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Folic acid stability in hydrogen peroxide–potassium thiocyanate-treated milk

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Hydrogen peroxide, individually, or in combination with potassium thiocyanate, inactivated milk folic acid (free and total) significantly. Addition of hydrogen peroxide to milk caused inactivation of folic acid added to milk and this inactivation was prevented by the addition of potassium thiocyanate. Potassium thiocyanate *per se* did not inactivate folic acid in milk. Lactoperoxidase, in the presence of hydroperoxides or GSH-Mn(II), was also found to degrade folic acid *in vitro*. Potassium thiocyanate and high concentrations of hydroperoxides inhibited this degradation. The role of lactoperoxidase, hydroperoxides and potassium thiocyanate (antimicrobial system) on the folic acid stability in milk is discussed.

INTRODUCTION

Hydrogen peroxide is a strong oxidising agent that is widely used in food processing and preservation; for example, in the improvement of the solubility of oil seed protein in aqueous or acidic media (Jacks *et al.*, 1983), in the treatment of rape-seed flour to reduce glucosinolate content (Anderson *et al.*, 1975), in the elimination of typical moulds (*Aspergillus*, *Penicillium* and *Rhizopus*) on Polish brewing barley (Chelkowski *et al.*, 1980) and in the bleaching of fish protein concentrates (Rakesh *et al.*, 1972). It has been shown that H_2O_2 can be used to treat milk for cheese-making to prevent late blowing defect (El-Gendy *et al.*, 1980). Activation of milk lactoperoxidase through the addition of a source of H_2O_2 and thiocyanate can prevent bacterial spoilage of raw milk for a considerable time (Bjorck, 1978; Bjorck *et al.*, 1979).

Addition of H_2O_2 as a preservative to milk is known to accelerate oxidative deterioration, thereby decreasing the keeping quality of butter (Akar *et al.*, 1979). Partial loss of pyruvic acid (Buruiana *et al.*, 1980) and complete loss of methionine, tyrosine, tryptophan and cysteine/cystine in H_2O_2-preserved milk has also been described (Chaudhry & Qureshi, 1979). Losses of folic acid upon pasteurization and different types of heat-treatment of milk, are reported (Cerna & Kas, 1982; Rechcigl, 1982), but there is no information on the stability of folic acid in H_2O_2-treated milk. Enzymic degradation of folic acid was described using erythrocyte extracts (Braganca *et al.*, 1957), *Cicer arietinum* seedling preparations (Ghanekar & Braganca, 1960), horseradish peroxidase and red blood cell membrane oxidase (Innocentini & Duran, 1982). Recently, we have described the folic acid degradation due to C_9–N_{10} bond cleavage, catalysed by hemin and cytochrome C (Taher & Lakshmaiah, 1987*a*,*b*). The inactivation of folic acid by peroxide-dependent systems prompted us to investigate the stability of folic acid in milk, when milk lactoperoxidase is activated by the addition of H_2O_2 and KSCN. The possible role of lactoperoxidase in the degradation of folic acid is discussed.

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MATERIALS AND METHODS

Chemicals

Folic acid, α-naphthylethylenediamine, *tert*-butylhydroperoxide (TBHP), reduced glutathione (GSH) and lactoperoxidase (EC 1.11.1.7; mol. wt 93000; 90 purpurogallin units ng^{-1}) were purchased from Sigma (St. Louis, USA). Sodium nitrite was purchased from BDH (Poole, England), ammonium sulphamate from E. Merck (Darmstadt, Germany) and H_2O_2 from E. Merck (India).

Preparation of solutions

Folic acid, purified by the method of Blakeley (1957) was dissolved in glass distilled water by adding the

minimum amount of 0·1 N NaOH, and the pH was adjusted to 7·0 with 0·1 N HCl. The concentration of folic acid was determined spectrophotometrically (Chanarin, 1969). The folic acid solution thus prepared had less than 2% *p*-aminobenzoylglutamic acid (PABG) as contaminant. The concentration of H_2O_2 in stock solutions was routinely determined by titrating with a standard solution of $KMnO_4$ (Welcher, 1963) or by measuring the absorbance at 240 nm. The concentration of lactoperoxidase was calculated on the basis of a value of 114 for $E^{mM}_{1\,cm}$ at 412 nm (Morrison & Bayse, 1970).

