Biochemical characterisation of chlorophyllase from leaves of selected Prunus species — A comparative study

Hubert Śtytkiewicz, Iwona Sprawka, Paweł Czerniewicz, Cezary Sempruch, Bogumił Leszczyński and Marlena Sikora

Department of Biochemistry and Molecular Biology, Siedlce University of Natural Sciences and Humanities, Siedlce, Poland

Despite senescence-induced chlorophyll depletions in plants has been widely studied, the enzymatic background of this physiologically regulated process still remains highly unclear. The purpose of this study was to determine selected biochemical properties of partially purified fractions of chlorophyllase (Chlase, chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14) from leaves of three Prunus species: bird cherry (Prunus padus L.), European plum (Prunus domestica L.), and sour cherry (Prunus cerasus L.). Secondarily, this report was aimed at comparing seasonal dynamics of Chlase activity and chlorophyll a (Chl a) content within investigated plant systems. Molecular weight of native Chlase F1 has been estimated at 90 kDa (bird cherry) and approximately 100 kDa (European plum and sour cherry), whereas molecular mass of Chlase F2 varied from 35 kDa (European plum) to 60 kDa (sour cherry). Furthermore, enzyme fractions possessed similar optimal pH values ranging from 7.6 to 8.0. It was found that among a broad panel of tested metal ions, Hg²⁺, Fe²⁺, and Cu²⁺ cations showed the most pronounced inhibitory effect on the activity of Chlase. In contrast, the presence of Mg²⁺ ions influenced a subtle stimulation of the enzymatic activity. Importantly, although Chlase activity was negatively correlated with the amount of Chl a in leaves of examined Prunus species, detailed comparative analyses revealed an incidental decrement of enzymatic activity in early or moderately senescing leaves. It provides evidence that foliar Chlase is not the only enzyme involved in autuminal chlorophyll breakdown and further in-depth studies elucidating this catabolic process are required.

Key words: chlorophyllase; chlorophyll catabolism; leaf senescence; Prunus genus

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INTRODUCTION

Chlorophyllase (Chlase, chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14) is a chloroplast membrane protein that has been considered to be involved in catalyzing dephytilation reaction of chlorophyll molecules into specific chlorophyllide (Chlide) forms (Arkus et al., 2005; Okazawa et al., 2006; Harpaz-Saad et al., 2007; Yi et al., 2007). This enzyme was isolated and partially purified from tissues of a broad range of higher plants and algae, and its catalytic activity under in vitro conditions was intensively studied by many researchers (Hornero-Méndez & Mínguez-Mosquera, 2001; Todorov et al., 2003; Tsuchiya et al., 2003; Arkus et al., 2005; Okazawa et al.; 2006; Lee et al., 2010; Gupta et al., 2011; Gupta et al., 2012). It has been elucidated that young leaves of tea and tobacco possess higher levels of Chlase activity when compared to their respective mature organs (Kuroki et al., 1981; Todorov et al., 2003b). Additionally, Todorov et al. (2003a) revealed that upper rosette leaves of Arabidopsis thaliana are characterised by lower activity of Chlase and higher amounts of chlorophylls in relation to the inferior ones. It should be underlined that many authors reported an elevated activity of Chlase in plants subjected to a wide spectrum of environmental stressors such as water or manganese deficiency (Majumdar et al., 1991; Saidi et al., 2012), heavy metal exposure (Mihailovic et al., 2008), low and high temperature stress (Johnson-Flanagan & McLachlan, 1990; Todorov et al., 2003b), aphid infestation (Giepiela et al., 2005), and phytotoxicogenic infections and wounding (Stangarlin & Pascholati, 2000; Kariola et al., 2005). Interestingly, Todorov et al. (2003a) demonstrated higher Chlase activity in leaves of the ethylene-insensitive mutant (eti5) of A. thaliana when compared to the wild-type plants. Furthermore, changes in the enzyme activity in response to low and high temperature stress were more significant in leaves of eti5 mutants in relation to the wild-type ones (Todorov et al., 2003b). According to these authors, participation of ethylene is essential in stress-induced enhancement of Chlase activity in leaf tissues. Despite the fact that catabolic breakdown of green pigments is a developmentally controlled process occurring during leaf senescence or fruit ripening, the sophisticated regulatory mechanisms underlying diminution in the content of chlorophylls in vivo are still not well understood. Importantly, Schenk et al. (2007) evidenced that chl1 and chl2 mutants of A. thaliana with double knockout of two chlorophyllase (AtCHL1 and AtCHL2) genes were still prone to chlorophyll degradation. Additionally, a new pathway of senescence-related chlorophyll depletions in plant tissues that involves a pheophytinase (PPH) has been recently recognized, a novel plastid enzyme converting pheophytin (Pheo) into pheophorbide (Pheide) (Schelbert et al., 2009).

