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ORIGINAL ARTICLE

Inactivation of *Escherichia coli* and *Shigella* in acidic fruit and vegetable juices by peroxidase systems

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Abstract

Aims: To study the bactericidal properties of the lactoperoxidase (LPER)-thiocyanate and soybean peroxidase (SBP)-thiocyanate systems at low pH, their efficiency for inactivation of *Escherichia coli* and *Shigella* in acidic fruit and vegetable juices, their effect on colour stability of the juices and interaction with ascorbic acid.

Methods and Results: Three-strain cocktails of *E. coli* and *Shigella* spp. in selected juices were supplemented with the LPER or SBP system. Within 24 h at 20°C, the LPER system inactivated both cocktails by ≥5 $\log_{10}$ units in apple, 2–5 $\log_{10}$ units in orange and ≤1 $\log_{10}$ unit in tomato juices. In the presence of SBP, browning was significant in apple juice and white grape juice, slight in pink grape juice and absent in orange or tomato juice. Ascorbic acid protected *E. coli* and *Shigella* against inactivation by the LPER system, and peroxidase systems significantly reduced the ascorbic acid content of juices.

Conclusions: Our results suggest a different specificity of LPER and SBP for SCN^-, phenolic substrates of browning and ascorbic acid in acidic juices. The LPER system appeared a more appropriate candidate than the SBP system for biopreservation of juices.

Significance and Impact of the Study: This work may open perspectives towards the development of LPER or other peroxidases as biopreservatives in acidic foods.

Introduction

Growing consumer awareness of the health benefits of raw or minimally processed fruit and vegetables and related products has not only increased the consumption of these products but also the risk of foodborne illnesses at the same time (Beuchat 1996). The number of documented outbreaks of pathogenic bacteria associated with these products has increased in recent years. Outbreaks of *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella* and *Shigella flexneri* infection have been linked to products as unpasteurized apple juice and apple cider, tomatoes and tomato juice and unpasteurized orange juice (Parish 1997, Krause *et al.* 2001). From a microbial ecology point of view, the presence of these pathogenic bacteria in soil, air and water and thus the contamination of raw fruits and vegetables cannot be completely avoided. Moreover, fresh-cut fruits and vegetables and their juices are of special concern because the removal of the peel at the same time also eliminates the external barrier for invading bacteria (Leverentz *et al.* 2001).

Several pathogenic bacteria may resist the inherent acidity fruit juice and develop adaptive mechanisms that greatly enhance their survival and sometimes even their ability to grow in acidic environments. In this respect, Bagamboula *et al.* (2002) demonstrated high acid tolerance for *Sh. flexneri* and *Shigella sonnei*, which survived for at least 14 days in apple juice (pH 3·3) and tomato juice (pH 4·0) at 7°C. Ryu and Beuchat (1998) observed a high tolerance for *E. coli* O157: H7 in apple cider (pH 4·0) and orange juice (pH 3·8) held at 5°C or 25°C for up to 42 days and even reported growth in apple cider at

25°C. Effective measures to reduce or eliminate vegetative pathogens in fruit or vegetable products, especially those with a low infective dose, are thus required.

In 1998, the Food and Drug Administration took regulatory action requiring a 5-D reduction of vegetative pathogens in low pH products (pH ≤4·5) such as fruit and vegetable juices (Uljas and Ingham 1999). For this purpose, preservation technologies causing a minimal loss of nutritional and sensorial properties emerged as alternatives for thermal pasteurization, the latter affecting flavour and aroma profiles of fruit juices (Su and Wiley 1998). In this respect, the demand for less 'heavily' preserved, higher quality and more 'natural' preserved foods, however, has encouraged the use of natural antimicrobial agents or systems for food preservation such as peroxidase-based enzyme systems (Kussendrager and van Hooijdonk 2000).

Peroxidases are enzymes that catalyse the oxidation by hydrogen peroxide (H_2O_2) of a wide range of substrates, yielding short-lived oxidation products with antimicrobial properties (De Wit and van Hooijdonk 1996). Well-studied in this respect is lactoperoxidase (LPER), but also plant enzymes such as soybean peroxidase (SBP) receive increased attention. Both LPER and SBPs catalyse the oxidation of thiocyanate (SCN^-) by H_2O_2 to predominantly hypothiocyanite ($OSCN^-$) that becomes protonated to hypothiocyanous acid (HOSCN) at acid pH (≤5·3) (Thomas 1981). These oxidation products exhibit antimicrobial properties to a wide range of Gram-positive and Gram-negative bacteria, particularly at low pH (Tenovuo *et al.* 1991). The widespread occurrence of peroxidases in the human body and in many foods (O'Brien 2000) indicates that their application as biopreservative in naturally occurring concentrations may be considered as safe. Several applications, mainly of LPER, already exist in the health product industry, but also applications in the dairy industry have been suggested (Kussendrager and van Hooijdonk 2000). As the LPER system is mostly bacteriostatic towards vegetative pathogens (Garcia-Graells *et al.* 2003), combinations with other biopreservatives such as nisin (Elotmani and Assobhei 2004), lactoferrin and/or lysozyme (Banks *et al.* 1986) or preservation treatments such as high pressure (Garcia-Graells *et al.* 2003) have been tested and found to be very effective to enhance its antibacterial efficiency. Combinations of peroxidases with low pH have also been suggested to be effective against *Streptococcus mutans* and *Lactobacillus* spp. in the oral environment (Tenovuo *et al.* 1991) but have never been tested in food matrices and against foodborne pathogens.

