Chlorophyll reduction in rape seeds and it's influence on the bleaching earth consumption during refining

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Abstract

It is well known, that chlorophyll in edible oils reduces their oxidation stability, taste and shelf-life. In rape seeds chlorophyll is newly synthesised about 3 – 6 weeks after pollination. Short vegetation times, dryness, coldness or early winter onset influence the chlorophyll metabolism negatively.

The effect of the chlorophyll content in rape seeds on the bleaching earth consumption during the refining process was measured under lab scale conditions. It could be shown, that even a low decrease of the crude oil chlorophyll content in harvested rape seeds can lead to considerable bleaching earth savings.

Therefore different strategies to reduce chlorophyll in rape seeds were investigated.

These are classical plant breeding methods as well as the development of transgenic plants by inactivation of chlorophyll producing enzymes or over expression of chlorophyll degrading enzymes.

Finally it was tried to remove chlorophyll from crude rape seed oil by enzymatic treatment using recombinant proteins under refining conditions.

Key words: Rapeseed, oil, chlorophyll, refining, bleaching earth, plant breeding, transgenic plant, enzymatic process

Introduction

Chlorophyll is the green pigment in chloroplasts of plants and essential to photosynthesis. Therefore, there are small quantities of chlorophyll found in nearly every type of vegetable oil. But, the level of chlorophyll in rapeseed oil is mostly higher than that found in other vegetable oils. Especially in the case of freezing before the rapeseed has significantly ripened, the chlorophyll content in the crude rapeseed oil is very high and can be a problem for the refineries. We know this from Canada, North and East Europe. The reason is, that chlorophyll is synthesised again within the rape seed about 3 to 6 weeks after pollination.

Chlorophyll in edible oils reduces their oxidation stability, taste and shelf-life. To achieve stable oils, it is necessary to decrease the chlorophyll content in the oil to 0.05 ppm. Although up to 30 % of the total amount can be removed by neutralisation in the case of chemical refining, bleaching is the most important chlorophyll reducing refining step.

Worldwide about 45 mill. t/a rapeseed, or about 16 mill. t/a rapeseed oil, are produced. If it is assumed, that 0.7 % bleaching earth is needed for rapeseed oil bleaching, about 110.000 t/a of bleaching earth worldwide is applied for rapeseed oil refining.

The first aim of the studies was to determine to which amount bleaching earth can be saved only by reducing the chlorophyll content in the crude rapeseed oil (resp. the rape seeds).

At second, different strategies to reduce the chlorophyll content in rape seeds were investigated:

- classical plant breeding,
- development of transgenic plants, by
  a) inactivation of chlorophyll producing enzymes or
  b) overexpression of chlorophyll degrading enzymes.

Finally, the use of recombinant proteins to develop an enzymatic chlorophyll removal process from the oil was studied. The main questions of the investigations were:

- Are the enzymes active in oil?
- Which are the optimal reaction conditions?
- Where (regarding to the refining process) the enzyme is to put into the oil?
- Is the enzyme able to harm the oil?

Materials and Methods

1. Refining experiments in laboratory scale were carried out. Because, there were no crude rapeseed oils with high chlorophyll content available, model oils were made. For that, to the oil either pure chlorophyll or refined rapeseed oil was added to increase or decrease the initial chlorophyll content of the oil to a range between about 2 and 20 ppm. These oils were bleached under different conditions. Bleaching earth consumption, bleaching temperature and time as well as the citric acid concentration were varied. After that, the oil was normally deodorised and later analysed.

2. Chlorophyll kinetics in the seeds of 39 different winter and 33 summer rape accessions were measured.

3. There are two enzymes involved in the synthesis of chlorophyll a: glutamat-1-semialdehyd-aminotransferase (GSA-AT) and magnesium-chelatase. The genes for these enzymes were amplified and isolated by PCR. Gene constructs for reduced gene expression by RNA interference were generated and used for rape hypocotyl transformation by *Agrobakterium tumefaciens.*

There are four different enzymes involved in the chlorophyll catabolism: chlorophyllase, Mg-dechelatase, pheophorbide-a-oxygenase and RCC reductase. First, only the chlorophyllase genes were amplified and cloned from *Citrus clemente*. Gene constructs were generated and used for rape transformation in the same way described before.

