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Folate assay of foods by traditional and tri-enzyme treatments using cryoprotected *Lactobacillus casei*

Ashok K. Shrestha, Jayashree Arcot *, Janet Paterson

Department of Food Science and Technology, The University of New South Wales, Sydney 2052, Australia

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Abstract

Variables affecting the efficacy of the microbiological assay of folate in foods were examined. Spinach, fortified bread and two ready-to-eat breakfast cereals were extracted with or without autoclaving and centrifugation. Autoclaving and centrifugation lowered the yield of total folate in all foods. The food sample, after digestion with protease and α-amylase was deconjugated with chicken pancreas or human plasma (tri-enzyme treatment) or simply with conjugase alone (tradition single enzyme treatment). The tri-enzyme treatment was a significant improvement over the single enzyme treatment only in fortified bread. Deconjugation with chicken pancreas gave a significantly higher folate value than did human plasma in all foods except spinach. Folate assay by cryoprotected frozen *Lactobacillus casei* was compared with serially sub-cultured inocula. Using the cryoprotected frozen inoculum took a shorter time, was less tedious, gave better reproducibility and was more economical than using the conventional serial culture. The effects of the size of test tubes on the growth of culture and the wavelength at which turbidity was measured to achieve maximum detection were also investigated. *L.casei* grew faster in small tubes than in larger ones. The absorbance peak at 540 nm was higher than that at 620 nm.

Keywords: Folates; *Lactobacillus casei*; Cryoprotected culture; Tri-enzyme extraction

1. Introduction

'Folate' refers to a group of water-soluble vitamins that occur naturally in foods as various derivatives of folic acid, pteroyl glutamic acid (PGA). The most common natural folates are polyglutamates: tetrahydrofolic acid, 5-methyl tetrahydrofolic acid and 5-formyl tetrahydrofolic acid. Tetrahydrofolate, the biologically active form, is an essential coenzyme in the biosynthesis of nucleotides. Its deficiency reduces the concentration of nucleic acids for DNA synthesis, thus restricting cell division, causing congenital neural tube defects and megaloblastic anaemia. The increased plasma homocysteine concentration observed in folate deficiency causes arteriosclerosis. (Czeizel & Dudas, 1992; Medical Research Council [MRC] Vitamin Group, 1991; Selhub et al., 1995).

The assay of folates from foods generally involves three steps: liberation of folates from the cellular matrix; deconjugation from the polyglutamate to the monoglutamate forms; and the detection of the biological activity or chemical concentration of the resulting monoglutamate forms, mostly as PGA. Incomplete liberation from the cell matrix results in a low estimate of folates. Hence an increase in the apparent folate from a sample food is taken to indicate an improved assay method.

In the most common method for determining food folate activity deconjugation is achieved using γ-glutamyl hydrolase (EC 3.4.19.9) (also referred to as conjugase), most commonly from chicken pancreas or hog kidney. Conjugase from human and rat plasma, rat pancreas and rat liver have been used to a lesser extent (Keagy, 1985). The most common detection method is a microbiological assay relying on the turbidimetric bacterial growth of *Lactobacillus casei* (ATCC 7469) (Hawke & Villota, 1989).

There have been several improvements to the standard method. A number of researchers have reported that treatment with folate conjugase alone is usually insufficient to liberate folates bound to complex food matrices. Treatment of foods high in protein with protease and treatment of foods high in starch and glycogen with

* Corresponding author. Tel.: +61-2-9385-5360; fax: +61-2-9385-5931.

E-mail address: j.arcot@unsw.edu.au (J. Arcot).

