In vitro studies of temperature and pH influence on chlorophyll degradation by horseradish peroxidase: Spectroscopic and HPLC studies

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Abstract

In vitro chlorophyll *a* degradation by horseradish peroxidase in the presence of the resorcinol was investigated in this paper, and the influence of pH and temperature was particularly studied. Chlorophyll *a* degradation was followed by UV–Vis and HPLC. Chlorophyll *a* was degraded when hydrogen peroxide was added into reaction mixture containing chlorophyll fraction, horseradish peroxidase, resorcinol and phosphate buffer. HPLC analysis has identified the main degradation product of chlorophyll *a* as 13^2-hydroxychlorophyll *a*. The degradation was traced at different temperatures and pH values. The increasing temperatures led to increase of chlorophyll *a* degradation, with a maximum at 37 °C. The degradation also increased with increasing pH values, reaching maximum at pH 6.

Keywords: chlorophyll, horseradish peroxidase, resorcinol, temperature, pH, HPLC.

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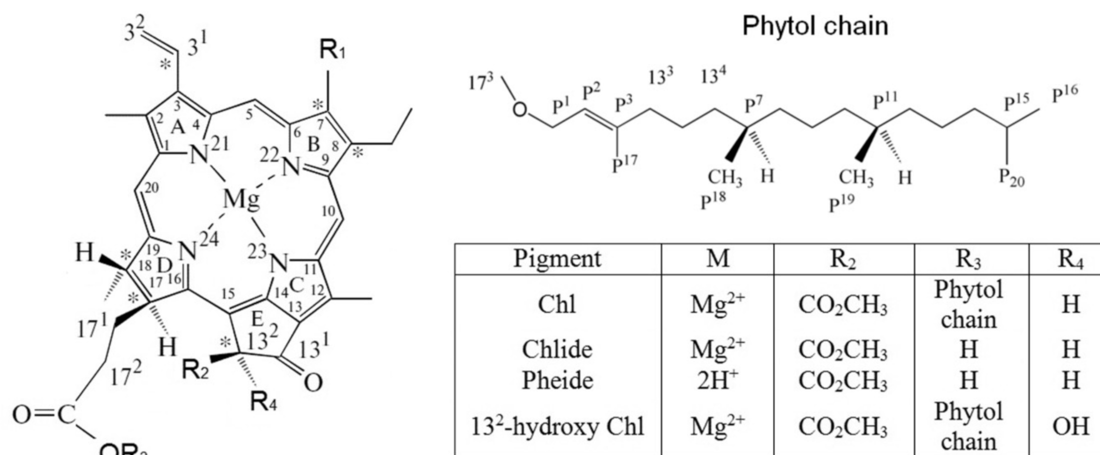
Chlorophylls (Chls) are the most ubiquitous pigments of all natural pigments, which are responsible for the color of all green plants. Biosynthetically, they are derived from protoporphyrin IX [1]. Structurally, chlorophylls are cyclic tetrapyrroles with isocyclic cyclopentanone ring, fused at the edge of the right-bottom pyrrole ring [2], as shown in Figure 1. products [3]. The previous studies have shown that the enzymatic Chl degradation may include several enzymes, such as chlorophyllase, Mg-dechelatase, Chl oxidase and peroxidase [4,5]. The role of chlorophyllase is in removal of phytol tail from chlorophyll and the formation of chlorophyllide (Chlide). On the other hand, Mg-dechelatase removes not only phytol tail, but the



Pigment	M	R_2	R_3	R_4
Chl	Mg^{2+}	CO_2CH_3	Phytol chain	H
Chlide	Mg^{2+}	CO_2CH_3	H	H
Pheide	$2H^+$	CO_2CH_3	H	H
13^2-hydroxy Chl	Mg^{2+}	CO_2CH_3	Phytol chain	OH

Figure 1. Chlorophyll structure. The C-atoms are numerated according to IUPAC nomenclature rules. In position C-7, $–R_1$ corresponds to: $–CH_3$ and –CHO in the cases of Chl a and Chl b, respectively.

Chls are susceptible to many chemical or enzymatic degradation reactions. The pathway of chlorophyll (Chl) breakdown can be provided by simultaneous actions of enzymes, weak acids, oxygen, light and heat, which can lead to the formation of a large number of degradation central Mg atom as well. Chl oxidase and peroxidase are indirectly included in the chlorophyll *a* (Chl *a*) degradation, because their action requires presence of phenolic compound. Both of them, peroxidase and Chl oxidase, as degradation products yield 13^2-hydroxychlorophyll *a* (Chl *a*-1) [6,7]. The role of peroxidase in the mechanism of Chl degradation *in vivo* is not completely understood. Still, it is known that during storage of plants the activity of chlorophyllase is slight decreased while, on the other hand, the activity of Chl

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degrading peroxidase is increased, implying a great role of peroxidase in Chl degradation [8].

