# Carotenoid-Cleavage Activities of Crude Enzymes from *Pandanous amryllifolius*

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Carotenoid degradation products, known as norisoprenoids, are aroma-impact compounds in several plants. *Pandan wangi* is a common name of the shrub *Pandanus amaryllifolius*. The genus name '*Pandanus*' is derived from the Indonesian name of the tree, *pandan*. In Indonesia, the leaves from the plant are used for several purposes, *e.g.*, as natural colorants and flavor, and as traditional treatments. The aim of this study was to determine the cleavage of $\beta$-carotene and $\beta$-apo-8′-carotenal by carotenoid-cleavage enzymes isolated from pandan leaves, to investigate dependencies of the enzymatic activities on temperature and pH, to determine the enzymatic reaction products by using Headspace Solid Phase Microextraction Gas Chromatography/Mass Spectrophotometry (HS-SPME GC/MS), and to investigate the influence of heat treatment and addition of crude enzyme on formation of norisoprenoids. Crude enzymes from pandan leaves showed higher activity against $\beta$-carotene than $\beta$-apo-8′-carotenal. The optimum temperature of crude enzymes was 70°, while the optimum pH value was 6. We identified $\beta$-ionone as the major volatile reaction product from the incubations of two different carotenoid substrates, $\beta$-carotene and $\beta$-apo-8′-carotenal. Several treatments, *e.g.*, heat treatment and addition of crude enzymes in pandan leaves contributed to the norisoprenoid content. Our findings revealed that the crude enzymes from pandan leaves with carotenoid-cleavage activity might provide a potential application, especially for biocatalysis, in natural-flavor industry.

**Introduction.** – Pandan leaves (*Pandanous amryllifolius*) are traditional herbal leaves which have been used in cooking and also for traditional herbal treatment of several illnesses in Southeast Asian countries [1]. Pandan leaves have been used to confer aroma and flavors in several traditional food. Since pandan leaves have been investigated for potential natural flavor with vanilla-like flavor, they were sometimes also called as 'vanilla of the east' [2]. Pandan leaf flavor has been also applied to rice-starch coating [3]. Supercritical $CO_2$ extraction from pandan leaves also has been investigated as a novel application in food flavorings [4][5]. Nowadays, various drying methods such as spray drying are applied to several products such as ice cream, yogurt, soup, cake, tea, rice, and coconut jam [6]. A tissue culture of pandan leaves was investigated to search for aroma-impact compounds [7]. Pandan leaves exhibited an excellent heat-stable antioxidant property and may be a good natural alternative to existing synthetic antioxidants in the food industry [8].

Carotenoids have an important role as natural colorants, and they are responsible for the attractive color of several foods including pandan leaves [9][10]. Besides the carotenoids, pandan leaves also contain several antioxidants, *e.g.*, tocopherol [10]. The products of the breakdown of carotenoids, known as carotenoid-derived aroma

compounds, have been identified as good potential natural flavor compounds which can be used in flavor industry. Several carotenoid-derived aroma compounds are extremely powerful, *e.g.*, the fruity signature of $\beta$-ionone is recognizable even at concentrations as low as 0.007 ppm, and the rose and raspberry-like aroma of $\beta$-damascenone at even lower concentrations of 0.002 ppm [11].

Specific enzymatic cleavage of carotenoids produces various types of norisoprenoids [12]. Several norisoprenoids derived from carotenoids such as $\beta$-ionone, $\beta$-cyclocitral, geranylacetone, and pseudoionone are important components of flavor and aroma in many fruits, vegetables, and ornamental plants [12]. The formation of norisoprenoids as aroma-impact compounds by enzymatic reaction have been identified in several plants, *e.g.*, star fruits, nectarines, quince, rose, osmanthus flower, seaweed, tomato, petunia flower, melon, coffee, citrus, maize, sorgum, and rice [13–21]. The aim of this study was to determine the cleavage of $\beta$-carotene and $\beta$-apo-8′-carotenal by carotenoid-cleavage enzymes isolated from pandan leaves, to investigate dependence of enzymatic activities on temperature and pH, and, to determine the enzymatic reaction product by using HS-SPME GC/MS technique. Then, we investigated the influence of heat treatment and addition of crude enzyme to the $C_{13}$-norisoprenoids formation in pandan leaves.