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α-amylase, in addition to folate conjugase (the tri-enzyme treatment), result in a significant increase in the apparent total folate concentration in foods (Cerna & Kas, 1983; DeSouza & Eitenmiller, 1990; Martin, Lauden & Soliman, 1990; Pederson, 1988). Similarly Tamura, Mizano, Johnson and Jacob (1997), Pfeiffer, Rogers and Gregory (1997), Rader, Weaver and Angyal (1998) and Aiso and Tamura (1998) reported a significant increase in total folate content in a range of foods treated with three enzymes compared with the values from folate conjugase treatment alone. These recent findings provide strong evidence for the need of tri-enzyme treatment in folate analysis. Relatively few studies have been reported on the use of human plasma conjugase, despite its advantages over conjugase from other sources. It is cheaper, is easily available, is required in a small quantity, contains very low endogenous folate and produces the monoglutamate form (Goli & Vanderslice, 1992). The time and labour required for the maintenance and standardisation of the *L.casei* culture and the variability between inocula have been reduced by the development of a cryoprotected frozen culture (Grosswicz, Waxman & Schreiber, 1981; Wilson & Horne, 1982). The objectives of the present investigation were to determine the effect: of cryoprotected culture versus conventional serial culture as inoculum; of autoclaving and centrifugation in extraction; of using the traditional single enzyme treatment or the tri-enzyme treatment; of deconjugation with chicken pancreas deconjugase or human plasma; of varying assay tube size; and of measuring turbidity at 620 or 540 nm, in folate analysis in a variety of foods.

2. Materials and methods

2.1. Enzymes

Desiccated chicken pancreas conjugase (No. 0459-12-2, Difco Laboratories, Detroit, MI 48232-7058) solution was prepared according to Kirsch and Chen (1984) (5 mg/ml). α-Amylase (A-3176, Sigma Chemical Co., St. Louis, MO 63178) (20 mg/ml) and protease (Megazyme, subtilisin A from *B. licheniformis*) (2 mg/ml) were prepared according to Rader et al. (1998).

2.2. Inoculum preparation

Cryoprotected *Lactobacillus casei* subsp. Rhamnosus (ATCC 7469) was obtained from the Department of Microbiology, The University of New South Wales, Sydney, Australia. Inoculum for the traditional microbial assay was prepared using serial transfer of the organism as described by Phillips and Wright (1982). Glycero-cryoprotected *L. casei* was also prepared as described by Wilson and Horne (1982).

2.3. Sample extraction

Fresh spinach (*Spinacia oleracea*) was purchased from a local supermarket. The vegetable was washed, patted dry, and stems removed. A sample of exactly 10 g fresh leaves was homogenized in a Waring blender with 100 ml of extraction buffer (0.1 M potassium phosphate, 1% ascorbic acid and pH 6.1). The homogenate was autoclaved at 121°C for 10 min, immediately cooled, and centrifuged at 1000 *g* for 15 min. Aliquots of supernatant (10 ml) were stored at −18°C (autoclaved sample). The same sets of sample were also extracted with neither autoclaving nor centrifugation (unautoclaved sample). Fortified white bread, breakfast cereal I (whole grain wheat cereal) and breakfast cereal II (oat, corn and wheat cereal) were purchased from the local supermarket. Ten grams each of the samples were also extracted using the autoclaved and unautoclaved procedures and stored at −18°C.

2.4. Determination of conjugase concentration

The unfortified durum wheat semolina obtained from Vetta Company, Sydney, Australia, was used for the determination of the optimum conjugase concentration needed to liberate folate from the food. Pooled human plasma obtained from the Prince of Wales Hospital, Sydney, Australia, was stored at −18°C in 10-ml aliquots. The same lot of chicken pancreas conjugase and human plasma were used to avoid the possible error caused by variation between batches.

2.4.1. Chicken pancreas concentration

Chicken pancreas (0.2, 0.5, 1.0 and 1.5 ml) preparation (5mg/ml) were added to 1 ml of sample extract and the volume was adjusted to 10 ml using phosphate ascorbate buffer, pH 7.2. The mixture was incubated at 37°C for 3 h. The samples were heated at 100°C for 5 min to de-activate the enzyme, cooled and analyzed by microbial assay.

