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Activation and Stabilization of The Hydroperoxide Lyase Enzymatic Extract from Mint Leaves (*Mentha spicata*) Using Selected Chemical Additives

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Abstract The effects of selected lyoprotecting excipients and chemical additives on the specific activity and the thermal stability of the hydroperoxide lyase (HPL) enzymatic extract from mint leaves were investigated. The addition of KCl (5%, *w*/*w*) and dextran (2.5%, *w*/*w*) to the enzymatic extract, prior to lyophilization, increased the HPL specific activity by 2.0- and 1.2-fold, respectively, compared to the control lyophilized extract. From half-life time ($t_{1/2}$), it can be seen that KCl has enhanced the HPL stability by 1.3- to 2.3-fold, during long-period storage at −20 °C and 4 °C. Among the selected additives used throughout this study, glycine appeared to be the most effective one. In addition to the activation effect conferred by glycine, it also enhanced the HPL thermal stability. In contrast, polyhydroxyl-containing additives were not effective for stabilizing the HPL enzymatic extract. On the other hand, there was no signification increase in HPL activity and its thermal stability with the presence of Triton X-100. The results also showed that in the presence of glycine (10%), the catalytic efficiency of HPL was increased by 2.45-fold than that without additive.

Keywords Hydroperoxide lyase · Chemical additives · Lyoprotectants · Stabilization · Kinetics

Introduction

The six-carbon volatile aldehydes and alcohols are organoleptically known to possess "green character," which is of major importance in fragrance and flavor industries [1]. Nowadays, there is an increasing demand for natural aroma compounds, produced via

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biotechnology pathway, instead of the synthetic ones. In higher plants, the C6 aldehydes are formed via lipid oxidation pathway by the sequential action of lipoxygenase (LOX) and hydroperoxide lyase (HPL) [2, 3]. In the first step, linoleate oxygen oxidoreductase (EC 1.13.11.12) catalyses the dioxygenation of various polyunsaturated fatty acids (PUFAs), containing 1(Z),4(Z) pentadiene moiety, into various regio-isomers of hydroperoxides of PUFAs; these hydroperoxides (HPODs) can be subsequently converted by HPL into short-chain aldehyde and oxo-fatty acid products by cleaving the c-c bound adjacent to the hydroperoxy group. As a result, the sequential actions of LOX and HPL have been recognized as a relevant biocatalytic approach for the enzymatic synthesis of a wide variety of aroma compounds [2–4].

The plant tissues are the major sources for HPLs. According to their substrate specificity, two groups of HPL can be distinguished. The first converts 13-HPODs to produce C6-aldehydes and C12-oxo-fatty acids, whereas the second cleaves 9-HPODs into C9-aldehydes and C9-oxo-fatty acids [3, 4]. Soybean seeds/seedlings have been widely studied and reported to contain both 13- and 9-HPL activities [3], while a high level of HPL activity, with a high specificity toward 13-HPODs, was detected in the enzymatic extract from mint leaves [4–6]. However, the use of HPL from mint in vitro is limited by its low thermal stability. In addition to immobilization, the addition of selected stabilizing additives, including salts, sugars/polyols, and polymers to the enzymatic extracts has been considered as an efficient mean for the stabilization of biocatalysts as well as for the enhancement of their activities [7–9]. The protective effect of several additives was attributed to the modification of the enzyme's microenvironment, specifically its water activity [7, 10] and/or to the molecular interactions between proteins and additives. HPL has been stabilized by a variety of chemical compounds, including detergents [11], glycerol [12], dithiothreitol [13], β-mercaptoethanol [11], and polyvinyl polypyrrolidone [14]. However, because of the nature of the source of the enzyme, there is a wide range of discrepancy in the reported experimental findings.

The overall objective of the present study was to investigate the stabilization of the HPL enzymatic extract from mint by the addition of selected chemical additives. This research work is aimed at valorizing mint leaves (*Mentha spicata*) as biocatalyst for the production of green notes with higher added value. The first objective was to evaluate the efficiency of selected lyoprotecting excipients for preserving the HPL activity of the enzymatic extract from mint during the lyophilization and enhancing its long-term storage stability. The study was also aimed at the investigation of the effects of selected additives on the HPL activity, during the enzymatic reaction, as well as on its thermal stability. Kinetic parameters of the HPL enzymatic extract, including K_m and V_{max} values as well as catalytic efficiency, were also determined in the presence and absence of additives.

