublethal stress agents including freezing (Montville, 1997). Injured cells require a recovery period before rapid growth occurs, thus resulting in lower yields compared to uninjured cells. The lower yield of glycerol-cryoprotected cultures prepared using the standard method suggests a

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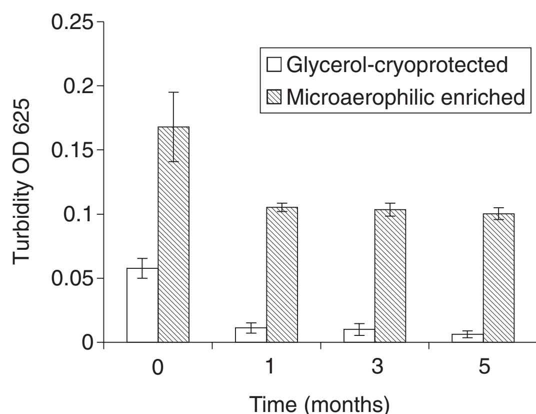

Figure 1 Comparison of growth response of cryoprotected cultures used for standard method to those enriched under microaerophilic environment.

period of recovery is required that limits the yield of viable bacteria in the inoculum and thus may decrease the growth response of *L. rhamnosus* to folate. Because *Lactobacillus* bacteria in general are nutritionally fastidious and preferentially grow in low oxygen and high carbon dioxide atmospheres (Kandler & Weiss, 2000), it is suggested that the greater yield of cells using the microaerophilic enrichment treatment is attributed to recovery of injured cells. The authors have successfully used this method to recover *L. rhamnosus* in glycerol-cryoprotected stock cultures for over 2 years with good results.

Folate analysis

Infant formula used as an internal standard contained 1.18 mg kg^{-1} and was within the certified value of 1.29 $\pm$ 0.28 mg kg^{-1} thus confirming the validity of the assay. Protease contained a negligible amount of endogenous folate (0.02 ng mg^{-1} of solid). However, α-amylase contained 0.93 ng folate per mg of solid and this value was subtracted from the final calculated level for folate in spinach.

Because the microaerophilic enrichment method resulted in a higher yield of bacteria compared to the standard method, it was necessary to determine the appropriate inoculum level that would result in the most sensitive assay. Growth response significantly increased ($P \leqslant 0.05$) when inoculum levels decreased from 150 to 50 μL (Table 1)

Table 1 Response of *Lactobacillus rhamnosus* to 5-formyl tetrahydrofolate standard at inoculum levels of 50, 100 and 150 μL

Inoculum level (μL)	Slope of the standard curve ± SD
50	0.47 ± 0.01
100	0.40 ± 0.02
150	0.32 ± 0.04

indicating a more sensitive assay at the lowest inoculum level. Although the growth response between inoculum levels of 30 and 70 μL was not significantly different ($P > 0.05$) (data not shown), a 50-μL inoculum level was chosen for subsequent assays to facilitate dilutions.

Optimization of enzyme reaction conditions

The effect of α-amylase and protease between pH 3.0 and 7.0 on measured folate is shown in Fig. 2.