2.4.2. Human plasma concentration

For human plasma, 2.75 ml of phosphate ascorbate buffer at pH 4.5 and 1 ml of 100 mM mercaptoethanol were incubated at 37°C for 10 min. 1 ml of sample extract and 0.1, 0.25, 0.5 and 1.0 ml of human plasma were added to the buffer and incubated at 37°C for 3 h. The enzyme was de-activated, cooled, diluted to 10 ml using the same buffer and assayed for folate.

2.5. Tri-enzyme treatment — hydrolysis

The pH of the extract was first adjusted to 4.5 using 0.1 M HCl. To a 10 ml sample, 1.6 ml of protease preparation was added and incubated at 37°C for 16 h. The reaction mixture was heated for 5 min in a boiling water

bath to stop enzymatic activity. It was further treated with 1.6 ml of α-amylase for about 4 h at 37°C.

2.6. Tri-enzyme treatment — deconjugation

The pH of enzyme hydrolysed extract was adjusted to 7.2 and 10 ml of hydrolysed extract was treated with 1.0 ml of chicken pancreas and incubated at 37°C for 3 h (Rader et al., 1998). The deconjugated extract was heated in a boiling water bath for 5 min, cooled, centrifuged at 1000 g for 10 min and the supernatant was stored at −18°C. The tri-enzyme treatment was also carried out using human plasma for deconjugation. Food extracts were first digested with protease and α-amylase. Before deconjugation, 2.75 ml buffer (0.1 M potassium phosphate, 1% ascorbic acid, pH 4.5) and 1 ml of 100 mM 2-mercaptoethanol were dispensed into test tubes and incubated at 37°C for 10 min. The sample extracts (1 ml) and 0.25 ml of human plasma were added to the buffer and mixed in a vortex mixer (Goli & Vanderslice, 1992). The deconjugation mixture was incubated at 37°C for 3 h in a water bath and the enzymatic reaction was stopped by boiling for 5 min. The pH was adjusted to 6.0 and the sample was diluted to a volume of 10 ml using extraction buffer. The mixture was centrifuged at 1000 *g* for 10 min to remove any suspended solids and the deconjugated extracts were stored at −18°C before use.

2.7. Single enzyme treatment

Food samples were also deconjugated by the traditional single enzyme method using chicken pancreas (Phillips & Wright, 1982) or human plasma as a source of conjugase (Goli & Vanderslice, 1992), using the same combination of conjugase and sample extract as in tri-enzyme treatment. Extraction did not involve protease and α-amylase.

2.8. Folate assay

Folic acid (F-7876, Sigma Chemical Co., St. Louis, MO, 63178) standard solutions and preparation of standard and sample tubes were done according to Keagy (1985). A suitable volume of inoculum (50 μl) was added to standard and sample tubes and incubated at 37°C for 16–18 h. Turbidity was measured as absorbance value in a spectrophotometer (UV-1601, Shimadzu Corporation, Kyoto, Japan) set at wavelength 540 nm.

2.9. Effect of test tube size and wavelength

The effect of test tube size (12×110 mm versus 16×150 mm) on the growth of the organism and the response of two different wavelengths, 540 and 620 nm, to the turbidity measurement in the given set of conditions were also checked for optimization of the method.

To avoid any contamination with bacteria, which may synthesize folate during the lengthy incubation period, test tubes were cleaned with detergent and antibacterial liquid and dried at 150°C and fresh distilled water was used throughout the assay.

2.10. Quality control

Preliminary analysis showed that protease and human plasma contained negligible amounts (2.6 ng/ml and 15 ng folic acid per ml of plasma) of endogenous folate but α-amylase and chicken pancreas contained significant amounts: 690 and 8300 ng folate per gram of solid enzyme, respectively. Hence, enzyme blanks were analyzed along with the samples and the endogenous folate values were subtracted from the calculated folate when these enzymes were present in the assay. Each microbial assay was accompanied by recovery studies. The assay with recovery level below and above the range of 95–105% was not considered for the study and simply discarded. A Standard Reference Material, SRM 1846, was obtained from NIST for quality control, and analyzed for total folate along with other samples.

