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

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Despite senescence-induced chlorophyll depletion 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, Hg^{+2}, Fe^{+2}, and Cu^{+2} cations showed the most pronounced inhibitory effect on the activity of Chlase. In contrast, the presence of Mg^{+2} 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 autumnal 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 (Ciepiela *et al.*, 2005), and phytopathogenic 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 *clh1* and *clh2* 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 depletion 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-Yaakov *et al.*, 2006; Schenk *et al.*, 2007; Schelbert *et*

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Abbreviations: Chl, chlorophyll; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Chlase F1, fraction 1 of chlorophyllase; Chlase F2, fraction 2 of chlorophyllase; Chlide, chlorophyllide; DEPC, diethylpyrocarbonate; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; Pheide, pheophorbide; Pheo, pheophytin; PMSF, phenylmethanesulfonyl fluoride.

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 preparates 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 ontogenetic 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, and 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 5 000 × *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 12 000 × *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 (Fang *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 10 000 × *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 $cm^3 \cdot min^{-1}$. 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:

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

The molecular weight (kDa) of native Chlase preparates was estimated with the use of experimentally outlined calibration curve showing a relationship between K_{av} (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 preparate, 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 12 000 × *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):

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

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 $mg \cdot cm^{-3}$.

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

Table 1. Purification of Chlase preparates extracted from leaves of the investigated *Prunus* species.

Purification procedure	Protein content ($mg \cdot cm^{-3}$)	Specific enzyme activity#	Purification (*n*-fold)
Bird cherry			
Homogenate	1.65	0.15	1.00
Ammonium sulfate precipitate	1.14	0.22	1.46
Dialysate	0.60	0.56	3.73
Sephadex G-200 gel filtration			
Fraction 1	0.09	5.53	36.86
Fraction 2	0.05	7.42	49.47
European plum			
Homogenate	2.40	0.68	1.00
Ammonium sulfate precipitate	1.85	1.32	1.94
Dialysate	1.36	1.48	2.18
Sephadex G-200 gel filtration			
Fraction 1	0.18	19.47	28.63
Fraction 2	0.12	16.92	24.88
Sour cherry			
Homogenate	2.52	0.51	1.00
Ammonium sulfate precipitate	2.05	0.95	1.86
Dialysate	1.70	1.40	2.75
Sephadex G-200 gel filtration			
Fraction 1	0.15	14.75	28.92
Fraction 2	0.11	9.52	18.67

#specific Chlase activity is defined as nanomole of substrate (Chl *a*) hydrolyzed per minute per mg of protein.

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^{+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+}, Bi^{3+}, Fe^{3+}) and the functional groups' modifiers (2-mercaptoethanol, diethylpyrocarbonate, dithiothreitol, 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 $p \leq 0.01$ were considered as statistically significant.

RESULTS

Results regarding the efficiency of isolation and partial purification of Chlase preparates 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 preparates 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