The objective of this study was therefore to study the efficiency of the LPER and SBP systems for inactivation of *E. coli* and *Shigella* spp. in several fruit and vegetable juices with inherent acidity. In addition, the effect of these two peroxidase systems on the colour stability and ascorbic acid content of the juices was assessed.

Materials and methods

Preparation of bacterial suspensions in fruit and vegetable juices

Escherichia coli K12 strain MG1655 (Hauben *et al.* 1997), EPEC strain ATCC12014 (American Type Culture Collection, Manassas, VA, USA), serotype O157:H7 strain ATCC43888 (American Type Culture Collection) (strain that does not produce either Shiga-like toxin I or Shiga-like toxin II and does not possess the genes for these toxins) and *Sh. flexneri* strains 6 (Bagamboula *et al.* 2002) and LMG10472 (Belgian Coordinated Collection of Micro-organisms, Gent, Belgium) and *Sh. sonnei* CIP8249 (Institut Pasteur Collection, Paris, France) were used in this study. Bacterial cultures were grown to stationary phase for 21 h with shaking (200 rev min^{-1}) at 37°C in 20 ml Luria–Bertani broth containing 10 g l^{-1} tryptone (Lab M, Amersham, England), 5 g l^{-1} yeast extract (Biokar Diagnostics, Pantin, France) and 5 g l^{-1} NaCl. Cells were harvested by centrifugation (3000 ***g***, 5 min) and resuspended to a concentration of approx. 10^6 CFU ml^{-1} in pasteurized commercial apple juice (pH 3·3; Minute Maid, Houston, TX, USA), freshly extracted apple juice (pH 3·4), commercial pasteurized orange juice (pH 3·8) (Looza, Tockwith, York), freshly extracted orange juice (pH 3·7) or commercial pasteurized tomato juice (pH 4·1) (Carrefour, Brussels, Belgium). For experimental purposes, 10^6 CFU ml^{-1} cocktails of the three *E. coli* strains (cocktail 1) and the three *Shigella* strains (cocktail 2) were prepared. The terms '*E. coli*' and '*Shigella*' in Results and Discussion sections refer to the respective three-strain cocktails, unless otherwise mentioned.

The freshly extracted juices were extracted with a household vegetable extractor from fresh apples and oranges that were first surface sterilized with 70% ethanol (Janisiewicz *et al.* 1999), rinsed with sterile water and peeled with a sterile knife. The aerobic plate count was approx. 3×10^2 CFU ml^{-1} for both juices. Freshly extracted apple juice was centrifuged (3000 ***g***, 5 min) to remove the pulp. This centrifugation step reduced the background microflora in this juice to undetectable levels. Juices were stored at 4°C and used within 24 h after extraction.

Application of peroxidase systems

A 10 mg ml^{-1} (1000 U ml^{-1}) stock solution of LPER (EC 1.11.1.7; Sigma, Bornem, Belgium) and SBP (EC 1.11.1.7; Sigma) was prepared in 50% (w/v) glycerol in phosphate-buffered saline (0·1 mmol l^{-1} potassium phosphate buffer,

pH 6·0; 150 mmol l^{-1} NaCl). For both peroxidases, one unit was defined as the amount of enzyme that forms 1·0 mg of purpurogallin from pyrogallol in 20 s at pH 6·0 at 20°C (Gladek and Modarski 1983). Aqueous 25 mmol l^{-1} stock solutions of the substrates of the LPER system, KSCN and H_2O_2, were sterilized by passage through a 0·22-μm Millex®-GS Filter Unit (Millipore S.A.S., Molsheim, France) and stored at 4°C. Working concentrations in the inactivation experiments were 30 μg ml^{-1} for both enzymes and 1 mmol l^{-1} for both substrates. In each experiment, a sample without any additions and one with only H_2O_2 were included to distinguish the antimicrobial effects of the complete LPER system and of H_2O_2 alone.

Peroxidase enzyme activity at different pH values was measured using the method of Shindler *et al.* (1976) with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) as chromogenic substrate. Therefore, 0·05 U (0·5 μg ml^{-1}) LPER or SBP was added to 1 ml 0·1 mol l^{-1} Na-acetate buffer of different pH values in the presence of 1 mmol l^{-1} ABTS and 0·05 mmol l^{-1} H_2O_2. The increase in absorbance at 412 nm was used as a measure for enzymatic activity. As the buffer capacity of the Na-acetate buffer diminished notably above pH 6·0, part of the experiment was repeated in 0·1 mol l^{-1} Na-phosphate buffer pH 6·0–7·0, but no significant effect of the buffer type was detected (data not shown).

Determination of microbial survival

Peroxidase-treated suspensions were serially diluted in peptone water, consisting of 10 g l^{-1} bacteriological peptone (Oxoid, Basingstoke, UK) and 5 g l^{-1} NaCl. Bacterial cocktails were subsequently plated onto tryptone soy agar (Oxoid) with an extra 0·6% yeast extract (Biokar Diagnostics) to stimulate recovery. Plates were incubated for 24–48 h at 37°C. To take into account the background microflora in the freshly extracted juices, a non-inoculated juice sample was also treated with LPER or SBP and the evolution of viable counts during 24 h storage analysed. The evolution of viable counts of *E. coli* or *Shigella* was then expressed as $\log_{10}(N_0 - N)$ with N_0 the total colony count in the peroxidase-treated sample and N the remaining background microflora after peroxidase treatment. The detection limit of plating was 20 CFU ml^{-1}. In time course experiments, the first data point was taken immediately after the addition of peroxidase enzyme systems, others at 3, 6 and 24 h after storage at 6°C or 20°C. All inactivation experiments were conducted in triplicate and representative results are shown. Differences at the 5% significance level were determined by Student's *t*-test.