2.11. Statistical analysis

Five replicates were carried out for each determination. Data were analysed using the Analysis of Variance (ANOVA) by Statgraphics package (Statistical Graphics Corporation, 1993, Manguistics Inc., USA). The multiple range test LSD (Duncan multiple range test), with significance level at $P < 0.05$, was applied to the results to test the significance.

3. Results and discussion

3.1. Inoculum preparation and testing

Fig. 1 shows the curves for assay of standard folic acid (PGA) solution with either *L. casei* maintained by conventional serial transfer method or cryoprotected method. These assays were done at different intervals of time but finished within one month of commencement (the values presented in the figure are average of 14 assays by cryoprotected method and 12 assays by conventional serial dilution). The blank value was lower, the assay response was higher and the standard deviation was lower with cryoprotected *L. casei* than with the conventionally prepared inoculum: the cryoprotected culture method was more sensitive and reproducible. Moreover, the use of cryoprotected culture reduced the chances of contamination and reduced the time required for each assay for culture preparation from 72 to 24 h, and proved cheaper too. The cryoprotected frozen culture was found to be viable for 3 months.

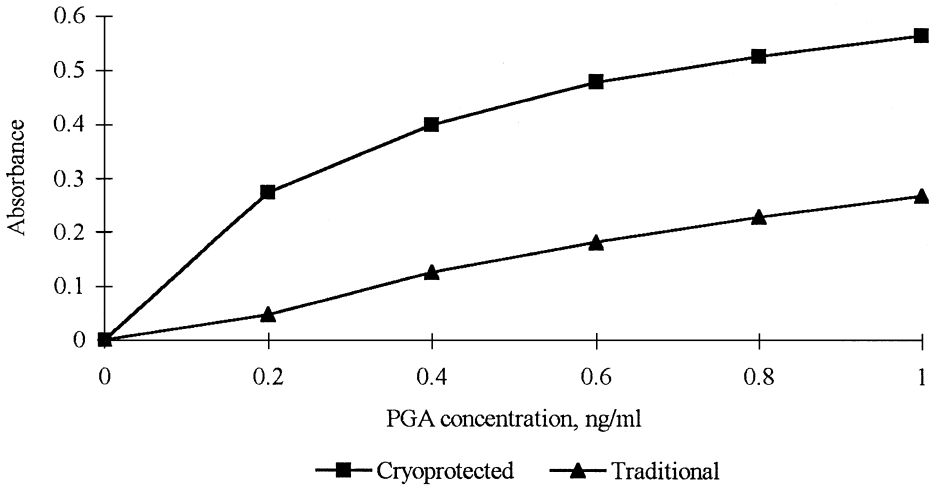

Fig. 1. Comparison of standard curve from traditional serial dilution and cryoprotected culture of *L. casei*.

3.2. Concentration of conjugase preparation

The optimization study showed that deconjugation of the semolina extract using 1 ml (5 mg) of chicken pancreas preparation was sufficient to liberate folate from the food (Table 1) under the given condition. Effect of length of time on liberation of folate for unfortified semolina was not studied as Rader et al (1998) have already shown there was no increase in folate value when incubation time increased from 2 to 4 h. In this study an average 3 h incubation time was used. Human plasma at 0.25 ml level liberated the highest amount of folate (Table 1). Therefore, 1 ml chicken pancreas preparation and 0.25 ml human plasma were used throughout the study.

The Standard Reference Material, SRM 1846, a milk based infant formula, was analyzed by the tri-enzyme procedure using chicken pancreas as conjugase. The folate content of the reference material was found to be 1.53±0.2 mg per kg, which was within the certified range of 1.29±0.28 mg per kg.