Previous studies have shown that phenol, 2,4-dichlorophenol and resorcinol were effective in Chl degradation. In the Chl degradation mechanism, peroxidase oxidizes the phenolic compound, with the hydroxyl group at the *p*-position and forms the phenoxy radical or/and superoxide anion. After, the obtained radical or superoxide anion attack on Chl *a* to form Chl *a*-1 [9], shown in Figure 2. Yamauchi and collaborators (2004) demonstrated that the other phenolic compounds, like *p*-coumaric acid, apigenin, apigetrin, naringenin are highly effective in the peroxidase – hydrogen peroxide system, having electron attracting groups at the *p*-position [8–10].

Funamoto and collaborators [6] have shown that Chl content in stored broccoli decreased significantly after 4 days storage at 15 °C, while the content in broccoli treated at 50 °C for 2 h has remained almost intact during storage [6]. Similary, Martínez *et al.* [11] have examined the influence of temperature and pH on degradation of chlorophyll with peroxidase from strawberry fruits in the presence of *p*-coumaric acid and the obtained results have indicated the biggest degradation at 35 °C and pH 5.2 [11].

The subject of this work is to study the influence of temperature and pH on *in vitro* degradation of Chl *a* from spinach in the presence of horseradish peroxidase (HRP) and resorcinol, as phenolic compound, including the identification of the major degradation product by UV–Vis spectroscopy and HPLC. As opposed to previous studies, this research is the first report about influence of pH and temperature on *in vitro* degradation of Chl *a* from spinach by HRP in the presence of resorcinol as phenolic compound.

EXPERIMENTAL

All experiments, beginning with extraction, were performed under dim light as far as possible, and inside vessels and equipment covered with aluminum foil or black cloth, preventing pigments exposure to light [12].

Extraction of plant pigments

Extraction of plant pigments from spinach leaves, *Spinacia oleracea* L. (found in the local market), was performed by using already published method [13]. The final extract was a mixture of pigments containing large amounts of various Chl forms, as well as accessory pigments, carotenoids (carotenes and xanthophylls).

Chlorophyll fraction

The Chl fraction – the purified mixture, *e.g.*, Chl *a* and chlorophyll *b* (Chl *b*), was isolated from the pigment extract by using column chromatography with silica gel as the adsorbent (silica gel 60, Merck, 0.063–0.200 mm) and *n*-hexane/acetone eluent mixture [13].

Enzyme

HRP (298 U/mg) was purchased from Sigma (Germany). A 2 μM stock solution of HRP was prepared by dissolving the 0.34 mg of the solid HRP in 10 ml of cold 50 mM phosphate buffer pH 6. The enzyme concentration was calculated using a ε_{403} = 102.0 $mM^{-1} cm^{-1}$ [14].

Chlorophyll degradation reaction

Chl degradation was determined as described by Yamauchi and Minamide with slight modifications [4]. The reaction mixture contained 0.2 ml Chl fraction in ethanol solution, 50 μl ethanol solution of resorcinol, 0.1 ml 1% Triton X-100, 0.1 ml 0.3% hydrogen peroxide, 40 μl HRP and 2.0 ml 100 mM phosphate buffer (pH