Materials and Methods

Materials

The additives, including glycine, mannitol, and glycerol, were purchased from BDH (Toronto, ON, Canada). While dextran (72.2 kDa), Triton X-100, linoleic acid (*cis,cis*-9,12-octadecadienoic acid), bovine serum albumin, xylenol orange salt, and commercial purified soybean lipoxygenase (Type 1B, 150,000 U mg^{-1} solid) were obtained from Sigma-Aldrich Chemical (St-Louis, MO, USA). Potassium chloride, mono- and dibasic potassium phosphate, as well as ferrous sulfate were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Preparation of 13-Hydroperoxide of Octadecadienoic Acid

The 13-hydroperoxide of octadecadienoic acid (HPOD) was prepared according to the method described by Gargouri et al. [4]. The reaction medium was composed of 200 mL of glycine buffer (0.2 M, pH 9.0), 200 mg of linoleic acid, and 10 mg of the commercial purified soybean lipoxygenase. The reaction mixture was stirred for 1 h at 25 °C under a constant flow of oxygen (0.1 bar). The HPODs formed were then extracted with diethyl ether (twice the same volume). The organic phase was then dried with $MgSO_4$ and evaporated under vacuum. Hydroperoxide concentration was determined using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} [15]. The separation of the 13-HPOD from other HPOD regio-isomers was accomplished using normal phase high-performance liquid chromatography (NP-HPLC), according to the procedure described by Hall et al. [16].

Preparation of HPL Enzymatic Extract

The HPL was extracted from mint leaves according to the procedure described by Suurmeijer et al. [11]. All steps of extraction were conducted at 4 °C. Fifteen grams of fresh mint leaves were homogenized in a blender with 50 mL of potassium phosphate buffer (0.1 M, pH 6.5). The mixture was filtered through four layers of cheesecloth; 2% (*v*/*v*) of $CaCl_2$ solution (1 M) was then added to the filtrate. After 2 h of agitation, the suspension was centrifuged at 30,000×*g* for 20 min. The pellet was resuspended in potassium phosphate buffer (0.02 M, pH 6.5) containing 0.1% (*w*/*v*) of Triton X-100 and 0.01 M of β-mercaptoethanol; the suspension was clarified by centrifugation at 15,000×*g* for 30 min. The supernatant recovered was used as the crude HPL enzymatic extract. Protein concentration of the enzymatic extract was determined according to a modification of the Lowry method [17]. Bovine serum albumin was used as a standard for the calibration curve.

HPL Enzymatic Assay

The HPL activity was determined using the Xylenol orange assay, according to a modification of the procedure described by Vega et al. [18]. The Xylenol orange assay is based on the oxidation of ferrous ions (Fe^{2+}) by HPODs into their ferric counterparts (Fe^{3+}), which complex with the Xylenol orange salt to form a chromophore that absorbs at 560 nm [19].

The reaction mixture consisted of 160 µL of 13-HPOD stock solution (0.6 mM), 100 µL of HPL enzymatic extract (4 to 30 µg protein/mL), and 1.24 mL of potassium phosphate buffer (0.02 M, pH 6.5). After 3 min of incubation at 20 °C, an aliquot of the reaction mixture of 200 µL was taken. The reaction was halted by the addition of 1 mL of xylenol orange reagent, which is composed of ferrous sulfate (0.25 mM), perchloric acid (85 mM), and xylenol orange salt (0.10 mM) in distilled deionized water. The developed color of the remaining 13-HPOD was measured spectrophotometrically after 10 min at 560 nm (15.3 10^{-9} M^{-1} cm^{-1}) using a Beckman DU-650 spectrophotometer (Beckman Instruments, San Raman, CA, USA). HPL specific activity was defined as micromoles of converted 13-HPOD per milligram protein per minute. All experiments were performed in triplicate trial. A control assay, containing all the components with denatured enzyme (95 °C/90 min), was run in tandem with these trials.