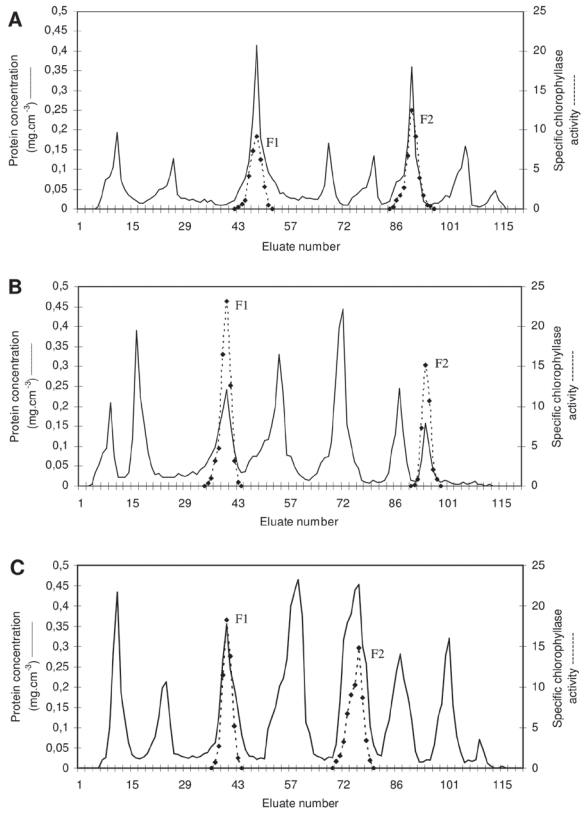

Figure 1. Gel filtration (Sephadex G-200) chromatograms of analysed Chlase preparates.
(**A**) bird cherry, (**B**) European plum, (**C**) sour cherry. Specific Chlase activity (------) was defined as nanomole of substrate (Chl *a*) hydrolyzed per minute per mg of protein. Data points are presented as the mean (n=3).

sour cherry demonstrated lower activity (13.1 and 35.5%, accordingly) in comparison with the corresponding fraction 2. Furthermore, it was found that the activity of both studied Chlase fractions from European plum and sour cherry reached higher values than the activity of corresponding enzyme samples of *P. padus* (Table 1).

The relative molecular weight (M_r) of native Chlase fractions was determined by the gel filtration (Sephadex G-200) chromatography of pooled eluates showing the enzyme activity (Table 2). The molecular mass of studied enzyme fractions was calculated using the calibrating curve (plot of experimentally derived K_{av} *versus* the logarithm of M_r for standard proteins in the range 12.3–205.0 kDa). It was estimated that Chlase F1 from leaves of the investigated *Prunus* species possessed higher values of molecular masses in relation to fraction 2. Detailed analyses revealed that Chlase F1 from European plum and sour cherry demonstrated very similar M_r levels (approx. 100 kDa), whereas the fraction from bird cherry had a slightly lower molecular mass (90 kDa). Additionally, it was ascertained that molecular weights of Chlase F2 varied from 35 kDa (European plum) to 60 kDa (sour cherry), depending on the tested plant system.

It was elucidated that the maximal activity of Chlase F1 isolated from *P. padus* was noted at pH 8.0, whereas the highest activity of this fraction from sour cherry and European plum was recorded at a slightly lower pH value (7.8). Furthermore, it was revealed that optimal pH for Chlase F2 varied in a broader range — from 7.6 (European plum), through 7.8 (bird cherry), to 8.0 (sour cherry). The substrate specificity of the analysed Chlase fractions was evaluated for both Chl *a* and Chl *b* (Table 3). The Lineweaver-Burk plots were used in order to designate the selected kinetic parameter (the Michaelis constant, K_m) of the tested enzyme preparates. Performed analyses proved that Chlase F1 isolated from leaves of all studied plants possessed higher affinity (lower K_m levels) for both tested substrates than fraction 2. The most significant differences in K_m values between the examined enzyme preparates (27 and 36% for Chl *a* and Chl *b*, respectively) were noted for Chlase extracted 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 *b* than Chl *a*.

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 activity of Chlase when compared to the control samples. Conversely, other examined metal ions caused a decline 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.

Plant species	Chlase fractions	Molecular weight (kDa)	K_{av}
Bird cherry	1	90	0.28
	2	40	0.44
European plum	1	~100	0.25
	2	35	0.46
Sour cherry	1	~100	0.25
	2	60	0.35

K_{av}, coefficient of partitioning designated using the gel filtration chromatography on Sephadex G-200 column.

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

Plant species	Chlase fraction	Chl *a*	Chl *b*
Bird cherry	1	2.8 ± 0.04	3.0 ± 0.07
	2	3.1 ± 0.06	3.5 ± 0.10
European plum	1	3.5 ± 0.12	3.9 ± 0.18
	2	4.8 ± 0.26	6.1 ± 0.45
Sour cherry	1	3.4 ± 0.09	3.7 ± 0.14
	2	4.2 ± 0.21	3.8 ± 0.16

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

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

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

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 ($p \leq 0.01$) by Duncan's Multiple Range Test.