In the context of contrary or inconclusive data published, concerning vague biological functions of Chlase in leaf tissues (Todorov et al., 2003b; Tang et al., 2004; Ben-Yakov et al., 2006; Schenk et al., 2007; Schelbert et al. 2009).
al. 2009), it is highly important to verify whether gradual chlorophyll breakdown during the progression of seasonal foliar senescence may be due to relevant changes in the levels of Chlase activity. Therefore, the ultimate purpose of performed analyses was to compare selected biochemical properties of partially purified Chlase pre pared from leaves of three phylogenetically related plant species belonging to the genus of Prunus: bird cherry (Prunus padus L.), European plum (Prunus domestica L.), and sour cherry (Prunus cerasus L.). Consequently, the present report was also aimed at evaluating specific patterns of Chlase activities and Chl \( a \) contents in leaves of the examined plants at different stages of their ontoge netic development (young, mature, and early, moderately, and progressively senescent leaves).

**MATERIALS AND METHODS**

**Plant materials.** Leaves of three representatives of Prunus genus: bird cherry (Prunus padus L.), European plum (Prunus domestica L.), and sour cherry (Prunus cerasus L.) were collected from shrubs that had grown in the Central-Eastern region of Poland (Siedlce district) during years 2006–2008. Five developmental stages of leaves (Y — young, M — mature, ES — early senescent, MS — moderately senescent, PS — progressively senescent) were included in the experiments regarding time-course changes in the activity of Chlase fractions and Chl \( a \) content. The age determination of examined plant organs was based on the observations of leaf blades and the content of foliar chlorophyll \( a \). Mature leaves of the examined plants were used to investigate the biochemical properties of studied chlorophyllases. The collected leaf samples were immediately frozen in liquid nitrogen and stored at −80°C until further analyses.

**Enzyme extraction.** The procedure of Chlase isolation was carried out using a modified method described by Todorov et al. (2003a). Frozen leaf tissues (30 g) were homogenized at 4°C for 5 min. in 400 cm\(^3\) of ice-cold 80% (v/v) acetone. The homogenate was filtered through two layers of gauze, and subsequently centrifuged at 5000 × \( g \) for 10 min. The supernatant was used for Chl \( a \) determination, whereas the pellet was resuspended in the extraction buffer (5 mM potassium phosphate buffer, containing 50 mM KCl and 0.24% Triton X-100, pH 7.0). The mixture was centrifuged at 12000 × \( g \) for 10 min., and thereafter the obtained supernatant was decanted and utilized as the crude enzyme extract in further biochemical analyses.

**Ammonium sulfate precipitation and dialysis.** The first purification phase of the obtained Chlase extracts was performed using ammonium sulfate precipitation (Yang et al., 1997). Solid ammonium sulfate (35.4 g) was added to each portion of the supernatant (100 cm\(^3\)) to receive 30% saturation. Precipitated proteins were centrifuged at 10000 × \( g \) for 20 min. The supernatant was decanted and ammonium sulfate was added to obtain 60% saturation. The mixture was centrifuged as described above. Then, the supernatant was discarded and the pellet was dissolved in the extraction buffer. Subsequently, the solution was subjected to dialysis against the extraction buffer at 4°C for 24 h.

**Gel filtration and molecular mass determination.** Further purification procedures comprised separation of Chlase fractions and determination of molecular masses of native enzymes. Dialysates (portions of 10 cm\(^3\)) were loaded onto a glass column (2 × 40 cm) filled with Sephadex G-200 bed and equilibrated with 200 cm\(^3\) of extraction buffer (pH 7.0). Enzyme elution was performed with the same buffer at the flow-rate of 0.3 cm\(^3\)·min\(^{-1}\). The eluates were collected in portions of 2 cm\(^3\), and subsequently, the content of protein was determined by means of a spectrophotometric method described by Layne (1957). Absorbance values were measured at 280 and 260 nm. Protein concentration (PC) in analysed eluates was calculated using the following empirical equation:

\[
PC \text{ (mg} \cdot \text{cm}^{-3}) = 1.55 \times A_{280} - 0.76 \times A_{260}
\]