Determination of hypothiocyanite concentration

Hypothiocyanite concentration was determined as described by Thomas *et al.* (1994). This method was based on the oxidation of the coloured 5-thio-2-nitrobenzoic acid (Nbs) to the colourless 5,5′-dithiobis (2-nitrobenzoic acid) (Nbs_2; Sigma). The assays were performed in a phosphate–NaCl buffer pH 7·2 (1·95 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 0·89 g l^{-1} $NaH_2PO_4 \cdot H_2O$, 8·19 g l^{-1} NaCl). A fresh 0·6 mmol l^{-1} Nbs solution, prepared by adding 20 mg Nbs_2 in 50 ml cold phosphate buffer pH 7·2 together with 0·1 mmol diethylenetriaminepentaacetic acid (Sigma) and 0·03 mmol 2-mercaptoethanol (Fluka, Buchs, Switzerland), was kept on ice. One millilitre of the sample to be analysed was brought to a volume of 4 ml with phosphate–NaCl buffer and 0·5 ml of the Nbs solution was added. The $OSCN^-$ concentration was calculated from the difference in absorbance at 409 nm between the control (sample in the absence of peroxidase enzyme system) and the peroxidase-containing sample as follows:

$$\frac{4{\cdot}5\ \text{ml} \times (A^{409}_{\text{control}} - A^{409}_{\text{sample}})}{0{\cdot}01405 \times 2\ \text{sample volume (ml)}} \qquad (1)$$

Determination of ascorbic acid concentration

Ascorbic acid concentration was determined by redox titration with potassium iodate (KIO_3) (Suntornsuk *et al.* 2002). An aqueous solution containing potassium iodate, and excess potassium iodide (KI) and hydrogen chloride (HCl) was prepared to initiate the following reaction:

$$KIO_3 + 5KI + 6H^+ \leftrightarrow 3I_2 + 6K^+ + 3H_2O \qquad (2)$$

Starch was added in excess to form an intensive blue coloured complex with iodine.

Iodine produced in (2) will then oxidize ascorbic acid in the samples to dehydroascorbic acid and the blue colour of the iodine–starch complex disappeared when all iodine was reacted out of the sample

$$C_6H_8O_6 + I_2 \leftrightarrow C_6H_6O_6 + 2I^- + 2H^+ \qquad (3)$$

The concentration of ascorbic acid in the sample could then be calculated based on the stoichiometric relationships of reactions (2) and (3).

Results

Enzymatic activity of lactoperoxidase and soybean peroxidase as influenced by pH and temperature

The enzymatic activity of LPER and SBP was determined at pH 3–7, a relevant pH range for fruit and vegetable juices and at ambient (20°C) and chill (6°C) temperature.

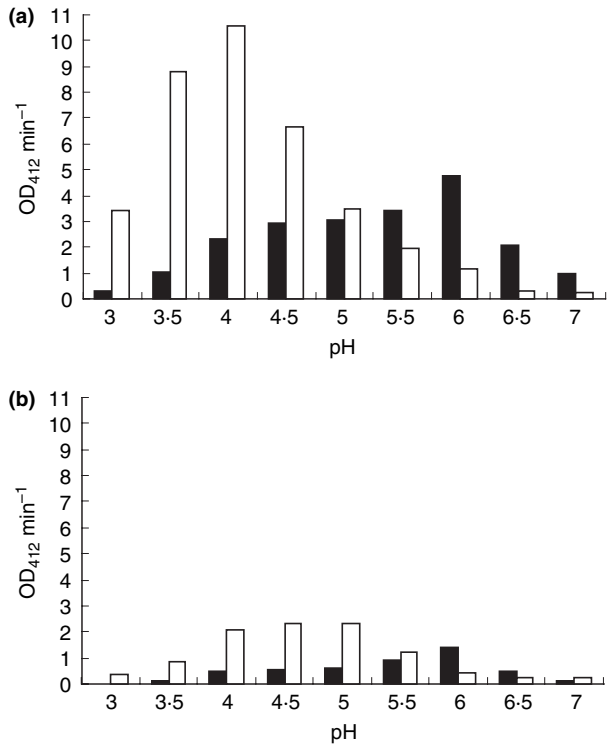

Figure 1 Enzymatic activity of lactoperoxidase (0·5 μg ml^{-1}) (■) and soybean peroxidase (0·5 μg ml^{-1}) (□) in 0·1 mol l^{-1} sodium acetate buffer pH (3–7) at 20°C (a) and 6°C (b) and with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) as chromogenic substrate. OD_{412}, optical density at 412 nm.