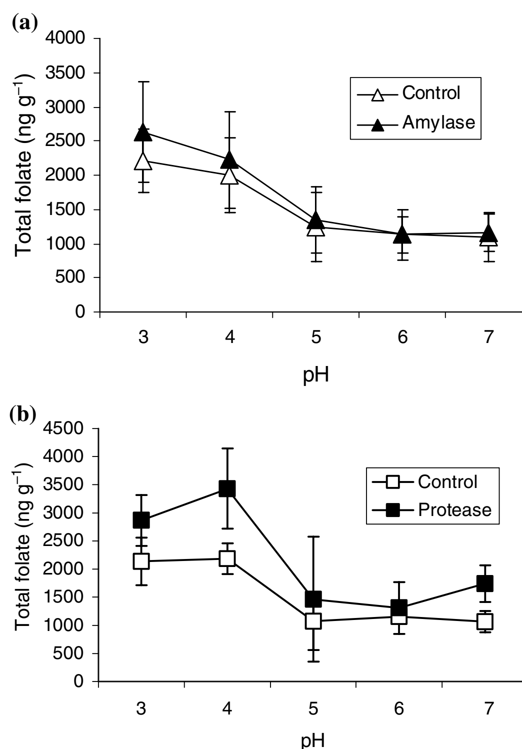

Figure 2 Effect of incubation pH for α-amylase (a) and protease (b) enzymes on folate content determined in fresh spinach ($T = 37$ °C). Each point represents the mean of three determinations ± SD.

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Folate measured in both control and α-amylase treated spinach samples increased ($P \leqslant 0.05$) as pH values decreased from 5.0 to 3.0 to a maximum level at pH 3.0 (Fig. 2a). However, folate values for α-amylase treated and control samples were not significantly different ($P > 0.05$) at all pH values. Although measured folate was not significantly affected by addition of α-amylase, an optimal incubation time of 6 h was determined by regression analysis of the combined data and this value was used in subsequent experiments where single-, dual- and trienzyme treatments were compared.

Measured folate in both control and protease treated spinach similarly increased ($P \leqslant 0.05$) with decreasing pH between 3.0 and 5.0 to a maximum level at 3.0 and 4.0 (Fig. 2b). However, in contrast to the results for α-amylase, protease at pH 3.0 and 4.0 significantly increased measured folate compared to the control. A pH of 4.0 for the protease reaction was chosen for subsequent assays because a smaller volume of buffer was needed to bring the extract to pH 7.0 for the reaction with conjugase.

Figure 3 shows the relationship between incubation time and α-amylase or protease activity under the optimal pH conditions previously determined for each enzyme. No significant differences ($P > 0.05$) were observed in folate determined with or without α-amylase between incubation times of 0 and 12 h (Fig. 3a), thus confirming the previous observation that release of bound folate in spinach is unaffected by α-amylase treatment. However, folate levels in protease treated spinach (Fig. 3b) did increase with increasing incubation time to a maximum of 8 h as determined by regression analysis.

Aiso & Tamura (1998) reported that both α-amylase and protease had little or no effect on the release of bound folate in spinach. Nevertheless, the authors proposed a trienzyme treatment using a pH of 6.0 for 1 h for α-amylase and a pH of 5.0 for 6 h for protease. Our results demonstrating a positive effect of protease on folate levels are not in agreement with the study by Aiso & Tamura (1998). The differences can be explained by variation in the composition of spinach samples in the two studies and thus the degree to which folate is bound to matrix constituents.

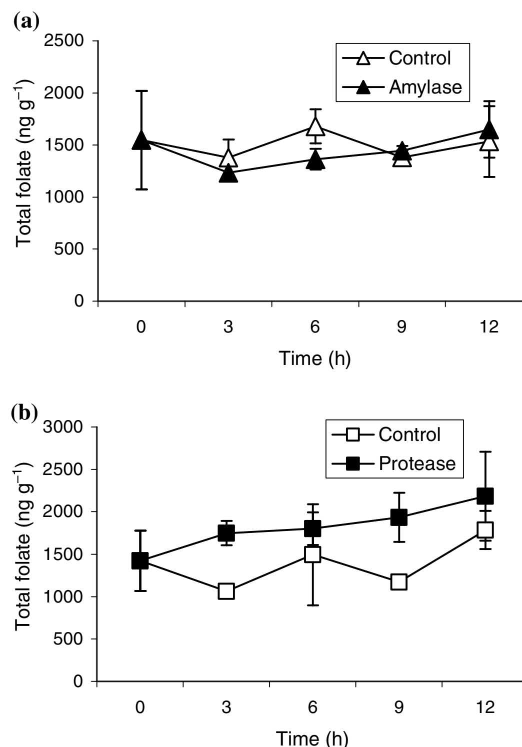

Figure 3 Effect of incubation time for α-amylase (a) and protease (b) enzymes on folate content determined in fresh spinach ($T = 37$ °C). Each point represents the mean of three determinations ± SD.

