1. Intention

Carotenoid derived aroma compounds are important flavor substances in the scent of fruit and flowers. These compounds are formed within three steps: the oxidative cleavage by carotenoid oxygenases, the enzymatic transformation to polar intermediates, and the acid catalyzed conversion into volatiles (Figure-1). In the last years the number of publications on carotenoid cleavage enzymes (subsequently also named as carotenases), involved in Step I, increased rapidly, but most of these carotenases are found solely on the basis of homologous clones. Constantly, differences between these homologous enzymes and enzymes isolated from natural sources, for example regarding the isoelectric point as well as molecular mass, were found.

The purpose of this thesis was the isolation of carotenoid oxygenases involved in Step I, from nectarine peel (*Prunus persica* var. *nucipersica*), flower petals of *Osmanthus* (*Osmanthus fragrans* var. *aurantiacus*), and Japanese Green Tea (*Camellia sinensis*). Therefore, kinetic, structural, and substrate specific aspects were investigated and final conclusions regarding Step I of carotenoid degradation with respect to differences and similarities between recombinant enzymes (based on homological clones) and enzymes isolated from natural sources were drawn.

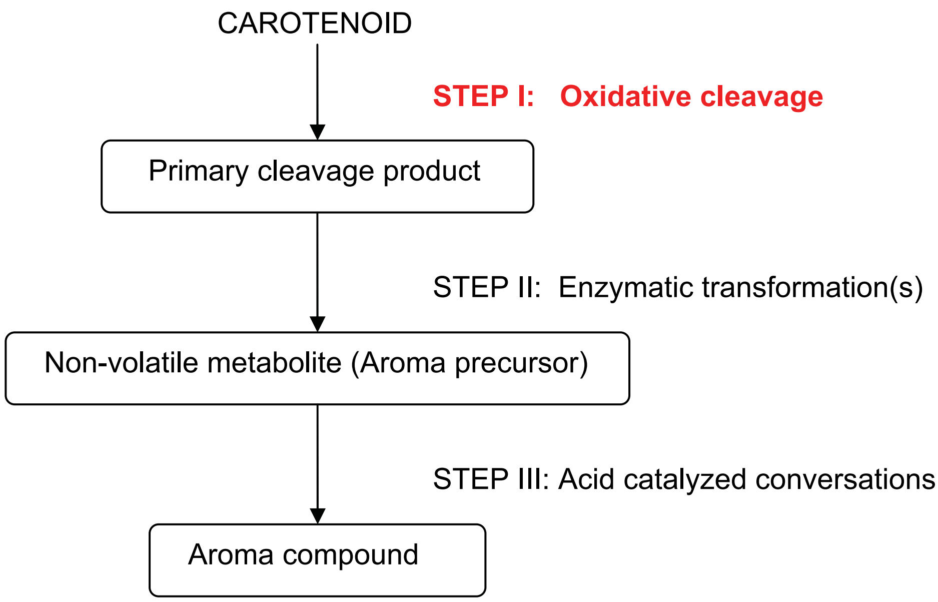

Figure-1: General steps for the conversion of carotenoids into flavor compounds [Winterhalter and Rouseff 2002]

2. *Background*

2.1 *Camellia sinensis*

Tea is a very popular drink and over 2.5 million tons of tea are produced annually.
The origin of *Camellia sinensis,* an evergreen plant, is the south and southeast of Asia, but today it is cultivated across the world in tropical and subtropical regions.
The leaf buds are used for tea production. Depending on the way of manufacturing and associated with the level of leaf oxidation, different teas are obtained. For the present study Japanese Green Tea of Sencha grade was chosen.
An overview on the processing from leaves to Sencha is given in Figure-2 [Fukatsu 1987]. The first step is the steaming of the fresh buds, followed by various rolling and drying steps in order to reduce moisture and to form the shape. Unlike Sencha, Black Tea is fermented and Chinese Green Tea is pan fried for approximately 10 sec at 280°C before drying and rolling. In case of Green Tea production, the first manufacturing step is done to inactivate enzymes, like oxidases, to preserve phenols, and chlorophylls.