Quantification of folic acid degradation in milk

Raw cow milk purchased from a local vendor was employed in all the studies. The degradation of endogenous milk folic acid was studied by a microbiological method using *Lactobacillus casei* (ATCC 7469) as the test organism. The degradation of exogenously added folic acid in the milk was measured by estimating PABG according to the Bratton–Marshall procedure (Bratton & Marshall, 1939). At the concentrations employed in these experiments, H_2O_2 and KSCN did not interfere with either microbiological or colorimetric procedures as ascertained by separate studies. After incubation, suitable aliquots were taken for further processing. For microbiological assay of folic acid the samples were processed and assayed according to the procedure described by Ramasastri (1965) in the medium described by Clegg *et al.* (1952). The total folate content, which includes polyglutamate forms, was estimated after treating an aliquot of the processed sample with 0·1 ml human plasma conjugase, as described earlier (Lakshmaiah & Ramasastri, 1980). For the colorimetric procedure, the milk samples (1·0 ml) were deproteinised by adding 1·5 ml of 40% trichloroacetic acid, filtered and an aliquot (usually 0·5 ml) of the filtrate was used for the assay of PABG.

Quantification of folic acid degraded by lactoperoxidase

Folic acid degraded by lactoperoxidase in the presence of GSH-Mn(II), H_2O_2 or TBHP was assayed by measuring the amount of PABG formed according to the Bratton–Marshall method. The enzyme reactions (details given in tables) were terminated by the addition of 0·5 ml of 40% trichloroacetic acid. Normally an 0·1 ml aliquot of reaction mixture was used for colour development. To this, 1·5 ml water and 0·6 ml of 10 N H_2SO_4 were added followed by 0·3 ml of 0·1% $NaNO_2$, 0·3 ml of 0·5% ammonium sulphamate and 0·2 ml of 0·1% α-naphthylethylenediamine (each at 5 min intervals with mixing). The solutions were then left at room temperature for 1 h. The azo-dye formed was measured at 556 nm, on a Shimadzu UV-190 double beam spectrophotometer. The concentration of PABG was calculated on the basis of a value of 50·4 for $E^{mM}_{1\,cm}$ at 556 nm (Shane, 1980).

RESULTS AND DISCUSSION

Degradation of endogenous folic acid in milk

For the activation of lactoperoxidase 8·5 mg of H_2O_2 and 10–15 mg of KSCN litre^{-1} milk are usually used (Dahlberg *et al.*, 1984). In the present investigation we used higher levels of H_2O_2 (10, 30 and 50 mg litre^{-1}) and KSCN (50 and 100 mg litre^{-1}) to elucidate their effects on the stability of milk folic acid. The per cent inactivation of endogenous free and total folic acid upon addition of H_2O_2 and KSCN to milk is shown in Table 1. At an H_2O_2 concentration of 10 mg litre^{-1} milk, the loss of free folate was 17·4% and that of the total folate was 3·9%. At a concentration of 30 mg H_2O_2 litre^{-1} milk, the loss of free and total folate amounted to about 20%. When 50 mg H_2O_2 litre^{-1} milk was used, the loss of free and total folate was about

Table 1. Effect of H_2O_2 and KSCN on stability of milk folic acid. (Milk (10 ml) was incubated at 30 ± 2°C for 12 h in the presence of H_2O_2 ± KSCN at the concentrations indicated. Folic acid was estimated microbiologically as described in the Materials and Methods section)

Preservative concentration (mg litre^{-1} milk)		Folic acid remaining (μg litre^{-1} milk)		Folic acid degraded (%)	
H_2O_2	KSCN	Free	Total	Free	Total
—	—	39·6	106	—	—
10	—	32·7	102	17·4	3·9
30	—	31·0	83·9	21·7	20·6
50	—	31·5	78·6	20·4	25·6
10	50	27·4	58·3	30·8	44·8
10	100	28·6	57·0	27·7	46·0
30	50	23·0	33·3	41·9	68·5
30	100	23·3	32·2	41·2	69·5
—	50	41·7	105	—	1·0
—	100	41·8	107	—	—

[a] Values are averages of three separate experiments.