Table 1
Folate contents (μg/100 g) of durum semolina using varying amounts of chicken pancreas and human plasma

Chicken pancreas[a] (5 mg/ml)	Human plasma[a]
25.0 (0.0)	25.0 (0.0)[c]
(22.5–27.5)	(22.5–27.5)
26.5 (0.2)[b]	25.7 (0.1)
(25.2–27.8)	(23.8–27.5)
36.5 (0.5)	29.0 (0.25)
(33.7–38.3)	(25.9–32.1)
50.9 (1.0)	27.5 (0.5)
(44.8–57.0)	(25.6–29.4)
49.9 (1.5)	27.5 (1.0)
(44.0–55.8)	(30.5–24.5)

[a] Values are means of duplicate analyses.
[b] The value in parenthesis indicates the amount in mL of conjugase used.
[c] 0 value in the parenthesis indicates undeconjugated samples.

3.3. Effect of heating and centrifugation

Heating and centrifugation of raw extract before tri-enzyme extraction caused significant loss of folates in most of the samples (Figs. 2 and 3). Tamura et al. (1997) also reported higher folate values when conjugase treatment was carried out before the heat treatment and centrifugation of the food mixture. The removal of undigested food residue after centrifugation is believed to be responsible for the loss in folate as the residue could have entrapped some folate.

3.4. Comparison between single and tri-enzyme methods

The tri-enzyme extraction did not necessarily give higher folate than did the single enzyme method (traditional method). Whether human plasma or chicken pancreas conjugase was used, the necessity for α-amylase and protease depended on the type of food matrix. In spinach, tri-enzyme (unautoclaved) and single enzyme treatment yielded significantly ($P < 0.05$) higher folate value than tri-enzyme (autoclaved) treatment with chicken pancreas as a source of conjugase (Fig. 2). With human plasma as conjugase, there was no significant difference ($P < 0.05$) in folate values between single enzyme and tri-enzyme (autoclaved) treated spinach samples but both were significantly lower than unautoclaved tri-enzyme treated sample (Fig. 3). Martin et al. (1990) and Aiso and Tamura (1998) reported a 22.3 and 51% increase in folate value in spinach respectively, when tri-enzyme treatment was compared with single enzyme method.

In bread, the apparent folate value obtained from tri-enzyme treated unautoclaved sample was significantly ($P < 0.05$) higher than single-enzyme treated sample, with both sources of conjugase (Figs. 2 and 3). The

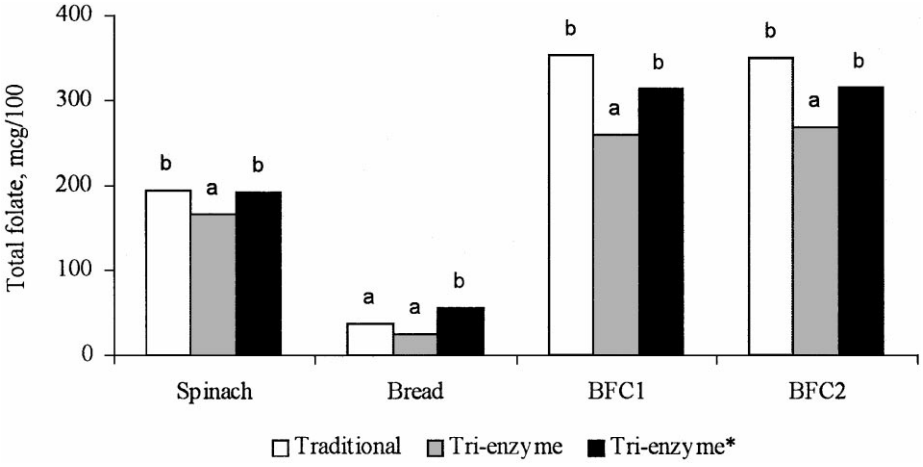

Fig. 2. Total folate content (mg/100 g) of foods using chicken pancreas as conjugase. Significance of LSD test of treatment mean $P < 0.05$. For a particular food, means followed by different superscripts are significantly different. BFC1 and BFC2 are breakfast cereals 1 and 2, respectively. * Indicates extract without autoclaving and centrifugation.