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 *a* 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

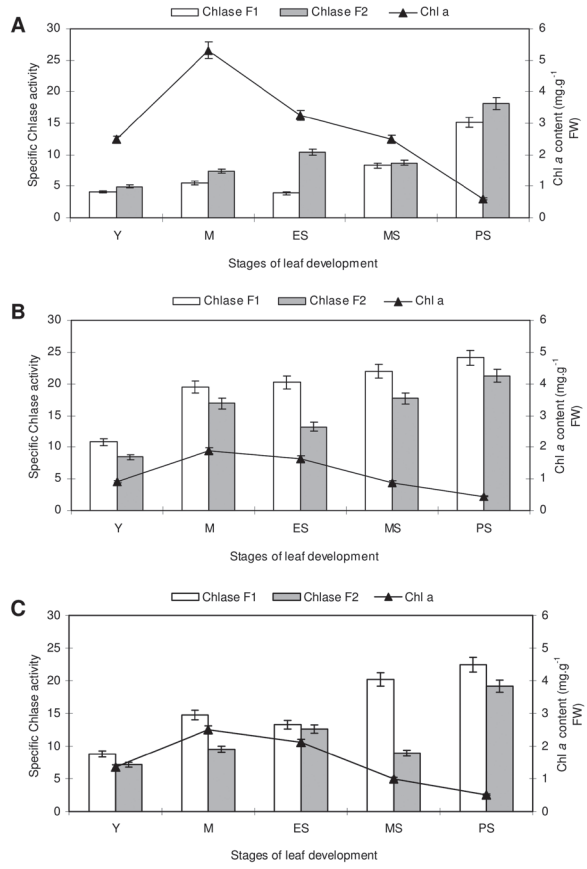

Figure 2. Seasonal dynamics of the specific activity of Chlase fractions and the content of Chl a in leaves of the examined *Prunus* species.
(**A**) bird cherry, (**B**) European plum, (**C**) sour cherry. FW — fresh weight; five developmental stages of leaves (Y — young, M — mature, ES — early senescent, MS — moderately senescent, and PS — progressively senescent) were included in the experiments regarding time-course changes in Chlase activity and Chl *a* content. The specific activity of Chlase was defined as nanomole of substrate (Chl *a*) hydrolyzed per minute per mg of protein. Data points are presented as the mean±S.D. (n=3).

—bird cherry and sour cherry). Furthermore, the highest content of Chl *a* 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 *a* in leaves of ex-

Table 5. Correlation between the levels of specific activity of analysed Chlase fractions and the content of Chl *a* in leaves of investigated *Prunus* species.

Plant species	R	
	Chlase F1–Chl *a*	Chlase F2–Chl *a*
Bird cherry	–0.710	–0.653
European plum	–0.295	–0.258
Sour cherry	–0.583	0.450

*$p<0.01$. The values of Pearson's correlation coefficient (R) were calculated using STATISTICA 9.0 software (StatSoft). Chlase F1–Chl *a* and Chlase F2–Chl *a* represent the correlation between specific activity of the relevant Chlase fractions and Chl *a* 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; Banaś *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; Hörtensteiner, 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 preparates 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 preparates 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 preparates. 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 preparates (Trebitsh *et al*., 1993; Tsuchiya *et al*., 1997; Arkus & Jez, 2006). It should be underlined that the final step of enzyme purification using the gel filtration (Sephadex G-200) chromatography revealed the presence of two protein fractions exhibiting Chlase activity. These findings are in agreement with results that have been previously reported in literature (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_r 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) (Trebitsh *et al*., 1993). Finally, it should be taken into consideration that high molecular weights of Chlase preparates 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*, accordingly). Kinetic analyses conducted by Hornero-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 isoform 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-contaminated materials (Karboune *et al*., 2005).