Comparison of single-, dual- and trienzyme treatments

A comparison of folate levels in spinach determined after single-enzyme (conjugase), dual-enzyme (protease + conjugase) or trienzyme (α-amylase + protease + conjugase) treatment is shown in Table 2. Our study showed that there was no difference in the measured folate between single- and trienzyme treatments. This result is

Table 2 Comparison of total folate determined in fresh spinach extracted with single-, dual- or trienzyme treatment before microbiological assay. Each value represents the mean of three determinations ± SD

Enzyme treatment	Total folate (ng g^{-1})
Conjugase only	1820 ± 560
Protease + conjugase	4100 ± 1370
Amylase + protease + conjugase	2450 ± 590

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confirmed by the findings of Shrestha *et al.* (2000) and Iwatani *et al.* (2003), who also reported no significant difference in the measured folate values in spinach treated by using either single- and trienzyme treatments.

The amount of folate measured in spinach using a dual-enzyme treatment (protease prior to conjugase) was over twice the amount determined using single-enzyme treatment (conjugase alone). However, folate values determined after trienzyme treatment were approximately 40% lower than that obtained using the dual-enzyme treatment. These results again confirm that protease is effective in releasing bound folate from spinach and that α-amylase is not effective. Moreover, the data show that the additional α-amylase incubation step as part of a trienzyme treatment decreased the amount of measured folate.

Variations in the amount of folate in spinach can occur depending on the cultivar used and growth conditions. Nevertheless, our data for conjugase-only extraction agree well with other reported literature values (1550–3020 ng g^{-1}) that were determined using a single-enzyme treatment (Babu & Srikantia, 1976; Perloff & Butrum, 1977; Klein and Kuo, 1981; Aiso and Tamura, 1998; Shrestha *et al.*, 2000; Iwatani *et al.*, 2003). However, the comparatively lower folate values for the trienzyme treatment compared to the dual-enzyme treatment found in this study are not consistent with earlier work by Aiso & Tamura (1998). In that study, the authors reported that a trienzyme treatment consisting of α-amylase (1 h at pH 6.0) and protease (6 h at pH 5.0) before conjugase resulted in higher folate values than dual-enzyme treatments (α-amylase + conjugase or protease + conjugase). Our trienzyme treatment, based on regression analysis of the data in Fig. 3a and 3b, included longer incubation treatments for α-amylase treatment (6 h) and protease (8 h). Because folates are subject to oxidative degradation during assay (Tamura, 1998), lower folate values for trienzyme treatment found in this study may be the result of the additional exposure to heat during α-amylase incubation with little or no gain in the release of bound folate. Shrestha *et al.* (2000), using breakfast cereals that are high in carbohydrates, similarly reported that a dual-enzyme treatment consisting of α-amylase followed by conjugase led to significantly higher values of folate compared to a trienzyme treatment. These results support their conclusion that optimal reaction pH, incubation time and choice of enzymes need to be evaluated for each product assayed in order to prevent underestimation of the vitamin.

Conclusions

Microaerophilic enrichment is an important step in folate analysis because it eliminates the problem associated with recovery of frozen cryoprotected microbial cultures stored for extended periods. Optimal extraction of folate in spinach samples was achieved by a dual-enzyme treatment consisting of a 37 °C incubation with protease for 8 h at pH 4.0 followed by treatment with conjugase at 37 °C for 3 h at pH 7.0. The release of bound folate from the sample matrix may be influenced by the chemical composition of the food and thus it is essential to determine assay parameters for individual foods.