Effect of Lyoprotecting Excipients on HPL Activity and Stability

Prior to lyophilization, two excipients, KCl (5%, *w*/*w*) and dextran (2.5%, *w*/*w*), were added to the fresh enzymatic extract (1.5 mg protein/mL). The effect of selected lyoprotectants

was investigated by measuring the HPL activity of the lyophilized enzymatic extracts with and without excipients. To assess the storage stability, lyophilized HPL enzymatic extracts were stored at −20 and 4 °C for a period of 5 weeks and assayed for HPL activity weekly according to the procedure previously described. The inactivation rate of lyophilized HPL activity was estimated by calculating the first-order inactivation constant ($K_{\text{inactivation}}$) on semilogarithm plots. The storage time (week) required to decrease half of the initial HPL activity ($t_{1/2}$) was calculated using the following equation:

$$t_{1/2} = \ln(2)/K_{\text{inactivation}}$$

Effect of Selected Additives on HPL Activity and Stability

The effect of selected additives on HPL activity was investigated by varying their concentrations in the reaction mixture from 0.3% to 1.5% (*w*/*v*) for Triton X-100, from 5% to 20% (*w*/*v*) for glycine, and from 5 to 20 mM for dithiothreitol. The effect of selected additives on the HPL thermostability was evaluated by preincubating the reconstituted lyophilized enzymatic extracts at a wide range of temperatures, varying from 0 °C to 65 °C for 1 h. The residual HPL activity was measured using the standard assay.

Results and Discussion

Effect of Selected Lyoprotecting Excipients

Selected lyoprotecting excipients were assayed for their efficiency to preserve the HPL activity of the enzymatic extract from mint during the lyophilization. Table 1 summarizes the HPL activity of selected lyophilized preparations, with and without excipients. The results show that the HPL specific activity of the lyophilized enzymatic extract was enhanced by 10% compared to that of the fresh one. However, the extent of enhancement of the HPL specific activity, upon the addition of lyoprotecting excipients to the enzymatic extract prior to the lyophilization, seems to be dependent on the nature of lyoprotectants. The addition of KCl and dextran at 5.0% and 2.5% (*w*/*w*), respectively, resulted in an increase in the specific activity of HPL by 2.0- and 1.2-fold compared to that of the free-excipient extract. Similarly, Hall et al. [16] reported that the addition of KCl to the enzymatic extract from *Penicillium camemberti* increased the HPL activity by 2.25-fold; however, an inactivation of this activity was obtained upon the addition of further dextran.

Table 1 Effect of lyophilization in the presence of KCl and dextran on the HPL activity from mint leaves.

Extract[a]	Additive concentration[b]	Specific activity[c]
Fresh	–	0.128 (±0.007)[d]
Freeze dried	–	0.138 (±0.028)[d]
Freeze dried with KCl	5	0.290 (±0.071)[d]
Freeze dried with dextran	2.5	0.171 (±0.021)[d]

[a] HPL-catalyzed reaction was conducted at 15 °C and pH 6.5 using 13-hydroperoxy-linoleic acid as substrate

[b] Additive concentration is expressed as 1 g of additive per 100 mg proteins of the enzymatic extract

[c] Specific activity is expressed as micromoles of converted HPOD per milligrams of protein per minute of reaction

