



Original article

Testing of folate conjugase from chicken pancreas vs. commercial enzyme and studying the effect of cooking on folate retention in Thai foods

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ABSTRACT

Crude enzyme from chicken pancreas as a source of folate conjugase was prepared in a lyophilised form. Homogeneity, stability and activities were checked against a commercial enzyme. Subsequently, the prepared crude enzyme was used to investigate the process of folate extraction in various food matrices and study the effect of cooking on folate retention in several Thai foods. The lyophilised enzyme was homogeneous and contained 4 µg endogenous folate per g of prepared lyophilised crude enzyme. The stability of the lyophilised enzyme and the diluted enzyme, kept at 4 °C, was at least 12 months and 3 days, respectively. The activity of the prepared lyophilised folate conjugase, used at the level of 20 mg/g sample, was evaluated by a microbiological assay of total folate in brown rice, egg, whole milk powder, soybeans and asparagus. It showed comparable results to that of the commercial enzyme. Single-enzyme treatment (folate conjugase alone) was sufficient for the determination of folate content in brown rice, soybeans and asparagus. However, for egg and whole milk powder, it was necessary to apply tri-enzyme treatment as this showed a significantly higher level (at $P < 0.05$) of total folate than that obtained using only single- or di-enzyme (protease + folate conjugase) treatments. Among the representative foods studied, the levels of total folate ranked in order of concentration, were soybeans > egg > asparagus > brown rice > steamed-mackerel (common form sold) (305, 117, 95, 41 and 24 µg/100 g wet weight basis, respectively). After cooking, the true retention of total folate in the cooked foods ranged from 60% in brown rice (cooked in an electric rice cooker) up to 82% in boiled asparagus. On a wet weight basis, the total folate content in boiled soybeans, boiled asparagus, boiled egg, fried mackerel and cooked brown rice was 100, 84, 72, 22 and 12 µg/100 g, respectively. One boiled egg (50 g) contributes 36 µg folate, which is equal to 18% of the Thai Recommended Daily Intake (RDI), and thus it can be considered as a good source of folate based on one serving size. A serving of boiled soybeans (70 g) or boiled asparagus (80 g) is an excellent source of folate, each contributing about 35% of the Thai RDI.

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1. Introduction

Folate is essential for purine and DNA synthesis, amino acid metabolism and formate oxidation in mitochondria and cytosol (Bowman and Russell, 2001). It has been well recognised that adequate folate intake plays a significant role in the prevention of neural tube disorders (Medical Research Council (MRC), Vitamin Study Research Group, 1991; van der Put et al., 2001; Mattson and Shea, 2003). Folate deficiency has been linked to elevated plasma homocysteine, which is an established independent risk factor for vascular disease morbidity and mortality (Refsum et al., 1998; Mattson et al., 2002; Moleerergpoom et al., 2004). Nowadays folic acid fortification in various food products such as milk powder, bread, cereal products, weaning foods, macaroni and so on has

become more popular in the world market (Dunn et al., 1999; Kloeblen, 1999; Rader et al., 2000; Arcot et al., 2002a; Pawlosky et al., 2003). Many studies have found that folate supplementation, either from foods or fortified products, protects against neural tube defects (Scott et al., 1995; Botto et al., 1999; Berry et al., 1999).

It is well recognised that folate data available in different databases are of different quality because they result from different methods for folate extraction and determination. This can lead to unreliable estimation of dietary folate intake (Bailey, 1995; Tamura, 1998; Arcot and Shrestha, 2005). Various aspects of folate determination in foods were extensively reviewed by Hyun and Tamura (2005) and Arcot and Shrestha (2005). The analytical process involves liberating folate from food matrices, deconjugation of folate polyglutamates to mono- or diglutamyl forms and then measuring total folate or their vitamers. Several methods have been used for the quantitative determination of folate, such as microbiological assay (AACC Method, 2000; AOAC, 2006), HPLC (Vahteristo et al., 1997; Stokes and Webb, 1999; Ndaw et al., 2001; Jastrebova et al., 2003;

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Doherty and Beecher, 2003; Ginting and Arcot, 2004), liquid chromatography–mass spectrometry (Stokes and Webb, 1999; Pawlosky et al., 2003; Leporati et al., 2005; Zhang et al., 2005; Alaburda et al., 2008), enzyme protein binding assay (EPBA) (Finglas and Morgan, 1994; Arcot et al., 2002a), and enzyme linked immunosorbent assay (ELISA) (Alaburda et al., 2008). Among these methods, the microbiological assay is the most widely used procedure for measurement of total folate in foods (Puwastien et al., 2005; Arcot and Shrestha, 2005; Hyun and Tamura, 2005). Several studies found that folate extraction by tri-enzyme treatment (α -amylase, protease and folate conjugase) is more effective than single-enzyme folate conjugase treatment alone (Johnston et al., 2002; Yon and Hyun, 2003; DeVries et al., 2005). However, Puwastien et al. (2005) reported in their international laboratory performance study that only nine out of 20 laboratories used tri-enzyme treatment. The limited use of the tri-enzyme treatment for folate extraction is likely due to the high cost of the required enzymes, especially for laboratories in developing countries where resources are limited and the procedure is time-consuming and labour-intensive.