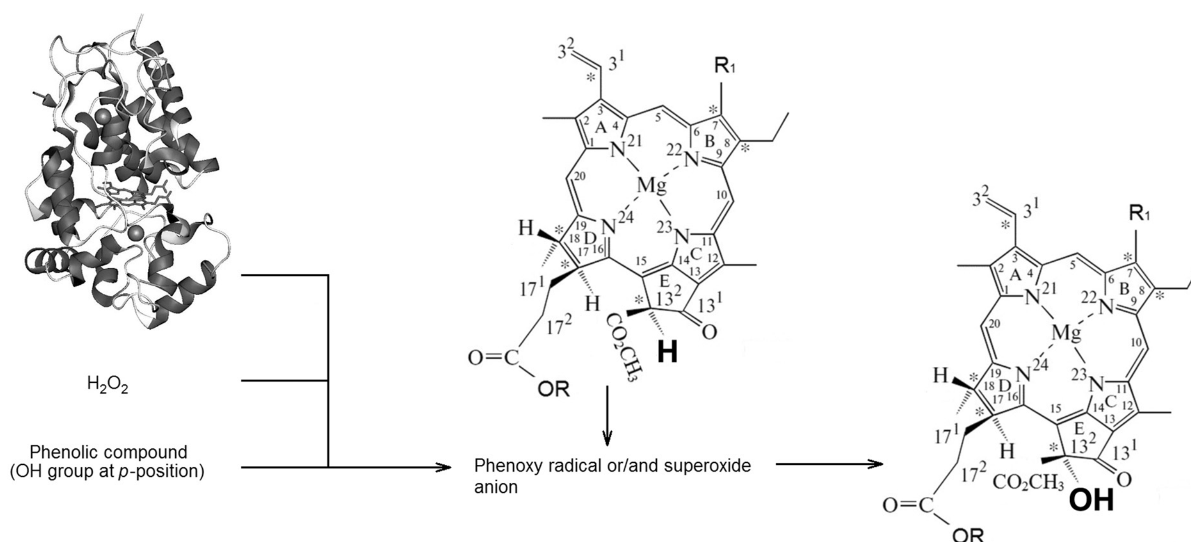

Figure 2. The mechanism of chlorophyll degradation by HRP in the presence of phenolic compound. (R corresponds to phytol tail; R_1 corresponds to: $–CH_3$ and –CHO in the cases of Chl a and Chl b, respectively).

6.0) in a total volume of 2.5 ml. The reaction was allowed to proceed for 10 min at defined temperatures and pH values, then stopped by the addition of 2.5 ml 96% ethanol. After, all presented Chls from the reaction mixture, were extracted from the reaction mixture by addition of 5.0 ml *n*-hexane.

UV–Vis spectroscopy

The spectrophotometric measurements were made on a Varian Cary-100 spectrophotometer equipped with 1.0 cm quartz cells. All spectra were recorded from 350 to 800 nm with 1.0 bandwidth. Spectra of all compounds are recorded in *n*-hexane solution. Chl concentration in the mixture was set in the range between 10^{-5} and 10^{-6} mol/dm^3 [15]. Chl *a* content in relation to Chl *b*, in Chl fraction, was 5:1.

HPLC Analysis

HPLC Analysis of Chl-containing reaction mixture was performed under isocratic conditions on Agilent 1100 Series set-up (Waldborn, Germany), on Zorbax Eclipse XDB-C18 column, by using diode array detector set at detection wavelength ($\lambda_{det.}$): 660 nm; the isocratic conditions were: mobile phase – acetonitrile/ /methanol/ethyl acetate, 60:20:20, flow rate – 1 ml/min, temperature 25 °C.

RESULTS AND DISCUSSION

HPLC Chromatograms of the degraded Chls, extracted from the reaction mixture, at the different temperatures are shown in Figure 3. The absorption spectra of the main compounds in the eluent mixture observed on the HPLC chromatograms at t_{ret} = 13 min (assigned as Chl *a*), and t_{ret} = 12 min (assigned as Chl *a*-1), are shown in the increments of the Figure 3, respectively; the spectra shown in the increments correspond to the two peaks.

HPLC chromatograms of the extracted Chls from the reaction mixture at different pH values are shown in Figure 4. The absorption spectra of all main compounds in the eluent mixture observed in the HPLC chromatograms at t_{ret} = 13 min (Chl *a*), and t_{ret} = 12 min (Chl *a*-1), are shown in the increments of the Figure 4, respectively; the spectra shown in the increments were taken correspond to the two peaks.

There are many studies about the effects of various phenolic compounds such are 2,4-dichlorophenol, phenol, *p*-coumaric acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyacetophenone, resorcinol and umbelliferone on peroxidase-mediated Chl oxidation [4,5]. On the other hand, the obtained results with *o*-diphenols and derivatives such as catechol,

