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Differences in the activity and concentration of elements of the antioxidant system in different layers of *Brassica pekinensis* head

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ABSTRACT

Differences in the activity of superoxide dismutase, catalase (CAT) and ascorbate peroxidase (APX) as well as in the concentration of ascorbate, tocopherol and hydrogen peroxide (H_2O_2) were found in leaves from different layers of the Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.) head. The youngest chlorophyll-deficient leaves from the most inner layers of the cabbage head were characterized by a high concentration of ascorbate, high activity of iron superoxide dismutase (FeSOD), cooper/zinc superoxide dismutase (Cu/ZnSOD) and a low content of H_2O_2. On the other hand, activity of CAT, manganese superoxide dismutase (MnSOD) and APX and tocopherol content were highest in chlorophyll-rich leaves from outer parts. The results of this work are interesting from the human nutrition standpoint, as the measured antioxidants have beneficial effects on human health. They can also be utilized to improve storage conditions due to an unequivocal function of antioxidant molecules in maintaining postharvest quality of vegetables.

Introduction

Plants from the *Brassicaceae* family are important dietary vegetables consumed in Europe. Leaves of *Brassica pekinensis*, also called Chinese cabbage, are abundant in protein (about 1.3%), minerals (calcium, phosphorus, iron) and vitamins such as vitamin C (ascorbate, AsA) and vitamin E (tocopherols) (Podsędek, 2007). The latter two compounds are of special interest due to their antioxidant activity and beneficial effect on human health. According to numerous reports, their consumption is associated with a reduced risk of many chronic diseases (Gaziano et al., 1995; Steinmetz and Potter, 1996; Agarwal and Rao, 2000). Antioxidants have been shown to fulfill many essential functions in all living organisms. In general, they form a sophisticated system maintaining redox homeostasis within the cells. Antioxidants include enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and small molecular, water soluble compounds such as ascorbate and lipid-soluble compounds such as the tocopherols. SOD dismutates superoxide radical ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), which is further reduced to H_2O by CAT and peroxidases such as APX. CAT was found to be present mainly in the peroxisomes/glyoxysomes and in the mitochondria, scavenging photorespiratory/respiratory H_2O_2 (Salin, 1988; Asada, 1992; Ádám et al., 1995; Willekens et al., 1997; Hegedüs et al., 2001) while APX operates in the cytosol and chloroplasts (Salin, 1988; Mittler and Zilinskas, 1994; Asada, 1992; Zhang and Kirkham, 1996; Hegedüs et al., 2001). Ascorbate protects the hydrophilic space of the cell (Havaux et al., 2005), and has an important role as a donor/acceptor in electron transport at the plasma membrane (Davey et al., 2000). In plants, it plays a major role in photosynthesis, acting in the Mehler peroxidase reaction with APX as a regulator of the redox state of photosynthetic electron carriers and as a cofactor for violaxanthin de-epoxidase, an enzyme involved in the photoprotective xanthophyll cycle. Ascorbate is also a cofactor for an enzyme that hydroxylates proline residues in the cell wall that is required for cell division and expansion (Smirnoff and Pallanca, 1996; Latowski et al., 2010). Tocopherols include four forms: α, β, γ and δ, of which α-tocopherol is the most abundant found in the green parts of plants (Surówka et al., 2009). Among the best characterized functions of tocopherols in cells is their action as antioxidants in chloroplasts, protecting plants from photoinhibition and photo-oxidative stress (Havaux

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Cu/ZnSOD, copper/zinc superoxide dismutase; FeSOD, iron superoxide dismutase; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide radical; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species.

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et al., 2005). The antioxidant functions of tocopherols are thought to include scavenging lipid peroxy radicals to terminate lipid peroxidation chain reactions and physically quenching $^1O_2$ or chemically scavenging $^1O_2$ thus controlling its levels (Szymańska and Kruk, 2008). α-Tocopherol may affect intracellular signaling in plant cells either directly, by interacting with key components of signaling cascades, or indirectly, through the prevention of lipid peroxidation or the scavenging of singlet oxygen. In the latter case, α-tocopherol may modulate the function of plant hormones, such as jasmonic acid, which control growth, development and plant responses to stress (Munné-Bosch and Alegre, 2002; Horvath et al., 2006). The Chinese cabbage head consists of leaves in different developmental stages differing in metabolic activity, due to natural etiolation. It presents a unique experimental model for investigating the antioxidant system in different phases of growth and development. It is well recognized that overproduction of reactive oxygen species (ROS) causes natural or induced senescence, leading to a significant loss in quality of vegetables during postharvest storage. Thus, uncovering the profile of abundance of antioxidants and enzyme activity in layers of the Chinese cabbage head may be utilized in improving vegetable storage strategies, thereby minimizing quality loss due to accelerated postharvest senescence. This is the first report describing the distribution pattern of selected antioxidants and its correlation with developmental stage and metabolic activity of leaves in the Chinese cabbage head. Reports describing the distribution of antioxidants in leaves varying in age, developmental stage and chlorophyll content are currently ambiguous (Lee and Kader, 2000; Nilsson et al., 2006), making the results of this work important to our understanding of this phenomenon.