[d] Standard deviation of triplicate

The differences between the lyoprotectants, KCl and dextran, may be due to their chemical structures and/or to their mechanism protective effects (Table 1). The mechanism behind the activation effect of KCl and dextran as lyoprotectants has been a matter of debate in literature [8, 20, 21]. It has been stated that the lyoprotecting excipients form a highly viscous glass around the protein molecules may prevent them from the unfolding and aggregation [22]. Indeed, large molecules, such as dextran, form a glass more readily and tend to increase the glass transition temperature (T_g) of the enzymatic preparation (T_g of >100 °C), whereas salts, such KCl, possess low T_g (~ 0 °C) [21]. On the other hand, Koeduka et al. [8] suggested that monovalent cations may enhance the activity of the lyophilized enzymatic extract through their direct global effect on the enzyme conformation. Furthermore, intermolecular proteins/excipients interactions may also contribute to the enzyme activation [20, 21]. Koeduka et al. [8] have reported that KCl excipient was an effective lyoprotectant, which has increased more than fivefold the activity of the purified HPL from recombinant barley compared to that of the free salt control; these authors suggested that this effect may be due to the spatial arrangement surrounding the heme in the HPL active site, as a result of the presence of the salt. Lindsay et al. [23] suggested that the activation effect of KCl (98%, *w*/*w*) on the activity of *Penicillin* amidase may be due to an ordering of water molecules around the enzyme (kosmotropicity).

Although KCl tends to form a glassy state less readily than dextran, it has the ability to form intimacy hydrogen bonding and ordering water molecules [21]. The higher activation effect obtained in the presence of KCl (Table 1) may suggest that the formation of intimacy hydrogen bonding is more effective for the protection of HPL from mint than the formation of a readily glassy state. On the basis of the experimental results, the addition of KCl (5%, *w*/*w*) was selected to be used throughout this study for further investigations.

Storage Stability of the Lyophilized HPL Enzymatic Extract

The stability of the lyophilized HPL enzymatic extract, with KCl and the free-salt one, during its storage is shown in Figure 1. After 5 weeks of storage at 4 °C and −20 °C, the free-salt extract showed 12.0% and 46.3% of the initial HPL specific activity, respectively.

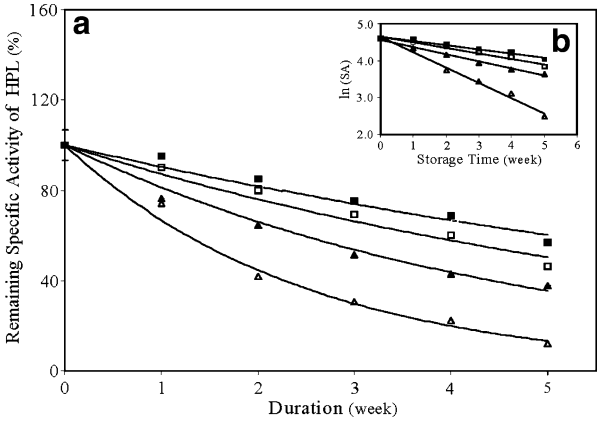

Fig. 1 **a** Thermal stability profiles of the HPL enzymatic extract from mint, where the (%) residual specific activity was determined for the lyophilized extract, without excipient added at 4 °C (*empty triangles*) and at −20 °C (*empty squares*) as well as for the lyophilized extract with KCl at 4 °C (*filled triangles*) and at −20 °C (*filled squares*); **b** The semi-logarithm plots of the thermal stability profiles

However, the presence of KCl increased 38.0% and 57.0% of the storage stability of the HPL enzymatic extract, after 5 weeks of storage at 4 °C and −20 °C, respectively (Fig. 1).

The semi-logarithmic plots of the inactivation kinetics at −20 °C and 4 °C (Fig. 1A) show straight lines, with high correlation coefficient values of $r^2 \geq 0.98$; these results indicate that the thermal inactivation of HPL in the presence and absence of KCl followed the first-order kinetic behavior. The half-life time ($t_{1/2}$) and the rate constant of inactivation ($K_{\text{inactivation}}$), estimated at different temperatures, are given in Table 2. The free-salt extract showed a $K_{\text{inactivation}}$ value of 0.41 and 0.15 at 4 °C and −20 °C, respectively, whereas that with KCl exhibited a $K_{\text{inactivation}}$ value of 0.19 and 0.11, respectively. The inactivation of the HPL enzymatic activity during storage may be due to the irreversible chemical degradation process, which was reported to be initiated by a small amount of water present in the lyophilized enzymatic extract [24]. The experimental findings (Table 2) also indicate that the lyophilization in the presence of KCl resulted in an increase of the $t_{1/2}$ value of the HPL by 1.3- to 2.3-fold at −20 °C and 4 °C, respectively, compared to that of the free-salt extract. Similarly, Hall et al. [16] reported that the stability of the HPL enzymatic extract from *P. camemberti* was greater in the presence of KCl than that without it. The stabilization of the HPL enzymatic activity in the presence of KCl may be ascribed to a decrease in the amount of water molecules in the enzyme's microenvironment and/or to an increase in the hydrophobic interactions, as a result of a high ionic strength of the homogenate [20]. In addition, these results are in agreement with those reported in literature [16, 22, 24], which have suggested that the higher the T_g of the preparation, the more stability will be displayed in the long-term.