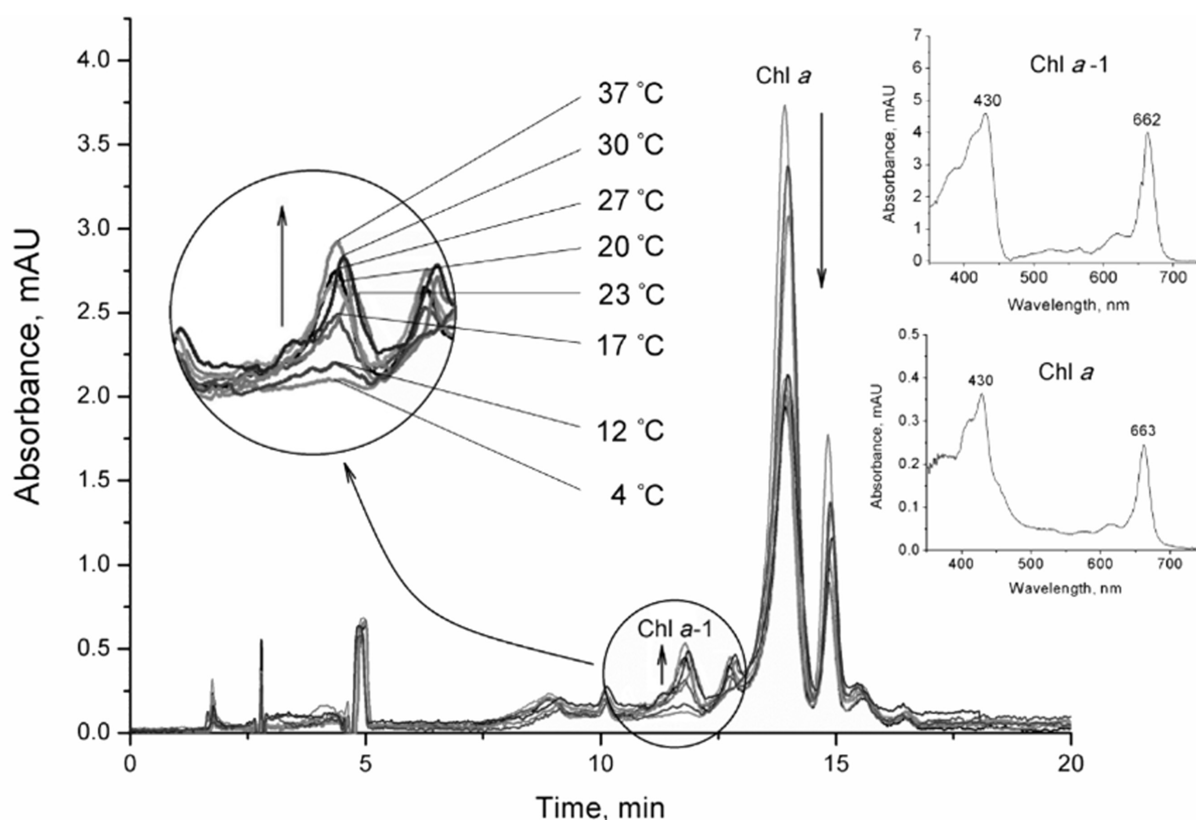

Figure 3. HPLC profile of the extracted chlorophylls from the reaction mixture at the different temperatures. The reaction medium contained 1 μM of Chl a, 16 nM HRP, 0.1 mM resorcinol, 50 mM sodium phosphate buffer, pH 6.0, and 0.1 mM H_2O_2. Detection was carried out at 660 nm.