The molecular weight (kDa) of native Chlase pre pared was estimated with the use of experimentally outlined calibration curve showing a relationship between \( K_c \) (coefficient of partitioning) and log\(_{10}\) molecular mass of the following standard proteins used: cytochrome \( c \) (12.3 kDa), trypsin (23.8 kDa), ovalbumin (45.0 kDa), bovine serum albumin (66.0 kDa), and myosin (205.0 kDa) (Sytykiewicz et al., 2008).

**Chlorophyllase activity assay and determination of Chl \( a \) concentration.** Determination of Chlase activity was performed according to the method of Yang et al. (2004), with minor modifications. The reaction mixture consisted of 0.35 cm\(^3\) of the enzyme prepare, 0.15 cm\(^3\) (0.1 µM final concentration) of Chl \( a \) from spinach dissolved in acetone (Sigma-Aldrich, Germany), and 1 cm\(^3\) of potassium phosphate buffer (5 mM, pH 7.0), containing 50 mM KCl and 0.24% Triton X-100. Samples were incubated in darkness at 30°C for 30 min. The reaction was stopped by addition of 2 cm\(^3\) of ice-cold acetone and 2 cm\(^3\) of \( n \)-hexane. The mixture was vigorously vortexed and centrifuged for 2 min. at 12000 × \( g \). Subsequently, both the residual content of substrate in the upper layer of this mixture and Chl \( a \) amount in acetone solution obtained during the enzyme extraction were determined by measuring the absorbance at three wavelengths: 644, 662, and 750 nm, using a Hewlett-Packard UV-Vis spectrophotometer (model 8453). The concentration of Chl \( a \) in tested samples was calculated using the following formula (Ihl et al., 2000):

\[
\text{Chl} \ a \text{ (nmol} \cdot \text{cm}^{-3}) = 11.30 \times (A_{662} - A_{644}) - 1.11 \times (A_{750} - A_{644})
\]

The specific activity of Chlase was defined as nanomole of substrate (Chl \( a \)) hydrolyzed per minute per mg of protein (Arriagada-Strodthoff et al., 2007). The concentration of protein was measured using the method of Lowry et al. (1951). The calibration curve was prepared for bovine serum albumin (BSA) in the range of amounts: 0.25–3.0 mg/cm\(^3\).

**Assessment of optimal pH.** Determination of the optimal pH of purified Chlase preparates was accomplished with the use of Theorell/Steinhagen buffer. The following pH values of the reaction mixture were included during the experiments: 3.0, 4.0, 5.0, 6.0, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8, 9.0, 10.0, 11.0, and 12.0. The reaction mixtures contained 0.35 cm\(^3\) of the enzyme extract, 0.15 cm\(^3\) of Chl \( a \) acetone solution (0.1 µM final concentration), and 1 cm\(^3\) of the corresponding buffer solution. The chlorophyllase activity was assayed as described above.

**Determination of kinetic parameters of the purified Chlase.** The Michaelis constant (\( K_m \)) was evaluated using the Lineweaver-Burk equation. To determine this parameter, the initial velocity (\( V_0 \)) of the enzymatic reaction catalyzed by Chlase was estimated. Substrate specificity of the analysed Chlase fractions was designated for both Chl \( a \) and Chl \( b \) in the concentration range: 0–50 µM. The reaction mixtures were incubated in the dark.
at 30°C for 5, 10, 15, 20, 30, and 40 min., and subsequently the specific activity of Chlase was determined.

**Effect of metal ions and functional groups’ modifiers on the enzyme activity.** To establish the impact of different metal chlorides and reagents modifying amino acids molecules on Chlase activity, the enzyme samples were preincubated for 24 h at 4°C in the presence of 10 mM concentrations of tested metal cations (Ag⁺, Co²⁺, Cu²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Zn²⁺, Al³⁺, Bi³⁺, Fe³⁺) and the functional groups’ modifiers (2-mercaptoethanol, diethylpyrocarbonate, diethiothreitol, and phenylmethanesulfonyl fluoride). After this stage, the reaction mixtures containing the effectors were incubated in the dark at 30°C for 30 min, and thereafter the specific activity of Chlase was assayed.