Effect of Selected Additives on HPL Activity

A wide range of additives, including dithiothreitol, glycine, sucrose, mannitol, glycerol, and Triton X-100, were evaluated for their ability to preserve the HPL enzymatic activity during the enzymatic reaction. Table 3 shows that the selected polyhydroxyl-containing additives were not effective for stabilizing the HPL activity of the enzymatic extract from mint. The decrease in the HPL activity may be due to the ability of polyhydroxyl-containing additives to strip off the water layer surrounding the enzyme molecules, which is essential for the enzyme activity [24]. A direct effect of the molecular structure of additive on the enzyme itself, by binding either in or near its active site, may also be an explanation for the decrease in the enzymatic activity [25]. Contrary to the HPL activity from mint, many other HPL from other sources showed an enhancement in the presence of polyols [20, 26]; it was suggested that the protective interaction between the enzyme and additives might imply a direct involvement of hydroxyl groups [26].

Table 3 shows that the most appropriate additive for the stabilization of HPL enzymatic activity was glycine. The results (data not shown) also indicate that the stabilization effect

Table 2 Stability of lyophilized HPL extract with and without KCl (5%, *w*/*w*) at −4 °C and −20 °C.

	Lyophilized extract		Lyophilized extract with KCl	
	4 °C	−20 °C	4 °C	−20 °C
$K_{\text{inactivation}}$[a]	0.414 (±0.022)	0.148 (±0.012)	0.194 (±0.009)	0.113 (±0.009)
$t_{1/2}$[b]	1.6	4.7	3.7	6.1

[a] The constant of inactivation ($K_{\text{inactivation}}$) was estimated from the slope of the plot of the ln percentage remaining specific activity versus the week number of storage at 4 °C and −20 °C

[b] The half-life time of the HPL enzymatic extract from mint is defined as the number of weeks at which 50% of the initial activity was obtained

Table 3 Effect of adding selected additives in the reaction medium on the specific activity of the HPL enzymatic extract from mint.

Additives[a]	Additive concentration	Specific activity[b]	Relative specific activity[c]
Control	–	0.139 (±7.0)[d]	100
DTT	15[e]	0.267 (±2.4)[d]	191.6
Glycine	10[f]	0.353 (± 9.6)[d]	252.8
Succrose	5[f]	0.070 (±13.3)[d]	50.5
Mannitol	5[f]	ND	ND
Glycerol	3[f]	0.126 (±7.5)[d]	90.2
Triton X-100	0.5[f]	0.181 (±6.8)[d]	129.6
Glycine + DTT	10[f], 15[e]	0.366 (±18.9)[d]	262.0
DTT + Triton	15[e], 0.5[f]	0.301 (±6.8)[d]	215.4

ND Not detected

[a] HPL-catalyzed reaction was conducted at 15 °C and pH 6.5 using 13-hydroperoxy-linoleic acid as substrate

[b] HPL specific activity is defined as μmol of converted HPOD per mg of protein per minute of reaction

[c] The relative specific activity was defined as the percentage of HPL specific activity in the presence of additive compared with that of the control (without additives)

[d] The assays were run in triplicate and relative standard deviations (%) are given