**Results and Discussion.** – *Carotenoid-Cleavage Activity.* Crude enzyme from pandan leaves showed carotenoid-cleavage activities by using two different carotenoid substrates ($\beta$-carotene and $\beta$-apo-8′-carotenal; *Figs. 1* and *2*). This crude enzyme with carotenoid-cleavage activity, isolated directly from the plant, is a cytosolic protein [13–15]. Several carotenoid-cleavage enzymes have been isolated directly from the plants, *e.g.*, starfruit, osmanthus, nectarines, quince, and seaweed [13–17]. Access of carotenoid-cleavage enzymes to its substrates is most likely limited to carotenoids located in outer envelope of the plastids, because the substantial amounts of $\beta$-carotene are present in the outer envelopes of plants, *e.g.*, pea and spinach [22–24].

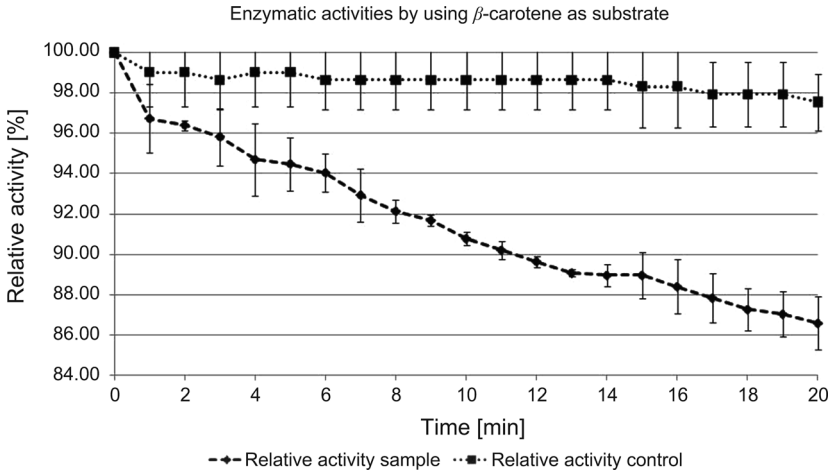



Fig. 1. *Carotenoid-cleavage activity of crude enzymes, at 30° and pH 7, by using β-carotene as substrate*

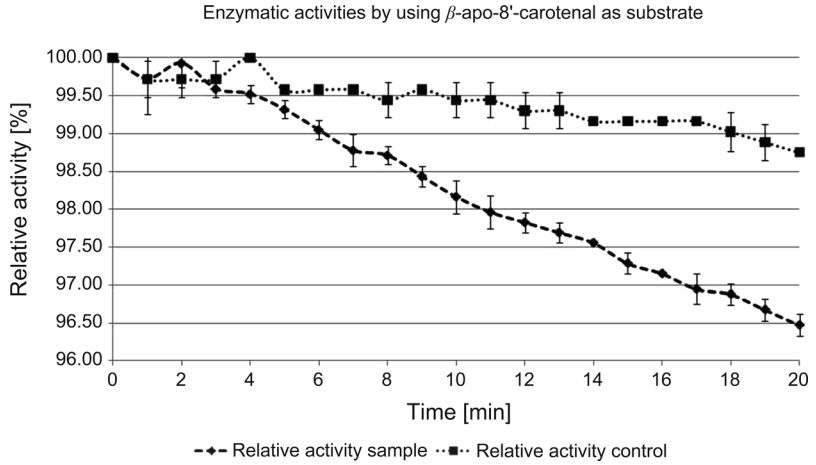



Fig. 2. *Carotenoid-cleavage activity in crude enzymes, at 30° and pH 7, by using β-apo-8′-carotenal as substrate*

The cleavage activity of the crude enzyme represents a higher chemical degradation at the same time (*Figs. 1* and *2*). We checked the control data where the oxidation was only caused by the nonenzymatic degradation, 'control' meaning that we studied also the solutions under the same reaction conditions at several temperatures but without protein or addition of crude enzymes to the solution. We studied the carotenoid-cleavage reaction continuously for 20 min by recording the absorption at a wavelength 505 nm. At this wavelength, it is possible to trace the substrate cleavage, uninfluenced from the spectral absorption of resulting reaction products [16][17].