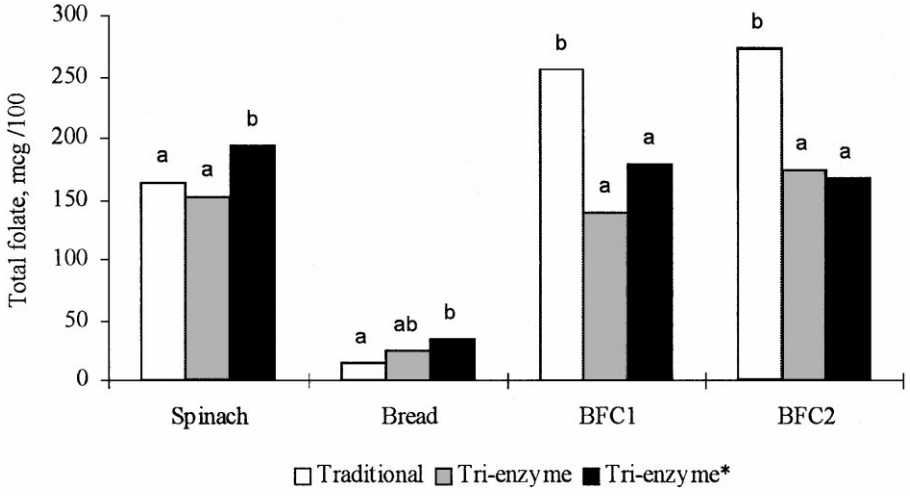

Fig. 3. Total folate content (mg/100 g) of foods using human plasma as conjugase. Significance of treatment means at $P < 0.05$. For a particular food, means followed by different superscripts are significantly different. BFC1 and BFC2 are breakfast cereals 1 and 2, respectively. * Indicates extract without autoclaving and centrifugation.

increase in folate value following tri-enzyme extraction for bread was 130% with human plasma and 47% with chicken pancreas. Aiso and Tamura (1998) reported 57% increase in folate value in white bread, after tri-enzyme extraction using rat plasma as conjugase at pH 7.0.

In breakfast cereals, there was significant ($P < 0.05$) decrease in folate, in samples extracted with the tri-enzyme method in all, except the unautoclaved samples deconjugated with chicken pancreas where the apparent decrease was not significant (Figs. 2 and 3). There was no consistent effect of tri-enzyme treatment on folate content of enriched breakfast cereals noticed, as the single enzyme method yielded more folate from the food matrices tested than did the tri-enzyme method. Previous research by Tamura (1998) also revealed that it might not always be necessary to perform all three enzyme treatments for every single food item. Rader et al. (1998) also reported an insignificant difference in folate content when a number of folate fortified foods including bread and ready-to-eat breakfast cereals were analysed by single enzyme and tri-enzyme methods using chicken pancreas conjugase. It may also be true that the folic acid added externally (as in fortified foods) might have mixed only physically and hence not be bound to protein and carbohydrate; hence enzymatic treatment may not be required.

Previous studies on the effect of tri-enzyme treatment of foods showed variable increase in folate values. Tamura et al. (1997) reported approximately 271% increase in mean folate content for food composites analyzed by tri-enzyme method as compared with the values obtained by using folate conjugase alone. Rader et al. (1998) reported a 12% increase (average value of

Table 2
Total folate content (μg/100 g) of foods human plasma or chicken pancreas conjugase[a,b]