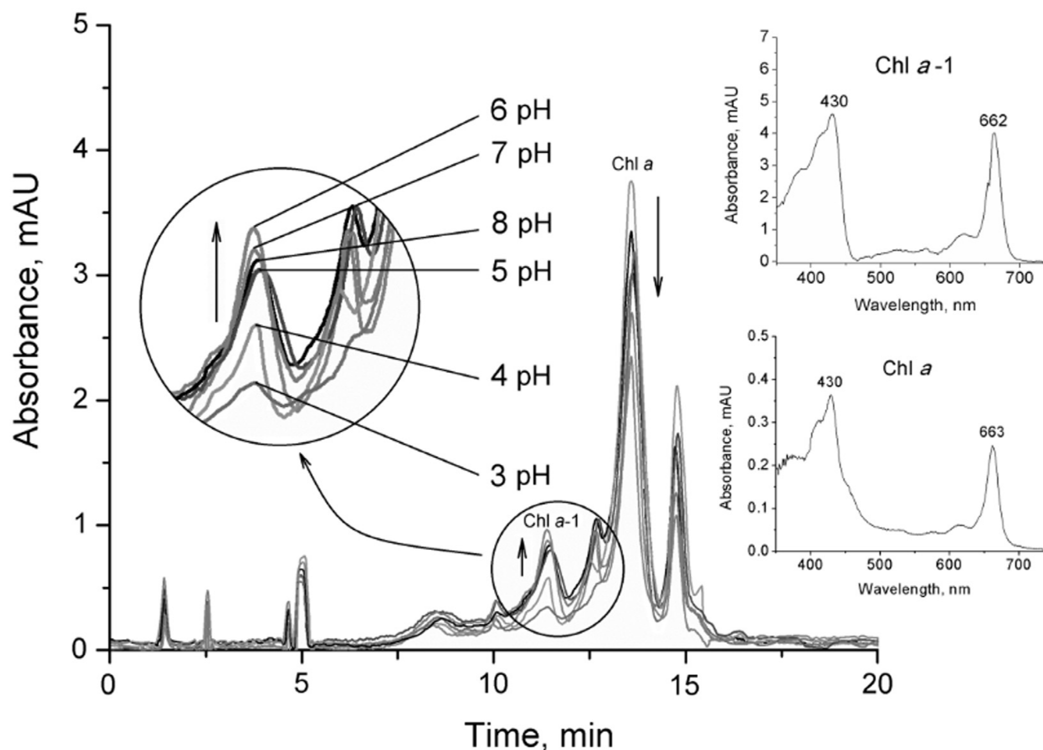

Figure 4. HPLC Profile of the extracted chlorophylls from the reaction mixture at different pH. The reaction was performed at 25 °C in medium contained of 1 μM of Chl a, 16 nM HRP, 0,1 mM resorcinol, 50 mM sodium phosphate buffer, pH 6.0, and 0.1 mM H_2O_2. Detection was carried out at 660 nm.

guaiacol, ferulic acid, caffeic acid and chlorogenic acid on degradation of Chl by peroxidase were negative, *e.g*., the degradation was not detected [16]. Based on these findings, it can be concluded that the phenolic compounds involved in Chl degradation could be monophenols whith a hydroxyl group at *p*-position. The flavonoids, with hydroxyl group in *p*-position in the B-ring, such as apigenin and its 7-glucoside derivative, as well as naringenin were also effective in peroxidase-mediated degradation of Chl [8]. Based on the presented data, we selected resorcinol to mediate Chl *a* degradation by HRP.

Bacause the peroxidase is capable of abstracting a labile hydrogen atoms from phenolic substrate [17], the interpretation of these results is that the enzyme is involved in production of Chl *a*-1 radicals (at t_{ret} =12 min – Figures 3 and 4) [18]. The absorption spectra of the Chl *a* and Chl *a*-1, given in the increments of Figures 3 and 4, showed good agreement with literature data [9,18–20]. The absorption spectra of Chl *a* and Chl *a*-1 are very similar, which is expected since they are both porphyrine type of components with only difference in C-13^2 position, so identification of Chl *a*-1 is based on the retention time on HPLC chromatogram, obtained under similar conditions [21]. As it is well known, Chls as the porphyrin derivatives have two major absorption bands in the visible range, due to extended π-delocalization at the edge of cyclic tetrapyrrole (porphyrin) skeleton (Figure 1): ''red'' (Q-) band and ''blue'' (Soret or B-) band [22–24]. The "red" and the "blue" bands of Chl *a* (assigned as Q_y- and Soret band) are located at 662 and 430 nm in acetonitrile, respectively [12,22], similar to the ones shown in the increments of Figures 3 and 4. The ratios of absorbance intensities for Soret- and the Q-band is ~1.3 for Chl *a* [22]. Of course, the bands intensities and their maximum absorption positions (λ_Q and λ_{Soret}) in the other solvents are different, and they are also influenced by many other factors like substitution, ligands, H-bonding, the surroundings [24].

Since the analysis of this type of compounds (such as Chls) predominantly uses C-18 column, retention time of the analyzed components always decreases in the same order: chlorophyll *a* > C13^2-hydroxychlorophyll *a* > chlorophyll *b* and predominantly depends on the polarity of the mobile phase [15,25]. The HPLC chromatograms obtained at the different temperatures (Figure 3), show decrease of Chl *a* with increase of temperatures from 4 to 47 °C. On the other hand, HPLC chromatograms obtained at the different pHs at constant temperature (Figure 4) also show decrease of Chl *a* with increase of pH. The amount of Chl *a*-1 is accumulated on the account of the degraded Chl *a*.