The data revealed that oxidation by enzymatic degradation is higher than the nonenzymatic degradation (*Figs. 1* and *2*). The total soluble protein in crude enzymes was $16.46 \pm 0.13$ mg/ml. $\beta$-Carotene was identified as the native carotenoid in several green-leave vegetables [25–27]. The carotenoid-cleavage activities were higher by using $\beta$-carotene as substrate than $\beta$-apo-8′-carotenal (*Figs. 1* and *2*). In arabidopsis, *in vitro* assays, with $C_{40}$ carotenoids such as $\beta$-carotene higher enzymatic activity was observed rather than with $\beta$-apo-8′-carotenal [28].

Economically attractive biocatalytic conversion of hydrophobic substrates are often prohibited by the stability of the enzyme [28–30]. The enzyme, sometimes not being sufficiently stable or active in the organic media, required to dissolve the hydrophobic substrates [28–30]. Biocatalytic conversion of carotenoids to produce natural flavors, *e.g.*, norisoprenoids, by carotenoid-cleavage enzymes from several plants, *e.g.*, star fruit, nectarines, quince, seaweed, and arabidopsis, have been investigated by using surfactant *Tween 40* [13][15–17][28]. Since carotenoids are insoluble in the aqueous medium used for enzyme activity, they can be dispersed with surfactans, *e.g.*, *Tween 40*. These compounds play an important role in the dispersion of substrate (increasing the specific surface area) [29].

*Influence of Temperature on the Enzymatic Activity.* Several factors influence the activity of carotenoid-cleavage enzymes, *e.g.*, temperature. Temperature profile of

carotenoid-cleavage activity of crude enzyme from pandan leaves showed an increase of the activity until the maximum temperature measured (*Fig. 3*). Also, the control solution was studied under the same reaction conditions at several temperatures but without protein or addition of crude enzymes to the solution.

The optimum temperature of this enzyme was 70° (*Fig. 3*). The optimum temperatures of enzymes with carotenoid-cleavage activities, called carotenoid-cleavage dioxygenases (CCDs), were *e.g.*, in nectarines and starfruit 45°, in tea 70°, and in seaweed 55° [13][15][17][31]. These high optimum temperatures of carotenoid-cleavage activity indicate that the enzyme can be an excellent alternative for food industry, especially for several thermal processes.

*Influence of pH on the Enzymatic Activity.* The optimum pH value of $\beta$-carotene degradation activity was found to be 6 (*Fig. 4*), while the optimum pH values for carotenoid-cleavage activity were reported in tea leaves as 7.4, in star fruit as 8.5, and in quince in the range of 6–9 [13][16][31]. The control with only nonenzymatic degradation displayed a relatively different pH profile.

Different climatic origins of the plants and biochemical modifications influence also the characterization of the enzyme especially for the optimum-pH activity [16][32]. Post-translation modifications, *e.g.*, phosphorylations carotenoid-cleavage enzymes from fresh tea, were also investigated and displayed a great influence on the isoelectric pH of native enzymes [32]. In autumn tea, the isoelectric point of native enzyme is much lower, *ca.* 1.8, whereas, in spring tea it is *ca.* 7.4 [31]. This is due to secondary phosphorylations of CCDs in tea leaves during seasonal changes [32].

*Determination of Enzymatic-Reaction Product by Using HS-SPME GC/MS.* HS-SPME GC/MS Experiments were performed to identify the major volatile products isolated from incubation of two different carotenoid substrates, $\beta$-carotene and $\beta$-apo-8′-carotenal, with crude enzymes from pandan leaves. By this method, the major

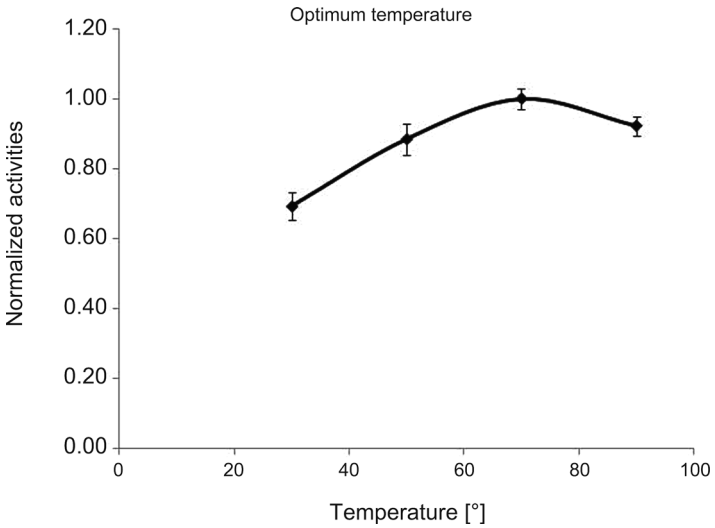



Fig. 3. *Temperature dependence of carotenoid-cleavage activity of crude enzymes*