Material and methods

Plant material

Chinese cabbage (cv. Orient Express F_1) seeds were sown into multipots filled with peat substrate and cultured in greenhouse conditions. One-month-old seedlings were planted in the field at the research farm of The University of Agriculture in Kraków, Poland. Plants were cultivated according to standard agriculture practices. Three-month-old healthy plants at the harvest maturity stage were selected and transferred to the laboratory. Cabbage heads were divided into three parts (outer, central, inner layers) and collected as shown in Fig. 1. Samples were frozen in liquid nitrogen, pulverized and stored at −80 °C. Analyses were performed on material collected from six cabbage heads.

Total chlorophyll content determination

Chlorophyll $a+b$ content was determined according to the method described by Vernon (1960). The tissue (0.5 g) was homogenized in chilled 80% acetone (1 ml). After centrifugation at 14,000 × *g* for 5 min at 4 °C, the supernatant was collected. The total content of chlorophyll $a+b$ was determined with a Beckman spectrophotometer at 663 and 645 nm using simultaneous equations based on the specific extinction coefficients 82.04 [l g^{-1} cm^{-1}] for chlorophyll *a* and 45.6 04 [l g^{-1} cm^{-1}] for chlorophyll *b*.

H_2O_2 concentration determination

The endogenous hydrogen peroxide (H_2O_2) level was investigated according to the modified method described previously by Brennan and Frenkel (1977). H_2O_2 was extracted by homogenization of 0.5–1 g of frozen, powdered tissue in 2 ml of cold acetone. After centrifugation (5 min at 12,000 × *g*) the pellet was discarded and 0.5 ml of the extract was collected. Titanium reagent (50 μl of 20% titanium tetrachloride in concentrated HCl, v/v) was added to 0.5 ml of the extract, followed by the addition of 0.1 ml of NH_3 aq. (25%) to precipitate the peroxide–titanium complex. After 5 min of centrifugation at 10,000 × *g*, the supernatant was discarded and the precipitate was repeatedly washed in 1 ml of acetone and centrifuged for 5 min at 10,000 × *g*. The precipitate was solubilized in 1 ml of 1 M H_2SO_4 and brought to a final volume of 2 ml. Absorbance of the obtained solution was read at 415 nm against a water blank. The concentration of H_2O_2 in the extract was determined by comparing the absorbance against a standard curve representing the titanium–H_2O_2 complex, over the range from 0.05 to 0.3 mmol in 1 ml. All H_2O_2 measurements were normalized to a fresh weight of tissue.

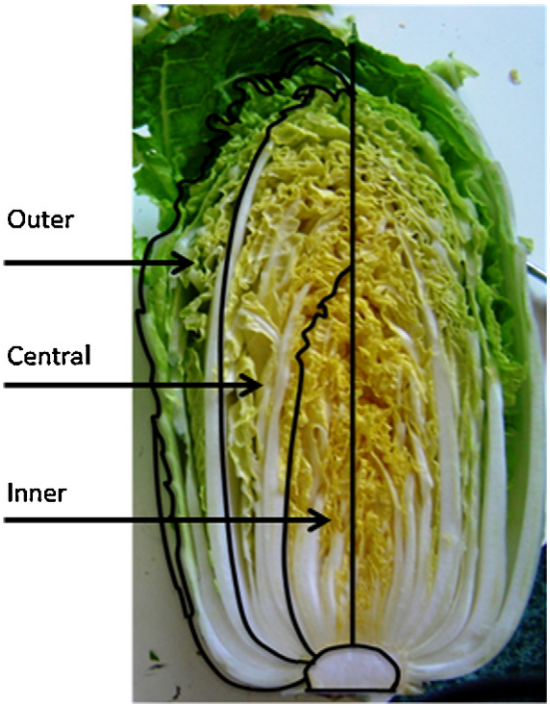

Fig. 1. Longitudinal section of *Brassica pekinensis* head with a schematic division of leaf layers taken for analysis.

Protein isolation

To isolate fractions of soluble proteins (later called crude extracts), plant material was homogenized (1.0 g fresh weight) at 4 °C in a 100 mM Tricine–Tris buffer pH 8.0, containing 100 mM $MgSO_4$, 1 mM dithiotreitol (DTT) and 3 mM EDTA. Non-soluble material was removed by centrifugation for 2 min at 3000 × *g*. The protein concentration was determined according to Bradford (1976) using the BioRad protein assay (Bio-Rad, Hercules, CA) with BSA (bovine serum albumin) as a standard.

Analysis of SOD by native PAGE

SOD bands were visualized on polyacrylamide gels using the activity staining procedure described by Beauchamp and Fridovich (1971), after protein separation from crude extracts in the non-contiguous laemmli buffer system, with a 4% stacking and 12% separating polyacrylamid gel (Laemmli, 1970). After 30 min of dark incubation in the staining buffer at room temperature, gels were exposed to white light until SOD activity bands became visible. For identification of SOD forms, selective inhibition of different isoenzymes was performed. Copper/zinc superoxide dismutase (Cu/ZnSOD) and iron superoxide dismutase (FeSOD) were inhibited by adding H_2O_2 to the staining buffer in a concentration of