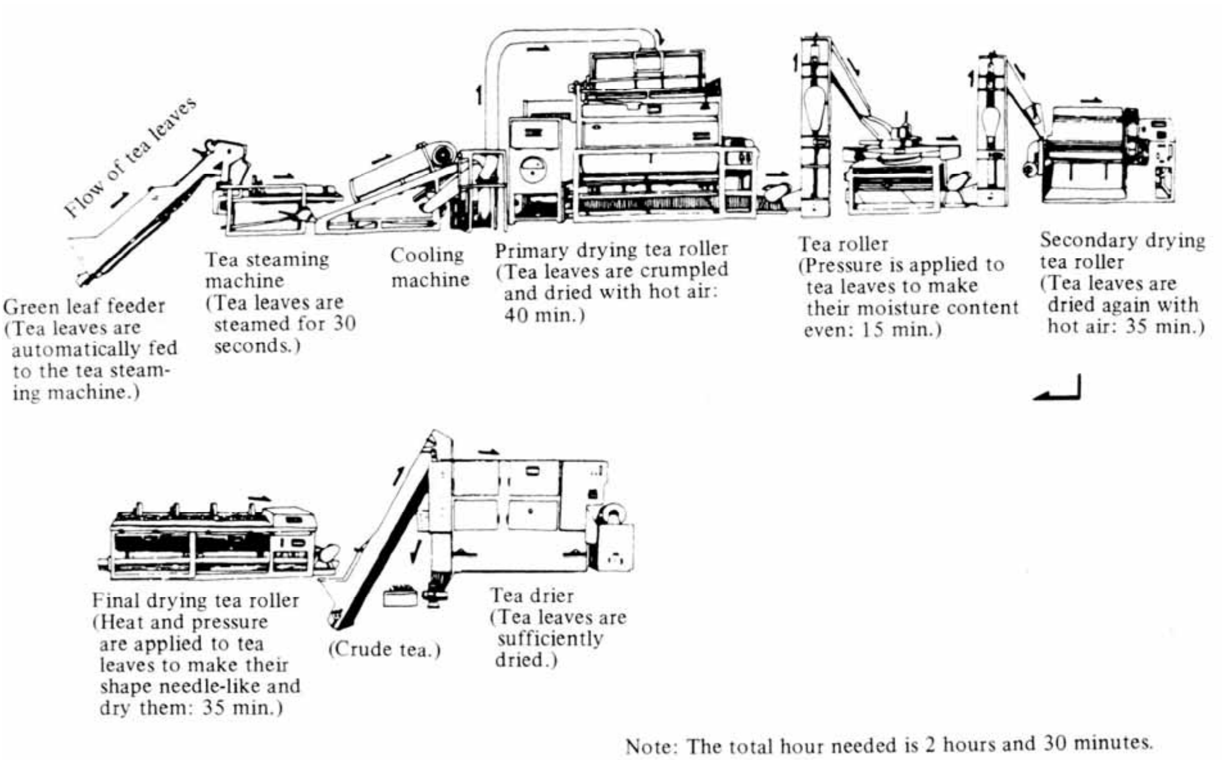

Figure-2: Sencha production [Fukatsu 1987]

2.1.1 Carotenoid composition

The carotenoid composition of different tea varieties was investigated by Suzuki and Shioi [2003]. In Sencha 12 carotenoids were found, among them β-carotene and the xanthophylls zeaxanthin, lutein, neoxanthin, violaxanthin, and auroxanthin. The highest level of β-carotene was found in Sencha among the tea types studied (Sencha, Gyokuro, Matcha, Hojicha, Ceylon, Tikuan-tin, Pu-erh). Furthermore, the structural change of carotenoids due to heat treatment by tea manufacturing was observed. In Sencha the amount of violaxanthin was reduced and two new auroxanthin species appeared, indicating a conversion of violaxanthin to auroxanthin. The level of *cis*-type carotenoids increased about 10 % compared to the fresh leaves [Suzuki and Shioi 2003].