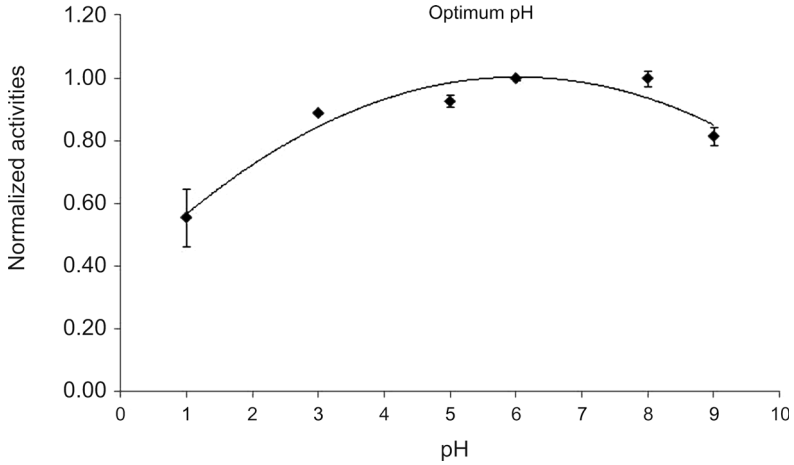



Fig. 4. *pH Dependence of carotenoid-cleavage activity of crude enzymes*

carotenoid breakdown in crude-enzyme solution was higher rather than that without enzymes. Also solutions (control) were studied under the same reaction conditions at several temperatures but without protein or addition of crude enzymes to the solution.

HS-SPME GC/MS-Analysis showed a $\beta$-ionone peak as major reaction product, and the concentration of $\beta$-ionone in sample is higher rather than in the control (*Fig. 5*). This result implies that the crude enzymes from pandan leaves have a capability to cleave $\beta$-carotene at the C(9)=C(10) and C(9′)=C(10′) bonds, with $\beta$-ionone being the dominant volatile reaction product (*Scheme*). The enzyme also can cleave $\beta$-apo-8′-carotenal at the C(9)–C(10) bond to produce $\beta$-ionone.

Enzyme catalysis employed to produce molecules for perfume, flavors, and fragrances has the advantage of allowing the products to be labeled as natural [33], so that, natural flavor of $\beta$-ionone from our study is a promising natural food ingredient. In recent years, several natural ingredients have been recognized as the health-impact compounds [6]. Therefore, several efforts have been devoted to prepare flavors from natural sources. The continued interest in natural compounds by consumers reflects their concern about their health as well as the quality of food they consume [6].

HS-SPME GC/MS Technique for determination of $\beta$-carotene breakdown products have been investigated in several crude enzymes from plants, *e.g.*, star fruits and seaweed [17][15]. Enzymes which have the ability to produce $\beta$-ionone from $\beta$-carotene and $\beta$-apo-8′-carotenal have been investigated in several plants [15][18][29], *e.g.*, roses, osmanthus flowers, arabidopsis, bitter melon, maize, sorgum, rice, petunia flower, grape, citrus, and they play important roles to produce norisoprenoids in these selected plants [14][21][34–39]. So far, we could not find other norisoprenoids as the enzymatic-reaction products. Several investigations established that a carotenoid-cleavage enzyme can cleave specifically the substrate to produce a specific enzymatic product [24]. $\beta$-Ionone as $C_{13}$ norisoprenoid possesses an antiproliferative and cells-cycle regulatory effect in breast cancer cells and can suppresses mammary carcinogenesis [40][41].