In a first set of experiments, activity measurements were conducted with ABTS, a commonly used chromogenic substrate for this purpose (Fig. 1a,b). The pH significantly influenced the enzymatic activity of both enzymes. At 20°C, LPER showed an optimal enzymatic activity at pH 6·0 and SBP at pH 4·0 (Fig. 1a). At 6°C, the LPER optimum was situated at pH 6·0 and SBP was optimally active at pH 4·5–5·0 (Fig. 1b). As could be expected, decreasing the temperature from 20°C to 6°C significantly reduced the activity of both enzymes.

In a second set of experiments, activity measurements were conducted with thiocyanate (SCN^-) as a substrate by measuring hypothiocyanite production (Table 1). When this assay was used, the SBP showed an optimum activity around pH 3·0, while two optima were found for LPER, around pH 3·5 and pH 6·5. Another remarkable feature, when the activity measurements with ABTS and with SCN^- are compared, is that SBP shows higher activity than LPER with the former substrate (Fig. 1), but LPER shows higher activity than SBP with the latter. To exclude the possibility that $HOSCN/OSCN^-$ was produced via a nonenzymatic mechanism, part of the experiment (pH ≤4·5) was repeated in the presence of H_2O_2 and SCN^- but without the soybean or LPER enzyme. We observed no difference in the absorbance compared with the control sample, indicating that $HOSCN/OSCN^-$ was not generated in the absence of the peroxidase enzymes.

Table 1 Amount of hypothiocyanous acid/hypothiocyanite [$HOSCN/OSCN^-$] (μ mol l^{-1}) generated by the lactoperoxidase and soybean peroxidase systems after 30 min in citrate–phosphate buffer pH 2·0–7·0. Initial concentrations were enzyme, 30 μg ml^{-1}; H_2O_2, 1 mmol l^{-1}; SCN^-, 1 mmol l^{-1}

	[$HOSCN/OSCN^-$] (μ mol^{-1})	
PH	Lactoperoxidase	Soybean peroxidase
2·0	72·6 ± 2·8[a]	26·8 ± 2·6[b]
2·5	74·6 ± 1·5[a]	26·1 ± 4·0[b]
3·0	76·2 ± 4·0[a]	28·9 ± 2·3[b]
3·5	90·4 ± 4·3[a]	19·4 ± 2·1[b]
4·0	79·8 ± 3·1[a]	12·1 ± 0·7[b]
4·5	37·6 ± 2·9[a]	8·9 ± 0·5[b]
5·0	17·4 ± 6·9[a]	9·6 ± 2·2[a]
5·5	15·6 ± 8·7[a]	11·9 ± 0·3[a]
6·0	74·3 ± 3·2[a]	7·2 ± 0·4[b]
6·5	83·8 ± 6·0[a]	5·7 ± 0·7[b]
7·0	14·0 ± 0·5[a]	4·3 ± 1·2[b]

Values in a row followed by a different superscript letter are significantly different ($P < 0·05$).

Effect of lactoperoxidase and soybean peroxidase systems on colour stability of different fruit and vegetable juices

The colour stability of commercial apple, grape, pink grape, orange, tomato and freshly extracted apple and orange juice in the presence of 30 μg ml^{-1} LPER or SBP and 1 mmol l^{-1} H_2O_2 and 1 mmol l^{-1} SCN^- was visually evaluated (Fig. 2a–g). The SBP system caused an immediate and significant colour change in commercial and freshly extracted apple juice (Fig. 2a,b) and grape juice (Fig. 2f), a slight change in pink grape juice (Fig. 2g) and no colour changes in commercial and freshly extracted orange juice (Fig. 2c,d) and tomato juice (Fig. 2e) upon storage for 2 weeks at ambient temperature (Fig. 2) or 6°C (data not shown). On the contrary, none of the seven juices of the test panel changed colour in the presence of the LPER system during 2 weeks of storage (Fig. 2a–g).

Antimicrobial effect of soybean peroxidase and lactoperoxidase system on Escherichia coli and *Shigella* in fruit and vegetable juices

The survival of a three-strain cocktail of *E. coli* (strains MG1655, ATCC 12014 and ATCC 43888) and *Shigella* spp. (*Sh. flexneri* strains 6 and LMG10472 and *Sh. sonnei* CIP8249) was evaluated in the presence of the LPER or