[e] Concentration of the additive is expressed in mM

[f] Concentration of the additive is expressed in % (*w*/*v*)

of glycine was 4.3-fold higher in the presence of KCl than that without it; these experimental findings suggest a beneficial interaction of KCl and glycine with HPL for its stabilization. Glycine has been reported to increase the hydrophobic interaction, as a result of the exclusion of the chemical additive from the protein surface [25]. The experimental results (Table 3) also indicate that Triton X-100 and dithiothreitol (DTT) enhanced the HPL activity by 1.2- and 1.9-fold, respectively, compared to that of the control. Many studies [8, 26–28] have reported that the non-ionic detergents are effective additives for the recovery of HPL from the cell membranes and for the enhancement of its activity. Koeduka et al. [8] suggested that the addition of detergents to the reaction mixture affected the binding of HPL to its substrate, leading, hence, to a decrease in K_m value. Noordermeer et al. [29] suggested that Triton X-100 induced a subtle conformational change in alfalfa HPL, which may result in an improved spin state of the heme. On the other hand, dithiothreitol has been used to maintain the sensitive sulfydryl residues of HPL in its reduced form [27, 30]. On the basis of the experimental findings (Table 3), glycine, Triton X-100, and dithiothreitol have been used as selected additives for all subsequent investigations.

Effect of Selected Additive Concentrations on HPL Activity

Figure 2 indicates that the effect of glycine and dithiothreitol on the HPL specific activity was dependent on their concentrations. Compared to the other investigated chemical additives, glycine seems to be the most stabilizer additive; it increased the HPL specific activity from 0.14 to 0.39 μmol converted HPOD/(min mg proteins), when its concentration was increased from 0% to 20%. This increase may be due to an increase in the hydrophobic interaction, as a result of the exclusion of chemical additive from the protein surface [25].

The experimental findings (Fig. 2) also show that increasing DTT concentration led to an increase in the HPL-specific activity to a maximum of 0.29 μmol converted HPOD/(min

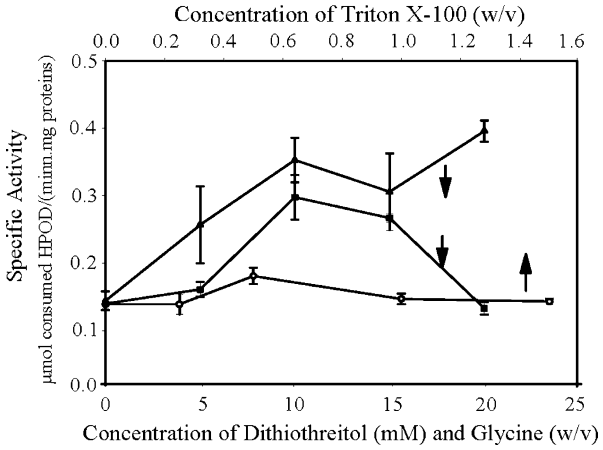

Fig. 2 Effect of additive concentration on the HPL activity of the enzymatic extract from mint: (*empty circles*) Triton X-100 (*w*/*v*); (*filled squares*) dithiothreitol (mM); and (*filled triangles*) glycine (*w*/*v*)

mg protein) at a DTT concentration of 10 mM; this increase was followed by a decrease in the HPL specific activity to 0.26 and 0.13 µmol converted HPOD/(min mg protein) at a DTT concentration of 15 and 20 mM, respectively. Thiol-containing additives have been reported to maintain the sensitive sulfydryl residues of HPL in the reduced form [27, 30]. However, the decrease in the HPL specific activity at a concentration above 10 mM may be due to the direct effect of the molecular structure of DTT on the enzyme itself, resulting hence in a conformational and/or an electronic distribution changes. Dithiothreitol is commonly added in HPL extracts at concentrations ranging from 1 to 7 mM [13, 30].

Compared to glycine and dithiothreitol, the effect of Triton X-100 was independent on its concentration; a specific activity of 0.14 µmol converted HPOD/(min mg protein) was obtained at a wide range of Triton X-100 concentrations, varying from 0.25% to 1.5% (*w*/*v*). Detergents are generally added to the HPL enzymatic extract at percentage ranging from 0.1% to 0.5% (*w*/*v*) [11, 13]; they are regarded as inactivators for HPL activity at a concentration greater than 1% (*w*/*v*) [31].