Samples	Traditional method[c]	Tri-enzyme method	Tri-enzyme method[d]
Spinach			
Human plasma	164±16m	152±14m	194±10m
Chicken pancreas	193±19m	164±23m	192±21m
Bread			
Human plasma	15±5a	25±5a	35±10a
Chicken pancreas	37±3b	25±7a	55±11b
Breakfast cereal 1			
Human plasma	256±27p	140±30p	179±20p
Chicken pancreas	354±11q	260±47q	313±13q
Breakfast cereal 2			
Human plasma	274±26x	173±22x	168±16x
Chicken pancreas	353±20y	269±31y	315±33y

[a] Values are mean ± standard deviation, based on 10 replicate determinations.
[b] Significance of LSD test of treatment means, at $P < 0.05$.
[c] Within columns for a particular food, means followed by different letters are significantly different.
[d] Indicates extraction without autoclaving and centrifugation.

37 different foods) and Martin et al. (1990) a 19% increase (average value of 12 different foods) in folate values when tri-enzyme method was applied in place of conjugase only.

However, literature values are not directly comparable with the values obtained from the present analyses, as there are differences in the steps of deconjugation, type of conjugase, incubation time, enzyme concentration and type of foods analysed. As the folic acid assay is highly method-dependent, an alteration of pH, buffer, incubation time and temperature, type and time of enzyme and conjugase addition can affect the apparent folate content. Although pH 4.5 is used for the tri-enzyme treatment, especially for protease and α-amylase, it is not known whether this is the pH optimum for the enzymes, as the affinity of the enzymes to substrates may be different, depending on the food items. Birktoft and Breddam (1994) and Aiso and Tamura (1998) stated that the optimal pH for enzyme treatment might be different for each type of food and recommended the identification of the optimum pH and a suitable incubation time for each food.

3.5. Comparison of conjugase sources

Conjugase from chicken pancreas, deconjugated folate from the given food products more effectively than did human plasma. Using the single enzyme method, chicken pancreas conjugase yielded significantly ($P < 0.05$) more folates than human plasma in all foods except spinach (Table 2). When the tri-enzyme method was used, chicken pancreas conjugase yielded significantly ($P < 0.05$) more folates than human plasma in all foods except spinach and one of the bread samples.

Several factors could have caused lower folate values in food samples deconjugated with human plasma. Lakshmaiah and Ramasastri (1975 a and b) recommended that pH 4.5 be maintained because the plasma conjugase has maximum activity at this pH. But, at this pH, folate is less stable (Keagy, 1985; Lund, 1994). Goli and Vanderslice (1992) reported a considerable loss of folate during deconjugation with human plasma at pH 4.5 compared with pH 6.0–7.0. Plasma folate conjugase activity can also be inhibited by urea or uric acid, sulphates, various urine metabolites and inhibitors from legumes (Livant et al., 1994).

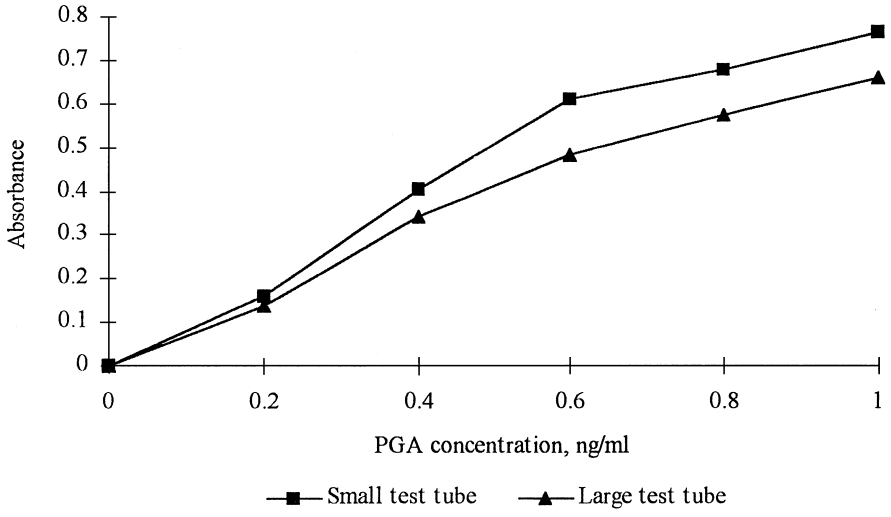

Fig. 4. Effect of test tube size on the growth of *L. casei*. Value expressed are menas of 4 replicate determinations.