References

Aiso, K. & Tamura, T. (1998). Trienzyme treatment for food folate analysis: optimal pH and incubation time for α-amylase and protease treatments. *Journal of Nutritional Science and Vitaminology*, **44**, 361–370.

Babu, S. & Srikantia, S.G. (1976). Availability of folates from some foods. *American Journal of Clinical Nutrition*, **29**, 376–379.

Doyle, M.P., Beuchat, L.P. & Montville, T. (1997). Microbial growth, survival, and death in foods. In: *Food Microbiology* (edited by M.P. Doyle, L.R. Beuchat & T.J. Montville). Pp. 23–29. Washington DC: ASM Press.

Green, R. & Jacobsen, D.W. (1995). Clinical implication of hyperhomocysteinemia. In: *Folate in Health and Disease* (edited by L.B. Bailey). Pp. 75–122. New York: Marcel Dekker.

Iwatani, Y., Arcot, J. & Shrestha, A.K. (2003). Determination of folate contents in some Australian vegetables. *Journal of Food Composition and Analysis*, **16**, 37–48.

Kandler, O. & Weiss, N. (2000). Regular, nonsporing gram-positive rods. In: *Bergey's Manual of Systematic Bacteriology* (edited by P.H.A. Sneath, N.S. Mair, M.E. Sharpe & J.G. Holt). Pp. 1208–1234. Baltimore: Lippincott Williams & Wilkins.

Klein, B.P. & Kuo, C.H.Y. (1981). Comparison of microbiological and radiometric assays for determining total folacin in spinach. *Journal of Food Science*, **46**, 552–554.

Martin, J.L., Landen, W.O., Soliman, A.G.M. & Eitenmiller, R.R. (1990). Application of a trienzyme extraction for total folate determination in foods. *Journal of Association of Official Analytical Chemists*, **73**, 805–808.

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Newman, E.M. & Tsai, J.F. (1986). Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. *Analytical Biochemistry*, **154**, 509–515.

Pederson, J.C. (1988). Comparison of γ-glutamyl hydrolase (conjugase; EC3.4.22.12) and amylasse treatment procedures in the microbiological assay for food folates. *British Journal of Nutrition*, **59**, 261–271.

Perloff, B.P. & Butrum, R.R. (1977). Folacin in selected foods. *Journal of American Dietetic Association*, **70**, 161–172.

Pffeifer, C.M., Rogers, L.M. & Gregory, J.F. (1997). Determination of folate in cereal–grain food products using tri-enzyme extraction and combined affinity and reversed-phase liquid chromatography. *Journal of Agricultural and Food Chemistry*, **45**, 407–413.

Rader, J.I., Weaver, C.M. & Angyal, G. (1998). Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal–grain products. *Food Chemistry*, **62**, 451–465.

Shrestha, A.K., Arcot, J. & Paterson, J. (2000). Folate assay of foods by traditional and tri-enzyme treatments using cryoprotected *Lactobacillus casei*. *Food Chemistry*, **71**, 545–552.

Tamura, T. (1990). Microbiological assay of folates. In: *Folic Acid Metabolism in Health and Disease*. *Contemporary Issues in Clinical Nutrition*, Vol. 13 (edited by M.F. Picciano, E.L.R. Stokstad, J.F. Gregory III). Pp. 121–137. New York: Wiley-Liss.

Tamura, T. (1998). Determination of food folate. *Journal of Nutritional Biochemistry*, **9**, 285–293.

Tamura, T., Mizuno, Y., Jonston, K.E. & Jacob, R.A. (1997). Food folate assay with protease, α-amylase, and folate conjugase treatments. *Journal of Agricultural Food Chemistry*, **45**, 135–139.

Wilson, S.D. & Horne, D.W. (1982). Use of glycerol-cryoprotected *Lactobacillus casei* for microbiological assay of folic acid. *Clinical Chemistry*, **5**, 1198–2000.

Yamada, M. (1979). Folate contents in milk. *Vitamins (Japanese)*, **53**, 221–227.

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