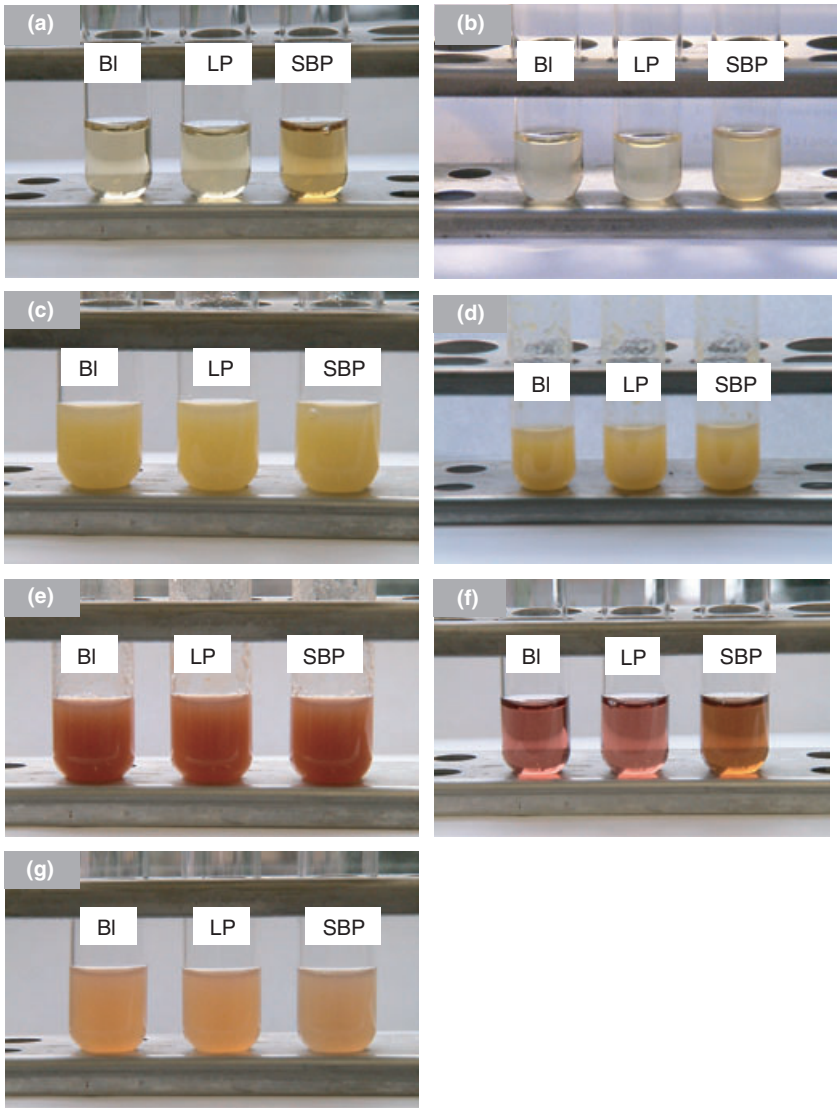

Figure 2 Effect of addition of lactoperoxidase (LPER) and soybean peroxidase (SBP) on colour stability of different fruit and vegetable juices. (a) Commercial apple juice pH 3·3; (b) freshly extracted apple juice pH 3·4; (c) commercial orange juice pH 3·8; (d) freshly extracted orange juice pH 3·7; (e) tomato juice pH 4·1; (f) grape juice pH 3·3; (G) pink grape juice pH 3·2. Bl: no additives; LPER and SBP: addition of 30 μg ml^{-1} LPER or SBP together with 1 mmol l^{-1} H_2O_2 and 1 mmol l^{-1} SCN^-. Photos were taken 2 weeks after the addition of the additives.

SBP system at 6°C and 20°C. The terms '*E. coli*' and '*Shigella*' hereafter refer to the respective three-strain cocktails, unless otherwise mentioned.

Each peroxidase system was applied in three different commercial juices with a different pH that did not significantly change colour in the presence of the soybean or LPER system, as shown in Fig. 2. For the LPER system, these juices were apple, orange and tomato and for the SBP system orange, tomato and pink grape juices. In addition, the LPER system was also applied in two freshly extracted juices, from apple and orange. In the absence of additives, *E. coli* and *Shigella* survived for at least 24 h at 6°C and 20°C, and a slight growth of 0·5 $\log_{10}$ units after 24-h storage was observed for *Shigella* in tomato juice at 20°C (data not shown). In the presence of 1 mmol l^{-1} H_2O_2, *E. coli* and *Shigella* populations were reduced by 2 $\log_{10}$ units or more within 24 h in commercial and freshly extracted apple juice at 6°C and 20°C but remained stable for all other juices (data not shown). In the presence of the LPER system (Fig. 3), both *E. coli* and *Shigella* were inactivated in commercial and freshly extracted apple juice at 6°C and 20°C, resulting in undetectable numbers after 24 h or less. At 20°C, both cocktails showed significantly less resistance to the LPER system in freshly extracted apple juice compared with its commercial counterpart, while at 6°C, no significant difference in sensitivity was observed. Furthermore, neither *E. coli* nor *Shigella* was inactivated in orange (freshly extracted or commercial) or tomato juice at 6°C, with only 1 $\log_{10}$ unit or less inactivation after 24 h. At 20°C in commercial orange juice, the viable count was reduced by 2 $\log_{10}$ units for *E. coli* and to undetectable numbers