20% and 25%, respectively. At a fixed concentration of H_2O_2 (10 mg litre^{-1} milk) addition of KSCN (50 mg and 100 mg litre^{-1} milk) resulted in greater losses of free and total folic acid without any dependence on the amount of KSCN. With 30 mg H_2O_2 litre^{-1} milk and 50 or 100 mg KSCN, the loss of free and total folate increased further, but again the percentage loss was not proportional to the dose of KSCN. Addition of 50 or 100 mg of KSCN litre^{-1} milk with no H_2O_2 resulted in minimal loss of folic acid.

Degradation of folic acid added to milk

The effect of preservatives on degradation of folic acid added to the milk was studied by estimating the PABG formed according to the Bratton–Marshall procedure. We did not observe any loss of folic acid in the absence

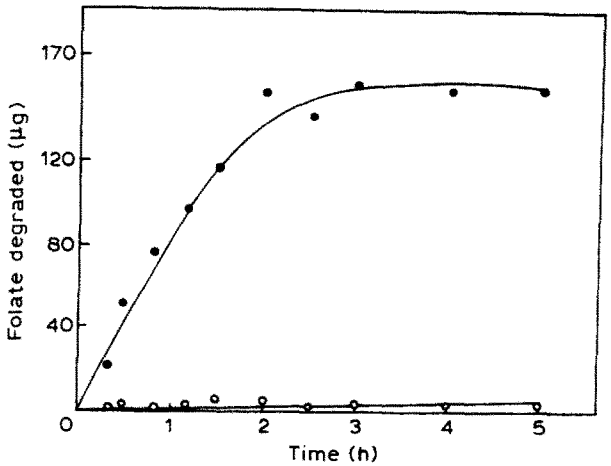

Fig. 1. Effect of H_2O_2 and KSCN on the stability of folic acid added to milk: 1 mg folic acid and 0·1 mg H_2O_2 (●); 1 mg folic acid, 0·5 mg KSCN and 0·1 mg H_2O_2 (○); incubated separately in 10 ml milk at 30 ± 2°C. At each time point, as indicated, PABG was estimated as described in the Materials and Methods section.

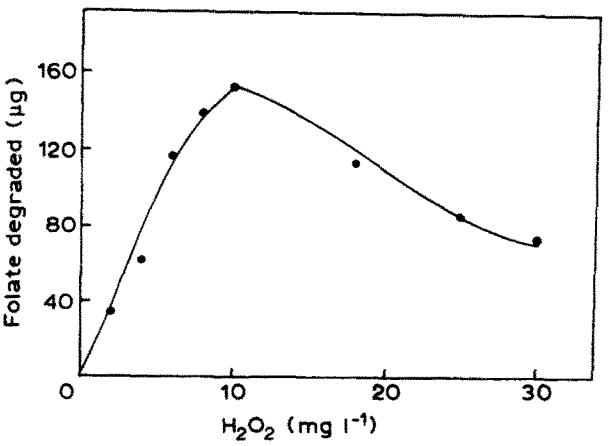

Fig. 2. Effect of H_2O_2 concentration on the stability of folic acid added to milk: 1 mg folic acid and H_2O_2 as indicated was incubated in 10 ml milk at 30 ± 2°C. After 2 h, PABG was estimated as described in the Materials and Methods section.