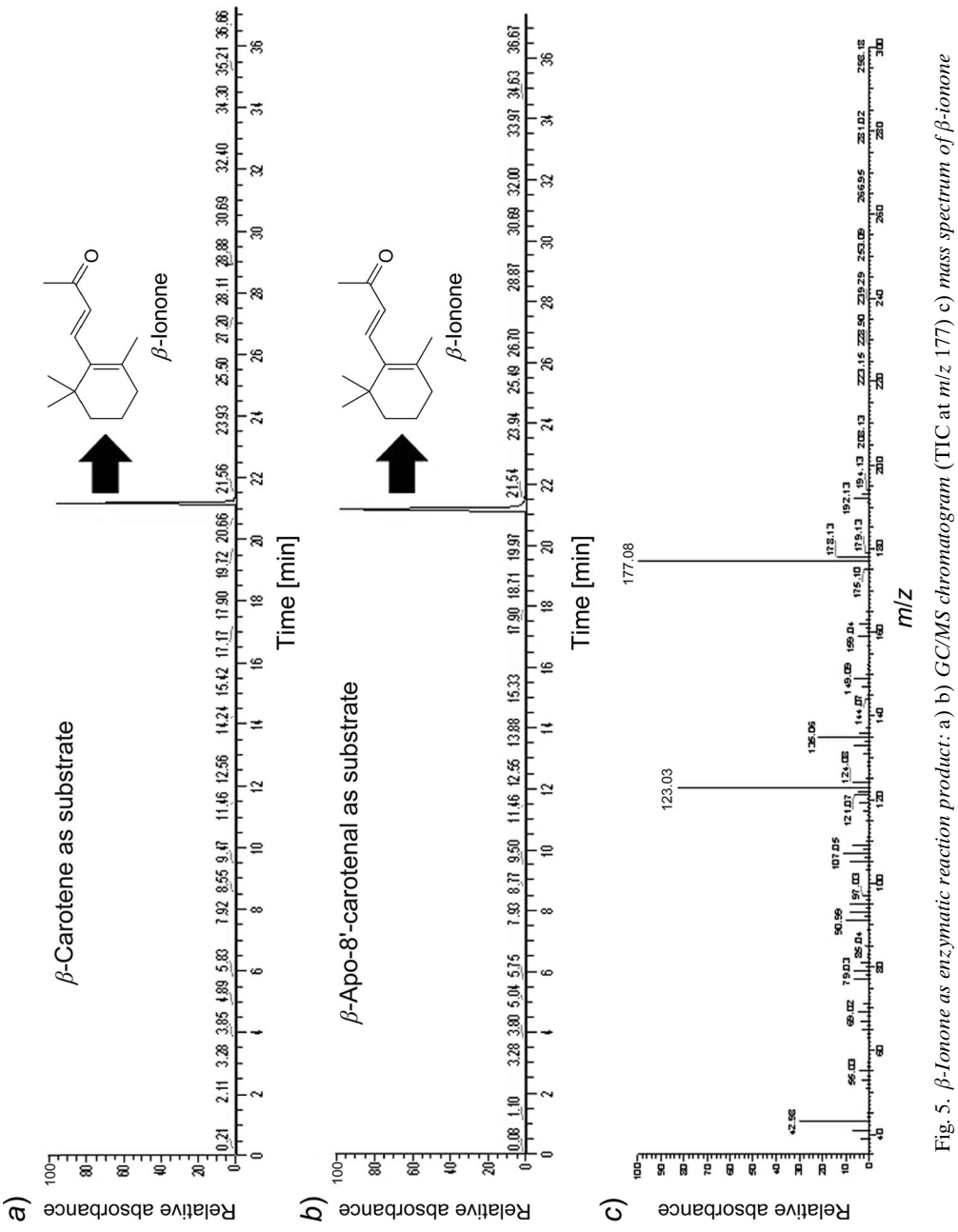
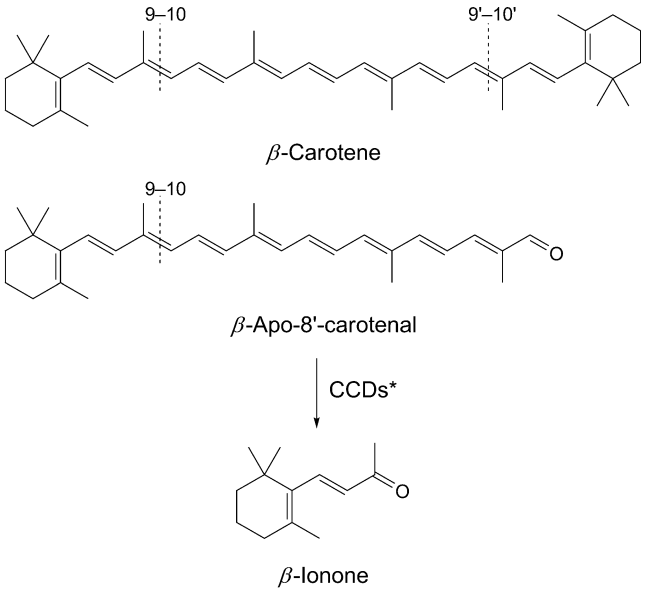