2.1.2 Flavor composition

Volatile compounds in Japanese Green Tea have been investigated by many research groups. Over 270 volatile compounds were identified [Kumazawa and Masuda 1999]. During tea processing the total loss of carotenoids is approximately 40 % [Kawakami and Kobayashi 2002] and many carotenoid derived aroma compounds like β-cyclocitral (1), β-ionone (2), 5,6-epoxy-β-ionone (3), β-damascenone (4), linalool (5), nerolidol (6), and dihydroactinidiolide (7) have been identified (Figure-3). Beside thermal degradation various enzymes (polyphenol oxidase, lipoxygenase, β-carotene bleaching enzyme) are involved in the aroma formation [Kawakami and Kobayashi 2002].

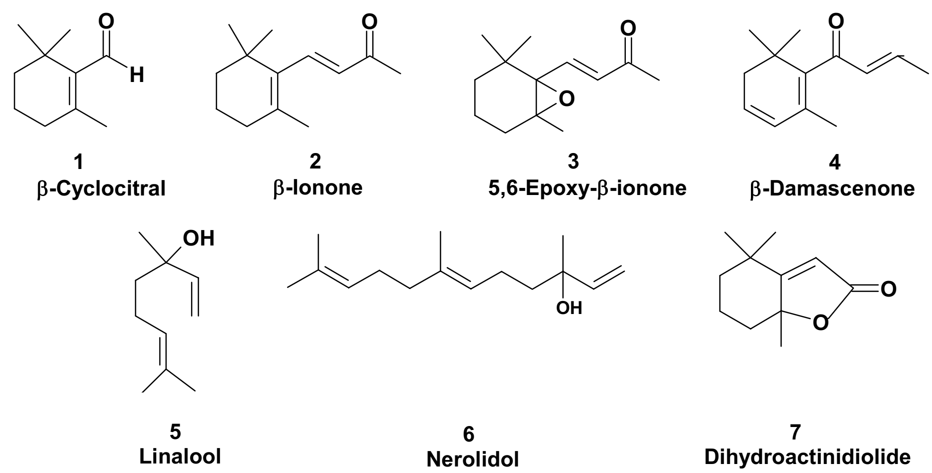

Figure-3: Carotenoid derived aroma compounds in Japanese Green Tea

2.1.3 Enzymes

Tea enzymes can be classified into four groups:

- Enzymes related to the tea flavor
- Enzymes involved in the catechin biosynthesis
- Enzymes of the nitrogen metabolism
- Other enzymes

The best studied enzyme of tea is the tea polyphenol oxidase. This enzyme causes an oxidative degradation of amino acids, carotenoids and lipids. Its seasonal change of activity is of particular interest. In young leaves the enzymatic activity is higher compared to the mature leaves. High enzymatic activity was detected after plucking and during storage, whereas an inactivation was detected during processing. A second oxidizing enzyme is the tea peroxidase, whose metabolic role still is not completely understood. Active enzymes were found in plucked leaves and injured leaves. The role of the peroxidase, contributing to color and tea flavor, is discussed. C_6-aldehyde producing enzymes are the lipoxygenase and hydroperoxid lyase. The oxidation of the plant aldehydes to their corresponding alcohols is due to an alcohol dehydrogenase activity. Enzymes involved in the catechin metabolism are L-phenylalanine ammonialyase and enzymes of the shikimi acid biosynthesis. Enzymes concerned with the nitrogen metabolism are involved in the assimilation of nitrate and in the amino acid metabolism. Among all of these tea enzymes, the ribonuclease and the endonuclease show a surprisingly high temperature optimum of 70°C. Further tea enzymes studied so far are malate dehydrogenase, chlorophylase and acid phosphatase [Jain and Takeo 1984].

In addition to these known enzymes, more recent studies related to the presence of a carotenoid cleavage enzyme in fresh leaves of Japanese Green Tea, which is also characterized by an exceptionally high temperature optimum and seasonal changes, are carried out by Fleischmann and Yamamoto [Fleischmann et al. 2004, Yamamoto 2003]. This was the basis for more thorough investigations on the enzymatically induced formation of carotenoid derived aroma compounds during tea manufacturing.