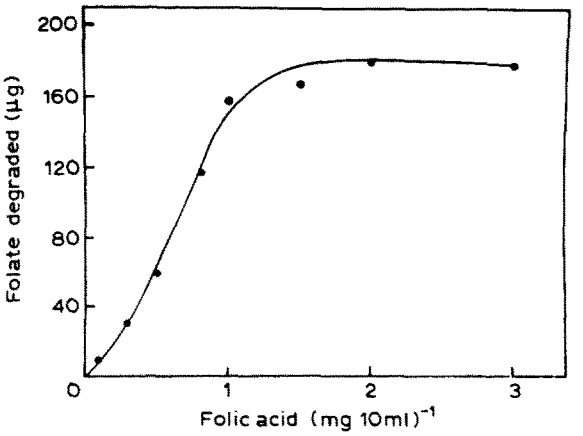

Fig. 3. Effect of folic acid concentration on the stability of folic acid added to milk: 0·1 mg H_2O_2 and folic acid as indicated were incubated in 10 ml milk at 30 ± 2°C. After 2 h, PABG was estimated as described in the Materials and Methods section.

of H_2O_2 and KSCN. Potassium thiocyanate alone also did not have any effect on the folic acid stability. When H_2O_2 was added, the degradation of folic acid showed a progressive increase for a period of 2 h and addition of KSCN inhibited this degradation (Fig. 1). The degradation of folic acid as a function of H_2O_2 concentration in milk is shown in Fig. 2. The degradation increased progressively at low concentrations of H_2O_2 (up to 10 mg litre^{-1}), but higher concentrations of H_2O_2 were inhibitory. This kind of inhibition may be due to inactivation of lactoperoxidase by excess H_2O_2 (Chung & Wood, 1970). Figure 3 shows degradation of folic acid as a function of its concentration. The degradation increased with increasing concentration up to 1·5 mg folic acid 10 ml^{-1} milk, but above this concentration there was no change in degradation.

Degradation of folic acid by lactoperoxidase

The *in vitro* degradation of folic acid by lactoperoxidase was also studied in the presence of hydroperoxides (H_2O_2/TBHP) or GSH-Mn(II). The diazotization procedure of Bratton and Marshall was used to estimate PABG. As shown in Table 2, the extent of degradation was more when GSH-Mn(II) was used in place of hydroperoxides. De Chatelet and Shirley (1981) reported that a hydroperoxide-generating system is more effective in the oxidation of formate than the reagent H_2O_2 added as a single bolus. In this reaction, also, GSH-Mn(II) probably serves as a hydrogen peroxide-generating system in the presence of lactoperoxidase. The inhibition of KSCN may be due to competition with folic acid at the enzyme active site (Chung & Wood, 1970). High concentrations of hydroperoxides (H_2O_2 and TBHP) also inhibited lactoperoxidase-mediated folic acid degradation. Chung and Wood (1970) have reported that high concentrations of H_2O_2 inactivate lactoperoxidase (Table 1). Thus, the endogenous milk-

Table 2. Degradation of folic acid by lactoperoxidase in the presence of hydroperoxides or GSH-Mn(II), and its inhibition by KSCN. (Reaction mixtures in a final volume of 1 ml contained 50 mM phosphate buffer, pH 5·5, and other constituents as indicated. Incubation was carried out at 37°C for 20 min; reactions were terminated by addition of 0·5 ml of 40% trichloroacetic acid; PABG was estimated as described in the Materials and Methods section)

Folic acid (mM)	Lactoperoxidase (μM)	H_2O_2 (mM)	TBHP (mM)	GSH (mM)	Mn(II) (mM)	KSCN (mM)	Folic acid degraded (mM)
0·5	0·5	—	—	1·0	0·5	—	0·122
0·5	1·0	—	—	1·0	0·5	—	0·251
0·5	0·5	—	—	1·0	0·5	0·25	0·003
0·3	0·75	0·025	—	—	—	—	0·051
0·3	0·75	0·05	—	—	—	—	0·030
0·3	0·75	0·6	—	—	—	—	0·000
0·3	1·5	0·025	—	—	—	0·25	0·000
0·3	1·0	—	1·0	—	—	—	0·029
0·3	1·0	—	0·6	—	—	—	0·064
0·3	1·0	—	0·6	—	—	0·25	0·000

folic acid degradation appears to be due to oxidative destruction of reduced folic acid derivatives and not to the action of lactoperoxidase. However, the degradation of folic acid added to milk shows the characteristics of lactoperoxidase-mediated degradation; that is, inhibition by KSCN and high concentrations of H_2O_2.

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