Fig. 5. *β-Ionone as enzymatic reaction product:* a) b) *GC/MS chromatogram* (TIC at *m/z* 177) c) *mass spectrum of β-ionone*

Scheme. *Cleavage of β-Carotene and β-Apo-8′-carotenal to Produce β-Ionone as the Enzymatic Reaction Product*




*Influence of Several Treatments on the Norisoprenoid Formation in Pandan Leaves.* We investigated the influence of denaturing conditions or heat treatment, and addition of crude enzyme to the norisoprenoids, *e.g.*, β-cyclocitral, α-ionone, and β-ionone formation in pandan leaves. The GC/MS chromatograms of control, and of samples after heat treatment and addition of external crude enzymes are shown in *Fig. 6, a–c*. Degradation of carotenoids can be observed by heat treatment, together with the enzymatic oxidation by carotenoid-cleavage enzyme [11]. Thermal or chemical degradation of β-carotene to produce norisoprenoids, *e.g.*, β-ionone, have been investigated in several food models [42][43]. Comparing with the specific enzymatic cleavage, the thermal treatment led to higher norisoprenoid contents than the enzymatic reaction.

The norisoprenoid content after enzyme addition was almost the same with the control treatment (*Fig. 6, d*). The limited access of the enzyme to the carotenoid substrate inside the pandan leaves may be the reason of this result. The challenge to increase the enzymatic carotenoid-cleavage has been investigated by optimization process in carotenoid-cleavage enzymes from arabidopsis [28–30]. The kinetics of enzymatic reactions performed with hydrophobic substrates is highly dependent on the properties delivery system. Since the carotenoids as substrates are located inside the plastids of pandan leaves, the delivery of the external enzyme to the substrate can be hindered.

*Application of Isolated Enzymes with Carotenoid-Cleavage Activities to the Production of Natural Flavors.* Carotenoids as the precursor and norisoprenoids as

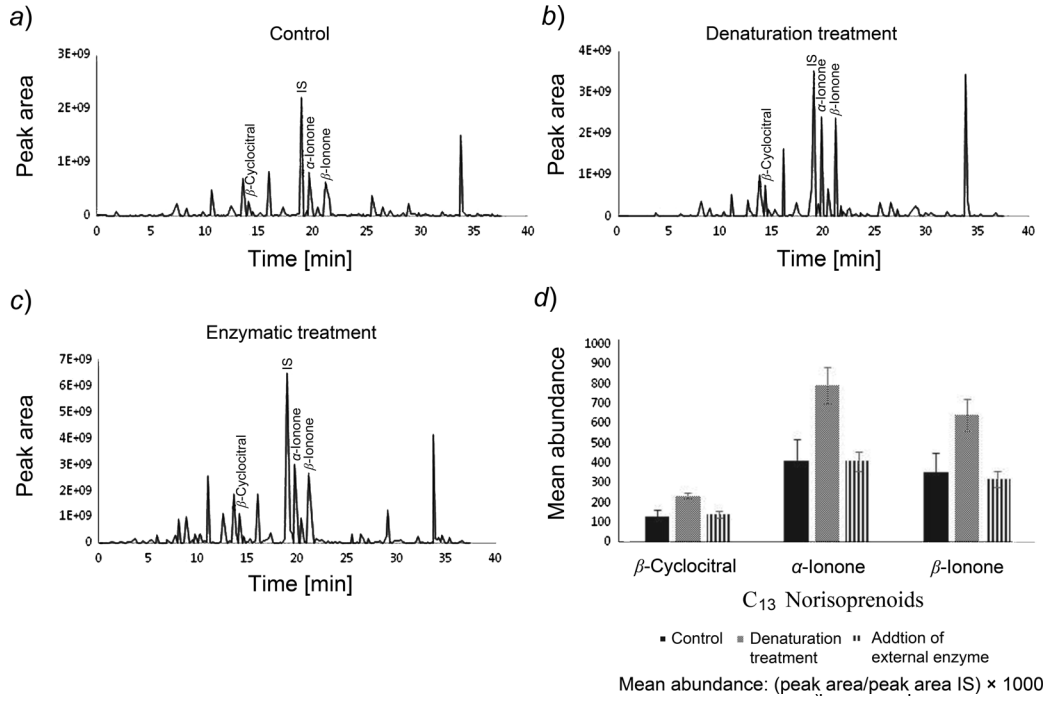



Fig. 6. *GC/MS:* a) *Control,* b) *after heat treatment at 100°,* c) *after addition of external crude enzyme, and* d) *quantification of $C_{13}$ norisoprenoids*