**Statistical analyses.** The experiments regarding Chlase isolation, purification, and determination of its biochemical properties were performed in a completely randomized design. All analytical procedures were conducted in three independent replicates (n=3) and the results are expressed as an average ± S.D. (standard deviation). The empirical results were analysed using STATISTICA 9.0 software (StatSoft). Interdependence between the activity of Chlase fractions and Chl a concentration in leaves of tested Prunus species was evaluated by calculating the Pearson’s correlation coefficient (R). Significance of differences between the mean values of analysed Chlase parameters (relative activity of the enzyme in the presence of tested metal ions and functional groups modifiers) was subjected to a one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT). Values of p≤0.01 were considered as statistically significant.

**RESULTS**

Results regarding the efficiency of isolation and partial purification of Chlase preparations from leaves of the investigated Prunus species are presented in Table 1. It was established that crude extracts of Chlase from European plum demonstrate the highest enzyme activity, while the cell-free leaf homogenates of bird cherry possessed the lowest Chlase activity. The performed sequence of enzyme purification techniques led to a gradual increment in the specific Chlase activity in parallel with a decrease in the content of protein. Elution profiles of Chlase preparations that were purified using the gel filtration chromatography on a Sephadex G-200 column revealed the presence of 7–8 protein fractions, depending on the examined plant species (Fig. 1). Importantly, the assay of Chlase activity proved the occurrence of two fractions of this enzyme in leaves of all tested plant systems. The two peaks of Chlase activity were designated fractions F1 and F2 by the order of elution.

It was demonstrated that Chlase F2 from tissues of bird cherry had higher activity (34.2%) than the fraction 1. Conversely, Chlase F1 from European plum and
sour cherry demonstrated lower activity (13.1 and 35.5%, accordingly) in comparison with the corresponding fractions from European plum and sour cherry. It was found that the activity of Chlase F2 from sour cherry demonstrated lower activity (13.1 and 35.5%, accordingly) in comparison with the corresponding fraction from European plum. Moreover, it was found that both Chlase fractions extracted from leaves of the investigated plant species were characterised by higher $K_m$ values for Chl $b$ in comparison with the kinetic data obtained for Chl $a$. An opposite tendency was ascertained only in case of Chlase F2 from sour cherry that had a lower $K_m$ level (approx. 10%) estimated for Chl $a$ than Chl $b$.

It has been revealed that the presence of analysed metal ions and functional groups’ modifiers in the reaction mixtures led to very similar changes in the relative activity of Chlases isolated from leaves of the investigated representatives of Prunus genus (Table 4). It should be underlined that among eleven tested metal ions, only magnesium cations evoked a slight increase (2–9%, depending on the enzyme preparates) in the relative activity of the analysed Chlases. The ultimate high suppression of Chlase activity was demonstrated in the case of Hg$^{2+}$, Fe$^{2+}$, and Cu$^{2+}$ ions (75–80, 65–71 and 58–66% loss of the enzyme activity, respectively).

**Table 2. Molecular mass (kDa) of the analysed Chlase fractions.**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Chlase fractions</th>
<th>Molecular weight (kDa)</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird cherry</td>
<td>1</td>
<td>90</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40</td>
<td>0.44</td>
</tr>
<tr>
<td>European plum</td>
<td>1</td>
<td>~100</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35</td>
<td>0.46</td>
</tr>
<tr>
<td>Sour cherry</td>
<td>1</td>
<td>~100</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>60</td>
<td>0.35</td>
</tr>
</tbody>
</table>

$K_m$ values (µM) are presented as the mean ± S.D. (n=3).

**Table 3. The Michaelis constant (µM) of the studied Chlase fractions calculated for Chl $a$ and Chl $b$ as substrates.**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Chlase fraction</th>
<th>Chl $a$</th>
<th>Chl $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird cherry</td>
<td>1</td>
<td>2.8±0.04</td>
<td>3.0±0.07</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.1±0.06</td>
<td>3.5±0.11</td>
</tr>
<tr>
<td>European plum</td>
<td>1</td>
<td>3.5±0.12</td>
<td>3.9±0.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.8±0.26</td>
<td>6.1±0.45</td>
</tr>
<tr>
<td>Sour cherry</td>
<td>1</td>
<td>3.4±0.09</td>
<td>3.7±0.14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.2±0.21</td>
<td>3.8±0.16</td>
</tr>
</tbody>
</table>