Effect of Additives on HPL Thermal Stability

The effect of some chemical additives, including glycine (10 and 20%, *w*/*v*) and Triton X-100 (0.5%, *w*/*v*), on the thermal stability of the HPL enzymatic extract from mint was investigated. Figure 3 illustrates the remaining HPL activity, in the presence and absence of additives, after 1 h incubation at a wide range of temperatures varying from 0 °C to 65 °C. The overall results indicate an increase in the thermal stability of the HPL enzymatic extract in the presence of additives compared to that of the control assay. The HPL activity of the control assay showed a residual specific activity of 93%, 74%, 46%, and 29% after 1 h incubation at 25 °C, 35 °C, 45 °C, and 55 °C, respectively; however, it was completely inactivated at 65 °C. In contrast, the HPL activity, assayed in the presence of 10% (*w*/*v*) glycine, remained constant after 1 h incubation at temperatures ranging from 0 °C to 35 °C; at 45 °C and 65 °C, it showed 59% and 21% of the initial HPL activity, respectively. At a high concentration of glycine 20% (*w*/*v*), the HPL thermal stability profile showed a slow decline in the enzyme residual activity from 100% to 70 and 37% at 45 and 65 °C, respectively. Triton X-100 appeared also to stabilize the HPL activity at temperatures

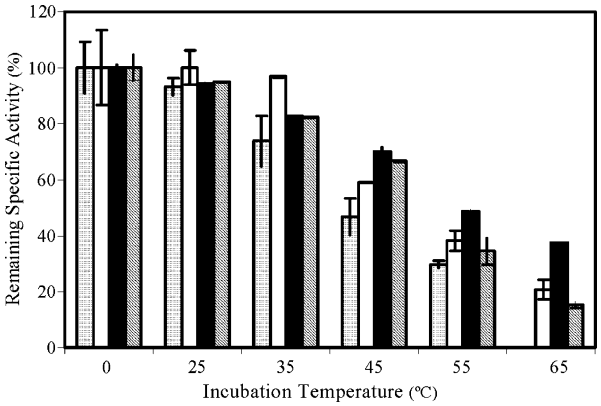

Fig. 3 Effect of selected additives on the thermal stability profile of the HPL enzymatic extract from mint, after temperature treatments of 1 h, where the (%) residual specific activity of HPL was determined for trials containing: (▨) without excipient, (*empty squares*) 10% glycine, (*filled squares*) 20% glycine, and (▨) 0.5% (*w*/*v*) Triton X-100

ranging from 25 °C to 45 °C; however, at higher temperatures of 55 °C and 65 °C, a rapid decrease in the HPL activity was obtained compared to that of the control assay. The thermal stability of the HPL enzymatic extract from mint at 55 °C in the presence of glycine (20%) was 1.4-fold higher than that observed with Triton X-100.

Monsan and Combes [10] have suggested that the protective effect of several chemical additives on the enzyme thermal stability is attributed to their effect on the water activity; indeed, the high interaction of additives with water molecules will result in the formation of water clusters and then in the reduction of the amount of free water [31, 32]. The thermal stability profiles of HPL are in agreement with those reported for HPL activity from other sources [16, 31, 33]. Kim and Grosch [33] reported that the pear HPL activity lost 100% of its initial activity after 1 h incubation at 50 °C. The sunflower HPL activity, incubated at 4 °C for 48 h, showed a loss of more than 50% of its activity [31].

Kinetic Parameters of HPL Enzymatic Extract

Kinetic parameters of the HPL activity expressed in the enzymatic extract from mint were determined and compared in the presence and in the absence of salt and/or glycine. The correlation between the activity of the HPL enzymatic extract from mint versus 13-HPOD substrate concentration resulted by a hyperbolic curve, suggesting Michaelis–Menten kinetics (Fig. 4a). These results were confirmed by the linearity of the Lineweaver Burk plots (Fig. 4b). For all investigated enzymatic extract, a plateau was obtained in the range of substrate concentrations, 100 to 200 μM. Table 4 shows the kinetic parameters (V_{maxapp} and K_{mapp}), estimated by linear regression.