Totally, thirty nine transgenic rape lines were generated.

4. The use of recombinant proteins for an enzymatic chlorophyll removal from the oil was examined.

At first, the chlorophyllase gene was amplified and cloned from clementine. The protein was then expressed in *E. coli.* The soluble, insoluble and total protein fractions were assayed for enzymatic activity tests, carried out at different conditions of pH, temperature, substrate concentration and enzyme concentration. The effects were measured by thin layer chromatography.

After that, it was tried to transform these results into a process for enzymatic chlorophyll removal under laboratory conditions. Basis was the classical chemical edible oil refining process. Because there was no rapeseed oil rich in chlorophyll available, pure chlorophyll a were added to the crude oil. After degumming and neutralisation a buffer with pH 7.8 and a liquid chlorophyllase extract were added. The system was stirred at fifty five degrees for 2 hours. Then it was tried to separate the formed chlorophyllide into the water phase.

In addition, the gene for phaeophorbide-a-oxygenase was amplified and cloned from tomato and the gene for RCC-reductase was delivered from the University of Bern. Both were expressed in *E. coli*. Enzyme activities of soluble and unsoluble assays were proved.

Results

1. The crude oil chlorophyll content very strong influences the bleaching earth amount, which is needed to reach a target chlorophyll content in the refined oil. At higher crude oil chlorophyll contents significantly more bleaching earth is needed as at lower crude oil chlorophyll contents to reach the same chlorophyll content in the refined oil.

2. Winter rape lines contain generally lower chlorophyll than summer rape lines. Different types of kinetics indicate a potential for further breeding activities to develop rape lines with lower chlorophyll content in the seeds.

3. The GSA-AT RNAi gene constructs showed no effect. Two plants with Mg-chalatase-RNAi constructs were extremely chlorotic. Chlorophyll was not only removed from the seeds, but also from the leaves. All other plants showed no effect. In 4 plants with chlorophyllase overexpression gene constructs reduced chlorophyll contents were measured at the 42 day after pollination. In summary, the strategy of chlorophyll overexpression seems to be more promising, than the strategy of inhibition of the chlorophyll synthesis.

4. Enzymatic activity tests showed, that chlorophyllase from *Citrus* can be used in oily buffers, has a high thermostability, which is higher in oil than in water, and has low activity losses at pH values different from the optimum.

Lab scale enzymatic refining, using chlorophyllase, showed that the chlorophyllide can be formed, but not effectively separated. Within the activity tests acetone was used as a solubiliser. Because the use of acetone in edible oil refining is not possible, it was looked for other substances. Using a mixture of 1 part water and 1 part isopropanol, a slight separation was possible. Other systems, using ethanol or glycerine, failed.

Activity tests showed, that the used phaeophorbide-a-oxygenase and RCC-reductase are active, but the fluorenscent chlorophyll catabolite is only formed in the case of presence of both enzymes. Because both enzymes need some co-factors for working, a successful application into the edible oil refining process is not much probable.

Conclusions

Even low reductions of the chlorophyll content in crude rapeseed oil lead to a considerable decrease in refining expense, especially to a lower bleaching earth consumption.

Different chlorophyll reduction strategies were investigated.

Recombinant chlorophyllase from *Citrus clementii* is able to split the chlorophyll into chlorophyllide and phytol under normal edible oil refining conditions. For the separation of the hydrophilic chlorophyllide, an acetonic system is necessary.

The activity of recombinant pheaophorbide-a-oxygenase and RCC-reductase is proved in vitro. Because of the need of some co-factors, their application in an edible oil refining process is not to expect.

There were 39 transgenic plants generated. 4 of them showed significantly lower chlorophyll contents at the 42 day after pollination.

The chlorophyll kinetics of 35 summer and 39 winter rapeseed lines during ripening were measured. Large differences between them indicate a potential for classical plant breeding of rapeseed lines with low chlorophyll content.