$K_m$ values (µM) are presented as the mean ± S.D. (n=3).
whereas a moderate decrement of Chlase activities was influenced by the presence of Zn\(^{2+}\), Al\(^{3+}\), and Bi\(^{3+}\) cations (41–49, 44–51 and 42–48%, accordingly). Furthermore, the lowest inhibition of the relative activity of the examined Chlase preparates was caused by the addition of Ag\(^{+}\) ions (11–18%). It has also been noted that the activity of chlorophyllases from the tested plant species was inhibited by phenylmethanesulfonyl fluoride (PMSF, 31–42%) and diethylpyrocarbonate (DEPC, 17–30%). On the contrary, the presence of 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) in the reaction mixture led to a subtle increase (1–5 and 2–8%, respectively) in the enzyme activity when compared to the non-treated samples.

In this report, the specific activity of both studied Chlase fractions and Chl\(\alpha\) concentrations were monitored within leaves of the tested Prunus species collected at five stages of their ontogenetic development (Y — young, M — mature, ES — early senescent, MS — moderately senescent and PS — progressively senescent). Performed biochemical analyses proved that Chlase F1 and F2 isolated from European plum displayed the highest level of activity, whereas the lowest values of the enzymatic activity were demonstrated in case of Chlase preparates from bird cherry leaves (Fig. 2). Importantly, the following three patterns of time-course variations in the specific activity of Chlase fractions have been identified: i) slight continuous increment in the enzyme activity (fraction 1—European plum); ii) constant increase in Chlase activity with an incidental decline at ES stage (fraction 1—bird cherry and sour cherry, fraction 2 — European plum); iii) steady elevation in the catalytic activity with a one-time decrement at MS stage (fraction 2 —bird cherry and sour cherry). Furthermore, the highest content of Chl\(\alpha\) was ascertained in bird cherry leaves, while concentrations of the analysed green pigments in foliar tissues of European plum and sour cherry were detected at comparable levels. It has been found that seasonal changes in the content of Chl\(\alpha\) in leaves of examined Prunus species are significant.

### Table 4. The influence of tested effectors (10 mM) on the activity of chlorophyllase.

<table>
<thead>
<tr>
<th>Metal ion/reagent</th>
<th>Relative activity (%)</th>
<th>Bird cherry</th>
<th>European plum</th>
<th>Sour cherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag(^{+})</td>
<td>85 ± 6a</td>
<td>89 ± 8a</td>
<td>82 ± 5a</td>
<td></td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>72 ± 5a</td>
<td>78 ± 6a</td>
<td>75 ± 6a</td>
<td></td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>34 ± 2b</td>
<td>42 ± 3a</td>
<td>39 ± 2a</td>
<td></td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>35 ± 3a</td>
<td>29 ± 2b</td>
<td>31 ± 2b</td>
<td></td>
</tr>
<tr>
<td>Hg(^{2+})</td>
<td>20 ± 1b</td>
<td>25 ± 2a</td>
<td>22 ± 1a</td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>106 ± 4a</td>
<td>109 ± 5a</td>
<td>102 ± 3a</td>
<td></td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>73 ± 2a</td>
<td>78 ± 3a</td>
<td>72 ± 2a</td>
<td></td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>54 ± 2a</td>
<td>59 ± 3a</td>
<td>51 ± 2a</td>
<td></td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>49 ± 4a</td>
<td>56 ± 3a</td>
<td>52 ± 4a</td>
<td></td>
</tr>
<tr>
<td>Bi(^{3+})</td>
<td>55 ± 2a</td>
<td>58 ± 3a</td>
<td>52 ± 2a</td>
<td></td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>78 ± 2a</td>
<td>71 ± 1a</td>
<td>73 ± 2a</td>
<td></td>
</tr>
<tr>
<td>2-ME</td>
<td>105 ± 5a</td>
<td>102 ± 5a</td>
<td>101 ± 4a</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>102 ± 4a</td>
<td>108 ± 5a</td>
<td>103 ± 5a</td>
<td></td>
</tr>
<tr>
<td>DEPC</td>
<td>72 ± 2b</td>
<td>70 ± 2b</td>
<td>83 ± 3a</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>65 ± 3a</td>
<td>58 ± 2b</td>
<td>69 ± 3a</td>
<td></td>
</tr>
</tbody>
</table>

Empirical data are given as the mean ± S.D. (n=3). The relative activity of Chlase is expressed as percentage changes in relation to non-treated enzyme samples (control=100%). Different letters in rows indicate significant differences (p≤0.01) by Duncan’s Multiple Range Test.