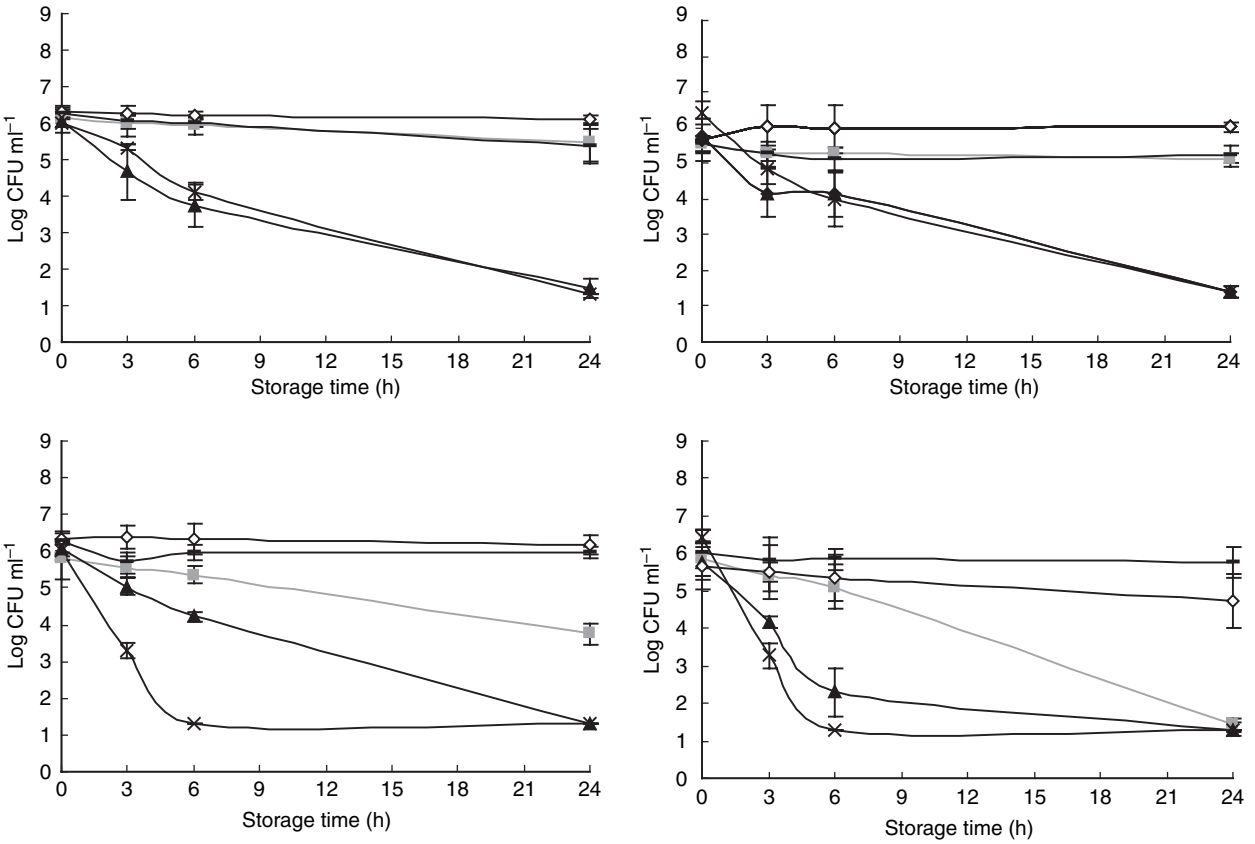

Figure 3 Evolution of viable counts of mixtures of *Escherichia coli* strains MG1655, O157:H7 (ATCC 43888) and ATCC12014 (left) and *Shigella flexneri* LMG10472, *Sh. flexneri* 6 and *Shigella sonnei* CIP8249 (right) during 24-h storage at 6°C (top) and 20°C (bottom), respectively, in commercial apple juice pH 3·3 (▲), orange juice pH 3·8 (■), tomato juice pH 4·1 (◇) and freshly extracted apple juice pH 3·4 (×) and orange juice pH 3·7 (—) in the presence of the lactoperoxidase system (30 μg ml^{-1} lactoperoxidase enzyme, 1 mmol l^{-1} H_2O_2, 1 mmol l^{-1} SCN^-). Presented data are the mean value of three experiments ± standard deviation.

for *Shigella* within 24 h, while in tomato juice and freshly extracted orange juice, no or only slight (<1 $\log_{10}$ unit) inactivation was observed.

In the presence of the SBP system, viable counts of *E. coli* and *Shigella* diminished with only 1 $\log_{10}$ unit or less within 24 h in tomato, orange or pink grape juice (data not shown).

Interaction of ascorbic acid with the lactoperoxidase and soybean peroxidase systems

First, we studied the effect of the LPER or SBP system on the ascorbic acid content of freshly extracted orange juice containing 53 ± 3 mg ascorbic acid per 100 ml. After 24 h in the presence of the LPER and SBP system (30 μg ml^{-1} enzyme, 1 mmol l^{-1} of both H_2O_2 and SCN^-) at 20°C, the ascorbic acid content of the juice declined by 39 ± 5% and 52 ± 4%, respectively. No significant further decrease after prolonged storage for another 24 h at 20°C was observed. This decrease in ascorbic acid content was not directly related to the presence of $HOSCN/OSCN^-$ but also occurred in the presence of 1 mmol l^{-1} H_2O_2 alone (data not shown).

Secondly, based on the efficient inactivation of *E. coli* and *Shigella* in the presence of the LPER system in apple juice (Fig. 3) and the fact that commercial apple juice contained no ascorbic acid, this juice was chosen to study whether the antimicrobial efficiency of the LPER system was affected by the presence of ascorbic acid. Therefore, apple juice supplemented with 0, 35 and 50 mg ascorbic acid per 100 ml was inoculated with 10^6 CFU ml^{-1} *E. coli* or *Shigella*, and the evolution of viable counts was followed for 24 h at 6°C and 20°C after the addition of the LPER system (Fig. 4). In the absence of ascorbic acid, inactivation reached at least 5 $\log_{10}$ units for *E. coli* and *Shigella* within 24 h at 6°C and 20°C, while in the presence of 35 mg ascorbic acid per 100 ml, a strong protective effect was observed at 6°C for both *E. coli* and