Peroxidase-mediated Chl degradation activities at different temperatures and pH are shown in Figures 5

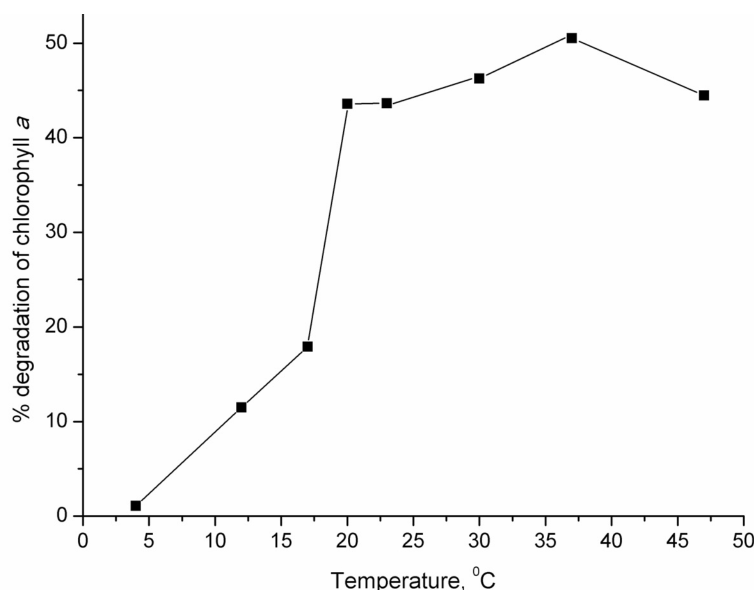

Figure 5. Temperature effects on Chl a degradation by HRP, in the presence of resorcinol at pH 6.

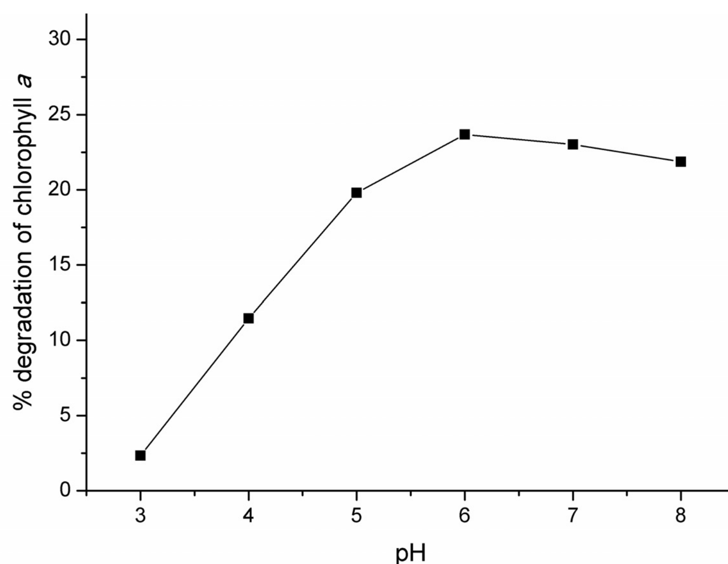

Figure 6. Effect of pH on degradation of Chl a *by HRP in the presence of resorcinol at 25 °C.*

and 6. The degradation rate of Chl a was determined using the absorption values from UV–Vis spectra at 662 nm.

The degradation of Chl *a* by HRP in the presence of resorcinol increases with increasing temperatures, but not in the linear manner in the whole range. An increase of Chl degradation by HRP in the presence of resorcinol was observed in 4–17 °C range, following by a strong rise in 17–20 °C range, a slight increase in 20––37 °C, and a slight decrease afterwards. The obtained results are in line with the results of degradation of Chl by HRP in the presence of *p*-coumaric acid, with optimal degradation temperature at 35 °C [11].

The results reflecting pH effects on degradation reaction of Chl *a* by HRP in the presence of resorcinol are shown on Figure 6. Two remarks might be drawn from it: a slight increase in pH range from 3 to 6, followed by a second region from pH 6–8 in which a slight decrease has been recorded. The achieved results are in line with the results of degradation of Chl *a* by HRP in the presence of *p*-coumaric acid with optimal pH value at 5.2 [11].

Having in mind the optimal temperature and pH ranges for HRP activity (30–40 °C and pH 6–8, depending on the medium), our results (of Chl degradation by HRP in the presence of resorcinol) look expected [26]. On the other side, the comparison with *Martinez et al.*, (2001) results leads to similar conclusions [11]. It means that *in vitro* degradation of Chl *a* by HRP needs similar conditions irrespective of the selected phenolic com-

pound, because activity of HRP depends more on pH values and temperature and not on structure of these two phenolic compounds (resocinol and *p*-coumaric acid).