It was experimentally demonstrated that optimal pH values of Chlase preparates 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 *Ailanthus altissima* 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 optima for both examined Chlase isoforms (BoCLH1 and BoCLH2) isolated from *B. oleracea* 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 Mg^{2+} 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 Mg^{2+} stimulated the activity of Chlase from *P. tricornutum* and *Piper betle* L. landrace Khasi Shillong (Terpstra & Lambers, 1983; Gupta *et al*., 2011). On the other hand, Hornero-Méndez and Mínguez-Mosquera (2001) revealed that magnesium cations caused a decrease (12%) in the activity of enzyme isolated from *C. annuum* fruits. Additionally, all studied Chlase preparates from *Prunus* plants were inhibited by other investigated metal cations. The highest detrimental effect was caused by Hg^{2+}, Fe^{2+}, and Cu^{2+} 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-Méndez & Mínguez-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. album* (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. Furthermore, Gupta *et al*. (2011) also demonstrated the detrimental impact of PMSF and DEPC on the activity of the enzyme from leaves of both tested *P. betler* landraces (Khasi Shillong and Kapoori Vellaikodi). In this study, the inhibition of Chlase activity was affected by DEPC, a chemical compound responsible for histidine modification, which suggests the importance of this amino acid in maintaining the catalytic activity of studied enzymes. Similarly, Hornero-Méndez and Mínguez-Mosquera (2001) demonstrated the activatory effect of 2-ME and DTT towards Chlase from *C. annuum*. Addition of these substances to the reaction medium might contribute to reducing of disulphide bonds into thiol groups and increasing of substrate binding to the active site of the enzyme. Interestingly, Arkus & Jez (2006) established that the activity of wheat Chlase decreased (2%) in the presence of 2-ME, whereas the presence of DTT caused an elevation (4%) of the enzyme activity.

Moreover, three various time-course patterns in the specific activity of Chlase within leaves of the tested plant species have been demonstrated. Permanent increase in the enzyme activity during leaf development was only ascertained in the case of fraction 1 from European plum. The other two types of Chlase activity profiles were asscociated with a continuous elevation with a single decrease occurring at early senescence (fraction 1–bird cherry and sour cherry, fraction 2–European plum) or moderately senescence (fraction 2–bird cherry and sour cherry). Moreover, it was elucidated that bird cherry leaves contained the highest amount of Chl *a*, whereas European plum and sour cherry demonstrated similar concentrations of the examined green pigments. Additionally, the congenial seasonal patterns of Chl *a* content in foliar tissues of the investigated plant systems were recorded. The highest Chl *a* amount was noted in mature leaves, whereas the progression of senescence was associated with a continuous decline in the concentration of the analysed compound. Performed tests referring to developmental patterns of Chlase activity provided indirect evidence that the studied enzyme is not the only biocatalyst involved with Chl breakdown in leaves of tested *Prunus* plants. However, according to Todorov *et al*. (2003b), it should be taken into consideration that the catalytic activity of Chlase under *in vitro* conditions may not reflect the levels of activity in the living cells. These authors proved that Chlase activity did not always correlate with Chl amounts in the examined samples of low- and high-temperature stressed *Arabidopsis* plants. Importantly, it has also been suggested that Chl catabolic processing may be associated with the action of an alternative biocatalyst to Chlase (i.e. chlorophyll peroxidase). Ultimately, Ben-Yaakov *et al*. (2006) identified diverse patterns of Chlase activity within senescing leaves of eleven plant species. It is of great importance that a decrement in Chlase activity occurred synchronically with Chl depletion in leaves of most investigated plant systems (*Petroselinum sativum* L., *Vinca rosea* L., *Cucurbita pepo* L., *Lycopersicon esculentum* L., *Brassica napus* L., *Nicotiana glutinosa* L., *Triticum aestivum* L. and *Helianthus annuus* L.). Conversely, high levels of Chlase activity were maintained when Chl content was significantly reduced in foliar tissues of *Melia azedarach* L. and *Tropaeolum majus* L. Furthermore, enzyme activity decreased in parallel with diminution in the amount of Chl in senescent leaves of *Hordeum vulgare* L. Interestingly, Pshibytko *et al*. (2004) also observed an age-related decrement in Chlase activity within seedling leaves of *H. vulgare*. Ben-Yaakov *et al*. (2006) speculated that distinct patterns of Chlase activity during leaf senescence may result from slight differences in the degrada-

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 isoforms 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, *AtCLH1* 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 *BoCLH1* broccoli transformants. However, no deceleration of Chl degradation in tissues of *BoCLH2* or *BoCLH3* transformants was observed.

Summarizing, the obtained results evidenced that studied Chlase samples from leaves of three investigated *Prunus* 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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