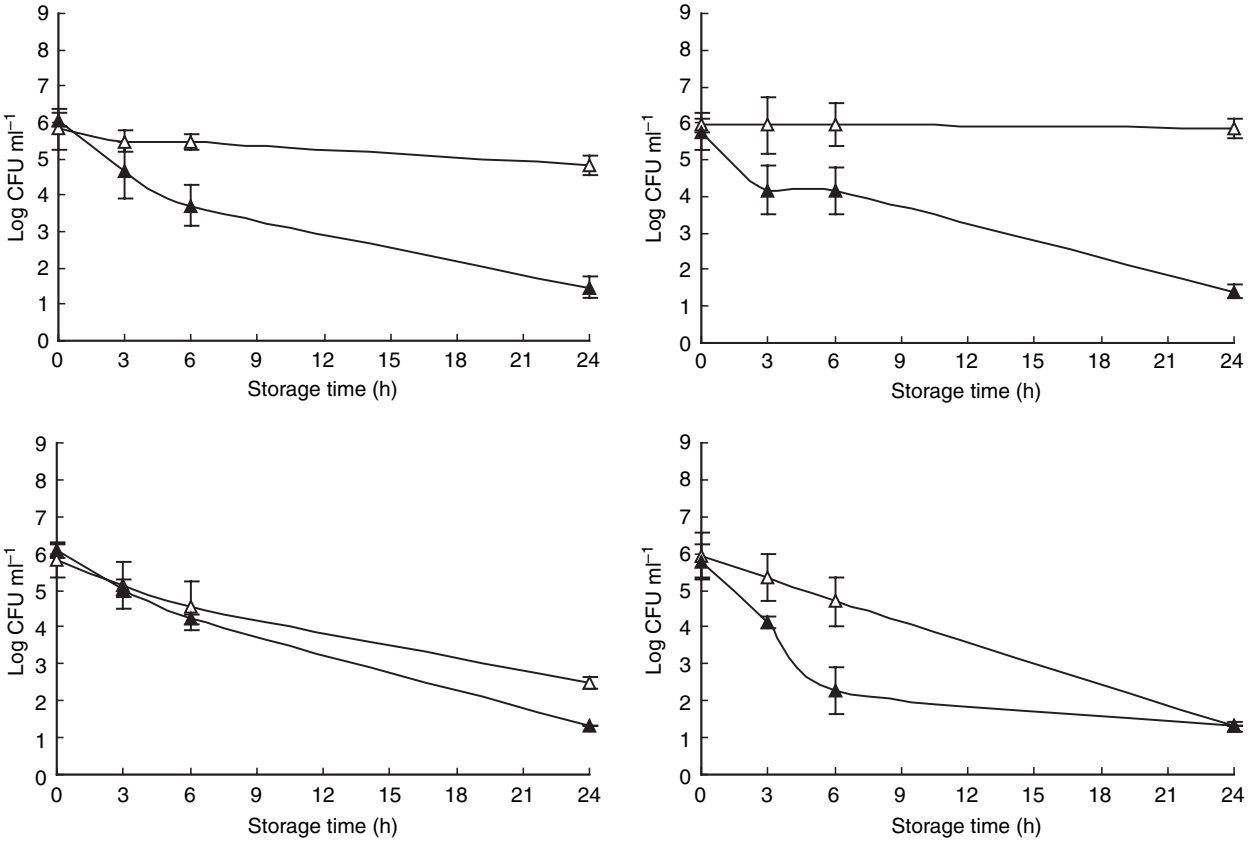

Figure 4 Evolution of viable counts of mixtures of *Escherichia coli* strains MG1655, O157:H7 (ATCC 43888) and ATCC12014 (left) and *Shigella flexneri* LMG10472, *Sh. flexneri* 6 and *Shigella sonnei* CIP8249 (right) during 24-h storage at 6°C (top) and 20°C (bottom), respectively, in the presence of the lactoperoxidase system (30 μg ml^{-1} lactoperoxidase enzyme, 1 mmol l^{-1} H_2O_2, 1 mmol l^{-1} SCN^-) in commercial apple juice pH 3·3 in the absence (▲) or presence (△) of ascorbic acid (35 mg 100 ml^{-1}). Presented data are the mean value of three experiments ± ±standard deviation.

Shigella, with 0·5 $\log_{10}$ units or less of inactivation after 24 h. At 20°C, this protective effect was less pronounced and both *E. coli* (3 $\log_{10}$ units) and *Shigella* (4 $\log_{10}$ units) were strongly inactivated after 24 h. In the presence of 50 mg ascorbic acid per 100 ml juice and at 20°C, similar inactivation levels were observed compared with 35 mg ascorbic acid per 100 ml apple juice (data not shown).

Discussion

The first objective of this work was to study the influence of pH and temperature on the enzymatic activity of LPER and SBP. Activity measurements were conducted with ABTS and SCN^- as substrates. The former is a commonly used chromogenic substrate for peroxidase activity measurements (Ozdemir *et al.* 2001), the latter more relevant in terms of antimicrobial activity establishment. With ABTS as substrate, we observed an optimal LPER activity in acetate buffer at pH 6 (Fig. 1). This is in line with reports by Ozdemir *et al.* (2001) and Kumar and Bhatia (1999) who found a similar pH optimum for LPER activity in McIlvaine buffer and milk. For SBP, we observed an optimal activity at more acidic pH 4·0. Decreasing temperature from 20°C to 6°C significantly decreased the enzymatic activity for both peroxidases but did not significantly change their pH optimum.