CONCLUSION

In conclusion, the main product of Chl *a* degradation by HRP, in the presence of resorcinol as phenolic compound, is Chl *a*-1 (t_{ret} = 12 min). Obtained results of temperature and pH influence on *in vitro* degradation of Chl *a*, have shown that the degradation increases with increasing temperature and has a maximum at 37 °C. On the other hand, Chl *a* degradation also increases with increasing of pH value and has a maximum at 6. It seems that *in vitro* degradation of Chl *a* by HRP needs similar conditions irrespective of the selected phenolic compound, because activity of HRP depends more on pH values and temperature and not on structure of these two phenolic compounds (resocinol and *p*-coumaric acid).

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REFERENCES

[1] W. Rüdiger, B. Grimm, in: B. Grimm, R.J. Porra, W. Rüdiger, H. Scheer (Eds.), Chlorophylls and Bacteriochlorophylls: biochemistry, biophysics, functions and applications, Springer, Dordrecht, 2006, p. 133.

[2] H. Scheer, in: B. Grimm, R. Porra, W. Rudigger, H. Scheer (Eds.), Chlorophylls and Bacteriochlorophylls, Springer, Dordrecht, 2006, p. 1.

[3] G.A.F. Hendry, J.D. Houghton, S.B. Brown, The degradation of chlorophyll-A biological enigma, New Phytol. **107** (1987) 255–302.

[4] N. Yamauchi, T. Minamide, Chlorophyll degradation by peroxidase in parsley leaves, J. Japan. Soc. Hort. Sci. **54** (1985) 265–271.

[5] Y. Akiyama, N. Yamauchi, Chlorophyll degrading enzymes in radish (*Raphanus sativus* L.) cotyledon chloroplasts and their changes in activities with senescence, J. Japan. Soc. Hort. Sci. **70** (2001) 453–457.

[6] Y. Funamoto, N. Yamauchi, T. Shigenaga, M. Shigyo, Effects of heat treatment on chlorophyll degrading enzymes in stored broccoli (*Brassica oleracea* L.), Postharvest Biol. Tec. **24** (2002) 163–170.

[7] S. Kaewsuksaeng, N. Yamauchi, Y. Funamoto, T. Mori, M. Shigyo, S. Kanlayanarat, Effect of heat treatment on catabolites formation in relation to chlorophyll degradation during storage of Broccoli (*Brassica olearacea* L. Italica Group) Florets, J. Japan. Soc. Hort. Sci. **76** (2007) 338–344.

[8] N. Yamauchi, A.E. Watada, Effectiveness of various phenolic compounds in degradation of chlorophyll by *in vitro* peroxidase-hydrogen peroxide system, J. Japan. Soc. Hort. Sci. **63** (1994) 439–444.

[9] N. Yamauchi, Y. Funamoto, M. Shigyo, Peroxidase-mediated chlorophyll degradation in horticultural crops, Phytochemistry Rev. **3** (2004) 221–228.

[10] Y. Funamoto, N. Yamauchi, M. Shigyo, Involvement of peroxidase in chlorophyll degradation in stored broccoli (*Brassica oleracea* L.) and inhibition of the activity by heat treatment, Postharvest Biol. Tec. **28** (2003) 39–46.

[11] G.A. Martínez, P.M. Civello, A.R. Chaves, M.C. Añón, Characterization of peroxidase-mediated chlorophyll bleaching in strawberry fruit, Phytochemistry **58** (2001) 379–387.

[12] S.W. Jeffrey, R.F.C. Mantoura, S.W. Wright, Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods, UNESCO Publishing, Paris, 1996.

[13] S.M. Milenković, J.B. Zvezdanović, T.D. Anđelković, D.Z. Marković, The identification of chlorophyll and its derivatives in the pigment mixtures: HPLC-chromatography, visible and mass spectroscopy studies, Advanced technologies **1** (2012) 16–24.

[14] M. Yan, J. Ge, Z. Liu, P. Ouyang, Encapsulation of Single Enzyme in Nanogel with Enhanced Biocatalytic Activity and Stability, Am. Chem. Soc. **128** (2006) 11008–11009.

[15] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Method Enzymol. **148** (1987) 350–382.

[16] M. Kato, S. Shimizu, Chlorophyll metabolism in higher plants VI. Involvement of peroxidase in chlorophyll degradatio, Plant Cell Physiol. **26** (1985) 1291–1301.