### Table 5. Correlation between the levels of specific activity of analysed Chlase fractions and the content of Chl\(\alpha\) in leaves of investigated Prunus species.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>R</th>
<th>Chlase F1–Chl(\alpha)</th>
<th>Chlase F2–Chl(\alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird cherry</td>
<td>-0.710</td>
<td>-0.653</td>
<td></td>
</tr>
<tr>
<td>European plum</td>
<td>-0.295</td>
<td>-0.258</td>
<td></td>
</tr>
<tr>
<td>Sour cherry</td>
<td>-0.583</td>
<td>0.450</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.01. The values of Pearson’s correlation coefficient (R) were calculated using STATISTICA 9.0 software (StatSoft). Chlase F1–Chl\(\alpha\) and Chlase F2–Chl\(\alpha\) represent the correlation between specific activity of the relevant Chlase fractions and Chl\(\alpha\) concentrations in leaves of the tested plants.
amined Prunus species were quite similar. Moreover, an increase in the concentration of Chl a during the process of leaf maturation was noted. Conversely, gradual chlorophyll depletion occurred in parallel with the progression of leaf senescence. Conducted statistical analyses revealed non-significant negative correlations between the specific activity of Chlase fractions and the content of Chl a in leaves of the tested plants (Table 5). Furthermore, it should be emphasized that the values of Pearson's correlation coefficient calculated for fraction 1 of the studied Chlases reached slightly lower levels when compared to the relevant samples of fraction 2.

**DISCUSSION**

In recent years, intensive efforts have been focused on deciphering the complex biological functions of Chlase and other crucial enzymes involved in chlorophyll turnover and maintaining its homeostasis at the physiological state of the cell, as well as during the adaptation of plants to a wide variety of biotic and abiotic stressors (Fernandez-Lopez et al., 1992; Karboune et al., 2005; Arkus & Jez, 2006; Azoulay-Shemer et al., 2008; Barry, 2009; Cowan, 2009; Beisel et al., 2010; Azoulay-Shemer et al., 2011; Banas et al., 2011; Büchert et al., 2011; Sytykiewicz et al., 2013). Surprisingly, numerous results regarding the participation of Chlase in senescence-induced chlorophyll diminution within different plant systems were often divergent or inconclusive (Wang et al., 2005; Ben-Yaakov et al., 2006; Criado et al., 2006; Hörtenstein et al., 2006; Barry, 2009; Distefano et al., 2009; Büchert et al., 2011; Gómez-Lobato et al., 2012). In order to clarify these discrepancies, there is a necessity to perform comprehensive studies on the biochemical and molecular properties of Chlase prepurposes from leaves of phylogenetically related plants, on the one hand, and to compare the patterns of seasonal dynamics of the enzyme activity in the context of time-course changes in chlorophylls' content, on the other hand. To the best of our knowledge, there are no published studies referring to the isolation, purification, and biochemical characterisation of foliar chlorophyllases from tissues of plant species classified within Prunus genus. Therefore, the experimental design of this report included extraction and a sequence of purification procedures of Chlase prepurposes from leaves of three investigated plant systems (bird cherry, European plum, and sour cherry). It was demonstrated that homogenization conditions for the enzyme isolation (5 mM potassium phosphate buffer, containing 50 mM KCl and 0.24% Triton X-100, pH 7.0) and applied purification techniques (ammonium sulfate precipitation, dialysis, and Sephadex G-200 gel filtration chromatography) were sufficient to obtain nearly homogeneous Chlase prepurposes. The enzyme is closely associated with protein complexes of the chloroplast membrane, therefore the presence of Triton X-100 (a nonionic surfactant) in the extraction buffer not only improved the cell membrane disruption, but also enhanced the efficiency of Chlase isolation. Similar analytical procedures were successfully applied by many authors in order to obtain partially purified Chlase prepurposes (Trebish et al., 1993; Tsuchiya et al., 1997; Schenk et al., 2007). On the other hand, Lee et al. (2010) identified the occurrence of three Chlase isoforms (BoCLH1, BoCLH2, and BoCLH3) in florets of broccoli (Brassica oleracea). However, the isozyme BoCLH3 possessed extremely low catalytic activity under in vitro conditions when compared to other detected isoforms of Chlase.