Interestingly, the pH dependency of both enzymes was changed when $HOSCN/OSCN^-$ production from $HSCN/SCN^-$ as a substrate was measured (Table 1). The pH optimum of SBP was shifted to pH 3·0, and LPER even showed two optima, at pH 3·5 and pH 6·5. A similar finding has been reported by Lumikari *et al.* (1991). An interesting observation is that at any fixed pH, the LPER produced significantly more hypothiocyanite than SBP. Comparison of the data in Fig. 1 and Table 1 suggests that LPER is more specific for SCN^- than for ABTS as a substrate compared with SBP. Similar to our findings, Ozdemir *et al.* (2001) reported different pH optima for LPER with different substrates, namely pH 6·0 for ABTS,

pH 7·5 for *p*-phenylenediamine and catechol, pH 8·0 for epinephrine and pH 6·8 for pyrogallol. Kamal and Behere (2003) reported also different pH optima (5·0–6·4) for SBP using various substrates.

The second objective of this study was to evaluate the effect of peroxidase addition on the colour stability of different fruit and vegetable juices (Fig. 2). In the presence of SBP, significant browning of commercial and freshly extracted apple juice and commercial grape juice. Nicolas *et al.* (1994) and Tomás-Barberán and Espin (2001) explained this browning susceptibility by the abundance of phenolic compounds in the juices, acting as natural substrates of oxidative enzymes such as polyphenol oxidase and peroxidase. The total phenol concentrations detected in apple, orange and tomato juices (respectively 239, 22 and 16 mg per 100 ml) (Scalbert and Williamson 2000) were in line with this observation. Conversely, Fig. 2 clearly illustrates that the LPER system could be applied in all seven juices of the test panel without affecting colour stability. The previous statements by Nicolas *et al.* (1994) and several other authors should thus be modified to 'not all peroxidases cause the same extent of browning'. The observation that browning reactions occur in the presence of SBP and not in the presence of LPER may also reflect the different substrate specificity of these two enzymes and suggests a correlation between the specificity of LPER and SBP for ABTS and phenolic substrates of browning reactions.

In the second part of this work, the viability reduction of a three-strain cocktail of *E. coli* and *Shigella* in different acidic fruit and vegetable juices in the presence of the LPER (Fig. 3) or SBP system (data not shown) was studied. An important observation from Fig. 3 is that the inactivation of *E. coli* and *Shigella* cocktails was clearly affected by temperature and juice type. Moreover, the pH of the juice may play an important role in the sensitization of *E. coli* and *Shigella* for the LPER system for three reasons. First, pH affects enzymatic production of HOSCN/$OSCN^-$ (Table 1), with an optimum at pH 3·5 for LPER. Secondly, low pH may sublethally injure vegetative pathogens (Smelt *et al.* 2002). Together with the findings by Van Opstal *et al.* (2003) who suggested a link between sublethal injury induced by pressure and sensitization to the LPER system, we postulate that also sublethal injury induced by low pH may induce increased sensitivity for the LPER system. Thirdly, at lower pH (≤5·3), hypothiocyanite occurs predominantly as an uncharged molecule, which can pass more easily through the bacterial membranes to conduct its antimicrobial action (Tenovuo *et al.* 1991). The SBP system caused little (0·5 $\log_{10}$ units within 24 h) or no inactivation of *E. coli* or *Shigella* cocktails in the respective juices, which may be explained by the significantly lower production rate of hypothiocyanite (3–6 times lower compared with the LPER system) at pH 3·0–4·0 (Table 1).

Another important issue was the interaction of the soybean or LPER system with ascorbic acid, a predominant vitamin in fruit juices. The significant loss in ascorbic acid content observed for orange juice in the presence of the LPER or SBP system (respectively 39 ± 5 and 52 ± 4%) is a considerable drawback for the application of peroxidase systems in fruit and vegetable juices. On the contrary, heat pasteurization of orange fruit juice also causes partial loss of ascorbic acid (Haddad 1977). Moreover, the decrease in ascorbic acid content was not directly related to the formation of HOSCN/$OSCN^-$ but occurred in the presence of H_2O_2. Furthermore, Mehlhorn *et al.* (1996) reported that ascorbic acid is a natural substrate for plant peroxidases. Our results also suggest a lower specificity of LPER for ascorbic acid compared with SBP. This finding may open new perspectives for the search of other, ascorbic-acid-inactive peroxidases, which do, however, have strong antimicrobial effectiveness because of efficient oxidation of (H)SCN.

In the next set of experiments, we also tested whether ascorbic acid affected the antimicrobial efficiency of peroxidase systems. Figure 4 clearly demonstrates a protective effect of ascorbic acid against the inactivation of *E. coli* and *Shigella* by the LPER system in apple juice, especially at 6°C.

In conclusion, in this work we demonstrated that the LPER system, more than the SBP system, has some interesting properties as a biopreservative in acid foods. An important drawback of adding peroxidase-based enzyme systems to fruit juices is that the ascorbic acid content is significantly reduced and, vice versa, that the presence of ascorbic acid significantly reduces the antimicrobial efficiency. Finally, our results suggest a different substrate specificity of LPER and SBP, with LPER being more specific for SCN^- than for ABTS, phenolic substrates and ascorbic acid. These findings may open perspectives towards the development of LPER or perhaps other peroxidases as biopreservatives in acidic foods.

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