putative enzymatic reaction products are attractive compounds especially for food industry, and they can be used as aroma compounds, colorants, and vitamins [9][11]. Enzymatic production based on natural carotenoid sources and CCDs promise significant productivity boots and improved cost efficiencies compared to the current isolation of natural flavors *via* extraction and distillation, or chemical synthesis [28]. The possible *in vitro* conversion and the unique properties of the enzymes with carotenoid-cleavage activities render them attractive for investigation on the substrate recognition and delivery procedures, as well as for industrial applications [28]. First, according to current knowledge the ability to cleave carotenoids region-specifically is only possible using CCDs that have been identified in many plants [12]. Second, molecular $O_2$ as the cheapest available oxidant is used for oxidation. Third, no cofactors are needed to catalyze the cleavage reaction [44]. So far, no industrial application of CCDs has been developed, but possible applications are investigated, *e.g.*, enzymatic conversion of $\beta$-carotene to valuable flavors and fragrances [44].

Future design by using potential substrates such as plant or algal biomass with high carotenoid content is promising to produce natural norisoprenoids, *e.g.*, $\beta$-ionone [28]. Some potential substrates such as carrot pomace from juice production are waste streams and would, therefore, be available at low cost and in sufficient amount, decreasing substrate expenses significantly [28]. Optimization of the production of natural norisoprenoids as carotenoids degradation product also have to be assessed.

Carotenoid-cleavage activity with optimum high temperature has indicated that the enzyme can have a good stability to be applied in food processing.

The economic benefits of manufacturing natural flavor compounds will surely remain limited to premium food products, as long as they are significantly higher priced than those of their chemically synthesized analogs [33][45]. Biocatalysis to produce natural flavors, *e.g.*, norisoprenoids from natural precursors like carotenoids, is increasingly becoming the driving force for its application and for the ongoing research activities in this field. Conducting under specific conditions to protect unstable products, *i.e.*, green processes, and functionalization of even complex precursor molecules in a highly specific way are two important factors allowing biocatalysis to outplay chemical approach in certain cases [45]. The health effect of several norisoprenoids with drive the research increasingly in the future. Several compounds from this class have been proved to be promising as cancer-protective constituents [46]. Individual isoprenoids suppressed the proliferation of leukemia cells with several degrees of potency [47]. Blends of several isoprenoids suppressed also the cancer-cell proliferation with efficacies equal to the sum of the individual impacts. Research exploiting these advantages of flavor biocatalysts will certainly be intensified in the future [45].

**Conclusions.** – The enzymatic carotenoid-cleavage activity plays an important role in $C_{13}$ norisoprenoid formation in pandan leaves. The optimum temperature for enzymatic carotenoid-cleavage is 70°, whereas the optimum pH value of carotenoid-cleavage activity is 6. Carotenoid-cleavage of $\beta$-carotene and $\beta$-apo-8′-carotenal as substrates furnished $\beta$-ionone as the major product, so that, in the nearer future, it seems realizable to produce flavor and fragrance compounds bearing the desired label 'natural' according to US and European food and safety regulations from pandan leaves. Nowadays, due to increased consumer demand for 'organic' or 'bio' products, 'natural' $\beta$-ionone has a higher market value than the synthetic form [28]. Our findings revealed that the crude enzymes from pandan leaves with carotenoid-cleavage activity have a potential to be used in flavor industry.

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## Experimental Part

*General.* Fresh pandan leaves were purchased from a local market. A UV/VIS spectrophotometer *Hitachi U 2900* with 1-cm matched cells was used for all absorbance measurements. A water bath (*GFL*) was used to determine the influence of temp. on enzymatic activity. For identification of the enzymatic reaction products, a GC/MS instrument was composed to *TRACE GC Ultra 2000* version 1.4 SR1 (*Thermo Corporation*, Austria), coupled with mass-spectrometric detector (*Thermo Corporation*, Austria), equipped with cap. column *HP-5* (*Agilent USA*, 30 m, 0.25 mm i.d., 0.25 μm film thickness). All chemicals used for the assay were of anal. grade.