On the basis of our results, it was estimated that molecular weights of Chlase F1 isolated from European plum and sour cherry were almost identical (approx. 100 kDa), while fraction 1 of the analysed enzyme from P. padus demonstrated a slightly lower M, value (90 kDa). Furthermore, it was established that molecular masses of Chlase F2 from the leaves of tested plant systems reached lower levels (35 kDa — European plum, 40 kDa — bird cherry, 60 kDa — sour cherry) when compared with fraction 1. Several studies demonstrated that molecular masses of chlorophyllases extracted from different plant species varied between 23.5 and 158 kDa (Fernandez-Lopez et al., 1992; Tang et al., 2004; Arkus et al., 2005; Lee et al., 2010; Azoulay-Shemer et al., 2008). Importantly, Arkus et al. (2005) claim that Chlase isolated from plant tissues may undergo aggregation to high molecular weight structures. Such situation probably occurred in the case of Chlase from Citrus sinensis fruits, which relative molecular mass estimated with the use of gel filtration had a very high value (376 kDa) (Trebish et al., 1993). Finally, it should be taken into consideration that high molecular weights of Chlase prepurposes may also indicate interactions of the enzyme with other proteins, and consequently, further in-depth molecular studies uncovering these possible protein-protein associations, as well as amino acid sequencing, are highly recommended.

Determination of the Michaelis constant ($K_m$) allows for evaluating the enzyme affinity to its substrate (lower $K_m$ values indicate a high affinity and a low catalytic efficiency). It was evidenced that Chlase F1 from leaves of the investigated Prunus species hydrolyzed the tested substrates more rapidly in comparison to fraction 2. Additionally, the studied enzyme fractions possessed higher $K_m$ values calculated for Chl b than for Chl a (with the exception of Chlase F2 from sour cherry). The results are coherent with findings reported by Tsuchiya et al. (1997). These authors revealed that $K_m$ levels of Chlase 1 isolated from Chenopodium album leaves were 4.0 and 3.1 µM for Chl a and Chl b, respectively. Furthermore, they demonstrated that Chlase demonstrated lower affinity towards both types of chlorophyll molecules ($K_m = 4.6$ and 4.4 µM for Chl a and Chl b, respectively). Kinetic analyses conducted by Homero-Méndez and Mínguez-Mosquera (2001) also confirmed more efficient bioconversion of Chl a than Chl b by Chlase isolated from Capsicum annum fruits ($K_m = 10.7$ and 4.04 µM for Chl a and Chl b, respectively). Importantly, Lee et al. (2010) postulated that various Chlase isoforms in plant tissues may participate in different catabolic pathways involved with degradation of chlorophylls. Furthermore, these authors evidenced that isozyme BoCLH1 in broccoli is responsible for Chl transformation into Phein, whereas isof orm BoCLH2 catalyzes Chl conversion into Chlide or Phein. It should be noted that Arkus et al. (2005) proved that substrate affinity depends on the purity of enzyme samples. According to these investigators, the highly purified recombinant Chlase of Triticum aestivum expressed in Escherichia coli system possessed 10–50-fold higher $K_m$ value (63 µM) in comparison to the enzyme extracted from cell lysates or those subjected to partial purification. Identifying the kinetic aspects of Chl catabolic process-
ing by Chlase isolated from different plant sources may be utilized in formulating strategies for delaying natural degradation of these pigments during fruit storage and contribute to improving their elimination from commercially available vegetable and fruit oils (i.e. refining of rapeseed, soybean, and palm oils). Additionally, Chlase may be used in enzymatic decolorization of chlorophyll-containing materials (Karbourne et al., 2005).

It was experimentally demonstrated that optimal pH values of Chlase preparations ranged from 7.6 to 8.0, depending on the characterised fraction and tested species. Coherent results were obtained by Fernandez-Lopez et al. (1992) in studies on foliar Chlase from Citrus limon L. and McFeeters et al. (1971), who analysed Chlase extracted from leaves of Allanthus alisitima Mill. According to these authors, maximal enzyme activity was observed at pH 7.8 and 8.0, respectively. Lee and coworkers (2010) revealed that pH 7.8 optima for both examined Chlase isoforms (BoCLH1 and BoCLH2) isolated from B. oleacea were 7.0 and 8.0, correspondingly. In addition, Tsuchiya et al. (1997) have found that two Chlase isoforms from leaves of C. album were characterised by higher activity towards Chl a than Chl b when a more alkaline reaction medium was used.