As shown in Table 4, V_{maxapp} of the HPL enzymatic extract, containing KCl, is 1.57-fold higher than that of the free-salt extract; however, the K_{mapp} for the HPL enzymatic extract containing salt (38.4 μM) was higher than that for the free-salt (9.7 μM). The presence of the salt seems to decrease the affinity of HPL toward its substrate; these results may be due to the effect of KCl on the binding of substrate in the active site and/or its partition in the enzyme's microenvironment. The experimental findings also indicate that the addition of glycine (10%) to the enzymatic assay led to a 2.45-fold increase in the catalytic efficiency, $V_{maxaapp}/K_{mapp}$, of the HPL enzymatic extract containing KCl. These results may indicate

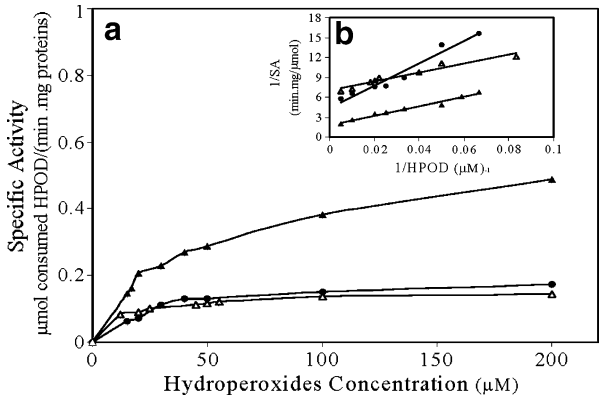

Fig. 4 **a** Kinetic studies of free salt HPL enzymatic extract from mint (*empty triangles*), as well as that with KCl (5%, *w/w*) carried out in the absence of glycine (*filled circles*), and in the presence of 10% (*w/v*) glycine (*filled triangles*); **b** The Lineweaver Burk plots

the synergistic action of glycine and KCl additives for the stabilization and the activation of HPL from mint.

In addition to the use of crude enzymatic extract, the variability of the enzymatic assays reported in literature made it difficult to compare the K_{mapp} values of HPL from different sources. However, the K_{mapp} value (26 μM) of HPL from tomato fruit [11] and that (50 μM) from watermelon leaves [13] were higher than that (9.7 μM) obtained for the HPL from mint (Table 4). Furthermore, the catalytic efficiency (5.7×10^{-3} L/mg min) for the HPL enzymatic extract from mint, containing KCl, is lower than that (2.94 L/min) reported for the HPL from strawberry fruit in the presence of 1 M of KCl [34].

Conclusion

The overall findings showed that the HPL enzymatic extract from mint was activated and stabilized by the addition of selected chemical additives. KCl excipient was not only found to be effective for protecting the HPL enzymatic extract from mint during lyophilization, but also for activating and stabilizing its specific activity. On the other hand, glycine, added

Table 4 Kinetic parameters of the HPL enzymatic extract from mint.

Enzymatic extract	K_M[a]	V_{max}[b]	Catalytic efficiency[c]
Lyophilized[d]	9.7 (±1.4)	0.14 (±0.004)	14.4×10^{-3}
Lyophilized + KCl (5%, w/v)[e]	38.4 (±4.2)	0.22 (±0.010)	5.7×10^{-3}
Lyophilized + KCl (5%, w/v) + glycine (10%, w/v)[f]	38.5 (±4.5)	0.54 (±0.024)	14×10^{-3}

[a] K_M values were defined as μM of substrate.

[b] V_{max} values were defined as μmol of converted hydroperoxides per mg of protein per minute of reaction.

[c] Catalytic efficiency was defined as V_{max}/K_M.

[d] HPL enzymatic extract was lyophilized without KCl added.

[e] HPL enzymatic extract was lyophilized with KCl.

[f] Glycine was added to the reaction medium of HPL. The enzymatic extract was freeze-dried with KCl.

in the enzymatic assay, was the most appropriate chemical additive for the HPL activity as well as for its thermal stability. The experimental data obtained throughout this study could be of interest for the potential biotechnological use of the HPL enzymatic extract from mint for the biosynthesis of selected flavoring compounds.

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