*Crude-Enzyme Extraction.* Crude enzymes were prepared by a method described before. Fresh leaves were grounded with mortar and pestle, then homogenized with a buffer soln. containing 100 mM *Tris*, 125 mM KCl, 5 mM $MgCl_2$, 1 mM dithiotreitol (DTT) (5 ml buffer per 1 g of fresh leaves) [17]. The homogenate was filtered through filter paper, then centrifuged at 1000 rpm for 10 min at 25°. Protein concentration was measured using the *Bradford* assay with bovine serum albumin as standard [48]. All experiments were performed in triplicate.

*Enzymatic-Activity Measurement.* The measurement of carotenoid-cleavage activity of the crude enzyme extracts was carried out as described in [17]. The degradation of the substrate (*β*-carotene and *β*-apo-8′-carotenal) was measured with a UV/VIS spectrophotometer at 505 nm. For all enzymatic assays, a mixture containing crude enzyme extract, carotenoid/*Tween 40* soln. as substrate and buffer was incubated. The buffer soln. contained *Tris* (pH 7; 100 mM), 125 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT. Carotenoid/*Tween 40* soln. as substrate was prepared as described in [17]. *β*-Carotene (1 mg) was dissolved in acetone and then added to 1 g of *Tween 40* dissolved in acetone. The soln. was mixed well, and the solvent was evaporated. The residue was diluted in 10 ml of $H_2O$ and filtered through 0.45-μM filter.

The enzymatic activity was investigated by recording the absorbance of each sample (250 μl of crude enzyme, 1675 μl of *Tris* buffer, 75 μl of carotenoid substrate) and control (1925 μl of *Tris* buffer and 75 μl of carotenoid substrate) for 20 min at 505 nm, started immediately after adding the substrate. The degradation of the substrate was followed by time-course measurements for 20 min. The difference in absorbance before and after incubation was determined as relative enzymatic activity. All experiments were performed in triplicate.

*Influence of Temp. on the Enzymatic Activity.* The effect of temp. on carotenoids-cleavage activity was evaluated by incubating the enzymatic reaction mixture at several temps. for 10 min. Then, the activity was spectrophotometically determined as described above. All experiments were performed in triplicate.

*Influence of pH on the Enzymatic Activity.* The effect of pH on carotenoid-cleavage activity was evaluated at several pH values of the *Tris* buffer containing 100 mM *Tris*-base, 125 mM KCl, 5 mM $MgCl_2$, 1 mM DTT. The method was identical to that described above. All experiments were performed in triplicate.

*Identification of Enzymatic-Reaction Products by HS-SPME GC/MS.* The enzymatic-reaction products were determined by using HS-SPME GC/MS. A SPME fiber coated with 75 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB; *Supelco*, Bellefonte, PA, USA) was introduced into headspace vial containing 1 ml of enzymatic reaction mixture and 1 ml of sat. NaCl, and then the mixture was incubated for 35 min at 80°. The reaction products were analyzed by GC/MS with a cap. *HP-5* column (*Agilent USA*, 30 m, 0.25 mm i.d., 0.25 μm film thickness); GC and MS conditions: oven program, 50° (3 min); 50–190° at 5°/min; 190–240° at 20°/min; injection mode, splitless; ion source temp., 250°; transfer line temp., 250°. The extracted volatile compounds were identified by comparison of their spectra with those in the *NIST 2005* library, and, finally, relative retention indices (*RI*s) were evaluated as well. All experiments were performed in triplicate.

*Influence of Several Treatments to the Norisoprenoid Formation in Pandan Leaves.* We investigated the influence of denaturing conditions or heat treatment, and addition of crude enzyme to the formation of norisoprenoids. For control treatment, 1 g of pandan leaves was grounded, and then 2 ml of $H_2O$ were added. For the heat treatment, 1 g of pandan leaves was grounded, 2 ml of $H_2O$ were added, and then incubated at 100° for 10 min. For the addition of crude enzyme, 1 g of pandan leaves was grounded, and then 1 ml of $H_2O$ and 1 ml of crude enzymes were added as described above for crude enzyme extraction. Then, the samples were treated as described above by using HS-SPME GC/MS. All experiments were performed in triplicate.

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