2.2 *Prunus persica nucipersica*

Nectarines belong to the Rosaceae family. With regard to the origin of these species contradictory theories are published. The most common one is that nectarines are a hybrid between peaches (*Prunus persica*) and plums (*Prunus domestica*). The pulp of nectarines varies from white over yellow to reddish. Many cultivars with different flavor and aroma compositions can be found on the market.

2.2.1 Flavor composition

The importance of carotenoid derived aroma compounds in white and yellow fleshed nectarines was reported by Aubert [Aubert et al. 2003a, Aubert et al. 2003b]. In white and yellow fleshed varieties aroma compounds of the ionone and damascone family could be detected. In yellow fleshed nectarines, which were also used for our experiments, major carotenoid derived volatiles were 3-hydroxy-β-damascone (8), 3-oxo-7,8-dihydro-β-ionone (9), 3-hydroxy-7,8-dihydro-β-ionone (10), 3-hydroxy-5,6-epoxy-β-ionone (11), 3-oxo-α-ionol (12), 3-hydroxy-β-ionone (13) and 3-hydroxy-β-ionol (14) (Figure-4) [Aubert et al. 2003a].

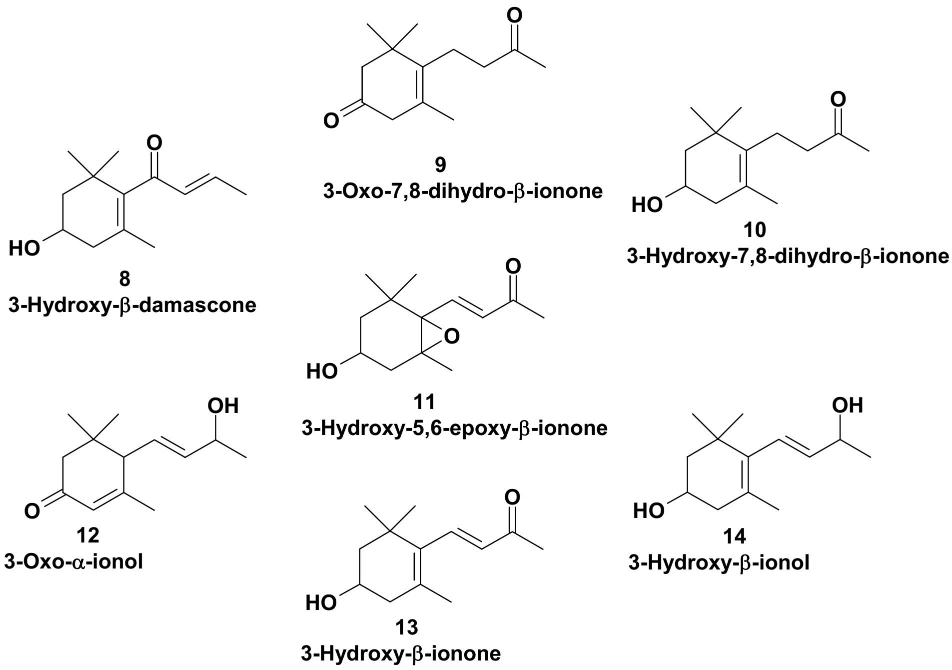

Figure-4: Structures of C_{13}-norisoprenoids in yellow fleshed nectarines

2.2.2 Carotenoid composition

The main carotenoids are β-carotene and β-cryptoxanthin [Gil et al. 2002]. Other carotenes found in *Prunus persica* varieties are lycopene, γ-carotene, ζ-carotene, phytoene, and phytofluene. Additional xanthophylls are zeaxanthin and lutein which are present as free xanthophylls or esters of fatty acids [Khachik et al. 1989].

2.3 *Osmanthus fragrans*

Osmanthus fragrans is a shrub native to East-Asia belonging to the Oleaceae family. Cultivars are found from Japan through China, Indo-China, Thailand, and India, to the Caucasus region. The flower petals of the evergreen shrub can vary from silver white (*Osmanthus fragrans* var. *latifolius*), via gold orange (*Osmanthus fragrans* var. *thunbergii*), to reddish (*Osmanthus fragrans* var. *aurantiacus*). Our samples were collected in Shizuoka prefecture, Japan, where *Osmanthus fragrans* var. *aurantiacus* is widespread. Because of the occurrence in this region the *Osmanthus* shrub is used as prefectural symbol.