During the experiments, incubation of the reaction mixture was carried out in a water bath at 30°C. Most authors reported that optimal reaction temperature for Chlase isolated from a wide panel of plant sources varied from 30 to 40°C (Ihl et al. 1998; Gaffar et al., 1999; Okazawa et al., 2006). Furthermore, Arkus et al. (2005) demonstrated that wheat Chlase may exhibit catalytic activity in the temperature range from 25 to 75°C, while total inactivation of the enzyme occurred at 85°C. Performed analyses uncovered that incubation of Chlase samples with 10 mM Mg2+ ions resulted in a subtle elevation in the relative enzyme activity when compared to the control. This finding is coherent with some reports indicating that the addition of Mg2+ stimulated the activity of Chlase from P. tricornutum and Piper betle L. landrace Khadi Shillong (Terpsstra & Lambers, 1983; Gupta et al., 2011). On the other hand, Hornero-Mendez and Minguez-Mosquera (2001) revealed that magnesium cations caused a decrease (12%) in the activity of enzyme isolated from C. annum fruits. Additionally, all studied Chlase preparations from P. tricornutum were inhibited by other investigated metal cations. The highest diminution of catalytic effect was caused by Hg2+, Fe2+, and Cu2+ ions, while Ag+ slightly suppressed the relative Chlase activity. Similar inhibitory effects towards the activity of Chlase obtained from different plant species have been previously described (Arkus & Jez, 2006; Yi et al., 2007; Hornero-Mendez & Minguez-Mosquera, 2007; Gupta et al., 2011).

Interestingly, published data evidenced that inhibition pertains to a wide range of concentrations of the ions used. For example, Gupta et al. (2011) used metal salts at a concentration of 5 mM, while Arkus & Jez (2006) tested 10 μM solutions of metal cations. Furthermore, conducted experiments revealed that presence of PMSF (inhibitor of serine hydrolases) and DEPC (histidine modifier) in reaction mixtures markedly inhibited Chlase activity, whereas the addition of 2-ME and DTT exhibited a tenuous stimulatory effect on the enzyme activity in comparison with the non-treated samples. Importantly, Tsuchiya et al. (2003) observed about 90% decrement in the activity of the recombinant Chlase from C. alinunum (CaCLH) expressed in E. coli as a result of adding PMSF to the reaction medium. Detailed mutagenesis studies performed by these authors confirmed the significance of serine residue for the activity of Chlase.
tion of chloroplast compounds. Nevertheless, Lee et al. (2010) postulated that a variety of plant systems might develop diverse mechanisms involved in the bioconversion of chlorophylls, as well as various modes of Chlase regulation. In fact, different Chlase isofoms may display a variety of biological functions in plant tissues. Kariola et al. (2005) demonstrated that Chlase 1 from A. thaliana counteracts the excessive accumulation of reactive oxygen species (ROS) within stressed plants. According to these authors, the expression of the AtCHL1 gene was notably up-regulated by necrotrophic pathogens: Erwinia carotovora and Alternaria brassicicola when compared to the control. On the other hand, AtCHL1 RNAi silenced plants characterised with impaired degradation of free Chl molecules. Likewise, Chen et al. (2008) demonstrated 1-2 days retardation in the process of postharvest yellowing within the antisense BoCHL1 broccoli transmamrants. However, no deceleration of Chl degradation in tissues of BoCHL2 or BoCHL3 transmamrants was observed.

Summarizing, the obtained results evidenced that studied Chlases samples from leaves of three investigated *P. sativus* species confirm similar biochemical properties. Experimental optimization of reaction conditions allowed for monitoring the specific Chlase activity during leaf development of tested plants, perform kinetic analyses regarding the substrate specificity, and reveal the influence of a diverse spectrum of effectors on the enzyme activity. These findings also provide a basis for further research including amino acid sequencing and modeling of three-dimensional structures of Chlase isozymes. At the molecular level, some genetic engineering techniques (i.e. DNA recombination and cloning) as well as relative quantification of gene expression should be applied in order to gain better insight into complex mechanisms underlying the transcriptional regulation of Chlase biosynthesis.

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REFERENCES