Chicken pancreas is one source of folate conjugase (pteroylpolyl-glutamyl hydrolase), which is the main enzyme required for the deconjugation of polyglutamate to the monoglutamate form, prior to total folate determination (Tamura, 1998). At present, the cost of the commercial desiccated chicken pancreas is increasing and often it is not available in the market. The pancreas is attached to part of the intestine that is normally discarded. Owing to the scarcity of commercial desiccated chicken pancreas, and to minimize the need for the imported enzyme, the preparation of lyophilised crude enzyme to be used as the source of folate conjugase was one of the objectives of this study. The characteristics of the prepared lyophilised enzyme, in terms of homogeneity, stability, activity and validity (compared to the commercial enzyme) were evaluated. Since crude enzyme from chicken pancreas contains not only the folate conjugase but also other enzymes such as protease and amylase, extraction of folate in various food matrices using single-, di- and tri-enzyme treatments was investigated. Previous studies by Areekul (1984) and by the Ministry of Public Health, Thailand (2006) analysed folate in raw foods using radiobinding assay and microbiological assay, respectively; they applied only single-enzyme extraction. Thus, another objective of this study was to determine the retention of folate in several foods after applying household cooking techniques, using tri-enzyme for sample treatment.

2. Materials and methods

2.1. Preparation and testing of folate conjugase from lyophilised chicken pancreas

2.1.1. Preparation of lyophilised crude enzyme from chicken pancreas

Chicken pancreas was purchased from a slaughterhouse supplied with birds from closely controlled indoor chicken houses. It was washed in tap water twice, rinsed in distilled water and then ground in a meat mincer. Then the crude enzyme was extracted by grinding with an equal volume of 0.2 M phosphate buffer, pH 7.2, using a glass mortar (Augustin et al., 1985). The tissue was separated by filtering through a double layer of cheesecloth and rewashed with the phosphate buffer. The obtained suspension was thoroughly mixed for about 1 h at low temperature (3–5 °C) using a magnetic stirrer at low speed. Aliquots of 5 ml of crude enzyme were packed into vaccine vials and lyophilised. To prevent contamination and moisture absorption, the vials were immediately sealed with rubber and aluminium caps and kept at 3–5 °C.

2.1.2. Homogeneity evaluation of the lyophilised crude enzyme

Seven vials of the prepared lyophilised crude enzyme were selected at random. Homogeneity was checked by microbiological

assay, in a random order, of total endogenous folate using *Lactobacillus casei*. Each vial was analysed in duplicate and all as one set under repeatable conditions, on the same day using the same reagents and conditions. The results obtained were statistically evaluated without removal of any values.

2.1.3. Stability evaluation of the lyophilised crude enzyme

The stability test of the crude enzyme during storage at 3–5 °C was performed in both forms—the lyophilised form during storage for 0, 1, 3, 6 and 12 months and the reconstituted form during storage for 0, 1, 3 and 5 days. The latter samples were prepared as an example of leftover enzyme solution. Five vials of the enzyme were randomly selected at each storage period. Each vial was used for a single analysis of folate in the in-house quality control samples (whole milk powder) by microbiological assay. If the enzyme was stable, the levels of total folate obtained should fall within the range of mean $\pm$ 2SD of total folate in whole milk powder at 0 day or 0 month.

2.1.4. Validation testing of folate conjugase activity in the lyophilised crude enzyme against the commercial enzyme

Folate conjugase activity in the lyophilised crude enzyme was validated against the commercial enzyme (Difco Co. Ltd., Sparks, MD, USA). The enzyme from the two sources was used to analyse total folate in five different food matrices by microbiological assay using single-enzyme extraction. The selected foods were whole milk powder, brown rice, egg, soybean and asparagus. About 500 g of each food was purchased from a representative market. Edible portions of the selected foods were prepared and homogenised. Six individual test portions of each food were sampled for total folate analysis. Three individual portions were treated with the prepared lyophilised crude enzyme and another set of three with the commercial enzyme. The amount of the lyophilised or the commercial enzyme used was 20 mg/g sample. To ensure the presence of conjugase activity in the lyophilised crude enzyme, non-deconjugated folate in the test materials was also determined.

2.2. Investigation of enzyme treatment for folate extraction from various food matrices

A second prepared set of common food commodities, different from those used in the enzyme validation study above, was used as the test materials for folate determination using single-, di- and tri-enzyme treatments for folate extraction. These were: brown rice as a representative for the cereal group, chicken egg for the egg group, asparagus for the vegetable group, whole milk powder for the dairy group and soybean for the legume, nut and seed group. Single- (folate conjugase from chicken pancreas) and tri-enzyme treatments (protease, amylase and folate conjugase; AACC Method 86–47, 2000) were applied to all the foods, and the di-enzyme treatment with amylase or protease, as appropriate, plus folate conjugase, was applied according to the main component of the test foods. For example, brown rice contains mainly starch, hence α -amylase and folate conjugase were used; whereas egg, milk and soybean contain high protein, so protease and folate conjugase were used. Asparagus contains negligible amounts of protein and starch, so the di-enzyme extraction was not conducted. For each food, three sub-samples were taken for each of the three treatments, followed by microbiological assay. The levels of total folate obtained are presented as mean $\pm$ SD ($n = 3$).

2.3. Effect of household cooking on folate retention in common Thai foods

Selected representative raw foods used to investigate the retention of folate after household cooking were brown rice, egg, soybean and asparagus. Steamed-mackerel, which is the common

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