[17] H.B. Dunford, in: J. Everse, K.E. Everse, M.B. Grisham (Eds.), Peroxidases in Chemistry and Biology, CRC Press, Boca Raton, Florida, 1991, p. 225.

[18] C.K. Lim, High-Performance Liquid Chromotography and Mass Spectrometry of Porphyrins, Choloropylls and Bilins, World Scientific Publishing Co., 2009.

[19] M.A. van Leewue, L.A. Villerius, J. Roggeveld, R.J. Visser, J. Stefels, An optimized method for automated analysis of algal pigments by HPLC, Mar. Chem. **102** (2006) 267–275.

[20] K. Abdelnaby, Extraction, purification and characterization of chlorophylls from spinach leaves, J. Agr. Food Chem. **40** (1992) 215–220.

[21] N. Yamauchi, A.E. Watada, Regulated chlorophyll degradation in spinach leaves during storage, J. Am. Soc. Hortic. Sci. **116** (1991) 58–62.

[22] A.J. Hoff, J. Amesz, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, FL, 1991, p. 723.

[23] M. Kobayashi, M. Akiyama, H. Kano, H. Kise, in: B. Grimm, R. Porra, W. Rudigger, H. Scheer (Eds.), Chlorophylls and Bacteriochlorophylls, Springer, Dordrecht, 2006, p. 79.

[24] H. Scheer, in: B.R. Green, W.W. Parson (Eds.), Light-Harvesting Antennas in Photosynthesis, Kluwer Academic Publishers, Dordrecht, 2003, p. 29.

[25] T. Watanabe, A. Hongu, K. Honda, M. Nakazato, M. Konno, S. Saitoh, Preparation of chlorophylls and pheo-

phytins by isocratic liquid chromatography, Anal. Chem. **56** (1984) 251–256.

[26] Z. Temoçin, M. Yiğitoğlu, Studies on the activity and stability of immobilized horseradish peroxidase on poly-(ethylene terephthalate) grafted acrylamide fiber, Bioprocess Biosystems Eng. **32** (2009) 467–474.

IZVOD

IN VITRO ISPITIVANJE UTICAJA TEMPERATURE I pH VREDNOSTI NA DEGRADACIJU HLOROFILA POD DEJSTVOM PEROKSIDAZE IZ RENA: SPEKTROSKOPSKA I HPLC ISPITIVANJA

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(Naučni rad)

Hlorofili su najzastupljeniji biljni pigmenti i pripadaju grupi porfirina. Njihova stabilnost je uslovljena brojnim faktorima. Prisustvo svetlosti, toplote, kiseonika, slabih kiselina i enzima, dovodi do formiranja velikog broja degradacionih proizvoda. U ovom radu je ispitivana *in vitro* degradacija hlorofila *a* iz spanaća pomoću peroksidaze iz rena u prisustvu rezorcinola (kao fenolnog jedinjenja), kao i uticaj pH vrednosti i temperature. Za degradaciju hlorofila pomoću peroksidaze neophodno je da fenolno jedinjenje koje u svojoj strukturi ima hidroksilnu grupu u *p*-položaju. Degradacija hlorofila *a* je praćena pomoću UV-VIS spektrofotometra i HPLC sistema. HPLC analizom kao glavni degradacioni produkt identifikovan je 13^2 -hydroxychlorophyll *a*. Tokom reakcije koncentracija formiranog produkta degradacije se proporcionalno povećava na račun smanjenja koncentracije hlorofila *a* u smeši. Dobijeni rezultati ispitivanja uticaja temperature na degradaciju hlorofila pomoću peroksidaze iz rena, pokazuju da se sa povećanjem temperature povećava degradacija hlorofila *a*, i dostiže svoj maksimum na 37 °C. Sa druge strane, povećanje pH vrednosti takođe povećava degradaciju hlorofila *a,* sa maksimalnim stepenom degradacije pri pH 6. Dobijeni rezultati ispitivanja degradacije hlorofila *a* pokazuju da degradacija hlorofila u najvećoj meri zavisi od vrednosti pH i temperature, što ukazuje na činjenicu da je za degradaciju hlorofila najviše odgovorna peroksidaza, jer se sa povećanjem temperature i pH vrednosti istovremeno približavamo optimalnim uslovima koji su neophodni za dejstvo peroksidaze iz rena.

Ključne reči: Hlorofil • Peroksidaza iz rena • Rezorcinol • Temperatura • pH • HPLC