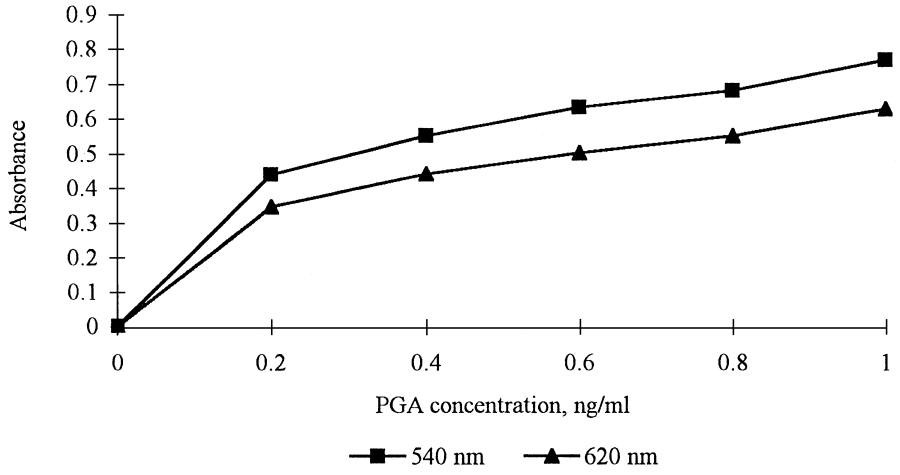

Fig. 5. Effect of wavelength on turbidity measurement of incubated media. Values expressed are means of 4 replicate determinations.

3.6. *Effect of test tube size and wavelength*

The conventional method uses larger volume of prepared media (5 to 10 ml) and bigger test tubes (e.g., 14×150 mm, 18×150 mm) (AOAC, 1995; Keagy, 1985; Kirsch & Chen, 1984; Pederson, 1988, method 992.05). Because of the cost of the medium, the volume of assay medium was lowered to 3 ml without noticeable effect on the growth of *L. casei* (result not shown). However, the changes in size of test tubes, 12×110 mm to 16×150 mm, had an effect on growth of the organism. The growth was slightly higher in smaller tubes than in larger ones (Fig. 4). Correlation with absorbance at 540 nm was less variable with the small tubes ($R^2 = 0.981$), than with the large tubes ($R^2 = 0.897$) (in log scale).

A range of wavelengths: 540 and 700 nm (Keagy, 1985; Wilson & Horne, 1982), 620 nm (Begum, Paterson & Arcot, 1997), 640 nm (Kirsch & Chen, 1984), 650 nm (Pederson, 1988), 660 nm (Klein & Kuo, 1981; Ruddick, Vanderstoep & Richards, 1978) have been used to record the turbidity of incubated media. The absorbance value of incubated media was consistently higher at 540 than at 620 nm (Fig. 5) and hence was the wavelength used for the assays.

4. Conclusions

Cryoprotected frozen *L. casei* inoculum was a more sensitive, quicker and reliable detector of folate than the *L. casei* culture prepared by the serial dilution method. Autoclaving and centrifugation of food mixture caused an under-estimation of folate in foods. The tri-enzyme treatment was not necessarily the best method for folate extraction for all foods; for fortified breakfast cereals, the single enzyme method was more suitable. Variations in the source of conjugase significantly affected the apparent folate content of foods: chicken pancreas yielded more folate than human plasma. The optimal pH for enzyme treatment might be different for each type of food. Further studies are needed to determine appropriate conditions for each type of food; otherwise incomplete hydrolysis with resulting under-estimation of folate content could easily occur.

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