Figure-5: Flowering *Osmanthus* shrub

The reddish petals are arranged cauliflory (Figure-5). During the short flowering period of only a few nights the color changes from reddish to yellowish.
Because of its unique scent commercial extracts are highly demanded. They can reach prices of up to 400 US $ per kilogram. In Asia the essential oils are used for flavoring tea, wine and cosmetics. In Europe extracts are mainly used by the perfume industry.

2.3.1 *Flavor composition*

Osmanthus varieties show by far the highest diversity of carotenoid derived aroma compounds among the flowering plants. Among the over 100 identified volatiles β-ionone (2), α-ionone (15), dihydro-β-ionone (16), dihydro-β-ionol (17), and *cis*- and *trans*-theaspirane (18, 19) are major components (Figure-6) [Kaiser 2002, Kaiser and Kraft 2001].

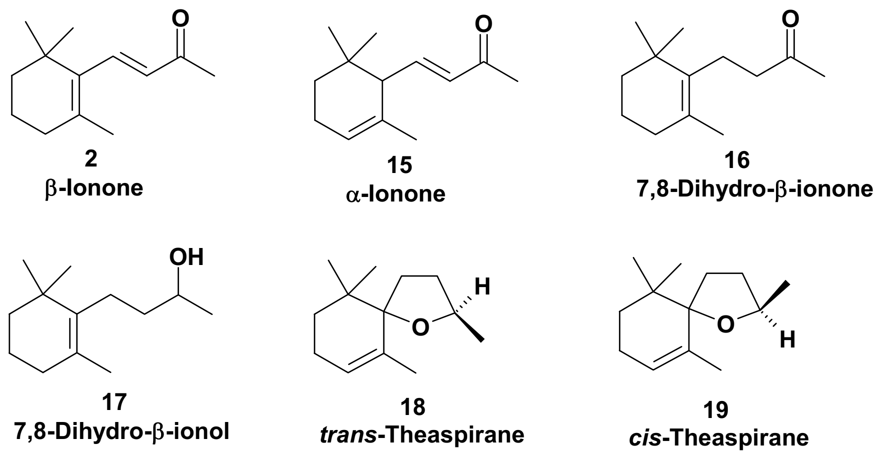

Figure-6: Main flavor compounds of *Osmanthus fragrans*

2.3.2 Carotenoid composition

So far no literature data about the carotenoid profile of *Osmanthus fragrans* is available. The identification of the major carotenoids was performed within this thesis.
It is well known that the color of yellow flower petals is often connected with high amounts of carotenoids, quite often with the major carotenoid β-carotene. Neverless, there is no proof so far that all of these yellow flowers produce ionone-like fragrances [Kaiser and Kraft 2001]. Only for some of these compounds, like in *Crocus sativus* or *Petunia hybrida* an enzymatic pathway has been established [Bouvier et al. 2003, Simkin et al. 2004a].

2.4 Carotenoids

Carotenoids are red and yellow pigments which are widely distributed in nature. The number of naturally occurring carotenoids reached 750. The majority are tetraterpenes composed of eight isoprene units. β-Carotene (20) is the best studied carotenoid which belongs to the class of carotenes. Whereas carotenes are only composed of hydrocarbons, xanthophylls are carotenoids that additionally contain oxygen. Beside β-carotene the most important carotenes are α-carotene (21), and lycopene (22). Carotenoids can also contain hydroxyl-, methoxy-, carboxyl-, keto-, aldehyd-, and carboxylic acid groups as terminal unit. Beside the carotenes mentioned, xanthophylls used within this study are lutein (23) and zeaxanthin (24) with hydroxyl-functions, canthaxanthin (25) with keto-functions, astaxanthin (26) with keto- and hydroxyl-function, and neoxanthin (27) with an expoxy- and hydroxyl-function (Figure-7). Additionally, carotenoids can be bound to glycosides, esters, sulfates, and proteins.